Fractionation and determination of phenolic and flavonoid compound from *Moringa oleifera* leaves

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**Abstract.** *Moringa oleifera* is one of the nutrient-dense multipurpose plants that grows in Indonesia. *M. oleifera* leaf extract contains many phenolic compounds and flavonoids, one of them is quercetin compounds. In this study, total phenolic, total flavonoid, and quercetin compounds were determined based on their fraction as free phenolic acids (FPA), phenolic acid ester (PAE), and phenolic acid glycosides (PAG) which separated by phenolic acid fractionation methods. Fractionation method was done by liquid-liquid extraction. Total Phenolic compounds and flavonoid were determined using spectrophotometric UV-Vis. The concentration of quercetin was determined using High-Performance Liquid Chromatography (HPLC). Total phenolics in the crude extract (CE), FPA, PAE, and PAG fractions respectively were 50.2042; 5.7963; 1.5260; and 0.0617 mg/g GAE. The total flavonoids in the CE, FPA, PAE, and PAG fractions were 25.6627; 1.7207; 1.0510; and 0.2843 mg/g QE. The quercetin compounds in the CE, FPA, PAE, and PAG fractions were 1.7123; 1.9607; 0.1921; and 0.5067 μg/g. *Moringa oleifera* is potential natural sources for phenolic and flavonoid, particularly of quercetin.

1. Introduction

*Moringa oleifera* is one of the nutrient-dense multipurpose plants that grows in Indonesia. *M. oleifera* leaves extract contains primer metabolic compound such as protein, lipid, carbohydrate, mineral, vitamin, and amino acid [1]. *M. oleifera* is standardized contain flavonoid, phenolic, and carotenoid which can use as antioxidant, one of them is quercetin [2]–[4].

Quercetin is a member of the class of flavonoids called flavonols. Quercetin provides many health-promoting benefits, including improvement of cardiovascular health, eye diseases, allergic disorders, arthritis, reducing the risk for cancers and many more [5]. Quercetin is a strong antioxidant, with multiple therapeutic properties. It has hypolipidemic, hypotensive, and anti-diabetic properties [6].

Quercetin founds in *M. oleifera* leaves as aglycone and glycone [7]. Phenolic compounds in the plants can be known by their existence as free phenolic acid and phenolic acid liberated from esters and glycosides [8]. In this study, will report total phenolic, total flavonoid, and quercetin compounds determined by their existence as free phenolic acids (FPA), phenolic acid liberated from ester (PAE), and phenolic acid liberated from glycosides (PAG) which separated by phenolic acid fractionation methods.
2. Materials and methods

2.1. Materials
Leaves of *M. oleifera* were obtained from Tengaran, Kabupaten Semarang, Jawa Tengah.

2.2. Preparation and extraction
The collected material washed and dried by drying cabinet 50°C for 24 hours then powdered. *M. oleifera* powder extracted five times by maceration with ethyl acetate. Then collected, combined, then filtered and evaporated using a rotary evaporator.

2.3. Fractionation of phenolic acid [8]
The crude extract acidified to pH=2 using 6 N HCl and extracted five times with diethyl ether (1:1, v/v) at room temperature. The ether extracts of phenolic acids (referred to as free phenolic acids) were combined and evaporated to dryness under vacuum at ≤40°C. The water phase was adjusted to pH=7 with 2 M NaOH and then evaporated to near dryness under vacuum at ≤40°C. The residue was treated with 20 ml 4 N NaOH under nitrogen for 4 hours at room temperature. The reaction mixture was then acidified with 6 N HCl to pH=2 and extracted with diethyl ether as described above. The ether extracts of phenolic acids are referred to as phenolic acids liberated from ester bonds. Following this, the water phase was again adjusted to pH=7 with 2 M NaOH and then evaporated to near dryness under vacuum at ≤40°C. The residue was heated with 50 ml of 2 M HCl for 30 min at 95°C, cooled to room temperature, and extracted with diethyl ether as described above. These ether extracts of phenolic acids are referred to as phenolic acids liberated from glycosidic bonds.

2.4. Purification of phenolic acid fractions
Each of the residues of phenolic acid fractions, obtained as described above, was dissolved in 50 ml of 5% NaHCO₃ (pH=8) and extracted five times with diethyl ether to remove residual fatty material. The water phase was then acidified with 6 N HCl to pH=2 and extracted with diethyl ether as described above.

2.5. Total phenolic [9]
The total phenolic content of the extract was determined by Folin-Ciocalteu reagent. A 0.5 ml portion of appropriately diluted extracts was added to a 10-ml volumetric flask. The extract was mixed with 0.4 ml Folin-Ciocalteau reagent and incubate for 5–8 minutes. Added 4 ml Na₂CO₃ 7% and diluted to the volume (10 ml) with aquades. Then, incubated till 2 hours and the absorbance was measured at 765 nm versus the prepared blank. The result of determination was expressed in terms of GAE of the extract.

2.6. Total flavonoid [9]
Total flavonoids were measured using a colorimetric assay. A 0.5 ml portion of appropriately diluted extracts was added to a 10-ml volumetric flask. The extract was mixed with 4 ml aquades and 0.3 ml NaNO₂ then incubated for 6 minutes. Added 0.3 ml AlCl₃ 10% and incubated for 5 minutes. Then, added 4 ml NaOH 10% and diluted to the volume (10 ml) with aquades. Then, incubated till 15 minutes and the absorbance was measured at 510 nm versus the prepared blank. The result of determination was expressed in terms of QE of the extract.

2.7. Identification quercetin using High-Performance Liquid Chromatography (HPLC)
The instrument used in this experiment was Knauer Smartline UV Detector, 2500 with column Eurospher II RP C18 (150x4.6 mm, 5 μm). The wavelength used for this UV detector was 370 nm. Quercetin were quantified using isocratic elution consists Acetonitrile : H₃PO₄ 0,1% (40:60). The flow rate of the mobile phase was 1 ml/min, and the injection volumes were 20 μL of the standards and sample extracts. All flavonoids were quantified using the external standard method. Quantification was based on peak area.
2.8. Data analysis
Quantitative analysis of total phenolic was done by calculating the line equation from the standard raw curve of gallic acid using absorbance obtained and expressed in Gallic Acid Equivalent (GAE). Quantitative analysis of total flavonoid was done by calculating the line equation from the standard raw curve of quercetin using absorbance obtained and expressed in Quercetin Equivalent (QE). Quantitative analysis of quercetin was performed by calculating the area of the chromatogram. Quercetin concentration can be known by calculating the line equation from the standard raw curve between the area of the chromatogram to the concentration of quercetin. The data obtained were analyzed descriptively.

3. Result and discussion

3.1. Extraction and fractionation phenolic acid
In this study, the maceration method was used to extract M. oleifera, because maceration is an extraction method that is still usually used to extract flavonoid from plants [10]–[13]. The solvent used in this study is still acetate because this solvent is better than ethanol to the extracted flavonoid. Fractionation was carried out according to previous studies [8]. From the fractionation, the yield for free phenolic acid (FPA), phenolic acid liberated from esters (PAE), and phenolic acid liberated from glycosides (PAG) is 73.52%; 37.82%; and 45.40%.

3.2. Total phenolic
The total phenolic content of the extract was determined by Folin-Ciocalteu reagent in an alkaline atmosphere. The principle of this method is the formation of complex blue compounds that can be measured at a wavelength of 765 nm. Folin-Ciocalteau reagent will oxidize phenol or phenolic-hydroxy groups to reduce heteropoly acid (phosphomolybdate-phosphotungstate) contained in Folin-Ciocalteau reagent into a molybdenum-tungsten complex [14].

In this measurement, the standard phenolic total is used in the form of gallic acid. Then, the result of determination was expressed in terms of GAE of the extract. It was calculated by the use of the linear equation obtained from the calibration curve of standard gallic acid as follows

\[ y = 0.0224x - 0.007 \]
\[ R^2 = 0.9993. \]

Where “y” is the absorbance of the sample, while “x” represents the amount of gallic acid in µg/ml.

Gallic acid and Folin-Ciocalteau reagents can form stable blue complexes [15]. The hydroxy group in gallic acid phenolics ion reacts with Folin-Ciocalteau reagents by reducing heteropoly acid (phosphomolybdate-phosphotungstate) to form a blue molybdenum-tungsten complex that can be detected by spectrophotometers [16]. In this reaction, the phenolic ion will reduce Mo\(^{6+}\) to Mo\(^{5+}\) and molybdenum will oxidize the hydroxy group present in phenolic ion compounds (Figure 1) [17].

Na\(_2\)CO\(_3\) is added to the mixture because the combined phenolic can only be supported by Folin-Ciocalteau reagents in an alkaline atmosphere so that protons dissociate in phenolic compounds into phenolic ions [14], [18]. During the reaction, the reaction will turn blue. The blue color formed will increase the concentration of phenolic ion formed, forming the greater the phenolic concentration, the more phenolic ions will reduce heteropoly acid so that the resulting blue color becomes more concentrated [19].
Figure 1. The reaction between gallic acid and molybdenum compounds in the Folin-Ciocalteau reagent [17].

From the result, CE revealed high phenolic acid followed by the fractions. Among the fractions, FPA was found to have the highest phenolic content with 5.7963 mg/g GAE, and the lowest was PAE with 0.0617 mg/g GAE (table 1). The fractions were found to have low phenolic content than CE. This difference may be caused by some lipophilic compound rinsed when purification.

The total phenolic content of crude extract (CE) on this experiment was higher than previous similar studies, with total phenolic content 30.83–35.51 mg/g GAE [20]. However, in other studies, it was found 107,209 mg/g GAE [21]. Hence this variation may be related to concentration and solvent differences.

| Table 1. Total phenolic in each fraction. |
|-----------------------------------------|
| Total Phenolic (mg/g GAE) | CE | FPA | PAE | PAG |
|---------------------------|----|-----|-----|-----|
|                           | 50.2042 | 5.7963 | 1.5260 | 0.0617 |

CE: Crude Extract  
FPA: Free Phenolic Acids  
PAE: Phenolic Acid Liberated From Ester  
PAG: Phenolic Acid Liberated From Glycosides

3.3. Flavonoid total

Aluminum chloride colorimetric method was used for flavonoids determination. This method has a measurement principle based on color formation. The principle of determining the flavonoid content of the colorimetric-AlCl₃ method is the formation of complexes between aluminum chloride and the keto group on C-4 atoms and hydroxy groups on neighboring C-3 or C-5 atoms of flavones and flavonols [22]. The formation of this complex will shift the wavelength in the visible direction so that the solution will turn yellow [23]. The use of NaNO₂ and NaOH will form a NaNO₂-AlCl₃-NaOH system complex which shows special colors based on the reaction of aluminum ions with flavonoids in alkaline environments to form complex compounds [24]. The compound used as a standard for determining the levels of flavonoids is quercetin because quercetin is a flavonoid of the flavonol group that has a keto group on C-4 atoms and also a hydroxyl group on neighboring C-3 and C-5 atoms (figure 2) [22].
In this measurement, the standard flavonoid total is used in the form of quercetin. Then, the result of
determination was expressed in terms of QE of the extract. It was calculated by the use of the linear
equation obtained from the calibration curve of standard quercetin as follows

\[ y = 0.0019x - 0.0035 \]

\[ R^2 = 0.9997 \]

Where “y” is the absorbance of the sample, while “x” represents the amount of quercetin in µg/ml.

From the result, CE revealed high total flavonoid followed by the fractions. Among the fractions,
FPA was found to have the highest flavonoid content with 1.7207 mg/g QE, and the lowest was PAE
0.2843 mg/g QE (table 2). The fractions were found to have low phenolic content than CE. This
difference may be caused by some lipophilic compound rinsed when purification.

The total flavonoid content of crude extract (CE) on this experiment was higher than previous similar
studies, with total flavonoid content 32.74–98.67 mg/g QE [20]. However, in other studies, it was found
359.53 mg/g QE [21]. Hence this variation may be related to concentration and solvent differences.

**Table 2. Total flavonoids in each fraction.**

|          | CE   | FPA  | PAE  | PAG  |
|----------|------|------|------|------|
| Total Flavonoid (mg/g QE) | 25.6627 | 1.7207 | 1.0510 | 0.2843 |

CE: Crude Extract  
FPA: Free Phenolic Acids  
PAE: Phenolic Acid Liberated From Ester  
PAG: Phenolic Acid Liberated From Glycosides

3.4. Quercetin

Quercetin measurement was carried out on using High-Performance Liquid Chromatography (HPLC).
Based on the identification results of quercetin using HPLC, in each fraction contained quercetin
compounds (table 3). At peak EK, FPA, PAE, and PAG fractions for quercetin compounds successively
appeared at retention time (tR) 3.783 minutes (figure 3); 3.667 minutes (figure 4); 3.650 minutes (figure
5); 3.650 minutes (figure 6).

**Table 3. Levels of quercetin in each fraction.**

|          | CE   | FPA  | PAE  | PAG  |
|----------|------|------|------|------|
| Quercetin (μg/g) | 1.7123 | 1.9607 | 0.1921 | 0.5067 |

CE: Crude Extract  
FPA: Free Phenolic Acids  
PAE: Phenolic Acid Liberated From Ester  
PAG: Phenolic Acid Liberated From Glycosides
The total quercetin in the crude extract was 1.7123 μg/g. The results obtained from this study are different from similar studies. In another study that used water solvents, 80% methanol, and 70% ethanol with sonication method for 5 minutes, quercetin on *M. oleifera* leaves ranged from 6.340–27.490 mg/g [11]. Whereas in the study used acidified methanol (methanol / HCl, 100: 1, v/v) with reflux method for 2 hours then centrifuged and the top layer was sonicated for 5 minutes, quercetin was found to be 0.207 mg/g in *M. oleifera* leaf extract [25]. Furthermore in the study that used methanol with sonication method for 5 minutes then added HCl which then refluxed for 2 hours found quercetin as much as 0.70–12.6 mg/g in a crude extract of *M. oleifera* leaves [26]. This is possible because of differences in extraction methods, solvents and the length of contraction time used in the study.

**Figure 3.** Chromatogram profile of crude extract (CE). Quercetin chromatogram is a chromatogram 4 (tR = 3.783 minutes).

**Figure 4.** Chromatogram profile of Free Phenolic Acids (FPA). Quercetin chromatogram is a chromatogram 2 (tR = 3.667 minutes).
Figure 5. Chromatogram profile of Phenolic Acid Liberated From Ester (PAE). Quercetin chromatogram is a chromatogram 2 (tR = 3.650 minutes).

Figure 6. Chromatogram profile of Phenolic Acid Liberated From Glycosides (PAG). Quercetin chromatogram is a chromatogram 2 (tR = 3.650 minutes).

The most quercetin compounds were found in the FP A fraction, slightly more than the EK fraction. This might be due to the initial fractionation process, the addition of strong acids was carried out with a high enough concentration. So that it is possible for the quercetin compound to be attached, the bond is cut off and turned into a free quercetin compound. In addition, in this identification, the standard quercetin used was quercetin aglycone so that only quercetin was detected in the form of aglycone instead of bonding.

The quercetin level in the PAG fraction was more than the PAE fraction. This is because in nature the presence of quercetin binds to more glycosides and is more frequently found compared to the presence of ester-bound quercetin. Glycosides themselves are derivatives of the main group of quercetin [27]. Quercetin obtained in each fraction is quite small, between 0.19–1.96 μg/g. Given the many factors that will influence during the isolation process and the quercetin levels obtained, the quercetin compounds that are present are not isolated.

The results of the identification are known in each fraction containing more than one compound characterized by the appearance of several peaks at some retention time. In the EK chromatogram profile 13 peaks appeared (figure 3), the FPA fraction profile chromatogram appeared 10 peaks (figure 4), PAE fraction chromatogram profile appeared 6 peaks (figure 5), and in the PAG fraction chromatogram profiles appeared 4 peaks (figure 6).
4. Conclusion
The conclusions that can be obtained from this study include:

1. The yield obtained in the FPA, PAE and PAG fractions were 73.52%; 45.40%; and 37.82%.
2. Total phenolic levels found in CE, FPA, PAE, and PAG respectively amounted to 50.2042; 5.7963; 1.5260; and 0.0617 mg/g GAE.
3. The total levels of flavonoids found in CE, FPA, PAE, and PAG were respectively 25.6628; 1.7207; 1.0510; and 0.2843 mg/g QE.
4. Quercetin levels found in CE, FPA, PAE, and PAG were 1.7123; 1.9607; 0.1921; and 0.5067 μg/g.

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