ORIGINAL RESEARCH ARTICLE

Current Research on Biosciences and Biotechnology **7** (1) 2025 10-20

Current Research on Biosciences and Biotechnology

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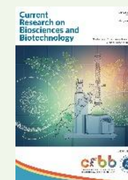

Liquid chromatography-mass spectrometric analysis of some bioactive compounds in commercial herbal products derived from *Moringa oleifera* L.

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ABSTRACT

Moringa oleifera is widely recognized as a “miracle tree” due to its nutritional, medicinal, and environmental benefits. Its diverse phytochemical content underlies significant pharmacological effects. However, conventional HPLC methods for analyzing multi-component compounds in *M. oleifera* are often time-consuming and lack sensitivity, particularly for compounds present at low concentrations. To address this, we developed and validated an analytical method using UPLC-ESI-MRM/MS combined with ultrasound-assisted extraction (UAE). Method validation covered linearity, sensitivity (LOD, LOQ), accuracy, and precision, while UAE was optimized using Response Surface Methodology (RSM) with Box-Behnken Design. Seven phytochemical compounds—rutin, hyperoside, nicotiflorin, astragalin, niazirin, quercetin, and kaempferol—were quantified in various *M. oleifera* products including dried powder, herbal tea, extracts, and capsules. The validated method showed linearity between 1.56–500 µg/mL, LOD values of 0.036–0.094 µg/mL, LOQ values of 0.0108–0.2850 µg/mL, with accuracy and precision within ±15%. Optimal extraction was achieved at a solvent ratio of 1:30, temperature of 40 °C, and extraction time of 17.85 min. UPLC-ESI-MRM/MS enabled rapid separation and detection of all seven compounds within 15 minutes, significantly improving resolution compared to UV-based HPLC. In addition, three abundant flavonoids were confirmed as the main flavonoids present in *Moringa*, mainly rutin, hyperoside and nicotiflorin. On the other hand, niazirin can be considered as the specific marker for *Moringa* leaves. These results demonstrate that UPLC-ESI-MRM/MS, coupled with optimized UAE, provides a sensitive, rapid, and reliable approach for profiling *M. oleifera* phytochemicals, supporting its quality assessment and potential applications in nutraceutical and pharmaceutical industries.

Article history:

Received 14 Jun 2025
Revised 18 Aug 2025
Accepted 22 Aug 2025
Available online 31 Aug 2025

Keywords:

Flavonoids
LC-MS
UAE
response surface methodology
niazirin

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DOI: 10.5614/crbb.2025.7.1/XR8PKR5P

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1. Introduction

Moringa oleifera L. is a plant originating from the plains along the sub-Himalayas, namely India, Pakistan, Bangladesh, and Afghanistan. This plant has spread widely to the east (lower part of China, Southeast Asia, and the Philippines) as well as to the west (Egypt, Africa, around the Mediterranean, and finally to the West Indies in America) (Meireles et al., 2020). *M. oleifera* comes from the Moringaceae family which is an annual tree that has been widely cultivated in the tropics and is easy to grow even in adverse conditions. Moringa is also known as the magic tree (Miracle tree) which has been indicated in traditional medicine for centuries and is estimated to have been used around 2000 BC (Meireles et al., 2020).

M. oleifera is the most promising tree and has been used for nutritional fulfilment, medicinal properties, environmental conservation, and several other purposes. Every part of this plant is edible and the leaves, roots, seeds, root bark, and bark have medicinal properties. Several studies explain that *M. oleifera* contain various kinds of flavonoid phytochemical compounds such as rutin, nicotiflorin, hyperoside, astragalin, quercetin, and kaempferol (Atawodi et al., 2010; Coppin et al., 2013; Mabrouki et al., 2020; Nizioł-Łukaszewska et al., 2020; Makita et al., 2016), as well as various other phytochemical compounds such as vitamins, phenolic acids, isothiocyanates, tannins, saponins (Vergara-Jimenez et al., 2017) to be rich in minerals such as calcium, potassium, iron, magnesium, zinc. and copper (Kasolo et al., 2012).

The development of extraction methods is essential for the optimal assessment of plant extracts. Commonly used separation methods from various types of plants are used such as maceration or soxhlet extraction (Tungmunnithum et al., 2020). In addition, many more selective methods have been developed to enrich plant extracts with certain phytochemical compounds such as ion-exchange chromatography (Banik et al., 2018), high-speed counter current chromatography (Deng et al., 2009), extraction with ultrasonic waves Ultrasonic-assisted extraction (UAE) (Wong et al., 2015), and microwave-assisted extraction (MAE) (Rodríguez-Pérez et al., 2016). Extraction with ultrasonic waves (UAE) is a technique based on the application of ultrasonic waves into a sample matrix immersed in a liquid medium to cause the cell wall to break and release the desired compound (Dey and Rathod, 2013). This extraction method is very useful and provides high extraction

yields, where the use of temperature can be controlled, the need for solvent in small quantities, and can combine other extraction methods such as maceration (Carreira-Casais et al., 2021).

The increasing knowledge about the effects of plant secondary metabolites on health causes the need for the importance of developing new techniques to separate the components of these phytochemical compounds, not only extraction methods but also methods of analysis of phytochemical components need to be developed. Several studies of qualitative and quantitative determination of the main phytochemical compounds in *M. oleifera* have been widely reported, such as the use of high-performance liquid chromatography with DAD detection (HPLC-UV/DAD) and high-performance liquid chromatography-electrospray ionization mass spectrophotometry (HPLC-ESI-MS) have been used in these studies (Makita et al., 2016; Shervington et al., 2018; Vongsak et al., 2013). However, multi-component analysis of the high-performance liquid chromatography (HPLC) method takes a long time and is not sensitive enough to analyze the components of phytochemical compounds in a mixture, especially those found at low concentrations (Liu et al., 2012; Moreno-González et al., 2020). Ultra-performance liquid chromatography (UPLC) combined with mass spectrophotometry in multiple reaction monitoring (MRM) modes have been reported to be a powerful approach for rapid analysis of a mixture in Traditional Chinese Medicine (TCM) (Liu et al., 2012; Li et al., 2021).

We therefore set out to develop an analytical method for active compounds in Moringa (*M. oleifera*) using UPLC-ESI-MRM/MS and optimization of the extraction method using UAE (Ultrasound-assisted extraction).

2. Materials and methods

2.1. Chemicals

Rutin, hyperoside, astragalin, nicotiflorin, niazirin, quercetin and kaempferol were purchased from MarkHerb® (Bandung, Indonesia). *Moringa oleifera* extract is obtained from company (A), herbal tea products, capsule and simplicia are obtained in the marketplace Indonesia.

2.2. Preparation of reference standards solution

The stock standard solution (1 mg/mL) of Rutin, hyperoside, astragalin, nicotiflorin, niazirin, quercetin and kaempferol were freshly prepared in methanol. The resulting solution was sonicated for 2 min and then filtered through 0,22 µm PTFE assembly.

2.3. Liquid chromatography-mass spectrometry analysis

2.3.1. Instrumentation

Analysis was performed on an Acquity UPLC H-Class system equipped with binary solvent manager, sample manager, and column heater (Waters, Milford, Michigan, USA) coupled triple quadrapole. The analyst software Masslynx operated the instrument and executed the data analyses.

2.3.2. Liquid chromatography conditions

The mobile phase consisted of solvent A: Water+0,1% formic acid and solvent B: ACN+0,1% formic acid. The elution was performed in gradient mode during a time course of 15 min as follows: 0-0.5 min, 10-20% B; 0.5-3.0 min, 20-28% B; 3.0-4.0 min, 28-30,3% B; 4.0-5.0 min, 30.3-33.3% B; 5.0-5.5 min, 33.3-50% B; 5.5-6.5 min, 50-70% B; 6.5-7.0 min, 70-80% B; 7.5-8.5, 80-100% B; 8.5-15.0 min, held to 100% B at flow rate 0,3 mL/min. Finally, the composition was returned to the initial (10% B) in 0,01 min. The column was equilibrated for 5 min before next injection. The column was operated at 40 °C and the sample/standards injection volume was 3 µL.

2.3.3. Mass spectrometry conditions

The detection was performed by multiple reaction monitoring (MRM). The ESI was operated in negative mode (ESI -) with the parameters in the source as follows: spray voltage, source gas flow (desolvation 900 L/hr), source temperature (desolvation temp 450 °C). The collision energies, capillary voltages, and cone voltages were optimized for each standard to give the best possible resolution and sensitivity. The conditions were optimized using direct infusion of 10 µg/mL standard solution of each compound.

2.4. Method validation

Validation of the proposed method was performed which includes linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ).

2.4.1. Linearity

A number of concentration series of rutin, nicotiflorin, hyperoside, astragalin, niazirin, quercetin, kaempferol as comparison were made, then analyzed using UPLC. Each concentration was analyzed three times (triplication). Linear least squares regression was used to calculate the slopes, intercepts and correlation coefficients. The acceptance requirement is the value of the correlation coefficients is 0.9950.

2.4.2. Precision and accuracy

The precision, defined as relative standard deviation (%RSD) was evaluated by analyzing the calibration standard prepared at low, medium and high concentration level of standard solution. Three independent calibration standards were prepared in different days to establish the intra-day and inter-day precision, respectively. Accuracy of the analytical method was calculated as the percentage of relative error the measured concentration with three levels at low, medium and high concentration for each standard. A limit of ±15% was considered acceptable for both precision and accuracy (European Medicine Agency, 2011).

2.4.3. Limit of detection (LOD) and limit of quantification (LOQ)

The LODs and LOQs were defined as the lowest concentrations of standard solutions or as the lowest spiked concentrations in fortified samples that produce a signal to-noise ratio of 3 and 10, respectively. The calculation of detection limit and quantification limit uses the following formula:

$$\text{LOD} = 3{,}3\ x\ \frac{SD}{a} \quad \text{Eq. (1)}$$

$$\text{LOQ} = 10\ x\ \frac{SD}{a} \quad \text{Eq (2)}$$

with:
SD = Standard Deviation
a = The slope of the calibration curve

2.5. Optimization of ultrasound-assisted extraction using response surface methodology

2.5.1. Optimization of ultrasound-assited extraction (UAE) procedure

UAE was performed in an ultrasonic device (ELMA ultrasonic cleaning type T760/DH) with 40 kHz frequency. The sample *M. oleifera* extract was powdered. The samples was extracted with MeOH 70% under different extraction times, extraction temperatures, and solid/liquid ratios. The obtained extraction solutions were filtered and then centrifuge using 4000 rpm for 5 min. The supernatant obtained was filtered using a 0.2 µm PTFE

filter and the concentration of the compound was determined using a validated analytical method.

2.5.2. Experimental design

The effects of three independent variables of temperature, solid/liquid ratios, and time to optimize the extracted number of compounds were investigated by using a Box-Behnken Design (BBD) for RSM. Based on the extraction time (10-30 min), extraction temperature (40-60 °C), and liquid to solid ratio (1:10-1:30 g/mL) were selected as the three factors of RSM design. A Box-Behnken design of three factors at three levels was established to perform RSM using Design Expert software (version 13.0, Stat-Ease Inc., Minneapolis, MN, USA). Three-factors, 3-level BBD was used. A total of 17 experiment runs with five centre points were generated by design expert software. Independent and dependent variables used in the Box-Behnken design for the optimization of *M. oleifera* show in Table 1.

2.6. Analysis of phytochemical properties of several herbal products made from *M. oleifera*

The samples *Moringa oleifera* extract is obtained from company (A), herbal tea products, capsule and simplicia are obtained in the marketplace Indonesia. Each sample was weighed as much as 1 gram and then extraction using UAE with the optimum parameters that have been obtained in the previous stage. The results of the extraction process and then centrifuge using 4000 rpm for 5 min. The supernatant obtained was filtered using a 0.2 µm PTFE filter and the concentration of the compound was determined using a validated analytical method.

3. Results and discussion

3.1. Optimization of MRM parameters

In the MRM mode, two parameters – cone voltage and collision energy – were optimized based on the target compounds' precursor and product ions, in order to maximize the signal of the selected fragment ion and the selectivity of its detection. The responses for all of the target compounds were best in negative ESI mode and then nitrogen is used for solution desolvation and argon for collision gas. The results of optimization of mass detection were carried out on ESI Negative Mode (M-H) ionization with a capillary voltage of 2.5 kV, source temperature 150 °C, desolvation temperature 450 °C, nitrogen gas flow rate 900 L/hr, cone 1 L/hr. The m/z ratios of the 7 standard compounds were obtained (Table 2).

Table 1. Independent and dependent variable used in the Box-Behnken design for the optimization of *M. oleifera* extraction

Factor Independent variables	Level used, actual code		
	Low (-1)	Medium (0)	High (+1)
X_1 = material-to-solvent ration (g/mL)	1/10	1/20	1/30
X_2 = ultrasonic tempheratur (°C)	40	50	60
X_3 = ultrasonic time (min)	10	20	30
Dependet variable			
Y_1 = content of rutin			
Y_2 = content of hyperoside			
Y_3 = content of nicotiflorin			
Y_4 = content of astragalin			
Y_5 = content of niazirin			
Y_6 = content of quercetin			
Y_7 = content of kaempferol			

Table 2. MRM Parameters for each standard

Compounds	Parent Ion (m/z)	Transition MRM		Cone Voltage (V)	Collision Energy (V)	
		Quantifier (m/z)	Qualifier (m/z)		Quantifier (V)	Qualifier (V)
Niazirin	324.08	44.736	132.05	20	11	11
Rutin	609.09	299.97	270.91	65	39	60
Hyperoside	462.97	299.90	270.91	55	25	45
Nicotiflorin	593.03	284.91	254.91	70	30	55
Astragalin	446.97	283.91	254.84	55	30	40
Quercetin	301.08	150.84	178.83	45	20	20
Kaempferol	284.78	92.83	116.86	65	40	45

3.2. Optimal condition of chromatography systems

Optimization of the chromatographic system was also carried out. Parameters optimized include: Mobile phase composition (gradient system), mobile phase pH, flow rate, and column temperature. The column selected was the Acquity UPLC® BEH C_{18} 1.7 m (2.1 x 100 mm) column.

Octadecyl silica (ODS or C_{18}) is the most widely used stationary phase because it is able to separate compounds with low, medium and high polarity (Gandjar and Rohman, 2007). The C_{18} column is used in the development of liquid chromatographic system methods due to its high retention and suitability for various analytical applications (Waters, 2008).

The development of a chromatographic system method covers all parameters and requires a series of analyzes to determine the best and effective separation conditions. In liquid chromatography, the elution system is one of parameter that needs to be considered. Under the optimum conditions obtained from the results of this study, the elution system applied is gradient elution (the composition of the mobile phase varies during the elution process). Gradient elution is used to increase the resolution of a complex mixture especially if the test sample has a wide polarity range (Gandjar and Rohman, 2007; Schellinger et al., 2008).

The chromatography system on the UPLC (Ultra Performance Liquid Chromatography) instrument is a modern liquid chromatography system, which has been modified from HPLC. UPLC works at high pressure in the range of 6,000 – 15,000 psi. The smaller particle size (less than 2 m diameter) in UPLC will provide better resolution, speed, and sensitivity compared to HPLC. Smaller particles can increase the pressure to 1000 bar or more which can increase the separation retention factor. Smaller injection volumes are also required in UPLC systems, which results in better efficiency and resolution. In addition, higher column temperatures can also reduce the mobile phase viscosity, diffusion coefficient and high flow rate without significantly reducing efficiency and increasing

back pressure on the column (Batool and Menaa, 2020; McShane et al., 2014; Rahman, 2018).

In view of the above, the mobile phase consisted of solvent A: Water+0,1% formic acid and solvent B: ACN+0,1% formic acid. The elution was performed in gradient mode during a time course of 15 min as follows: 0-0.5 min, 10-20% B; 0.5-3.0 min, 20-28% B; 3.0-4.0 min, 28-30,3% B; 4.0-5.0 min, 30.3-33.3% B; 5.0-5.5 min, 33.3-50% B; 5.5-6.5 min, 50-70% B; 6.5-7.0 min, 70-80% B; 7.5-8.5, 80-100% B; 8.5-15.0 min, held to 100% B at flow rate 0,3 mL/min. Finally, the composition was returned to the initial (10% B) in 0,01 min. The column was equilibrated for 5 min before next injection. This condition was finally chosen as it gave better ion intensities with ESI - and better peak resolution. Variations of gradient elution programs were tested in order to achieve the most efficient separation of the targeted compounds. The studies conducted for the selection of optimal separation conditions are represented in Fig. 1.

3.3. Method validation

Validation of analytical methods is the process used to ensure that the analytical procedures used for certain tests are in accordance with the intended use. The results of the validation of the analytical method can be used to assess the quality, reliability and consistency of the analysis results. There are several parameters that must be met in the validation of the analytical method, namely accuracy, precision, specificity, detection limit, quantification limit and linearity (Shrivastava et al., 2019).

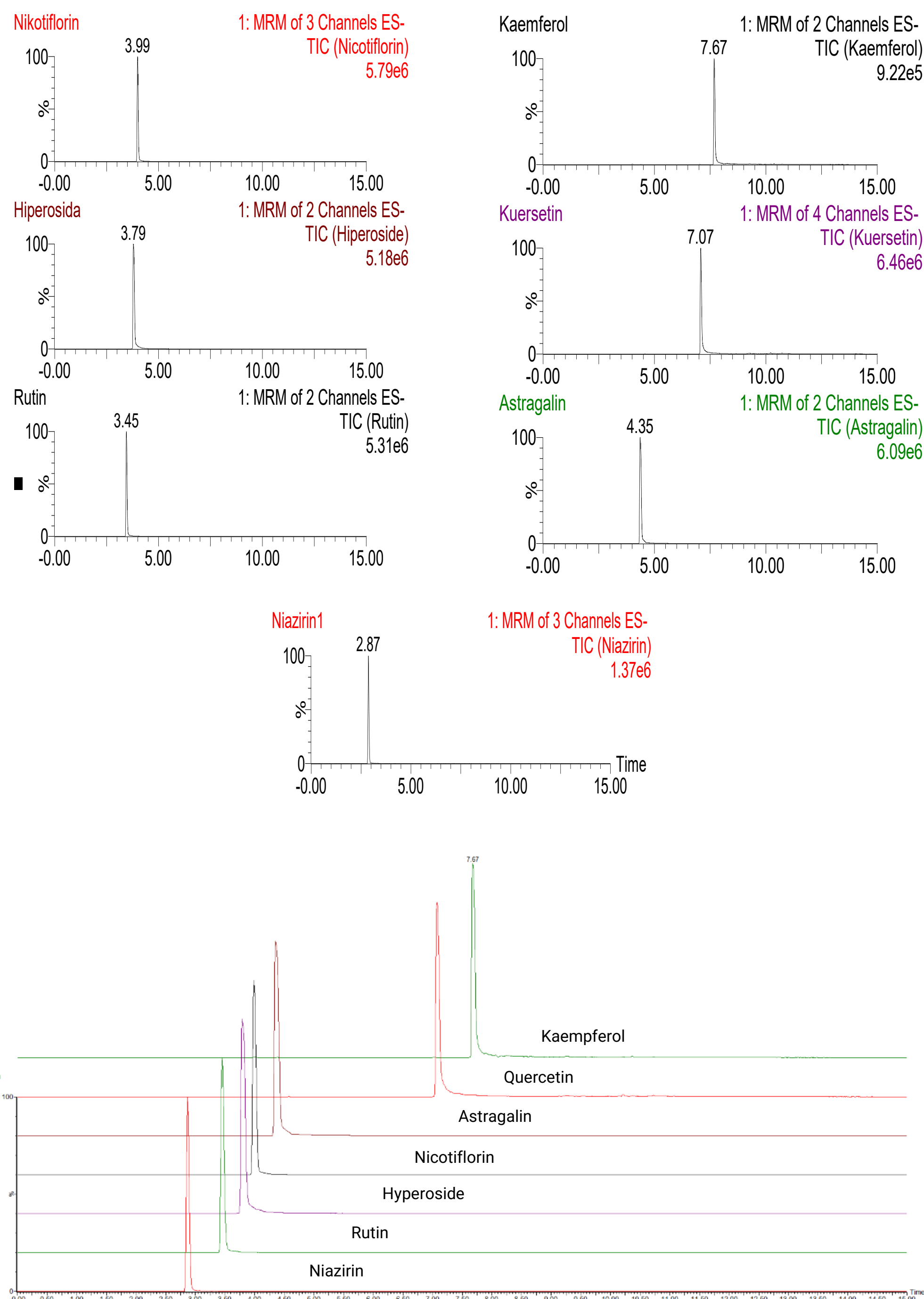

Fig. 1. MRM Chromatogram overlay 7 standard compounds (niazirin, rutin, hyperoside, nicoriflorin, astragalin, quercetin and kaempferol)

3.3.1. Linearity

Linearity is the ability of analytical methods that provide a direct response or with the help of a good mathematical transformation, proportional to the concentration of the analyte in the sample. Linearity validation was carried out on seven standard compounds (rutin, hyperoside, astragalin, nicotiflorin, quercetin, kaempferol and niazirin) with six concentration series for each standard. The results of linearity validation, the coefficient of determination or R^2 for each standard compound can be seen in Table 3. The value of the coefficient of determination or R^2 for seven standard compounds is >0.998. The requirement for acceptance of the coefficient of determination is 0.998 (Kazakevich and LoBrutto, 2007). These results indicate that the linearity test of seven standard compounds met the applicable requirements.

3.3.2. Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can still be detected and Limit of Quantification (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under specified experimental conditions. From the results of the study, that the LOD and LOQ values in each standard compound obtained high sensitivity. The results of the LOD and LOQ validation for each standard compound can be seen in Table 3.

Table 3. Validation parameters of the analytical method: linearity, and sensitivity

Compounds	Linearity			Sensitivity	
	Equations	(R^2)	Linear Range (mg/L)	LOD (mg/L)	LOQ (mg/L)
Niazirin	y= 143.4x + 1732.2	0.9998	15.625- 500.0	0.0650	0.1960
Rutin	y = 1006.2x + 3471.3	0.9993	3.90 – 125.0	0.0036	0.0108
Hyperoside	y= 1933.6x + 1301.9	0.9987	1.56 – 50.0	0.0520	0.1580
Nicotiflorin	y= 779.29x + 454.78	0.9990	3.90 – 125.0	0.0700	0.2100
Astragalin	y= 1049.3x + 3764.8	0.9986	6.25 – 200.0	0.0100	0.0290
Quercetin	y= 1092.2x + 2552.9	0.9994	1.56 – 50.0	0.0190	0.0590
Kaempferol	y= 261.57x + 524.62	0.9994	1.56 – 50.0	0.0940	0.2850

Table 4. Validation parameters of the analytical method: precision (%RSD), accuracy (%RE)

Compounds	Concentration (mg/L)	Intra-day		Inter-day	
		Precision RSD (%)	Accuracy RE (%)	Precision RSD (%)	Accuracy RE (%)
Niazirin	450	1.11	-3.99	1.17	2.97
	100	1.57	-0.01	0.95	3.76
	20	1.48	-4.13	1.67	4.41
Rutin	100	0.98	-5.12	1.96	0.89
	60	1.59	-4.88	0.23	1.56
	10	1.09	-0.61	1.96	0.22
Hyperoside	40	0.69	2.45	0.29	2.52
	20	0.81	0.07	0.98	2.31
	2.5	1.13	0.88	0.81	2.84
Nicotiflorin	100	0.26	2.80	0.78	1.93
	60	0.08	5.85	0.21	5.52
	20	0.61	3.38	0.84	2.56
Astragalin	140	0.95	-0.61	1.27	3.85
	70	0.54	4.96	0.32	4.67
	10	0.99	5.00	1.15	-0.06
Quercetin	4	1.12	2.70	1.71	-0.93
	25	1.13	5.61	1.88	-2.79
	40	1.56	3.86	1.53	-0.16
Kaempferol	3	1.92	-1.00	1.09	2.54
	25	0.23	2.13	0.28	2.26
	40	1.39	7.10	1.97	7.06

3.3.3. Precision and accuracy

Accuracy is an analytical method that describes the closeness of the value obtained by the method to the actual concentration value. While the precision describes the closeness of the repeatability of the test sample measurement. Accuracy and precision were carried out by testing 3 variations of concentration of low, medium and high concentrations. Accuracy and precision observations were carried out intra-day and inter-day with six replications for each concentration. Accuracy is indicated by the percentage value of relative error (%RE) while precision is indicated by the percentage value of relative standard deviation (%RSD). Accuracy and precision requirements are not more than ±15% (European Medicine Agency, 2011). The results of the validation of intra-day and inter-day accuracy (%RE) and precision (%RSD) can be seen in Table 4.

These results provide information that the validation of the analytical method for accuracy and precision tests provides results that are in accordance with applicable requirements, so that the developed method can be applied to assay or other applications.

3.4. Optimization of UAE with RSM

In order to study the combined effect of independent variables (material-to-solvent ratio, ultrasonic temperature, and ultrasonic time) on the extraction, experiments were performed for different combinations of parameters using statistically designed experiments, and results are shown in Table 5, which includes the

result of multi responses and the experimental values. Three factors at three-level Box-Behnken design were used in this study to investigate the influence of process variables such as material-to-solvent ratio, ultrasonic temperature, and ultrasonic time. From the developed model, the three-dimensional response surface was constructed to illustrate the main and interactive effects of independent variables on a response variable. The result of three-dimensional multi responses, show in Fig. 2-8.

Table 5. Observed response in the BBD for optimization of *M. oleifera* extraction with experiment value (multi responses)

Run	X_1	X_2	X_3	Rutin (mg/g)	Hyperoside (mg/g)	Nicotiflorin (mg/g)	Astragalin (mg/g)	Niazirin (mg/g)	Quercetin (mg/g)	Kaempferol (mg/g)
1	10	60	20	0.821	0.4	0.35	0.29	0.38	0.07	0.0089
2	20	50	20	1.86	1.06	0.77	0.76	0.34	0.146	0.0005
3	10	50	10	1.13	0.61	0.48	0.45	0.39	0.085	0.0081
4	20	40	10	2.27	1.37	1.03	0.99	0.5	0.207	0.0115
5	30	50	10	2.33	1.36	0.91	0.92	0.28	0.167	0.0002
6	20	50	20	1.93	1.09	0.82	0.78	0.34	0.143	0.0007
7	10	40	20	0.79	0.71	0.44	0.6	0.55	0.161	0.0203
8	20	60	10	1.01	0.54	0.41	0.38	0.16	0.074	0.0034
9	30	60	20	1.51	0.8	0.63	0.59	0.45	0.118	0.0127
10	20	50	20	1.91	1.12	0.8	0.79	0.33	0.139	0.0002
11	30	40	20	2.98	1.95	1.33	1.32	0.45	0.268	0.0132
12	20	40	30	2.13	1.45	1	1.08	0.56	0.238	0.0114
13	30	50	30	2.19	1.24	0.87	0.85	0.31	0.167	0.0033
14	20	60	30	1.25	0.64	0.52	0.46	0.35	0.104	0.0048
15	20	50	20	1.94	1.14	0.83	0.77	0.33	0.141	0.0003
16	10	50	30	1.16	0.65	0.52	0.49	0.44	0.089	0.0192
17	20	50	20	1.95	1.09	0.79	0.78	0.33	0.144	0.0002

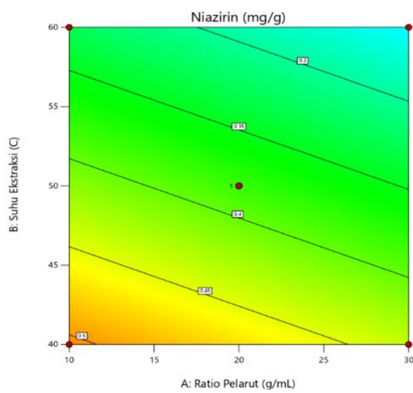

Fig. 2. Response surface model plot showing the effects of independent variables on niazirin content with effect the solvent ratio and extraction time.

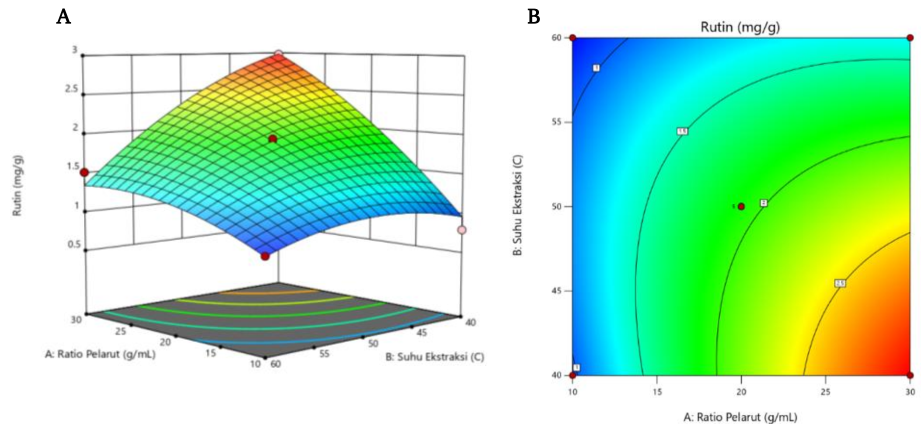

Fig. 3. Response surface model plot showing the effects of independent variables on rutin content. Panel (a) model 3D surface plot. Panel (b) Contour Plot

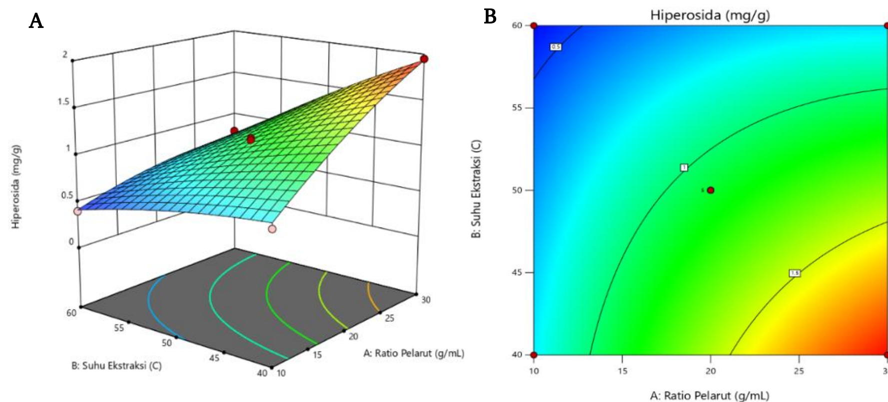

Fig. 4. Response surface model plot showing the effects of independent variables on hyperoside content. Panel (a) model 3D surface plot. Panel (b) Contour Plot.

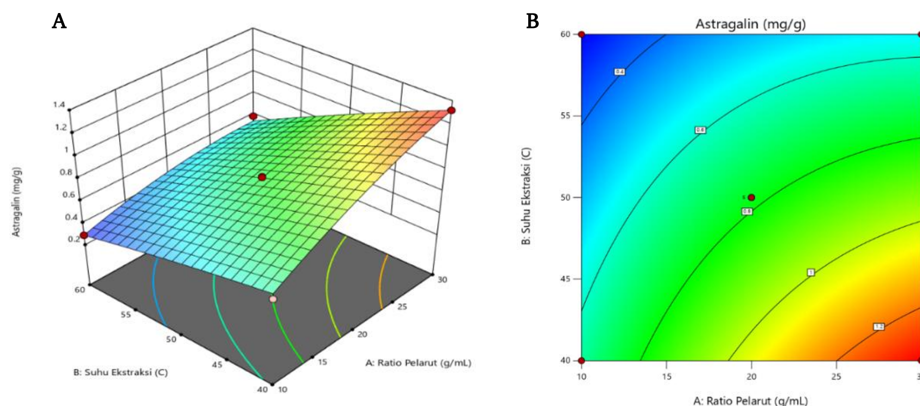

Fig. 5. Response surface model plot showing the effects of independent variables on astragalin content. Panel (a) model 3D surface plot. Panel (b) Contour Plot.

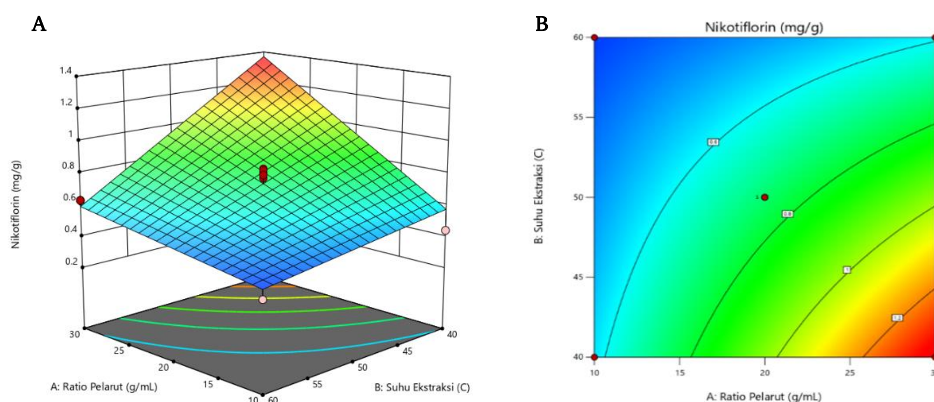

Fig. 6. Response surface model plot showing the effects of independent variables on nicotiflorin content. Panel (a) model 3D surface plot. Panel (b) Contour Plot.

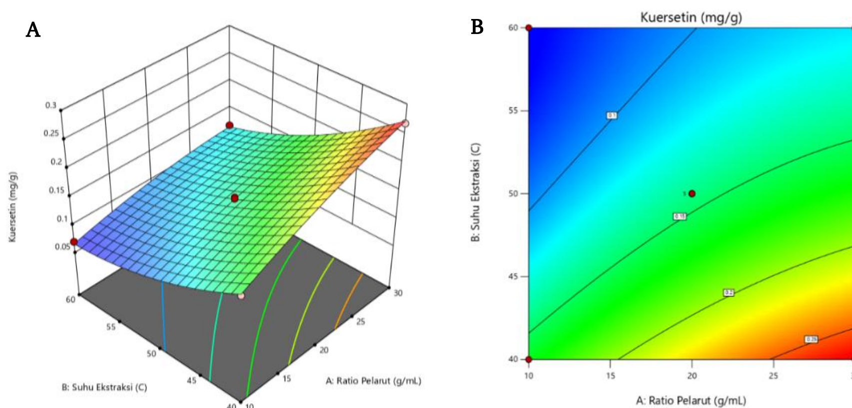

Fig. 7. Response surface model plot showing the effects of independent variables on quercetin content. Panel (a) model 3D surface plot. Panel (b) Contour Plot.

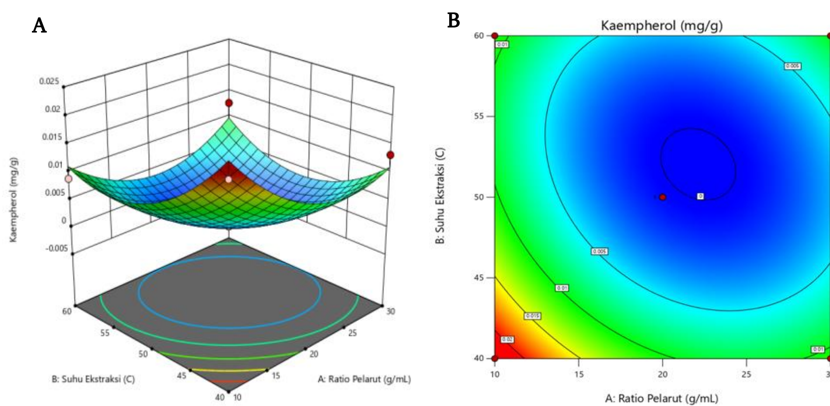

Fig. 8. Response surface model plot showing the effects of independent variables on kaempferol content. Panel (a) model 3D surface plot. Panel (b) Contour Plot

Fig. 2-8 are RSM model plots using surface plots. The color change on the graph shows that there are differences in the content of each compound with the combination of factors used (solvent ratio, temperature and extraction time). Colors that are closer to reddish indicate that the levels of these compounds are getting bigger, while colors that are closer to blue indicate that the levels of these compounds are getting smaller. In general, the analysis shows that each parameter affects each other's response to the amount of content of each compound.

Optimization using the RSM model to determine the optimum conditions is carried out using the desirability function. Derringer's desired function methodology was employed to optimize extraction process condition on the maximum extractive capacity of all compound (rutin, hyperoside, nicotiflorin, astragalin, niazirin, quercetin dan kaempferol) from *M. oleifera* show in Fig. 9 (material-to-solvent ration 1:30, extraction temperature of 40 °C, and extraction time (17.888 min). The desirability function provides information that is a transformation of the response variable to a scale of zero to one. The closer the value to one, the higher the value of the resulting optimization accuracy (Zhang et al., 2017; Nurmiah et al., 2013). All results are closely related to the data obtained from optimization analysis, which indicate that the Box-Behnken design could be effectively used to optimize the extraction parameters.

Ultrasound-assisted extraction (UAE) is mostly used for the extraction of natural ingredients. UAE can increase the extraction mass transfer rate caused by cavitation generated in the material (Prasetyaningrum et al., 2022). According to Rodríguez-Pérez et al. explaining that non-conventional extraction methods or extraction using environmentally friendly solvents are highly recommended for the extraction of phenolic compounds from Moringa leaf extract (Rodríguez-Pérez et al., 2015).

Extraction optimization is strongly influenced by three important factors, namely the solvent ratio. extraction temperature and time. According to Yuan et al. in their research to optimize the extraction solvent ratio in taxili plants, the results showed that the solvent ratio of 1:30 gave a higher yield percentage than other solvent ratio optimizations (Yuan et al., 2021). Another study conducted by Chahyadi and Elfahmi found that extraction with a solvent ratio of 1:30 gave the highest percentage yield of rutin compound, which was 20.38±0.66 g/kg (Chahyadi and Elfahmi, 2020).

The use of temperature on the optimization of the extraction method using the UAE also needs to be considered. The use of higher temperatures can result in a tremendous increase in the extraction efficiency of the phytochemical compound process. This is due to the fact that an increase in temperature encourages to disrupt the bonds in plant cell walls and break the matrix bonds leading to increased extraction of phytochemical compounds (Silva et al., 2020). However, increasing the extraction temperature will reduce antioxidant activity, especially for phenolic and flavonoid compounds (Elshreef et al., 2021). Based on research Prasetyaningrum et al. explained that the flavonoid content in Moringa (*M. oleifera*) plants will increase when the temperature ranges from 30 to 50 °C, this is because heat can damage the extracted plant cell tissue so that the active components are released and will also increase, but if there is an excessive increase in temperature will result in structural changes that cause degradation of the compound (Prasetyaningrum et al., 2022). This is supported by the optimization of Moringa plant extraction using UAE reported Wu et al. explaining that the optimum temperature of 40 °C produces a high percentage of yield for phenolic compounds and flavonoids (Wu et al., 2020).

In addition to the solvent ratio and extraction temperature, the extraction time is also an important factor in the extraction process of the target compound in a plant. The use of conventional tools in the extraction of a target compound takes a long time, while the use of modern extraction tools such as the UAE does not require a long time (Kumar et al., 2021). Based on research reported by Karunanithi and Venkatachalam that the optimum extraction of flavonoid compounds using UAE takes 17 minutes (Karunanithi and Venkatachalam, 2019). This is also supported by research Rahmatullah et al. that the optimum flavonoid compounds in Moringa were extracted using UAE for 20 minutes (Rahmatullah et al., 2021). Several other studies also explained that the flavonoid compounds quercetin and kaempferol through optimum extraction studies using UAE for extraction time of 15 minutes and 20 minutes, respectively (Jang et al., 2013; Wang et al., 2013).

The suggested RSM optimization method in Fig. 9 through a desirability function close to 1 will be verified. The verification in question is processing and analysis in accordance with the proposed extraction method design through the results of RSM optimization using Moringa extract which will be compared with the predicted response value of RSM optimization with experimental values. The results of the verification of the optimization results of RSM, can be seen in Table 6. Based on the information obtained from the Table 6, it is explained that the value of the good grade results in the prediction results through RSM optimization and the results through experiments are not too much different. The predicted levels obtained for all standard compounds were not much different from the results of the tests carried out under the optimum conditions used. So, based on these steps, it will be continued to determine the levels of several existing products on the market that contain Moringa plants, either in the form of dried simplicia, or other product forms.

Table 6. The results of the verification of the extraction optimization using the model RSM

Optimum Conditions	Compounds	Result of predicted and experiment (mg/g)	
		Predicted	Experiment
X_1 = material-to-solvent ration (g/mL) X_2 = ultrasonic tempheratur (°C) X_3 = ultrasonic time (min)	Rutin	3.014 ± 0.133	3.033 ± 0.021
	Hyperoside	1.940 ± 0.063	1.949 ± 0.019
	Nicotiflorin	1.377 ± 0.085	1.336 ± 0.015
	Astragalin	1.319 ± 0.045	1.316 ± 0.006
	Niazirin	0.429 ± 0.077	0.459 ± 0.008
	Quercetin	0.267 ± 0.008	0.225 ± 0.007
	Kaempferol	0.011 ± 0.003	0.014 ± 0.000

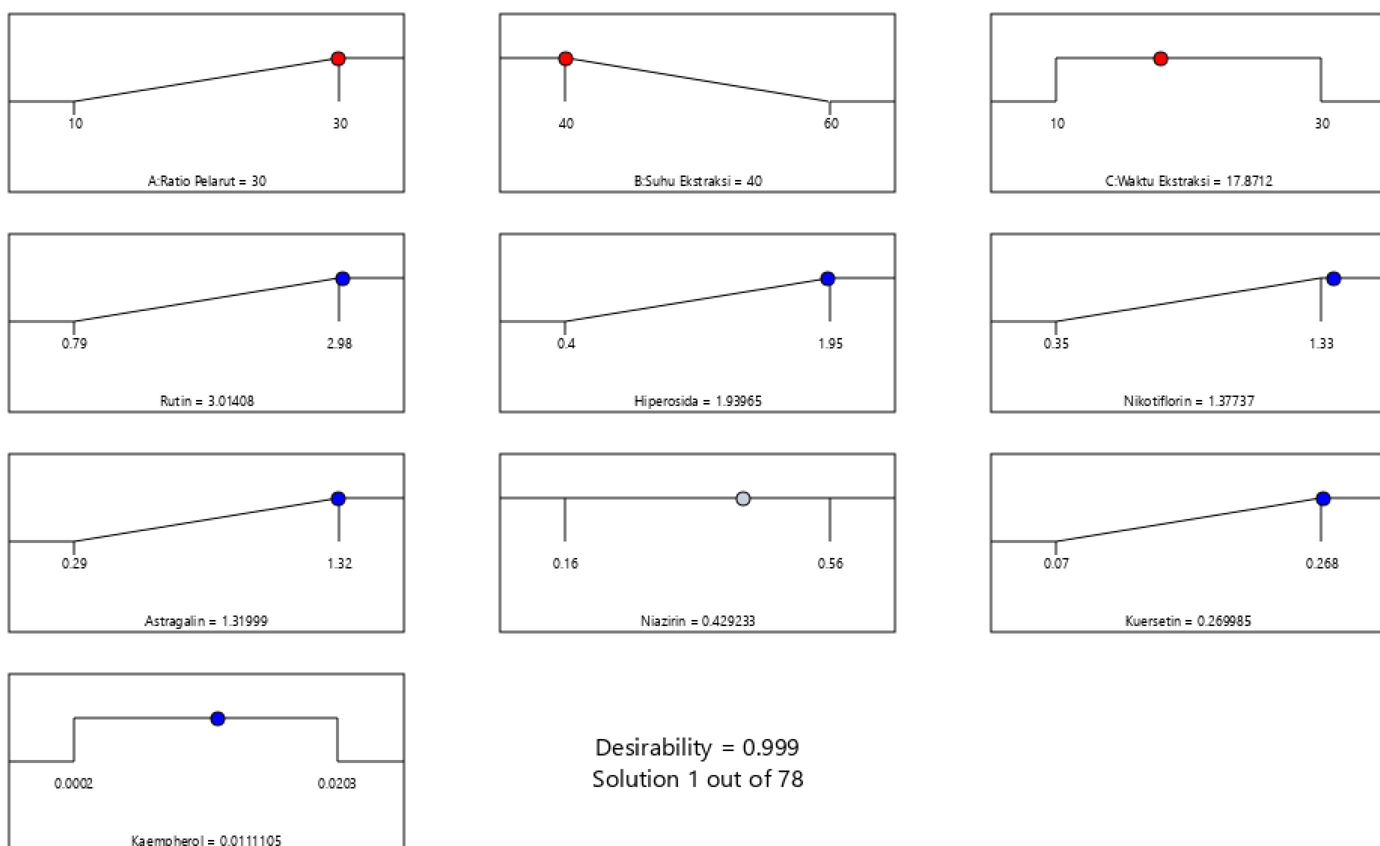

Fig. 9. Plot.Output Desirability for all response

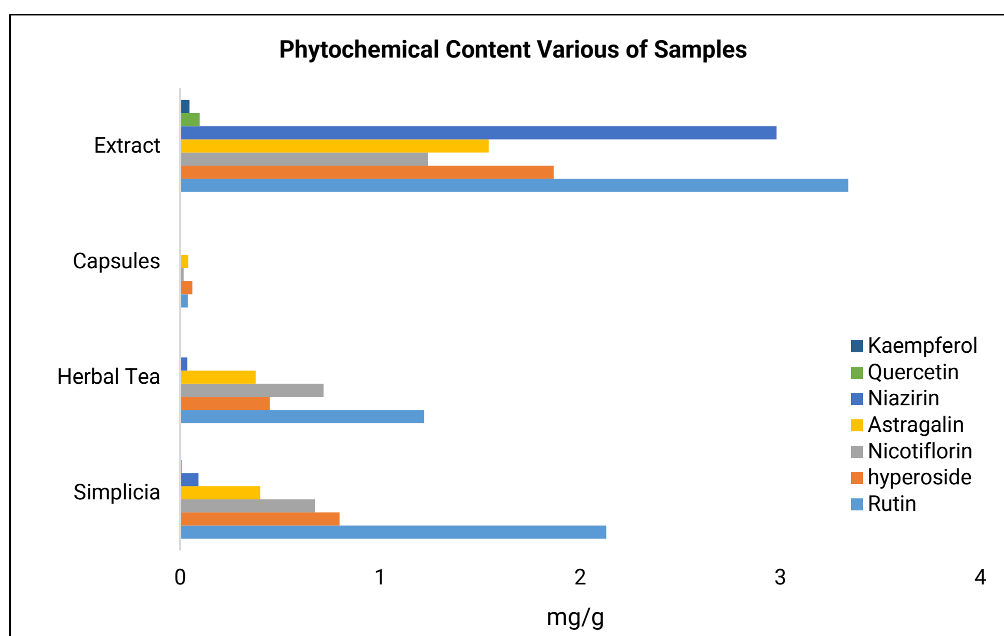

Fig. 10. Graph of active compound content of *Moringa* in several commercial products

3.5. Phytochemical properties of several herbal products made from *M. oleifera*

Moringa (*Moringa oleifera* Lam.) is the most widely cultivated species of the Moringaceae family. Moringa is a plant that can tolerate various environmental conditions, so it can easily grow even in extreme conditions such as high temperatures or in areas with light snow (Krisnadi, 2015). The genus Moringa contains many flavonoid compounds and their glycoside forms. Flavonoids that are often found are rutin, quercetin, rhamnetin, kaempferol, apigenin and myricetin as well as several other types of flavonoids (Abd Rani et al., 2018).

Currently, the lifestyle of returning to nature (back to nature) is a trend that brings people back to using natural materials, including treatment with medicinal plants. One of them is by utilizing the Moringa plant as an additional supplement. In this study, the determination of the levels of compounds presents in Moringa (rutin, hyperoside, nicotiflorin, astragalin, niazirin, quercetin and kaempferol) against existing products containing

Moringa plants in the form of dried simplicia, herbal tea products, extracts and capsules can be seen in Fig. 10.

The graph in Fig. 10 shows the active compound content of the Moringa plant in several existing products. There are 4 products used, namely products in the form of dry simplicia, herbal teas, capsules and extract. Determination of assay using optimum conditions that have been optimized previously, to obtain various levels of compounds. From the graphic information, it is explained that the content of compounds (rutin, hyperoside, nicotiflorin, astragalin, niazirin, quercetin and kaempferol) from extract is higher than other existing products. This could be due to the condition of the product that has gone through the extraction process. Extraction is a series of processes for separating substances from their mixture using a suitable solvent, so that it is possible to use a suitable solvent to produce higher levels of these compounds. Unlike simplicia products and herbal teas, which are dried moringa plants without going through any process.

The genus Moringa contains various phytochemical compounds such as alkaloids, saponins, tannins, steroids, phenolic acids, glucosinolates, flavonoids and terpenes. The diversity of phytochemical compounds in this genus contributes to its pharmacological activity (Abd Rani et al., 2018). The genus Moringa contains many compounds from the flavonoid group and its glycoside form. Flavonoids are often found such as rutin, quercetin, nicotiflorin, astragalin, hyperoside, rhamnetin, kaempferol, apigenin and myricetin as well as several other types of flavonoids (Abd Rani et al., 2018; Lin et al., 2019).

4. Conclusion

The conclusion of this study is that the seven active compounds (rutin, hyperoside, nicotiflorin, astragalin, niazirin, quercetin, and kaempferol) were measured using a validated method with a linearity range (1.56-500 g/mL), LOD (0.036-0.094 g). /mL), LOQ (0.0108-0.2850), acceptable precision and accuracy (<±15%). High resolution is obtained in a short time (within 15 minutes) which makes UPLC-ESI-MRM/MS a fast and sensitive method for the analysis of moringa extract levels. Optimization of the extraction method using Response surface methodology-Box Behnken Design with three variables (solid to-solvent ratio, extraction temperature and extraction time) obtained the optimum conditions for the extraction of seven phytochemical compounds, namely solvent ratio (1:30), extraction temperature (40 °C) and extraction time (17.85 minutes). Determination of the levels of the seven phytochemical component compounds was carried out on several existing products such as simplicia, herbal tea, extract and capsules containing *M. oleifera*. The levels of phytochemical compounds obtained varied, in which the extract product contained the highest phytochemical content compared to other products.

Acknowledgements

The authors thank to Endowment Fund for Education (LPDP) from the Ministry of Finance, Republic of Indonesia, for the scholarship and research cost.

Conflict of interest

The authors declare no conflict of interest in this research.

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