

Sequential Microwave-Ultrasound Assisted Extraction of Flavonoid from *Moringa oleifera*: Product Characteristic, Antioxidant and Antibacterial Activity

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Abstract: *Moringa oleifera* leaves contain secondary metabolites in flavonoid compounds known to prevent several diseases. Therefore, appropriate extraction methods are required to produce extracts with a high yield of flavonoids from *Moringa*. In this study, the extraction from *Moringa* leaves was carried out using the sequential microwave/ultrasound-assisted extraction (MUAE) method compared with sequential ultrasound/microwave (UMAE), microwave (MAE), ultrasound (UAE), and maceration (ME). The effects of the time, temperature, and percentage of ethanol were studied on total flavonoid content using AlCl_3 colorimetric assay. The extracts were analyzed by Scanning Electron Microscopy (SEM), Fourier Transforms Infrared Spectrophotometry (FTIR), and High-Performance Liquid Chromatography (HPLC). The antioxidant and antibacterial activities were tested using DPPH-scavenging and disc diffusion methods. The results of SEM surface analysis on various extraction methods show differences on each surface. The FTIR spectrum showed the presence of flavonoid O-H at 3200 cm^{-1} , C=O at 1621 cm^{-1} , and C-O at 1019 cm^{-1} . In the results of HPLC, MUAE extracts 16.70 mg/100 g flavonoid quercetin at the retention time of 4.5 min, with the highest total flavonoids (2.89 mg QE/g), the highest antioxidant activity (IC_{50} 72.31 $\mu\text{g/mL}$), and highest antibacterial activity (*S. aureus* 7 mm, *E. coli* 2 mm).

Keywords: *Moringa oleifera*; quercetin; microwave; ultrasound; antioxidant

■ INTRODUCTION

Moringa oleifera is a tropical and subtropical plant belonging to the Moringaceae family. *Moringa* is one of the most vital medicinal and pharmaceutical plants, containing various useful nutrients such as potassium, iron, phosphorus, calcium, vitamin A, vitamin D, and essential amino acids [1]. In addition, *Moringa* leaves extracts exhibit antioxidant, anti-hypertensive, and anti-cancer effects [2]. Tannins, steroids, triterpenoids, flavonoids, saponins, interquinones, and alkaloids are antioxidant agents found in *Moringa* leaves [3]. In addition, biologically active compounds such as flavonoids (myricetin, quercetin, kaempferol, 3-O-glucoside kaempferol) were also found in *Moringa* [4].

Conventional extraction methods such as maceration are popular for bioactive compound

extraction. However, a large amount of solvent and a long extraction time are required [5]. In recent years, several advanced extraction techniques have been developed. In order to extract flavonoids from natural sources, microwave, ultrasound, pulsed-electric field, pressured liquid, and supercritical fluid have been used [6-7]. Ultrasound-assisted extraction (UAE) is preferable for the extraction of natural products. UAE can increase the extraction mass transfer rate caused by the resulting cavitation in the material. The short extraction process is achieved as the solubility of the analyte in the extraction media increases as surface tension and solvent viscosity decrease, boosting extraction efficiency. The polymeric structure of the cell wall is destroyed, allowing more bioactive chemicals from plant material to enter the liquid extraction phase

[5]. Microwave-assisted extraction (MAE) has been widely used to extract secondary metabolites from plants due to the shorter extraction time, less solvent required, and high extraction yield. MAE is equivalent to other current extraction techniques, such as supercritical fluid extraction from a technical standpoint; however, it has economic and practical advantages because it is easy to operate and requires simple sample preparation techniques [8]. MAE can reduce solvent use and extraction time while increasing extract yield. Microwaves are used in MAE to heat and evaporate water from the cell. As a result, the cell swells, stretches, and ruptures, allowing the metabolic component to leave and be extracted by the solvent [9]. UAE and MAE can be considered as one of the most effective extraction strategies for extracting bioactive chemicals from plant materials, such as flowers, due to their high extraction efficiency [10], fruits [11], leaves [5], bark [12], seeds [13], and pods [14].

Combining two or more extraction techniques, such as microwave extraction (MAE) and ultrasound (UAE), can speed up processing time and be more efficient than single extraction [15]. In order to speed up the extraction process and liberate the targets from the matrix in a short amount of time, simultaneous irradiation with ultrasound and microwave energy can be used [16]. Ultrasound and microwave radiations could speed up the extraction procedure and enhance the extraction of bioactive compounds [17]. When the mass transfer mechanism is improved, the extraction time is reduced indeed [18]. Ultrasound/microwave-assisted extraction (UMAE) uses UAE as pre-treatment and is continued by MAE for simultaneous irradiation as an essential upgrade of UAE [19]. The acoustic cavitation effect of ultrasonic causes a higher content of bioactive compounds, followed by internal heating in plant cells with microwave irradiation [20]. The reverse version with MAE as pre-treatment and continued with UAE approach resulted in greater levels of extractable bioactive chemicals due to the effect of internal heating within the plant cells caused by microwave irradiation followed by sonic cavitation [21]. Microwave-ultrasound-assisted extraction (MUAE) is a cost-effective and time-saving extraction method that

employs ultrasound and microwave technology compared to other new extraction technologies such as pressurized liquid extraction, supercritical fluid extraction, enzyme-assisted extraction, and pulsed electric field extraction [22]. MUAE can provide the high activation energy or impact energy required for the extraction process while also limiting or preventing the degradation of the bioactive compounds in the extract [19].

To the best of our knowledge, no comprehensive study reported on the effects of sequential microwaves/ultrasound on the quality and quantitative properties of flavonoids extracted from Moringa leaves. Therefore, this study aims to determine the effect of various extraction methods such as UAE, MAE, UMAE, and MUAE on variations in extraction time, temperature, and percent solvent on the quantification of total flavonoids and quercetin in Moringa leaves. In addition, the effects of different extraction methods were studied on antibacterial and antioxidant activities.

■ EXPERIMENTAL SECTION

Materials

The leaves of Moringa leaf powder bought from Herbology (Bogor, Indonesia), The chemical such as ethanol (96% purity, CAS 64-17-5 Merck, Germany), AlCl₃ (CAS 7446-70-0 Merck, Germany), potassium acetate (CAS 127-08-2 Merck, Germany), DPPH (2,2-Diphenyl-1-picrylhydrazyl) (CAS 1898-66-4 Merck, Germany), quercetin (CAS 117-39-5 Merck, Germany), kaempferol (CAS 520-18-3 Merck, Germany), myricetin (CAS 529-44-2 Merck, Germany), rutin (CAS 153-18-4 Merck, Germany), apigenin (CAS 520-36-5 Merck, Germany), rhamnetin (CAS 90-19-7 Merck, Germany), luteolin (CAS 491-70-3 Merck, Germany), metylluteolin (20243-59-8 Merck, Germany), galocatechin (CAS 3371-27-5 Merck, Germany), gallic acid (CAS 149-91-7 Merck, Germany), nutrient agar (SKU: 70148-100G, Sigma-Aldrich, USA), *Escherichia coli* (FNCC media agar *E. coli*, Nanobio Laboratory, Indonesia), and *Staphylococcus aureus* (FNCC media agar *S. aureus*, Nanobio Laboratory, Indonesia).

Instrumentation

The equipment used in this study included a glass funnel, measuring cup, beaker, Erlenmeyer, test tube, vial bottle, glass stirrer, dropper pipette, 5 mL measuring pipette, measuring flask, separating funnel, ose needles, condenser, round bottom flask, vial bottles, analytical scales (OHAUS PA224), Microwave (MGC20100S, Beko, Polish), Ultrasound (JP-010S, Skymen, USA), SEM (JSM-6510 LA, JEOL, Japan). The characterization was used FTIR spectrophotometer (Spectrum Two L160000A, PerkinElmer, USA), UV-Vis spectrophotometer (Model ENF-24/F, Shimadzu, Japan), and HPLC (Prominence LC-20, Shimadzu, Japan). The experimental setup of microwave and ultrasound is depicted in Fig. 1.

Procedure

Extraction of Moringa leaf

Moringa powder was extracted at a solid/liquid 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. The ultrasound extractor (model JP-010S, Skymen, USA), a timer (0–30 min), an ultrasonic with an output of 80 Watts, and a frequency of 40 kHz. UAE and MAE applied are guided by Jitan et al. [23] with minor modifications. Sequential MUAE and UMAE are guided by the research of Yu et al. [10] by alternating microwave first pre-treatment and then continued with ultrasound and vice versa. Extraction variable such as time (0, 5, 10, 20, 25,

30 min), temperature (30, 40, 50, 60, 70, 80 °C), and the solvent concentration of ethanol (0, 10, 20, 30, 40, 50, 60, 70, 80, 90%). Immediately after treatment, the mixture of Moringa was cool at room temperature. Furthermore, the extract was then filtered using Whatman 42 filter paper. The extract was collected in amber glass bottles and stored at refrigeration temperature.

Scanning electron microscopy (SEM) analysis

A scanning electron microscope was utilized to examine the morphology of Moringa extract extracted from UAE, MAE, UMAE, and MUAE. At a magnification of 1000 times, the surface and cross-section of the Moringa extract were photographed for morphological purposes.

Fourier transform infrared spectroscopy (FTIR) analysis

FTIR was used to examine the functional groups of Moringa extract extracted from UAE, MAE, UMAE, and MUAE. The extract samples were ground, and KBr was used to press the highly dispersed powders. The formed pellets were then used for FTIR spectroscopy measurements at 4000–500 cm^{-1} .

Total flavonoids determination

Total flavonoids were analyzed using the aluminum chloride colorimetric method with slight modification, according to Mukhriani et al. [24]. Quercetin was used to make the calibration curve. First, 10 mg of quercetin was dissolved in ethanol 96% and

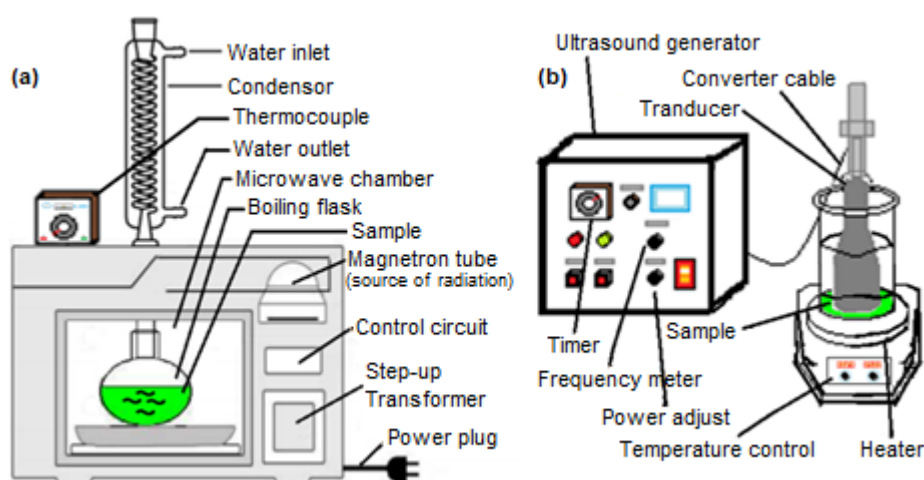


Fig 1. The schematic device of (a) Microwave extraction and (b) Ultrasound extraction

diluted to 100, 200, 300, 400, and 500 µg/mL. Next, 1 mL of each concentration of standard solutions, as well as 1 mL of UAE, MAE, UMAE, and MUAE extracts, were mixed with 1 mL of 2% aluminum chloride (AlCl₃) and 1 mL of 120 mM potassium acetate (CH₃COOK). The mixture was incubated at room temperature for 30 min. The absorbance was measured at 430 nm against a blank without AlCl₃ using UV-vis spectrophotometer. Total flavonoid content was calculated and expressed in quercetin equivalent (mg QE/g). Total flavonoid content = $(x \times v \times fp) / g$. Where x is the quercetin concentration from the standard curve (µg/mL), fp is the dilution factor, v is the extract volume (mL), and g is the extract weight (mg).

High-performance liquid chromatography (HPLC) analysis

Prominence LC-20 was used for the HPLC analyses. Shim-pack XR-ODSII column (150 mm × 3 mm ID) was used to separate the compounds at room temperature. Moringa extracts were injected into the HPLC column with a concentration of 0.10 g/L. The relative area was estimated using quercetin, kaempferol, myricetin, rutin, apigenin, rhamnetin, luteolin, metylluteolin, gallic acid (0.20 g/L) as the internal standard to explain probable changes in the instrument's analysis to the results of UAE, MAE, UMAE, and MUAE extracts.

Assays of antioxidant activity using the DPPH method

The antioxidant activity was determined using the DPPH scavenging technique modified from Tristantini et al. [25]. Initially, a 1 mM DPPH solution was prepared by dissolving 3.80 mg in 100 mL of ethanol. Next, 2 mL of UAE, MAE, UMAE, and MUAE extracts were added to 2 mL of 1 mM DPPH. The absorbance level was determined at 517 nm after 30 min of incubation in darkness. A UV-Vis spectrophotometer was used for analysis. A DPPH solution without adding the sample extract was utilized as a control. The results were expressed in percentage. The following formula was used to calculate the DPPH free radical scavenging assay: % DPPH scavenging activity = $(\text{absorbance of control} - \text{absorbance of sample}) / (\text{absorbance of control}) \times 100$. A calibration curve was used to determine the amount of DPPH in each well. The proportion of DPPH that remains is compared to the extract concentration to determine the

number of samples needed to achieve a 50% reduction in DPPH or inhibition concentration of 50% (IC₅₀). The IC₅₀ value of a very strong antioxidant activity is less than 50 g/mL [26].

Antibacterial test using disc-diffusion method

The antibacterial test was carried out by the disk diffusion method by calculating the inhibition zone produced from the extract against bacteria growing in a petri dish. In this test, *E. coli* as Gram-negative bacteria and *S. aureus* as Gram-positive bacteria were used. One loop of bacterial colonies (*E. coli* and *S. aureus*) from nutrient agar (NA) was diluted using sterile 0.9% NaCl solution to have turbidity, according to Mc. Farland (107–108 CFU/mL). A sterile cotton swab is inserted into a tube containing a bacterial suspension, then streaked evenly on the agar medium (in a petri dish). A total of 20 µL of Moringa sample solution (UAE, MAE, UMAE, and MUAE extract) was injected into blank disk paper using a micropipette. After the solution was completely absorbed, the sample's disc paper was placed on media nutrient agar (MNA) 1 containing *E. coli* and MNA 2 containing *S. aureus* test bacteria, and then incubated at 37 °C for 24 h. The clear zone formed around the disc indicates that the sample can inhibit bacterial growth, and its diameter can be known.

RESULTS AND DISCUSSION

Morphological Activity

The SEM results are shown in Fig. 2, which reveals the surface changes in the samples. Analysis of the solid characteristics of the extract from Moringa begins with surface analysis with SEM. SEM is used to see the effect of extraction technology on the surface of Moringa. The Moringa had distinct physical alterations due to the various extraction procedures. Moringa cell walls were slightly perforated after extraction by UAE, as shown in Fig. 2(a). UAE extract showed wrinkled surfaces and observable plant cell perforations. UAE can enhance extraction by using sonic cavitation, which breaks down cell walls, allowing solvents to penetrate plant material and release intracellular compounds. Fig. 2(b) shows the situation of more extensive cell wall rupture obtained from MAE.

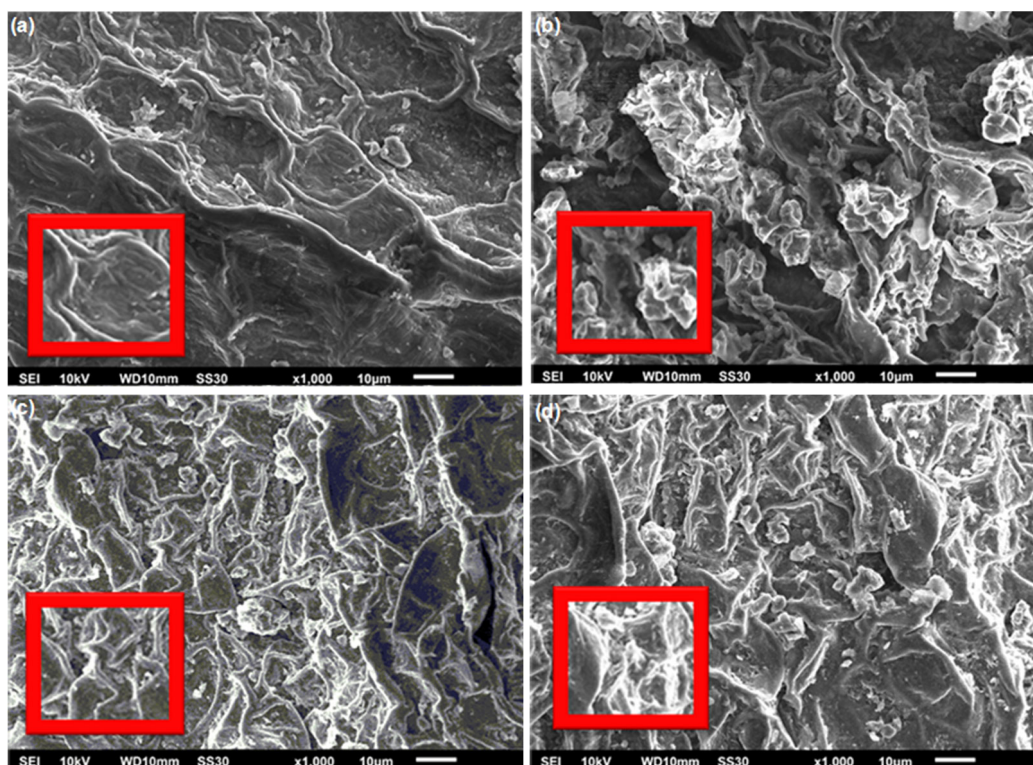


Fig 2. SEM results of UAE (a), MAE (b), UMAE (c), and MUAE extract (d)

Microwaves have a greater effect on cell wall rupture than ultrasound. In the MAE process, non-ionizing electromagnetic energy is applied directly to the raw material allowing rapid heating of solvents and suspensions due to the rapid energy delivery transformation. Ion conduction and dipole rotation cause dissolved ions to migrate due to this heating enhancing the solvent's penetration into the matrix, making it easier to retrieve the target chemical [7]. The microstructure of the ruptured tissue in the treatment using sequential UMAE and MUAE, as shown in Fig. 2(c, d), showed more

extensive damage or rupture of the cell wall indicated by a rougher surface. The diffusion rate of flavonoid compounds in cells increases on a rougher surface, and the solvent could completely extract flavonoid compounds [27].

FTIR Analysis

The functional group transformation of the functional groups in the UAE, MAE, UMAE, and MUAE, was also observed using FTIR spectra on the Moringa extract (Fig. 3). Table 1 and Fig. 3 show the FTIR

Table 1. Moringa FTIR wavenumber results compared with standard quercetin

Functional group	Wavenumber (cm ⁻¹)					
	Quercetin	Fresh Moringa	UAE	MAE	UMAE	MUAE
-OH (alcohol)	3340	3314	3278	3271	3314	3282
C=O	1711, 1666	1738	1737	1745	1738	1735
C=C	1656, 1510	1616	1648, 1605	1630	1652; 1625	1621
-OH (bend)	1379	1433	1413	1416	1427	1409
C-OC	1310, 1242, 1160	1319, 1113	1316, 1147	1325, 1158	1315, 1160	1317, 1162
C-H (aromatics)	938, 820, 600	628	594	595	614	595
C-H (stretch)	2924, 2880	2942, 2831	2917, 2849	2917	2940; 2834	2918; 2835
C-O (stretch)	1110, 1057	1021	1027	1022	1019	1019

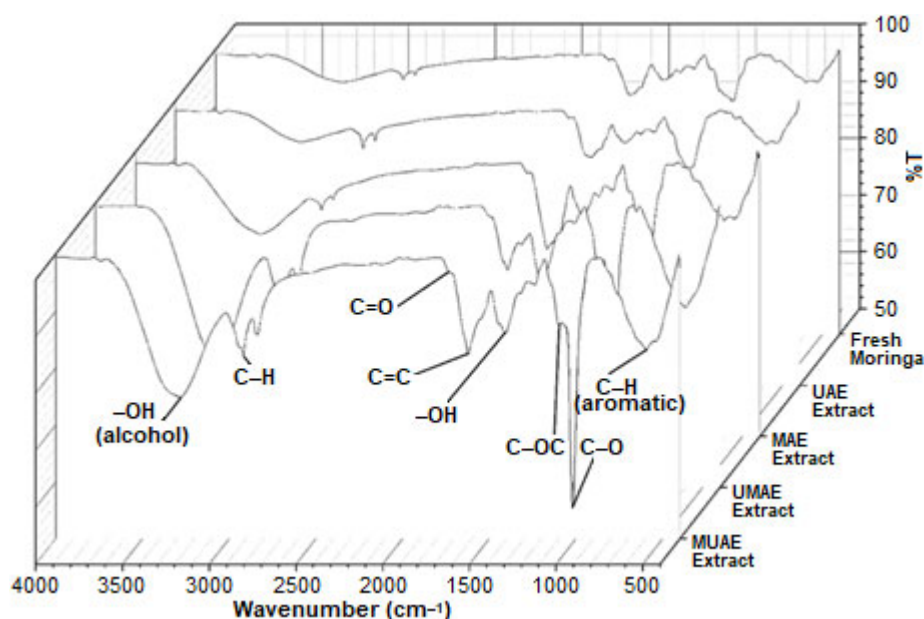
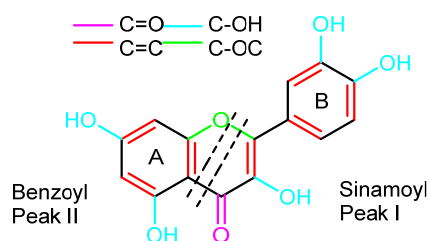


Fig 3. FTIR spectra on fresh Moringa, UAE, MAE, UMAE, and MUAE extract

result of Moringa extract. From the FTIR results in all extraction methods found the absorption of aryl ketonic strain (C=O) at 1735–1845 cm^{-1} , aromatic ring stretching (C=C) was found at 1605–1652 cm^{-1} , bending of the phenol (C–OH) at 1409–1433 cm^{-1} , and stretching in the aryl ether ring (C–OC) at 1113–1325 cm^{-1} .

For the flavonoid group, the presence of carboxylic acids (O–H) is indicated by peaks at 3000–2700 cm^{-1} , and the presence of an ester functional group (C=O) is indicated by one peak in the region 1770–1668 cm^{-1} [28]. The flavonoid structure of quercetin is shown in Fig. 4.

Moringa had phenolic O–H peaks at 3321.96 cm^{-1} , C=C alkene peaks at 1657.50 cm^{-1} , phenol C–OH stretching at 1237.05 cm^{-1} , and C–OC bending vibrations at 1057.68 cm^{-1} [9]. The stretching of the OH group was identified in the FTIR spectra of pure quercetin in its characteristic band at 3406 and 3283 cm^{-1} , while the phenol function's OH bending was detected at 1379 cm^{-1} . Aromatic ring stretching C=C was detected at 1610, 1560, and 1510 cm^{-1} . Absorption of aryl ketonic strain C=O was clearly visible at 1666 cm^{-1} . In-plane C–H flexural bands in aromatic hydrocarbons were detected at 1317 cm^{-1} . The bands at 1263, 1200, and 1165 cm^{-1} are caused by C–O stretching (the aryl ether ring, C–O in phenols, and C–OC). Out-of-plane flexural bands (C–H aromatics) were seen at 933, 820, 679, and 600 cm^{-1} , respectively [29].



3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one
Quercetin ($\text{C}_{15}\text{H}_{10}\text{O}_7$) = 302.2 g/mol

Fig 4. Functional groups in the flavonoid quercetin

It can be concluded that the difference in the extraction method has no significant effect on the flavonoid functional groups of Moringa plants. Functional group results from FTIR results show quercetin compounds in all Moringa leaf extraction methods

Effect of Extraction Time on Total Flavonoids

The time for extraction has an impact on the flavonoid content. Therefore, the extraction period is extended to allow the solvent to permeate more deeply into plant cells and extract more flavonoids. The results of the total flavonoids with variation extraction time are shown in Fig. 5.

The total flavonoids with variation extraction time are shown in Fig. 5. The longer the extraction takes, the extraction content will increase gradually and reach the highest value total flavonoids (for UAE of 2.26 mg QE/g,

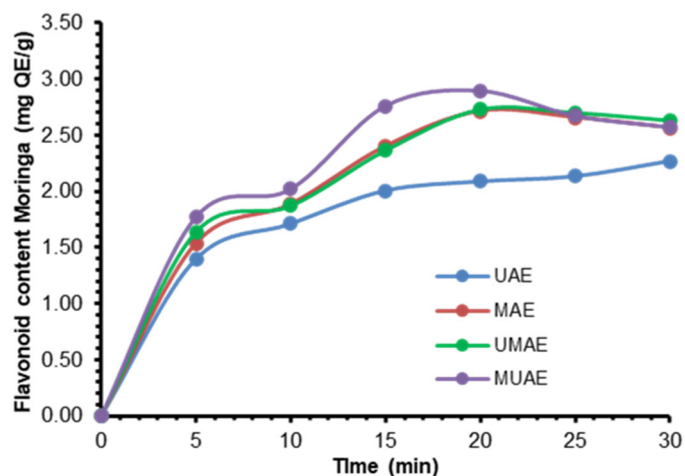


Fig 5. Extraction time effect on total flavonoids of UAE, MAE, UMAE, and MUAE extract

MAE of 2.71 mg QE/g, UMAE of 2.72 mg QE/g, and MUAE of 2.89 mg QE/g). According to total flavonoids results using the microwave, this technique can get extraction yield faster and more efficiently than ultrasound.

The increase in flavonoid content is due to the longer the solvent process, the higher the ability for the solvent to bind flavonoid compounds. In the ultrasound and microwave methods, cavitation and inner heat damage the plant cell wall so that the flavonoids present in the vacuole can be extracted out of the cell [30-31]. Sequential extraction techniques can increase efficiency because the fresh solvent is supplied in each extraction, which improves solubility [32]. However, generally, the flavonoid content in the extract is limited so that the extraction power will decrease at a certain time. It has been reported that the maximum time extraction in MAE was 15–20 min [33].

At the same yield, UMAE reduced the extraction time by 98.66% and 19.89%, respectively, compared to maceration and MAE [22]. UMAE is improved with the help of acoustic cavitation and certain fast microwave heating, the extraction time is shorter, the required volume of solvent required is less, and the yield UMAE is higher (97%) when compared with UAE (89%). It is because the internal heat of UMAE radiation breaks vacuole cells in plant cells faster, allowing flavonoids to be extracted more easily, as opposed to cavitation ultrasound

alone, which takes longer [34]. Sequential UMAE (UAE as pre-treatment) get higher yield (31.88%) when compared with MAE (27.81%), UAE (17.92%), and maceration (19.16%) [35]. MUAE extract (MAE pre-treatment) tended to be higher (7.85 mg QE/g) than UMAE (UAE pre-treatment) (6.55 mg QE/g), MAE (4.79 mg QE/g), and UAE (6.23 mg QE/g) [10].

Effect of Temperature on Total Flavonoids

From the results of time variations, the total flavonoid test at temperature variations was carried out at 20 min of the extraction process. The total flavonoids with variation temperature results are shown in Fig. 6. As the temperature rises, the extraction content rises with it, eventually reaching its maximum value total flavonoids (for UAE 2.26 mg QE/g, MAE 2.72 mg QE/g, UMAE 2.71 mg QE/g, and MUAE 2.89 mg QE/g).

As the solvent temperature rose, it came into contact with the free water molecules in Moringa, causing further cell damage and mass transfer. The flavonoid content increases 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 released will increase, but the subsequent increase results in structural changes resulting in a decrease in the detected compounds. Total flavonoids decreased when the temperature was higher than 50 °C [36]. Compared to UAE and MAE, sequential UAE + MAE was a much higher yield [20].

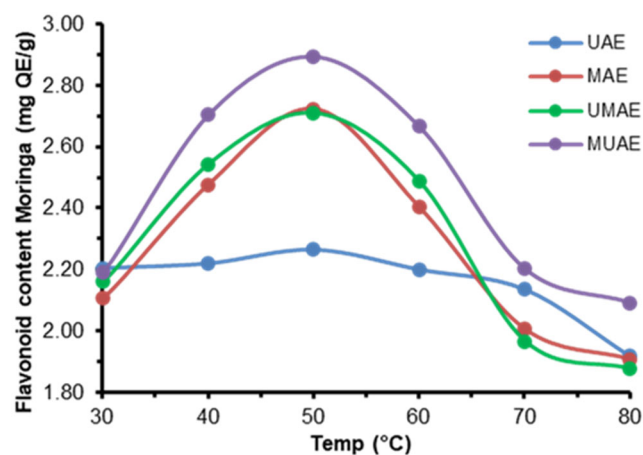


Fig 6. Temperature effect on the total flavonoids of UAE, MAE, UMAE, and MUAE extract

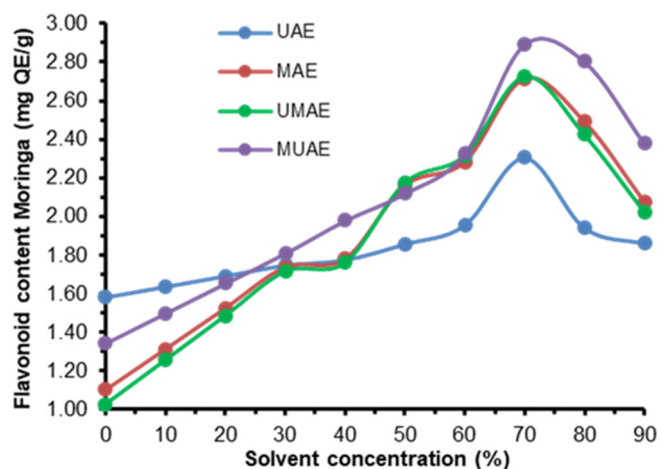


Fig 7. Effect of solvent concentration on the total flavonoids of UAE, MAE, UMAE, and MUAE extract

Effect of Solvent Concentration on Total Flavonoids

One of the driving factors for extraction is ethanol concentration, which is a crucial metric for assessing extraction efficiency. From the results of time and temperature variations, the total flavonoid test at ethanol concentration variations was carried out at 50 °C for 20 min of the extraction process. The total flavonoids with variation solvent concentration are shown in Fig. 7. In flavonoid test results, the ethanol concentration affected the total flavonoid content. The higher the extraction content will increase gradually and reach the highest value total flavonoids (for UAE of 2.30 mg QE/g, MAE of 2.71 mg QE/g, UMAE of 2.72 mg QE/g, and MUAE of 2.89 mg QE/g). The flavonoid content increases when the solvent concentration is between 50 and 70% (v/v). The best ethanol-water ratio was found in the range of 65–75% when Moringa was extracted at a temperature of 55–65 °C [37]. With a 70% solvent ratio in MAE, total flavonoids of 1.90 mg QE/g tended to be higher than 1.72 and 1.70 mg QE/g obtained by UAE and maceration, respectively [11]. The range of ethanol concentrations decreased significantly when ethanol was 70–80% (v/v) because chlorophyll was dissolved into the extract so that the yield of flavonoids that the solvent could extract was reduced. Higher solvent concentrations also can change the polarity of the solvent and affect the total solubility of flavonoids, resulting in a decrease [38]. If the solvent used is 96% concentrated ethanol, then the chlorophyll will

also be extracted, and the extract will become very thick so that the extraction rate is reduced [39].

HPLC Analysis

Furthermore, the extract of Moringa was analyzed using HPLC to determine the main components of the flavonoid quercetin in Moringa extract using the UAE, MAE, UMAE, and MUAE methods. Fig. 8 illustrates the HPLC spectra of various Moringa extraction processes.

The HPLC results for flavonoid quercetin in the UAE extract at a retention time (RT) of 4.21–4.60 min to get a yield of 39.58 ppm, for the MAE extract quercetin was found at RT 4.79 min to get a yield of 41.48 ppm, for the UMAE extract quercetin was found at RT 4.66 min got a yield of 11.97 ppm, and for the MUAE extract quercetin was found at RT 4.51 min to get a yield of 16.70 ppm. Research by Sukmawati et al. [40] found a quercetin range in RT 4.18–4.56 min, and RT is the minimum time required for the fluid mixture to be separated by gravity in the HPLC apparatus. The HPLC results for flavonoid kaempferol in the UAE extract at a retention time (RT) of 6.14 min to get a yield of 1.38 ppm. For the MAE extract, kaempferol was found at RT 6.02–6.42 min to get a yield of 14.81 ppm. For the UMAE extract, kaempferol was found at RT 5.23–6.43 min got a yield of 22.73 ppm, and for the MUAE extract, kaempferol was found at RT 5.10–6.33 min to get a yield of 22.84 ppm. Therefore, MUAE can extract more flavonoid kaempferol than UMAE, MAE, and UAE. In the sequential MUAE, it was found that the peak of other compounds at RT 3.54 was 289 ppm, which is thought to be the myricetin compound that was detected in HPLC analysis. This phenomenon was also reported in Shervington's study of flavonoid HPLC analysis from Moringa leaves, which found myricetin at RT 3.60 min, quercetin at RT 4.80 min, and kaempferol at RT 6.70 min [41]. Myricetin, quercetin, and kaempferol are the main flavonoids contained in Moringa. Other flavonoids such as apigenin, rhamnetin, and luteolin are found in smaller levels [42]. HPLC analysis of Moringa leaves revealed 26.20 ppm myricetin at 4.35 min, 5 ppm quercetin at 5.89 min, and 41.50 ppm kaempferol at 6.44 min [4]. Consequently, with the above information and the

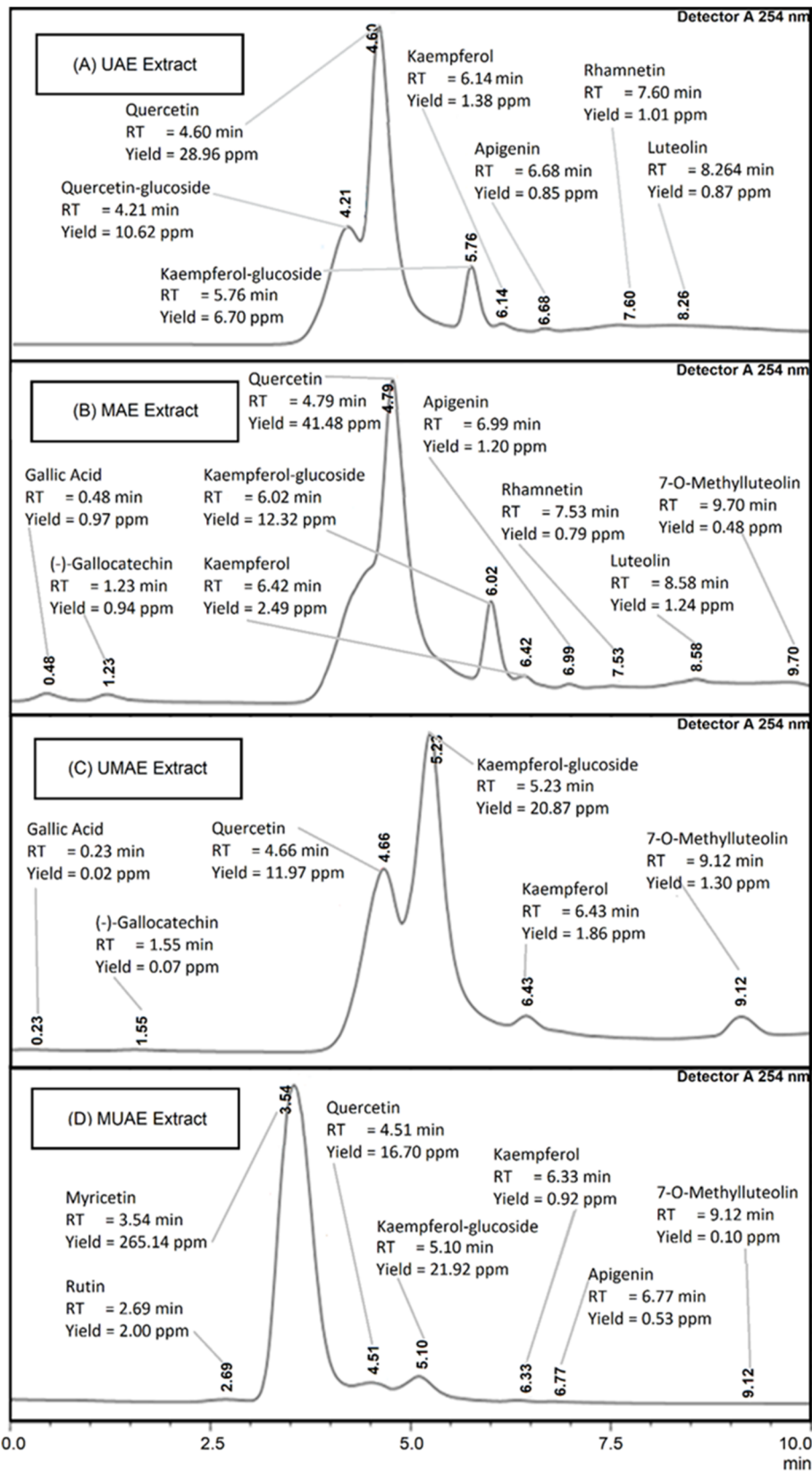


Fig 8. Spectra HPLC on UAE (A), MAE (B), UMAE (C), and MUAE (D) extract

comparison of the extraction method, we found that microwaves can extract more quercetin content, and sequential microwave-ultrasound can extract more flavonoids with reasonable efficiency.

Total Antioxidant Activity

UAE had the lowest antioxidant activity than MAE, UMAE, and MUAE, similar to the total flavonoid content results. Fig. 9 depicts the DPPH method's antioxidant test results.

Based on Fig. 9, the results of the extraction of Moringa by UAE, MAE, UMAE, and MUAE showed an increase in the % of antioxidants to the initial concentration of the sample. Table 2 shows the antioxidant activity and IC_{50} result of Moringa extract.

In this study, standard quercetin obtained IC_{50} of 39.34 $\mu\text{g/mL}$. The results of Moringa extraction by UAE ($IC_{50} = 87.83 \mu\text{g/mL}$), MAE ($IC_{50} = 80.21 \mu\text{g/mL}$), UMAE ($IC_{50} = 76.54 \mu\text{g/mL}$), and MUAE ($IC_{50} = 72.31 \mu\text{g/mL}$). All extraction methods yielded IC_{50} values of less than 100, indicating that the antioxidants extracted are potent antioxidants. The DPPH method was chosen because it is a method that is straightforward, easy, quick, and sensitive and only testing necessitates a tiny sample product. The DPPH method's quantitative evaluation of antioxidant activity is based on a change in the intensity of the purple color of the DPPH, which is proportional to the DPPH solution's concentration. The absorbance at the maximal wavelength of DPPH will change as a result of the color change when measured using UV-Vis spectrophotometry so that the value of free radical scavenging activity will be known, which is expressed by the IC_{50} value [43]. IC_{50} range of 150–200 $\mu\text{g/mL}$ is a weak antioxidant, the IC_{50} range of 100–150 $\mu\text{g/mL}$ is a

moderate antioxidant, the IC_{50} range of 50–100 $\mu\text{g/mL}$ is a strong antioxidant, and the IC_{50} range is < 50 $\mu\text{g/mL}$ is a very strong antioxidant [25]. Compared to maceration and MAE, UMAE increased antioxidant activity by 11.71% and 2.60%, respectively [22].

Antibacterial Test Using the Disc Diffusion Method

Bacteria such as *E. coli* (gram-negative) and *S. aureus* (gram-positive) were tested against UAE, MAE, UMAE, and MUAE extracts. Based on Table 3, the extract from Moringa extraction using UAE, MAE, UMAE, and MUAE have antibacterial activity on *E. coli* and *S. aureus*.

Table 3 shows the results of the inhibition zone diameter. The antibacterial test results of moringa extract with MUAE against *S. aureus* were found to be higher than other methods. In addition, MUAE extract can also affect Gram-negative bacteria such as *E. coli*. because the

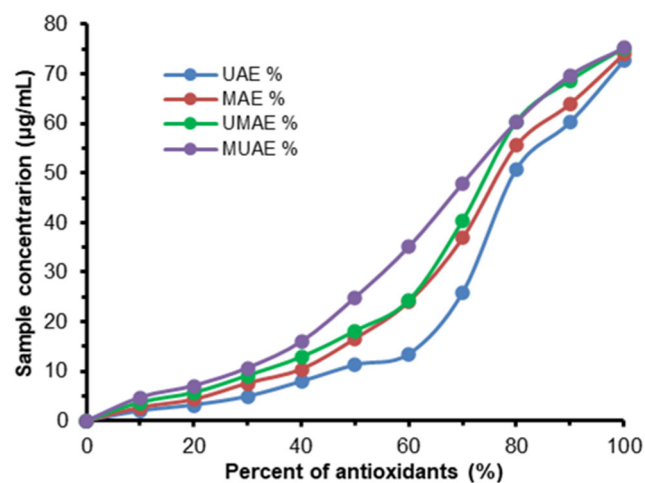


Fig 9. Antioxidant activity of UAE, MAE, UMAE, and MUAE extracts

Table 2. Results of antioxidant activity (%) and IC_{50} of Moringa extract

Concentration ($\mu\text{g/mL}$)	Antioxidant Activity (%)				
	Quercetin	UAE	MAE	UMAE	MUAE
60	71.43	13.48	24.05	24.30	35.11
70	71.77	25.88	36.87	40.34	47.87
80	77.54	50.79	55.55	60.30	60.31
90	81.06	60.25	63.94	68.65	69.67
100	82.44	72.66	73.93	75.14	75.21
IC_{50}	39.34	87.83	80.21	76.54	72.31

Table 3. Results of antibacterial activity of Moringa using the Disc Diffusion method

Bacteria test sample	Obstacles zone (mm)			
	UAE	MAE	UMAE	MUAE
<i>E. coli</i>	2	1	2	2
<i>S. aureus</i>	4	4	5	7

structure of the bacterial cell wall influences the sensitivity of bacteria to antibacterial. Gram-positive bacteria, as an example *S. aureus*, are more susceptible to antibacterial than Gram-negative bacteria because their cell walls are simpler than Gram-negative bacteria's, making it easier for antibacterial compounds to enter Gram-positive bacteria cells [44]. Therefore, the inhibition zone in *S. aureus* is larger than in *E. coli*.

■ CONCLUSION

The selection of the Moringa extraction method can affect the content of bioactive compounds and their biological activities. In total flavonoid content found that MUAE get more flavonoid content with 2.89 mg QE/g > UMAE with 2.73 mg QE/g > MAE with 2.71 mg QE/g > UAE with 2.30 mg QE/g. The best extraction conditions were found at 50 °C for 20 min and 70% ethanol. SEM scan of Moringa morphological extract showed cell damage or rupture of the results of cell extraction with ultrasonic and microwave indicated by a rougher surface, so it is expected that flavonoid compounds in cells (in plants located in vacuoles) can be extracted completely by solvents. In all samples, the FTIR spectrum of Moringa extract showed the presence of flavonoid C=O at 1621 cm⁻¹ and C-O at 1019 cm⁻¹, which was in accordance with the standard flavonoid quercetin spectrum. HPLC analysis found that MAE gets more quercetin content with 41.48 ppm > UAE with 28.96 ppm > MUAE with 16.70 ppm > UMAE with 11.97 ppm. Antioxidant test with DPPH showed that MUAE gets the highest antioxidant activity in the Moringa extract with an IC₅₀ of 72.31 µg/mL and the highest antibacterial with obstacles zone for 2 mm *E. coli*, and 7 mm *S. aureus*. Ultrasonic and microwave technology are beneficial for extracting flavonoids in Moringa.

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■ AUTHOR CONTRIBUTIONS

Aji Prasetyaningrum conceived and designed the experiments, Bakti Jos contributed reagents, materials, and analysis tools, Ratnawati analyzed and interpreted the data, Nur Rokhati revised the manuscript, Teguh Riyanto wrote the paper, and Gian Restu Prinanda performed the experiments. All authors agreed to the final version of this manuscript.

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