

## Optimization of Sequential Microwave-Ultrasonic-Assisted Extraction of Flavonoid Compounds from *Moringa oleifera*

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Received: 2 September 2021, Revised: 15 October 2021, Accepted: 22 October 2021, Published: 25 November 2022

### Abstract

Flavonoid are bioactive compounds that can be found in moringa leaves. Extraction of bioactive compounds using conventional method requires a long time and low extract yields. This study was aimed to determine the optimum process conditions of sequential microwave-ultrasonic-assisted extraction (MUAE) of total flavonoids (TF) from moringa. The response surface methodology (RSM) with Box-Behnken design (BBD) was employed to determine the optimum operating conditions of MUAE among the set of variables: Time (10 - 30 min), temperature (40 - 60 °C), and solvent concentration (60 - 80%). Furthermore, the flavonoids content was determined using high-performance liquid chromatography (HPLC) and the antioxidant activity was analyzed using the DPPH scavenging method. The RSM optimization showed that the predicted optimum total flavonoids content was 3.25 quercetin equivalent (QE) mg/g, achieved under operating conditions at a temperature of 49.76 °C, extraction time 20.68 min and solvent concentration of 71.16 %. These optimum operating conditions were validated, resulting in an average error of 0.546 %. The MS/HPLC results showed that the content of quercetin and myricetin were 0.167 and 2.89 g/ 100 mL, respectively. Moringa extraction product has a strong antioxidant activity with IC<sub>50</sub> of 74.58 - 74.62 µg/mL.

**Keywords:** *Moringa oleifera*, Microwave, Ultrasound, Extraction, Response surface methodology

### Abbreviations

TF: Total flavonoids  
MUAE: Sequential microwave-ultrasonic-assisted extraction  
UAE: Ultrasonic-assisted extraction  
MAE: Microwave-assisted extraction  
HAE: Heat-assisted extraction  
QE: Quercetin equivalents  
ANOVA: Analysis of variance  
RSM: Response surface methodology  
BBD: Box-Behnken design  
DPPH•: 1,1-diphenyl-2-picrylhydrazyl  
MS/HPLC: Mass spectrometry/High-performance liquid chromatography

### Introduction

*Moringa oleifera* is well-known as a nutrient-dense plant. Moringa has been used in herbal therapy to treat diseases, such as anti-tumor, anti-inflammatory, antihypertensive, antioxidant, antibacterial, antifungal and can be used as immune booster for dealing with Covid-19 [1]. It is due to the presence of bioactive compounds in the form of flavonoids (kaempferol, quercetin, myricetin, apigenin, niaziminin) in moringa leaves extracts. Quercetin acts as a free radical scavenger, and both *in vitro* and *in vivo* studies showed quercetin as a potent antioxidant [2]. The genetic variability, sample treatment methods, growing environment, and leaves maturity affect flavonoid concentration in moringa leaves samples [3].

The extraction process of flavonoid compounds from moringa has been intensively studied due to their outstanding benefits. Numerous parameters, such as solvents, techniques, and extraction time, influence bioactive chemicals extraction from plant materials. For large-scale production, the best extraction process should be effective and efficient [4]. Time and temperature are crucial in extracting flavonoid compounds [5]. At a temperature of 50 °C, it is relatively safe to prevent damage to certain secondary metabolites, especially flavonoids. Flavonoid are phenolic compounds that have a conjugated aromatic system. The conjugated aromatic system is easily damaged at high temperature [6]. Urias-Orona et al. reported that the ethanol solvent ratio affects the total flavonoids and antioxidant capacity [7]. A larger ethanol ratio tends to obtain more flavonoid compounds because flavonoids are polar, where the hydroxyl groups and sugars (glycoside bonds) in flavonoids tend to be easily soluble in alcohol and water [8].

Traditional extraction methods such as maceration, which takes time and spends solvent, have the disadvantage of causing oxidation, hydrolysis, and ionization damage to bioactive chemicals during the extraction process [9]. The use of ultrasonic-assisted extraction (UAE) and microwave-assisted extraction (MAE) is an alternative to improve the extraction process. The UAE process uses ultrasonic waves that are above the threshold of human hearing with a frequency greater than 16 - 20 kHz [10]. UAE is an environmentally friendly extraction method providing excellent repeatability in less time, easier manipulation, lower solvent consumption, and lower energy input without affecting the extraction yield [11]. Furthermore, the UAE process is a non-destructive treatment, so it is easy to be combined with other extraction techniques [12]. The UAE technique is faster than Heat-assisted extraction (HAE) or thermal technique to produce the yield product [13]. The MAE process uses microwave heat to separate the analyte from the sample matrix. The MAE technique has several advantages, including shorter extraction times and higher yields [14]. Compared to single extraction, combining 2 or more extraction techniques, such as sequential MUAE, can speed up the processing time and be more efficient [15]. The MUAE processes can produce more yield of flavonoid content [16]. Therefore, it is reasonable to investigate the MUAE treatment in improving the total flavonoids of moringa leaves extract. Optimization studies for the extraction process are very important to help in the natural resources business as well as to produce more efficient and cost-effective systems. The response surface methodology (RSM) is modelling in an empirical approach to determine the relationship and problems of several variables by finding the optimum value of response [17]. The development of the RSM has simplified optimization studies where multiple parameters can be studied simultaneously. The influence of variables on the behavior of the response system and the interdependence between variables can be seen more clearly by creating a 3-dimensional plot of the experimental data [18]. Surface response method with the Box-Behnken design (BBD) statistical model can be used to determine the optimum process conditions such as time, pH and temperature [19]. BBD designs generate higher-order response surfaces with fewer required runs than central composite designs, making them easier to work with the same number of factors. Take into account that the CCD design had better smallest values than the BBD design. The BBD design resulted in the lowest total cost [14,20].

Based on these understandings, this study aims to optimize the MUAE variables: Time, temperature, and ethanol concentration, on flavonoids concentration from moringa and to analyze the antioxidant activity of the MUAE extracts. The optimization process was conducted using RSM with Box-Behnken design (BBD) as an engineering tool useful for optimizing process conditions to obtain high total flavonoid yields. In this study BBD was chosen because the test is simple and requires fewer samples to run. The quercetin content was determined using mass spectrometry/high-performance liquid chromatography (MS/HPLC). Antioxidant activity was analyzed using the DPPH scavenging method.

## Materials and methods

### Materials

The plants of *Moringa oleifera* were reported as cultivated species in many regions in Java especially at NTT and East Java [21]. Several specimens were collected for determination at the Ecology and Biosystematics Laboratory, Department of Biology, Diponegoro University. **Figure 1** Show a specimen of the *Moringa oleifera* Lam.

In this study, the chemical such as ethanol (96% CAS 64-17-5), AlCl<sub>3</sub> (CAS 7446-70-0), potassium acetate (CAS 127-08-2), DPPH (2,2-Diphenyl-1-picrylhydrazyl) (CAS 1898-66-4), and standard quercetin (CAS 117-39-5) were purchased from Merck (Germany).

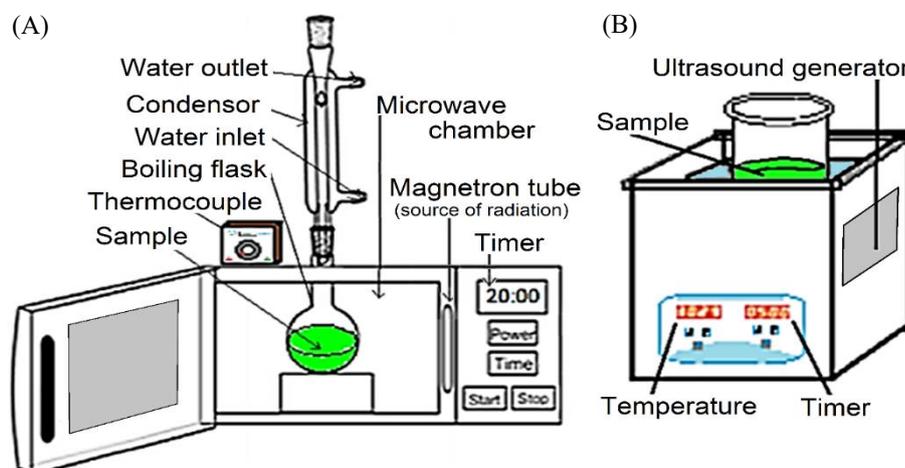


- Family Name: Moringaceae.
- Currently Accepted Name: Moringa oleifera Lam.
- Determined By: Mochamad Hadi.
- Determination at: Ecology and Biosystematics Laboratory, Diponegoro University
- Description: Tree with a height of 7-12 cm, white woody stem, simpodial branching, compound leaves, odd leaf progeny, oval leaf shape, 1-2 cm long, 1-2 cm wide.
- Habitat: In open site.
- Notes: Cultivated.
- Collector: Gian R. Prinanda.
- Date: July 2018.

**Figure 1** Specimen plant *Moringa oleifera* Lam.

### Flavonoids extraction from moringa leaves using sequential MUAE

The 100 g moringa leaves powder was extracted into 2.5 L ethanol solvent or solute/solvent ratio of 1:25 (g/mL). This study used a modified microwave extractor (MGC201000S, Beko, Poland) with a digital timer, temperature control, and a power of 200 W. This study used an ultrasonic extractor (model JP-010S, Skymen, USA) with a power of 80 watts and a frequency of 40 kHz. Sequential MUAE was following the research of Yu *et al.* [22] by alternating microwave first as pre-treatment and continued with ultrasound. Extraction was carried out with varied time (10 - 30 min), temperature (40 - 60 °C), and ethanol concentration as the solvent (60 - 80 %). After MUAE treatment, the mixture of moringa extract was immediately cooled at room temperature. Furthermore, the extract was then filtered using Whatman 42 filter paper. The extract was evaporated and obtained a thick extract of 10 g. Extracts were collected in amber glass bottles and stored in the refrigerator. The experimental setup of MUAE treatment is depicted in **Figure 2**.



**Figure 2** (A) microwave extraction, and (B) ultrasonic extraction.

### Total flavonoids (TF) determination

The aluminium chloride colourimetric method, modified from Mukhriani *et al.* [23], was used for flavonoids determination. The 1 mL moringa extract (extract with a solute/solvent ratio of 1:25 g/mL) was separately of aluminium chloride 2 % (CAS 7446-70-0), 1 mL potassium acetate 120 mM (CAS 127-08-2). It was kept at room temperature for 30 min; the absorbance of the mixture was measured at 430 nm against a blank without AlCl<sub>3</sub> using UV-vis spectrophotometer (Model ENF-24/F, Shimadzu, Japan). The calibration curve was prepared using quercetin standard solutions of 100 to 500 mg/mL in ethanol. Total

flavonoids values were expressed in terms of quercetin equivalent (mg QE/g sample) using the following formula:

$$\text{Total flavonoids} = \frac{(x \times v \times fp)}{g} \quad (1)$$

where  $x$  is the quercetin concentration from the standard curve (mg/mL).  $v$  is the extract volume (mL).  $fp$  is the dilution factor.  $g$  is the moringa extract weight (g).

#### High-performance liquid chromatography-Mass Spectrophotometry (HPLC-MS) analysis

The quercetin content of moringa extract was analyzed by HPLC (Shimadzu, Japan), using a Shim-pack GIST-HP C18 (150 mm (length) x 3 mm (ID), 3  $\mu$ m particle size). The column temperature was kept at 30 °C. Mobile phases which consisted of 0.1 % formic acid in water (A) and acetonitrile (B) were used in the following gradient elution method: 0 - 10 min, 10 - 85 % B; 10 - 13 min, 85 - 95 % B; 13 - 19 min, 95 - 95 % B. The flow rate was set at 0.3 mL/min, and the injection volume was 10  $\mu$ L. The sample was prepared for a concentration of 1 g of thick extract which was diluted into 100 mL of ethanol and then injected into the HPLC column. To explain the possible differences in the analysis of the instrument to the results of MUAE extract, the relative area was calculated using quercetin (2 g /100 mL or 2 %) as the internal standard.

The mass spectrometer was carried out in both positive and negative ionization multiple-reaction monitoring (MRM) mode. The source parameters were as follows: The capillary voltage set at 300 V for positive ionization mode and -300 V for negative ionization mode, the drying gas temperature was 320 °C, the flow was 11 L/min, and nebulizing gas pressure was 30 psi.

#### Antioxidant activity assays

The antioxidant activity was determined using the DPPH scavenging technique modified from Munteanu and Apetrei [24]. Initially, a 1 mM DPPH solution (2,2-Diphenyl-1-picrylhydrazyl) was prepared by dissolving 3.8 mg in 100 mL of ethanol. Next, 2 mL of each sample were added to 2 mL of 1 mM DPPH. The absorbance level was determined on the analysis of total flavonoids at an absorbance of 517 nm using a UV-Vis spectrophotometer (Shimadzu ENF-24/F, Japan). The following formula is used to calculate the DPPH scavenging test:

$$\%DPPH \text{ scavenging activity} = \frac{(A_0 - A_i)}{A_0} \times 100 \quad (2)$$

where  $A_0$  is the absorbance of the control and  $A_i$  is the absorbance of samples.

#### Experimental design

The flavonoid compounds of moringa that were extracted by MUAE were optimized utilizing RSM method with Box-Behnken design (BBD). In BBD the test sets on 15 runs, containing 12 factorial trials and 3 center point repetitions. The variation of time ( $X_1$ ), temperature ( $X_2$ ), and solvent concentration ( $X_3$ ) were chosen as independent variables. The independent variables were set at a time of 10 - 30 min, the temperature of 40 - 60 °C, and solvent concentration for 60 - 80 % ethanol, respectively. For the combination of these independent factors, the total flavonoids (TF) yield was chosen as the response. The real value of independent variables must be translated into coded variables (-1, 0, +1) to analyze using the response surface approach. **Table 1** shows the independent range and levels variables for extraction.

Each extraction response was used to construct a mathematical model that uses a second-order polynomial equation to link the TF yield to the independent variables, as shown in Eq. (3):

$$Y = \beta_0 + \sum_i^n \beta_i X_i + \sum_i^n \beta_{ii} X_i^2 + \sum_i^n \sum_j^n \beta_{ij} X_i X_j + \varepsilon \quad (3)$$

where  $Y$  is the total flavonoids;  $\beta_0$  is the constant coefficient;  $\beta_i$  is the linear coefficients;  $\beta_{ij}$  is the interaction coefficients;  $\beta_{ii}$  is the quadratic coefficients; and  $X_i, X_j$  are the coded values of the experimental variables.

In this study, 3 independent variables were investigated: Time ( $X_1$ ), temperature ( $X_2$ ), and ethanol concentration ( $X_3$ ), with  $Y$  was set as response variables (total flavonoids yield). Therefore, Eq. (3) can be rewritten as Eq. (4):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_1 X_1^2 + \beta_2 X_2^2 + \beta_3 X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (4)$$

**Table 1** Independent variables in the variation of time, temperature and solvent concentration.

| Levels | Independent variable |                  |                   |
|--------|----------------------|------------------|-------------------|
|        | Time (min)           | Temperature (°C) | Solvent conc. (%) |
| -1     | 10                   | 40               | 60                |
| 0      | 20                   | 50               | 70                |
| +1     | 30                   | 60               | 80                |

### Data analysis

Regressions with multiple linear variables were used to determine the best MUAE conditions using Statistica 10.0 software (Hamburg, Germany). Additional tests were carried out after the RSM optimization to confirm and verify the validity of the established model equation as well as the static experimental design. Furthermore, the software also made it possible to create a graph of surface and contour response values in a 3-dimensional plot. The confidence level for comparing the mean total flavonoids in multiple experimental runs was set at  $\alpha = 0.05$  or at a significant of 95 %.

### Results and discussion

#### Development of regression model

Flavonoid compounds were extracted from the powder of moringa leaves using the sequential MUAE method. After that, the RSM was used to fine-tune operating parameters: Time ( $X_1$ ), temperature ( $X_2$ ), and solvent concentration ( $X_3$ ). **Table 2** shows the design matrix for the experiment.

**Table 2** shows the whole design matrix of the trials, as well as the experimental and predicted yields of total flavonoids. As can be observed in **Table 2**, the TF yield obtained was in the range of  $1.43 \pm 0.006$  to  $3.26 \pm 0.011$  mg QE/g. The experimental error (pure error) and data reproducibility were determined using the centre point (run 13 - 15). In order to predict the total flavonoids (TF) yield, 2 equations, including 1<sup>st</sup> order (linear) and 2<sup>nd</sup> order (quadratic) equations, are assessed. **Table 3** shows the comparison of linear and quadratic equations regarding their results in predicting the TF yields with respect to the sum of squares analysis.

**Table 2** Experimental design using the Box-Behnken design to optimize flavonoids extraction from moringa using MUAE method.

| Run | Code pattern |    |    | Variable ( $X_1, X_2, X_3$ ) |            |             | TF yield (mg QE/g) |           |
|-----|--------------|----|----|------------------------------|------------|-------------|--------------------|-----------|
|     |              |    |    | Time (min)                   | Temp. (°C) | Solvent (%) | Experiment         | Predicted |
| 1   | +1           | 0  | -1 | 30                           | 50         | 60          | $2.16 \pm 0.011$   | 2.15      |
| 2   | +1           | 0  | +1 | 30                           | 50         | 80          | $2.26 \pm 0.008$   | 2.25      |
| 3   | 0            | -1 | +1 | 20                           | 40         | 80          | $2.40 \pm 0.004$   | 2.39      |
| 4   | 0            | +1 | +1 | 20                           | 60         | 80          | $2.32 \pm 0.002$   | 2.33      |
| 5   | -1           | 0  | -1 | 10                           | 50         | 60          | $1.88 \pm 0.008$   | 1.89      |
| 6   | 0            | -1 | -1 | 20                           | 40         | 60          | $2.30 \pm 0.002$   | 2.30      |
| 7   | +1           | -1 | 0  | 30                           | 40         | 70          | $1.68 \pm 0.013$   | 1.70      |
| 8   | +1           | +1 | 0  | 30                           | 60         | 70          | $1.62 \pm 0.006$   | 1.63      |
| 9   | -1           | +1 | 0  | 10                           | 60         | 70          | $1.38 \pm 0.013$   | 1.37      |
| 10  | -1           | -1 | 0  | 10                           | 40         | 70          | $1.43 \pm 0.006$   | 1.43      |
| 11  | 0            | +1 | -1 | 20                           | 60         | 60          | $2.23 \pm 0.004$   | 2.24      |
| 12  | -1           | 0  | +1 | 10                           | 50         | 80          | $1.97 \pm 0.011$   | 1.98      |
| 13  | 0            | 0  | 0  | 20                           | 50         | 70          | $3.26 \pm 0.010$   | 3.25      |
| 14  | 0            | 0  | 0  | 20                           | 50         | 70          | $3.25 \pm 0.008$   | 3.25      |
| 15  | 0            | 0  | 0  | 20                           | 50         | 70          | $3.23 \pm 0.014$   | 3.25      |

**Table 3** Sum of squares in the analysis of variance of flavonoid content.

|                       | Source              | Sum of squares | dF | Mean of square | F-value | F <sub>0.05</sub> | R <sup>2</sup> |           |
|-----------------------|---------------------|----------------|----|----------------|---------|-------------------|----------------|-----------|
| 1 <sup>st</sup> Order | SS <sub>reg</sub>   | 0.16718        | 3  | 0.05573        | 0.12    | 3.59              | 0.0311         | Aliased   |
|                       | SS <sub>error</sub> | 5.20720        | 11 | 0.47338        |         |                   |                |           |
|                       | SS <sub>total</sub> | 5.37438        |    |                |         |                   |                |           |
| 2 <sup>nd</sup> Order | SS <sub>reg</sub>   | 5.36950        | 9  | 0.59661        | 612.04  | 4.77              | 0.9991         | Suggested |
|                       | SS <sub>error</sub> | 0.00487        | 5  | 0.00097        |         |                   |                |           |
|                       | SS <sub>total</sub> | 5.37438        |    |                |         |                   |                |           |

According to the sum of square of models (**Table 3**), the 2<sup>nd</sup> order equation (quadratic equation) is found as the best match for the answer since it has the highest order polynomial with significant additional terms and is not aliased. The resulting flavonoids extraction equation in terms of coded factor is as follows:

$$Y = -29.108 + 0.4049X_1 + 0.7338X_2 + 0.2789X_3 - 0.009821X_1^2 - 0.007362X_2^2 - 0.001961X_3^2 - 0.000020X_1X_2 + 0.000032X_1X_3 - 0.000008X_2X_3 \quad (5)$$

where  $Y$  stands for the TF yield;  $X_1$ ,  $X_2$ , and  $X_3$  stand for the time, temperature, and solvent concentration, respectively.

The synergic effect is indicated by a positive sign in front of the phrases, and a negative sign indicates the antagonistic impact [18]. As a result, the terms  $X_1$ ,  $X_2$ ,  $X_3$ , and  $X_1X_3$  have a significant impact on TF yield, while the terms  $X_1^2$ ,  $X_2^2$ ,  $X_3^2$ ,  $X_1X_2$ , and  $X_2X_3$  have a minor impact. The influence of each independent variable has a favorable impact on the TF outcomes, according to the linear effect. The TF yield will grow to some level as the time, temperature, and solvent concentration increase. As shown in Eq. (5), the effect of the square and the interaction between independent variables has a negative effect, indicating that under certain variable conditions the TF value will tend to begin to decrease.

#### Fitting the model

The relevance and fitness of the quadratic model are also determined using analysis of variance (ANOVA) and significance was judged by the  $p$ -value calculated from the data [14]. **Table 4** shows the ANOVA for the response surface quadratic model.

It is well-known that when the  $p$ -value is smaller than 0.05, it indicates that the main factors significantly affect the response. The  $p$ -value (probability of error value) is a tool for determining the significance of each regression coefficient and its interaction impact, as well as the interaction effect of each variable. The greater the significance of the associated coefficient, the lower the  $p$ -value [25]. Based on the ANOVA in **Table 4**, it can be seen that linear models ( $X_1$ ,  $X_2$ ,  $X_3$ ) and quadratic models ( $X_1^2$ ,  $X_2^2$ ,  $X_3^2$ ) are significant terms. ANOVA analysis confirms that the linear effect of all independent variables significantly affects the TF yield since their  $p$ -values are lower than 0.05. On the other hand, the 2-way interaction models ( $X_1X_2$ ,  $X_1X_3$ ,  $X_2X_3$ ) have no effect on the TF yield since their  $p$ -value are higher than 0.05. The model's accuracy is shown by the high value of the R-square model at 99.91 %, which means that the model is significant or strongly correlated in representing the data. The difference was 0.09 %, indicating that the model was highly significant to the TF yield. The high value of correlation coefficient close to +1 indicated that a high correlation values between the experimental and prediction data [26]. At a 95 % confidence level, the model's F-value is 612.04 and the  $p$ -value is strongly lower than 0.05, indicating that the model is highly significant. **Figure 3** depicts the pareto diagram of the ANOVA analysis.

Based on pareto diagram (**Figure 3**), the linier and quadratic models of each independent variable is significant. Since the quadratic effect has a negative effect on TF yield, it can be concluded that further increase in independent variables decreases the TF yield. As explained in the previous section, it has been found that the linear effect of all independent variables positively affects the TF yield. Therefore, it is reasonable to conclude that the TF yield can be increased by increasing the value of independent variables since they positively and significantly affect the TF yield based on the statistical analysis.

The lack of fit in **Table 4** get an F-value of 1.9 and a  $p$ -value of 0.363 ( $p$ -value > 0.05 is not significant) indicated that the lack of fit was not significant compared to the pure error and that the model was successfully fitted to experimental data. Moreover, the F-value of the model is high, which is 612.04. This value is strongly higher than the F-table ( $F_{0.05} = 4.7725$ ). This fact confirms that the model developed highly

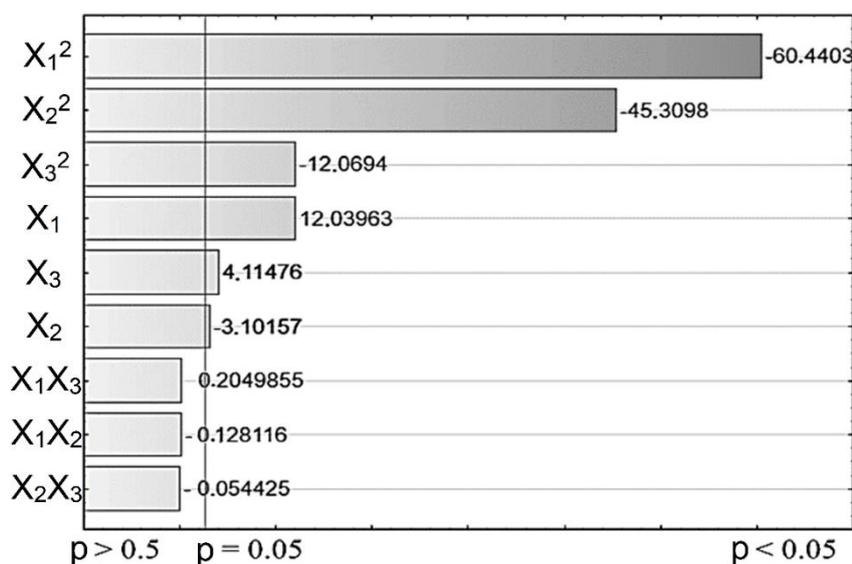
fits the experimental data. The comparison of the predicted and experimental values of TF yield is presented in **Figure 4**.

**Figure 4** shows that the experimental data highly fit the diagonal line confirming that the model developed perfectly predicts the value of TF yield. The points in this figure are on or near a straight line, which indicates that the remainder of this experiment is normally distributed and the model fitted by the response surface is accurate. Conversely, if the points on the graph cannot form a straight line, then it does not correspond to a normal distribution. Each scatter point is roughly distributed in a straight line and lies near the straight-line  $Y = X$ , indicating that it is an excellent approximation to the experimental yields [27].

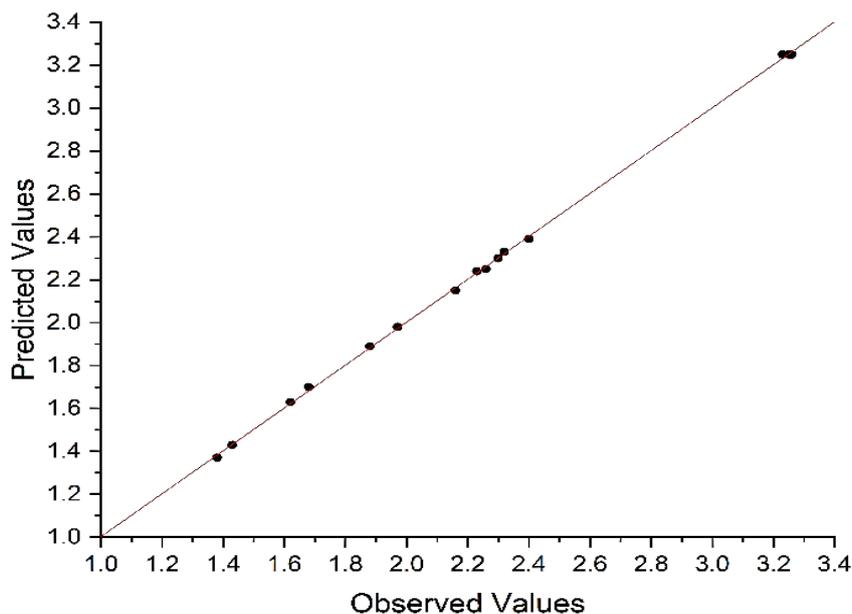
**Table 4** Analysis of variance (ANOVA) for response surface quadratic model.

| Parameter                     | dF | Adj SS  | Adj MS  | Reg. Coef. | T-value | F-value | p-value |        |
|-------------------------------|----|---------|---------|------------|---------|---------|---------|--------|
| Model                         | 9  | 5.36950 | 0.59661 | -29.108    | 180.38  | 612.04  | 0.000   |        |
| Linear                        | 3  | 0.16718 | 0.05573 |            |         | 57.17   | 0.000   |        |
| X <sub>1</sub>                | 1  | 0.14130 | 0.14130 | 0.405      | 12.04   | 144.95  | 0.000   | < 0.05 |
| X <sub>2</sub>                | 1  | 0.00938 | 0.00938 | 0.734      | -3.10   | 9.62    | 0.027   | < 0.05 |
| X <sub>3</sub>                | 1  | 0.01650 | 0.01650 | 0.279      | 4.11    | 16.93   | 0.009   | < 0.05 |
| Square                        | 3  | 5.20226 | 1.73409 |            |         | 1778.92 | 0.000   |        |
| X <sub>1</sub> <sup>2</sup>   | 1  | 3.56096 | 3.56096 | -0.009     | -60.44  | 3653.03 | 0.000   | < 0.05 |
| X <sub>2</sub> <sup>2</sup>   | 1  | 2.00124 | 0.65384 | -0.007     | -45.31  | 2052.98 | 0.000   | < 0.05 |
| X <sub>3</sub> <sup>2</sup>   | 1  | 0.14200 | 0.14200 | -0.002     | 12.07   | 145.67  | 0.000   | < 0.05 |
| 2 - way interaction           | 3  | 0.00006 | 0.00002 |            |         | 0.02    | 0.996   |        |
| X <sub>1</sub> X <sub>2</sub> | 1  | 0.00002 | 0.00002 | -0.000     | -0.13   | 0.02    | 0.903   | > 0.05 |
| X <sub>1</sub> X <sub>3</sub> | 1  | 0.00004 | 0.00004 | 0.000      | 0.20    | 0.04    | 0.846   | > 0.05 |
| X <sub>2</sub> X <sub>3</sub> | 1  | 0.00000 | 0.00000 | -0.000     | -0.05   | 0.00    | 0.959   | > 0.05 |
| Error                         | 5  | 0.00487 | 0.00097 |            |         |         |         |        |
| Lack-of-Fit                   | 3  | 0.00361 | 0.00120 |            |         | 1.90    | 0.363   | > 0.05 |
| Pure Error                    | 2  | 0.00127 | 0.00063 |            |         |         |         |        |
| Total                         | 14 | 5.37438 |         |            |         |         |         |        |

$S = 0.0312217$ ;  $R^2 = 0.9991$ ;  $R^2_{Adj} = 0.9975$



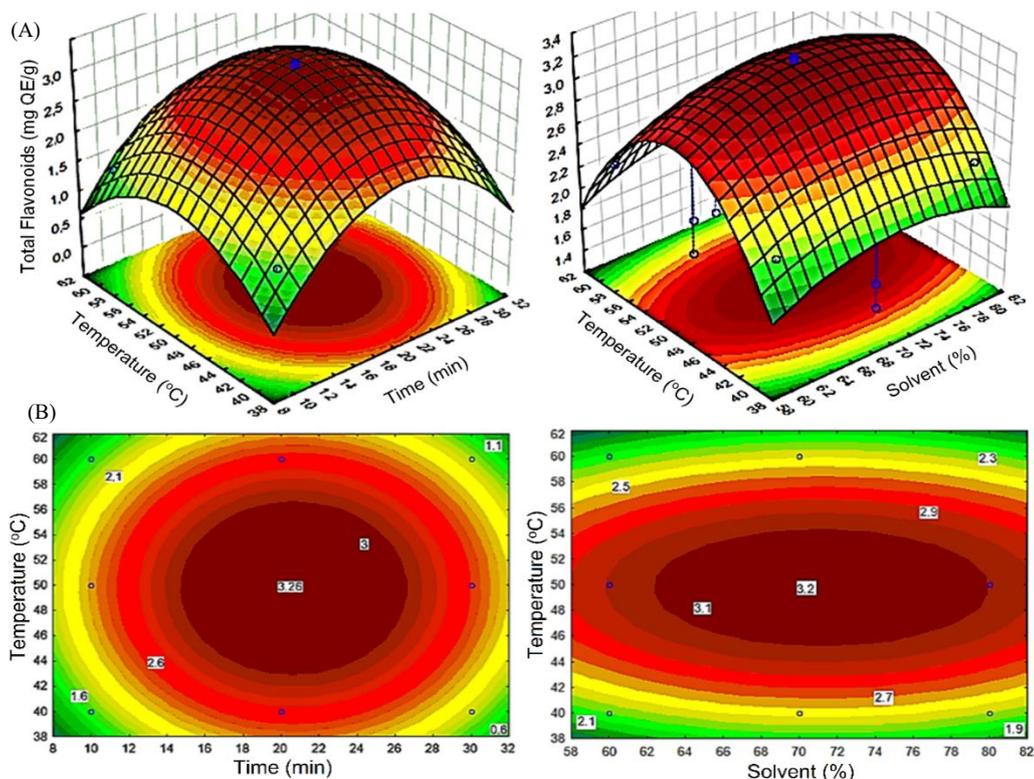
**Figure 3** The influence of time (X<sub>1</sub>), temperature (X<sub>2</sub>), and solvent concentration (X<sub>3</sub>) on the flavonoid content of the MUAE extraction is depicted in a pareto diagram.



**Figure 4** Predicted yield against observed (experimental) yield of total flavonoids.

**Optimization of flavonoids extraction from moringa through MUAE using RSM**

After the model is examined, we can use response plots in the form of surfaces and contours to determine the optimal value. The 3-dimensional (3-D) response surface and contour plots were the graphical representations of the regression Eq. (5). It provided a method to visualize the relationship between responses and experimental levels of each variable parameters and the type of interactions between 2 test variables. The 3-D response surface and contour plots are shown in **Figure 5**.



**Figure 5** (A) Surface, and (B) contour plots of the response of time ( $X_1$ ), temperature ( $X_2$ ), and solvent concentration ( $X_3$ ) to the flavonoid content.

Based on the surface response plots and contours (**Figure 5**), the characteristic of the stationary point in the flavonoid extraction experiment with MUAE was the maximum response point. Based on **Figure 5**, it can be observed that time and temperature have a proportional response to the TF results with a maximum point stationary form. While at the temperature and concentration of the solvent found a stationary form of the saddle point which shows that the solvent concentration does not give much difference to the TF results from solvent variations in the range of 60 - 80 % ethanol. The surface and contour plots show that higher total flavonoid yield (> 3 mg QE/g) can be obtained at 15 - 25 min, 45 - 55 °C, and 65 - 75 % solvent concentration. The highest TF results with high antioxidant activity can be obtained with optimum time and temperature conditions of 20 - 35 min and 40 - 55 °C, respectively [28]. Shorter extraction time may result in insufficient extraction of bioactive compounds, while longer extraction time may result in higher radiation and an increase in intermolecular temperature that can cause damage to bioactive components.

The solubility of flavonoid components as well as the diffusion coefficient can increase as the temperature rises. The ideal temperature range was discovered to be 45 - 55 °C. Moringa extraction with MAE increased yield at 50 °C, but yield decreased at 65 °C [29]. The breakdown of flavonoid molecules by heat and extended exposure to microwave/ultrasonic radiation resulted in a decrease in flavonoid yield at high temperatures. The effect of solvent concentration was found to be best at 65 - 75 %. The presence of chlorophyll which was also extracted at a high solvent concentration caused the total flavonoid extract to decrease at 80 % solvent concentration [30]. **Table 5** displays the optimum RSM values.

**Table 5** Estimation of optimum critical condition on MUAE treatment.

| Factor            | Observed Min | Critical Value | Observed Max |
|-------------------|--------------|----------------|--------------|
| Time (min)        | 10.00        | 20.67          | 30.00        |
| Temperature (°C)  | 40.00        | 49.76          | 60.00        |
| Solvent conc. (%) | 60.00        | 71.16          | 80.00        |

Predicted value on flavonoids concentration: 3.251 mg QE/g

All the maximum points were found as critical points within the experimental zone (**Table 5**), indicating that the critical point is the optimal value. As can be seen in **Table 5**, the optimum independent variables that gave the optimum TF yield were 20.67 min, 49.76 °C and 71.16 % of time, temperature and solvent concentration, respectively. This optimum independent variable gave the predicted optimum TF result at 3.251 mg QE/g. Based on the reference, the condition of the extraction ratio is 1:30 (g/mL); time 40 min; extraction temperature 50 - 55 °C; and solvent concentration 65 - 70 %; can produce a TF of 3.62 - 15.2 mg QE/g [31,32].

#### Verification of the predicted optimal extraction conditions

By calculating the regression equation from the given independents and evaluating the component contours and response surface plots, we can determine the optimal conditions. Furthermore, validation is carried out to ensure the model and optimization results. To determine the validity of the model, the experiment with the highest TF was selected for additional validation. The experimental results of model validation and predictive values are shown in **Table 6**.

**Table 6** Validation of experimental and predictive values under various operating conditions.

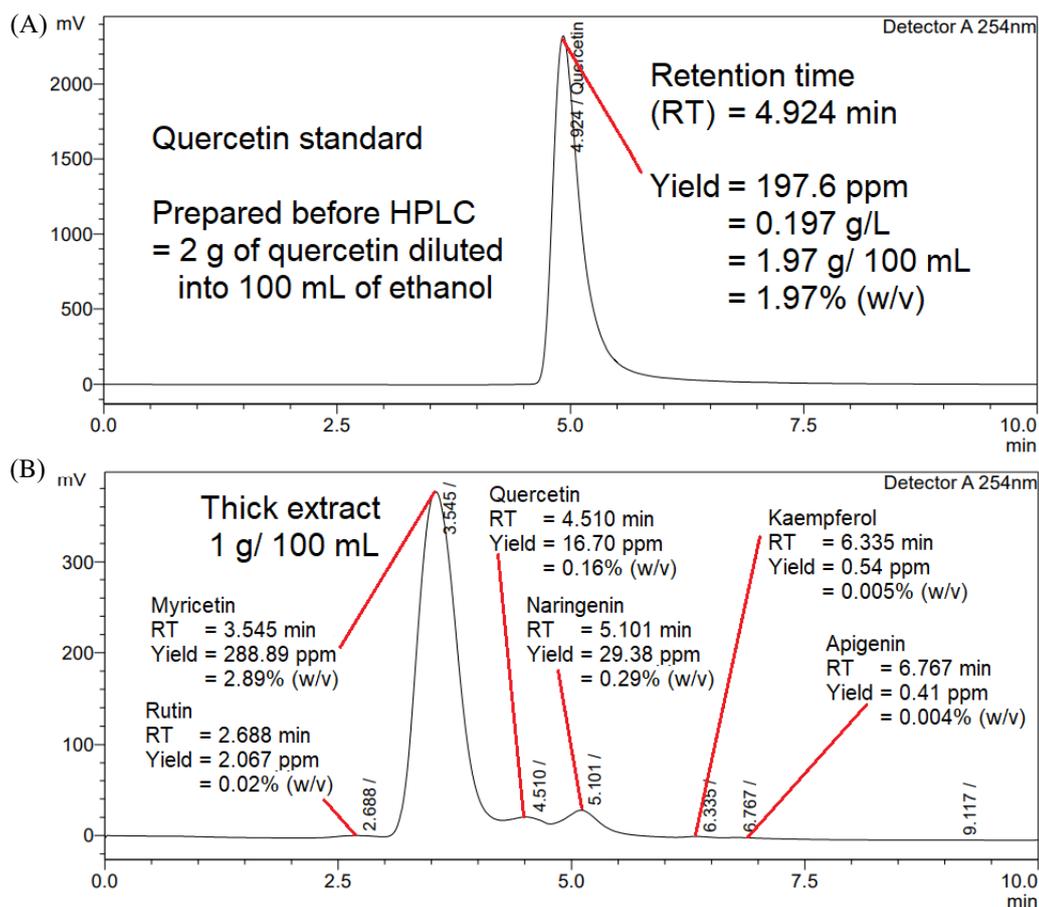
| Factor     |           |             | Response (mg QE/g) |           |          | Error (%) |
|------------|-----------|-------------|--------------------|-----------|----------|-----------|
| Time (min) | Temp (°C) | Solvent (%) | Observed           | Predicted | Residual |           |
| 21         | 50        | 71          | 3.27 ± 0.011       | 3.251     | 0.023    | 0.717     |
| 21         | 50        | 71          | 3.25 ± 0.001       | 3.251     | 0.003    | 0.102     |
| 21         | 50        | 71          | 3.22 ± 0.013       | 3.251     | -0.027   | 0.820     |
|            |           |             |                    |           | Average  | 0.546     |

As can be seen in **Table 6**, the experimental yield is close to the calculated yield using the model, with only a minor margin of error (0.546 %). The optimization results and simulation results show that the deviation between the predicted value and the actual value is below 5 %, indicating that the correlation is

good and the optimization of the response surface methodology for estimating the total flavonoid yield is reasonable and feasible [27].

#### High-performance liquid chromatography (HPLC) analysis of moringa extract

HPLC calibration curve was plotted as above-described method then chromatogram of 2 g standard quercetin and 1 g thick extract MUAE moringa are compared. The quercetin is identified at a specific retention time. Retention time (RT) is the minimum time required for the fluid mixture to separate by gravity in the separator, in this case, the chromatography device used in the HPLC apparatus. **Figure 6** shows the quercetin yield from the HPLC chromatogram result.



**Figure 6** (A) HPLC chromatogram of standard quercetin, and (B) thick extract MUAE moringa.

According to **Figure 6(B)**, the quercetin peak for the extracted moringa MUAE samples was at RT 4.51 min, which is close to the standard quercetin peak at RT 4.92 min (**Figure 6(A)**). The quercetin standard (**Figure 6(A)**) was found to yield 1.97 g/ 100 mL which was in accordance with the initial preparation before HPLC analysis with 2 g of quercetin dissolved in 100 mL of ethanol. These results are in accordance with the quercetin range at RT 4.183 - 4.563 min [33]. The yield of quercetin in moringa varied from 0.07 to 1.26 g/ 100 mL as indicated by the appearance of a peak at RT 4.0 min and on quantitative HPLC chromatograms [34].

In **Figure 6(B)**, from the MS/HPLC analysis we found the type of flavonoid rutin at RT 2.68 min with a yield of 0.02 g/ 100 mL; myricetin at RT 3.54 min with a yield of 2.89 g/ 100 mL; quercetin at RT 4.51 min with a yield of 0.16 g/ 100 mL; naringenin at RT 5.10 min with a yield of 0.29 g/ 100 mL; and kaempferol at RT 6.33 min with a yield of 0.005 g/ 100 mL. This event is in accordance with the reference to the extraction of quercetin from Iranian propolis using a microwave-ultrasound sequence, where in addition to the peak of quercetin at RT 5.13 min, other flavonoid peaks such as myricetin was also found at RT 3.71 min [35]. Myricetin, quercetin, and kaempferol are the main flavonoids contained in moringa, other flavonoids such as luteolin, apigenin are found in smaller levels [36]. In some references, myricetin

was found at a RT of 3.6 min at a level of 0.029 g/ 100 mL; quercetin was found at RT 4.8 min with a concentration of 0.11 g/ 100 mL; and kaempferol were found at RT 6 min with levels of 0.013 g/ 100 mL [37]. In the heat-assisted extraction (HAE) of moringa leaves, HPLC analysis at RT 4.35 min found 2.62 g/ 100 mL myricetin, at RT 5.89 min found 0.5 g/ 100 mL quercetin, and at RT 6.44 min found 4.15 g/ 100 mL kaempferol [38]. **Table 7** shows the comparison of the quercetin MUAE method with other extraction methods.

**Table 7** Comparison of quercetin yield in maceration, UAE, HAE, MAE and MUAE.

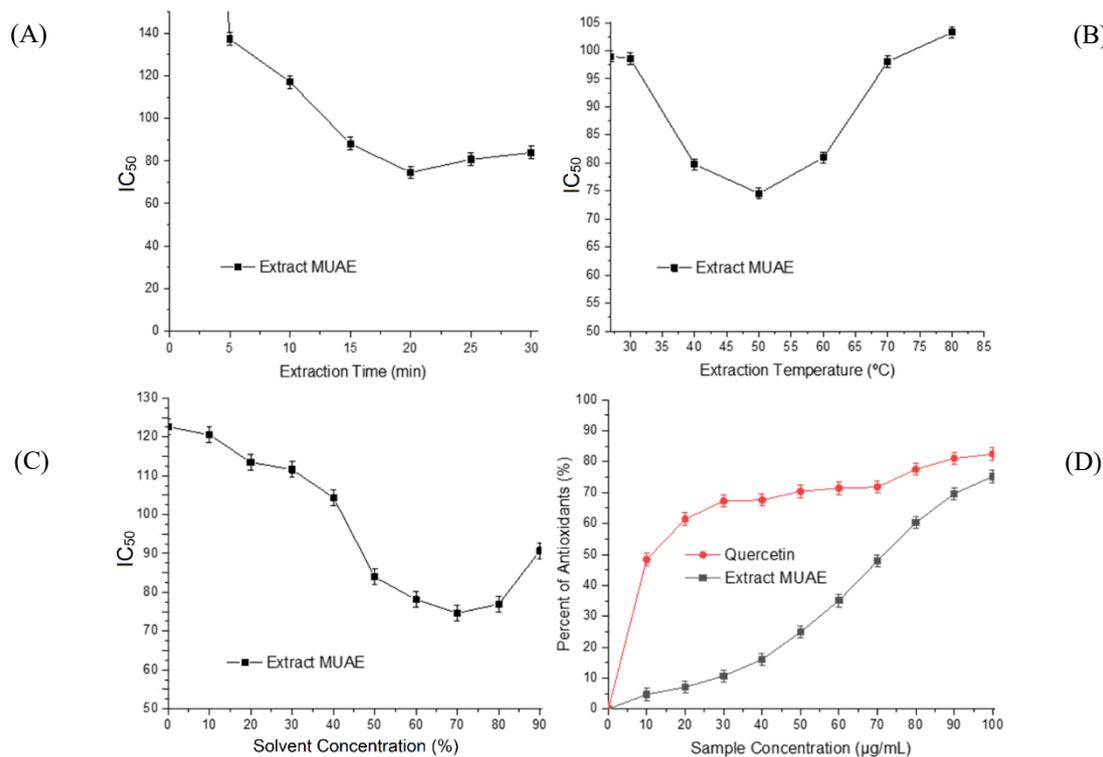
| Extraction methods          | Total Flavonoids<br>(mg QE/g) | Quercetin content<br>(g/100 mL) | DPPH Inhibition<br>(IC <sub>50</sub> ) |
|-----------------------------|-------------------------------|---------------------------------|--|
| Maceration (ME)             | 2.18                          | 0.06                            | 97.33                                  |
| Ultrasound (UAE)            | 2.26                          | 0.28                            | 87.83                                  |
| Heat (HAE)                  | 2.28                          | 0.50                            | 78.28                                  |
| Microwave (MAE)             | 2.72                          | 0.41                            | 76.54                                  |
| Microwave-ultrasound (MUAE) | 3.25                          | 0.16                            | 74.58                                  |

Based on **Table 7**, it shows that the extraction method that produces the highest antioxidant activity is microwave-ultrasonic sequential (MUAE) with the sequential MUAE > MAE > HAE > UAE > maceration. Microwave-assisted extraction can extract more flavonoid chemicals than heat-assisted extraction because microwave irradiation causes plant cells to rupture and cavitation can accelerate solvent diffusion [39]. Due to the greater radiation exposure, a prolonged extraction process can lead to higher thermal damage. Higher heat can damage the quercetin molecule, lowering quercetin content [40]. The reduced amount of quercetin detected in MUAE could be due to the breakdown of the quercetin molecule at high radiation. As a result, it can be concluded that the use of microwaves-ultrasound to extract more flavonoids but get lower quercetin content.

#### Effects of extraction variables on antioxidant activity

The results of the absorbance of DPPH compounds are sharp at the peak of 517 nm. These results are in accordance with the DPPH range at the absorbance of 515 - 520 nm. For comparison purposes, the antioxidant test was carried out on moringa extract extracted by sequential microwave-ultrasound-assisted extraction at various times, temperatures, and ethanol concentrations. The antioxidant results with the IC<sub>50</sub> value of the MUAE moringa extract are shown in **Figure 7**.

Based on **Figure 7**, the results antioxidant test of the moringa extracts from MUAE show a low IC<sub>50</sub>, indicating an increase in % antioxidant to the initial concentration of the sample. According to the results of the experiment, the lowest IC<sub>50</sub> values of moringa extract are 74.61, 74.55 and 74.62 µg/mL for independent variables of time at 20 min, the temperature at 50 °C and solvent concentration at 70 %, respectively. An increase in antioxidant occurred with increasing sample concentration, high antioxidant activity was found at 25 °C and a decrease in antioxidant power started at 45 °C, due to high temperature causing chemical degradation in the sample [41]. The inhibitory concentration (IC<sub>50</sub>) range of 150 - 200 µg/mL is a weak antioxidant, the IC<sub>50</sub> range of 100 - 150 µg/mL is a moderate antioxidant, the IC<sub>50</sub> range of 50 - 100 µg/mL is a strong antioxidant, and the IC<sub>50</sub> range of < 50 µg/mL is a very strong antioxidant [42]. The results of moringa extract obtained IC<sub>50</sub> of less than 100, indicating that moringa extract is classified as a strong antioxidant.



**Figure 7** IC<sub>50</sub> value of the MUAE extracts at the variation of (A) time, (B) temperature and (C) solvent concentration; (D) Effect of sample concentration on percent antioxidant.

## Conclusions

Extraction was the key step for the comprehensive utilization of total flavonoids from the moringa leaves. The sequential MUAE method was an efficient way to extract total flavonoids from moringa leaves. From the results of this study, it was concluded that the TF yield had been successfully modelled using RSM. The optimum extraction conditions were as follows: Extraction time (20.68 min), temperature (49.76 °C), solvent concentration (71.16 %). Under these conditions, the obtained TF yield was 3.25 mg QE/g. Furthermore, the validation of these optimum conditions gave an average error of 0.546 %, indicating that the model was sufficient to optimize the MUAE of moringa. In the MS/HPLC result, the quercetin was found in the moringa extract at RT of 4.51 min, which was close to the standard quercetin peak at RT of 4.92 min. Myricetin and kaempferol also was found at RT 3.545 and RT 6.335, respectively. The lowest IC<sub>50</sub> values from the results of the antioxidant activity test of moringa extract were in the range of 74.58 - 74.62 µg/mL. Based on these results, the obtained moringa extracts were classified as strong antioxidants because the IC<sub>50</sub> results were found to be less than 100 µg/mL.

## Acknowledgments

The authors acknowledge the Ministry of Research and Technology/National Research and Innovation Agency of Republic Indonesia (RISTEK/BRIN) for the financial support through the research project of Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2022, with contract number: 394-04/UN7.6.1/PP/2022.

## References

- [1] M Fajri. The potential of *Moringa oleifera* as immune booster against Covid 19. *IOP Conf. Ser. Earth Environ. Sci.* 2021; **807**, 022008.
- [2] FD Pierro, S Iqtadar, A Khan, SU Mumtaz, MM Chaudhry, A Bertuccioli, G Derosa, P Maffioli, S Togni, A Riva, P Allegrini and S Khan. Potential clinical benefits of quercetin in the early stage of Covid-19: results of a second, pilot, randomized, controlled and open-label clinical trial. *Int. J. Gen. Med.* 2021; **14**, 2807-16.

- [3] N Kaur, DS Arora, N Kalia and M Kaur. Antibiofilm, antiproliferative, antioxidant and antimutagenic activities of an endophytic fungus *Aspergillus fumigatus* from *Moringa oleifera*. *Mol. Biol. Rep.* 2020; **47**, 2901-11.
- [4] B Vongsak, P Sithisarn, S Mangmool, S Thongpraditchote, Y Wongkrajang and W Gritsanapan. Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method. *Ind. Crops Prod.* 2013; **44**, 566-71.
- [5] PTH Komala and A Husni. Pengaruh suhu ekstraksi terhadap aktivitas antioksidan ekstrak metanolik *Eucheuma spinosum* (in Bahasa Indonesia). *Jurnal Pengolahan Hasil Perikanan Indonesia* 2021; **24**, 1-10.
- [6] GS Agustien and SF Sucitra. Effect of different extraction method on total flavonoid contents of *Sansevieria trifasciata* P. leaves extract. *Galenika J. Pharm.* 2021; **7**, 143-51.
- [7] V Urías-Orona, G Gutiérrez-Soto, J Ruiz-Bautista, R Flores-Alonso, I Montiel-Ramos, GCG Martínez-Ávila, J Aranda-Ruiz and G Niño-Medina. Influence of extraction solvent on phenolic content and antioxidant capacity level of a commercial food supplement from *Moringa oleifera* leaves. *Archivos Latinoamericanos de Nutrición* 2017; **67**, 211-7.
- [8] SLRD Luna, RE Ramírez-Garza and SOS Saldívar. Environmentally friendly methods for flavonoid extraction from plant material: impact of their operating conditions on yield and antioxidant properties. *Sci. World J.* 2020; **2020**, 6792069.
- [9] B Kunarto, Sutardi, Supriyanto and C Anwar. Optimization of ultrasonic-assisted extraction of “melinjo kerikil” (*Gnetum gnemon* L. ‘Kerikil’) seeds using response surface methodology. *Jurnal Aplikasi Teknologi Pangan* 2019; **8**, 104-12.
- [10] MS Rahmatullah, S Suryono and JE Suseno. Instrumentation system of flavonoid compounds of moringa leaf using ultrasound assisted extraction (UAE). *Int. J. Progress. Sci. Tech.* 2021; **25**, 447-452.
- [11] X Lin, L Wu, X Wang, L Yao and L Wang. Ultrasonic-assisted extraction for flavonoid compounds content and antioxidant activities of India *Moringa oleifera* L. leaves: Simultaneous optimization, HPLC characterization and comparison with other methods. *J. Appl. Res. Med. Aromat. Plants* 2020; **20**, 100284.
- [12] M Sholihah, U Ahmad and IW Budiastira. Application of ultrasonic wave to increase extraction yield and effectiveness of antioxidant from mangosteen rind. *Jurnal Keteknik Pertanian* 2017; **5**, 161-8.
- [13] R Ratnawati, A Prasetyaningrum and DH Wardhani. Kinetics and thermodynamics of ultrasound-assisted depolymerization of  $\kappa$ -carrageenan. *Bull. Chem. React. Eng. Catal.* 2016; **11**, 48-58.
- [14] P Torabi, N Hamdami and J Keramat. Microwave-assisted extraction of sodium alginate from brown macroalgae *Nizimuddiniana zanardini*, optimization and physicochemical properties. *Separ. Sci. Tech.* 2021; **57**, 872-85.
- [15] T Wang, W Li and T Li. Microwave-ultrasonic synergistic extraction of crude se-polysaccharides from se-enriched tea. *Key Eng. Mater.* 2017; **737**, 360-6.
- [16] A Prasetyaningrum, N Rokhati, Y Dharmawan and GR Prinanda. Comparison study for extraction of bioactive flavonoids from *Moringa oleifera*, apple, onion, and ascorbic acid (orange) by using microwave-assisted, ultrasound-assisted and maceration methods. *IOP Conf. Ser. Mater. Sci. Eng.* 2021; **1053**, 012123.
- [17] I Ahmad, WC Prabowo, Y Nur, M Ardana, BP Rahayu and Herman. Optimasi metode ekstraksi berbantu mikrowave dengan pelarut hijau (asam sitrat-glukosa) terhadap kadar polifenol total dari daun kadamba (*Mitragyna Speciosa* Korth. Havil) menggunakan response surface methodology (in Bahasa Indonesia). *Majalah Farmasi dan Farmakologi* 2020; **24**, 11-6.
- [18] E Sap, ÜA Usca, MK Gupta, M Kuntoglu, M Sarikaya, DY Pimenov and M Mia. Parametric optimization for improving the machining process of Cu/Mo-SiCP composites produced by powder metallurgy. *Materials* 2021; **14**, 1921.
- [19] A Prasetyaningrum, R Ratnawati and B Jos. Optimasi proses ozonasi pada depolimerisasi  $\kappa$ -karagenan dengan metode respon permukaan. *Reaktor* 2017; **17**, 1-8.
- [20] CN Wang, NAT Nguyen and TT Dang. Memecahkan masalah perencanaan pesanan menggunakan heuristik pendekatan: Kasus di distributor bahan bangunan (in Bahasa Indonesia). *Appl. Sci.* 2020; **10**, 8959.
- [21] R Ridwan, H Hamim, N Hidayati and S Suharsono. Molecular and morphological analysis of indonesian drumstick tree (*Moringa oleifera* Lam.). *Asian J. Plant Sci.* 2021; **20**, 131-42.
- [22] J Yu, Q Lou, X Zheng, Z Cui and J Fu. Sequential combination of microwave- and ultrasound-assisted extraction of total flavonoids from *Osmanthus fragrans* Lour. flowers. *Molecules* 2017; **22**, 2216-32.

- [23] T Mukhriani, R Sugiarna, N Farhan, M Rusdi and MI Arsul. Total phenolic and flavonoid content of grapevine (*Vitis vinifera* L.) leaves ethanol extract. *ad-Dawaa' J. Pharm. Sci.* 2019; **2**, 95-102.
- [24] IG Munteanu and C Apetrei. Analytical methods used in determining antioxidant activity: A review. *Int. J. Mol. Sci.* 2021; **22**, 3380.
- [25] EW Gibson. The role of *p*-values in judging the strength of evidence and realistic replication expectations. *Stat. Biopharm. Res.* 2020; **13**, 6-18.
- [26] A Prasetyaningrum, M Djaeni, B Jos and Y Dharmawan. Optimasi proses ozonasi untuk penurunan kadar krom (Cr) dalam limbah cair elektroplating dengan metode respon permukaan. *In: Proceedings of the Seminar Nasional Teknik Kimia Kejuangan, Yogyakarta, Indonesia.* 2017, p. 1-7.
- [27] N Zhao and M Wang. Research on parameter optimization of the express warehousing and distribution system based on the Box-Behnken response surface methodology. *Adv. Civ. Eng.* 2021; **2021**, 8723017.
- [28] DW Dadi, SA Emire, AD Hagos and JB Eun. Effect of ultrasound-assisted extraction of *Moringa stenopetala* leaves on bioactive compounds and their antioxidant activity. *Food Tech. Biotechnol.* 2019; **57**, 77-86.
- [29] K Sin, WA Baraoidan and PD Gaspillo. Microwave-assisted extraction of phenolic compounds from moringa oleifera lam. leaves using response surface methodology as optimization tool. *Philippine Agr. Sci.* 2014; **97**, 36-42.
- [30] S Al-Hamimi, AA Mayoral, LP Cunico and C Turner. Carbon dioxide expanded ethanol extraction: Solubility and extraction kinetics of  $\alpha$ -pinene and cis-verbenol. *Anal. Chem.* 2016; **88**, 4336-45.
- [31] B Zhao, J Deng, H Li, Y He, T Lan, D Wu, H Gong, Y Zhang and Z Chen. Optimization of phenolic compound extraction from chinese *Moringa oleifera* leaves and antioxidant activities. *J. Food Qual.* 2019; **2019**, 5346279.
- [32] EN Fombang, P Nobosse, CMF Mbofung and D Singh. Optimizing extraction of antioxidants from roasted *Moringa oleifera* Lam. leaves using response surface methodology. *J. Food Process Preserv.* 2020; **00**, e14482.
- [33] Sukmawati, H Widiastuti and Miftahuljanna. Analisis kadar kuersetin pada ekstrak etanol daun miana (*Plectranthus scutellarioides* (L.) R.Br.) secara HPLC (high performance liquid chromatography) (*in Bahasa Indonesia*). *As-Syifaa Jurnal Farmasi* 2019; **11**, 38-44.
- [34] JP Coppin, Y Xua, H Chen, MH Pan, CT Ho, R Juliani, JE Simon and Q Wu. Determination of flavonoids by LC/MS and anti-inflammatory activity in *Moringa oleifera*. *J. Funct. Foods* 2013; **5**, 1892-9.
- [35] G Heidari, GD Najafpour, M Mohammadi and AA Moghadamnia. Microwave ultrasound assisted extraction: determination of quercetin for antibacterial and antioxidant activities of Iranian propolis. *Int. J. Eng.* 2019; **32**, 1057-64.
- [36] E González-Burgos, I Ureña-Vacas, M Sánchez and MP Gómez-Serranillos. Nutritional value of *Moringa oleifera* lam. leaf powder extracts and their neuroprotective effects via antioxidative and mitochondrial regulation. *Nutrients* 2021; **13**, 2203.
- [37] LA Shervington, BS Li, AA Shervington, N Alpan, R Patei, U Muttakin and E Mulla. A comparative HPLC analysis of myricetin, quercetin and kaempferol flavonoids isolated from gambian and indian *Moringa oleifera* leaves. *Int. J. Chem.* 2018; **10**, 28-35.
- [38] AA Kamarudin, N Saad, NH Sayuti, NAA Razak and NM Esa. Enhancement of phenolics and antioxidant activity via heat assisted extraction from *Moringa oleifera* using response surface methodology and its potential bioactive constituents. *Mal. J. Med. Health Sci.* 2020; **16**, 83-90.
- [39] Y Zhou, J Zheng, RY Gan, T Zhou, DP Xu and HB Li. Optimization of ultrasound-assisted extraction of antioxidants from the mung bean coat. *Molecules* 2017; **22**, 638.
- [40] LP Cunico, AM Cobo, S Al-Hamimi and C Turner. Solubility and thermal degradation of quercetin in CO<sub>2</sub>-expanded liquids. *Molecules.* 2020; **25**, 5582-92.
- [41] M Hanula, J Wyrwicz, M Moczowska, OK Horbańczuk, E Pogorzelska-Nowicka and A Wierzbicka. Optimization of microwave and ultrasound extraction methods of açai berries in terms of highest content of phenolic compounds and antioxidant activity. *Appl. Sci.* 2020; **10**, 8325.
- [42] NA Nugrahani, Z Auliyanti, and RP Rahayu. Perbandingan uji aktivitas antioksidan antara ekstrak buah kiwi dan apel secara *in vitro*. *Scientia Jurnal Farmasi dan Kesehatan* 2020; **10**, 90-6.