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Original Article

Total Phenolic, Total Flavonoid, Quercetin Content and Antioxidant Activity of Standardized Extract of *Moringa oleifera* Leaf from Regions with Different Elevation

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ABSTRACT

Context: *Moringa oleifera* is the famous plant that has been used as medicinal plant for diverse pharmacological activity. **Aims:** To evaluate the total phenolic, total flavonoid, quercetin content as well as the antioxidant activity of standardized extract of *Moringa oleifera* (Lamk) leaf, collected from three regions with different elevation. **Materials and Methods:** The leaves were extracted by maceration method using ethanol 96% and evaporated by rotary evaporator to obtain the viscous extract. The determination of total phenolic and total flavonoid were performed by spectroscopic method, while the quercetin concentration were determined by high performance liquid chromatography (HPLC). The antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. **Results:** Ethanolic extracts of *Moringa oleifera* leaf from region with medium altitude (Sigi regency) showed higher total phenolic, total flavonoid, quercetin concentration and antioxidant activity than regions with low and high altitude (Parigi and Palu cities). **Conclusion:** This study reveals the potency of standardized extracts of *Moringa oleifera* growing in medium altitude (Sigi regency) to be developed as antioxidant herbal medicine.

Key words: Kelor, *Moringa oleifera*, Antioxidant, Total phenolic, Total flavonoid.

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INTRODUCTION

Free radicals are mediator of some diseases that possessing high reactivity because of the unpaired electrons. The increasing amount of free radicals on human will lead to oxidative stress of the cells continuously directed to the emergence of degenerative diseases such as cancer, diabetic, inflammation and cardiovascular.[1-3] To overcome this, an antioxidants are indispensable to protect the cells from the negative effect of free radicals.[4] However, the use of synthetic antioxidants such as butyl hydroksianisol (BHA) and butyl hydroxyl toluene (BHT) cause some side effects which is cytotoxic for the lungs and liver and also carcinogenic.[5] Accordingly, many studies have been conducted on antioxidant compounds derived from plant sources which have more benefits than the synthetic.[6]

One of the medicinal plants that have been widely used in the world wide, particularly at Palu, Central Sulawesi, Indonesia, is *Moringa oliefera* leaf. It is used as vegetable soup and known as *uta kelo*. It has been reported that *M. oleifera* possessed high antioxidant activity contributed by the high presence of phenolic content.[7-11] Several classes of flavonoids were known as major compound that responsible for antioxidant activity. Some of them is quercetin. Quercetin has been known to have diverse pharmacological activity including antioxidant. Quercetin was also found as a major compound from *M. oleifera* leaf.[12-13] It is interesting to determine quercetin content of *M. oleifera* leaf and then correlate with its antioxidant activity. Moreover, variations of genotypes, growing regions, temperature, season, harvesting time, process and storage conditions are possibly to affect the phenolic profile and antioxidant property of *M. oleifera* leaf.[12,14]

Considering the broad medicinal effect consuming *M. oleifera* and the widespread of cultivation of this plants on different regions in Central Sulawesi, Indonesia, it is important to ensure the quality of standardized extracts based on physicochemical and phytochemical analysis that responsible for biological action. This study aims to evaluate the total phenolic, total flavonoids, quercetin content as well as the antioxidant activity of standardized extract of *Moringa oleifera* (Lamk) leaf, collected from three regions in Central Sulawesi, Indonesia, with different elevation.

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MATERIALS AND METHODS

Materials

The leaves of *Moringa oleifera* were collected on January 2017 from three growing regions chosen based on different altitude: Palu city, the capital city of Central Sulawesi Province, has high altitude of 150 m height above mean sea level (AMSL). Sigi regency (medium altitude = 70 m) and Parigi regency (low altitude = 15 m) are located at 20 and 65 km from Palu city, respectively. Quercetin, *1,1-difenil-2-pikrilhidrazil* (DPPH), gallic acid, ethanol 96%, methanol, ascorbic acid, sodium carbonate, aluminium chloride, aquadest and other chemicals and solvents (analytical grade) were purchased from Sigma Aldrich

Methods

Extractions

Moringa oleifera leaves were dried at room temperature and smashed manually. Each sample was extracted by maceration method with ethanol 96% at room temperature for 3 x 24 h. The maceration process is repeated 3 times. The liquid extract obtained is then evaporated with a rotary evaporator. The resulting extracts were freeze drying and used for further analysis.

Phytochemical Screening and Physicochemical Analysis

All extracts were subjected to phytochemical screening by thin layer chromatography (TLC) identification using different spraying reagent for particular compounds, such as dragendorf for alkaloids, $AlCl_3$ for flavonoid, $FeCl_3$ for tannin and phenolic, Lieberman Burchard for steroid and sulfuric acid for saponin/triterpenoid.[15] Beside that, the extracts were also analyzed for physicochemical properties, such as organoleptics, water and ethanol-soluble extractive matters, water content, total ash content and acid-insoluble ash content according to Indonesian Herbal Farmakope.[16]

Determination of Total Flavonoid

Total flavonoid was analyzed using aluminium chloride colorimetric method with slight modification according to Chang *et al.*[17] Quercetin was used to make the calibration curve. 10 mg of quercetin was dissolved in ethanol 96% and diluted to 2, 4, 6, 8 and 10 µg/mL. 1 ml of each concentration of standard solutions, as well as 1 ml of each sample solution, were mixed with 3 mL ethanol 96%, 0.2 mL of aluminium chloride 10%, 0.2 mL potassium acetate 1 M and 5.6 mL of distilled water. The mixture was incubated at room temperature for 10 min with intermittent shaking. The absorbance was measured at 376 nm against a blank without aluminium chloride using Cecil CE7410 UV-Vis spectrophotometer. Total flavonoid was calculated as mean ± SD (n = 3) and expressed as weight of quercetin equivalent (QE) at 100 mg extract.

Determination of Total Phenolic

Total phenolic content was determined with the Folin–Ciocalteu reagent according to Pourmorad *et al.*[18] with slight modification. A calibration curve was obtained by using gallic acid as standard. Different concentrations of gallic acid (5–125 mg/L) were prepared. 10 mg sample was diluted in 10 mL methanol on test tube. Both 0.5 mL of standards and samples were taken and mixed with 2.5 mL of Folin–Ciocalteu 50% and 2.5 mL of distilled water. After incubated for 5 min, 2 mL aqueous sodium carbonate solution (7.5%, w/v) was added. The final mixture was shaken and then incubated for 15 min in the dark at room temperature. The absorbance of all standards and samples were measured at 765 nm using Cecil CE7410 UV-Vis spectrophotometer and the results expressed as milligrams of gallic acid equivalents (GAE) per 100 mg of dry leaf weight.

Determination of Quercetin Concentration by HPLC

The determination of quercetin as a major compound on ethanolic extract was performed by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC). Sample was prepared by dissolving 25 mg dried ethanolic extract in 10 mL of methanol and sonificated for 15 min. Standard quercetin in methanol was prepared in concentration of 1.2, 2.4, 4.8, 9.6 and 19.2 µg/mL. Before injecting into the column, samples and standards solution were filtered through a 0.45 µm Millipore filter. In this study, HPLC Cecil CE4201 with UV visible detector was used for analysis with DataStream software system. The separation was carried out on a column C18 size 250 mm × 4.6 mm (inside diameter). Optimum efficiency of separation was obtained using methanol: water (90:10, v/v) with a flow rate 1 mL/min. Other parameters adopted were as follows: injection volume 20 µL and detection wavelength at 370 nm. Linear regression analysis using SPSS 17.0 (SPSS. Inc, Chicago IL, USA) was applied to correlate the peak area (Y) against the concentration of quercetin (X). The quantification of quercetin in the extract was quantified about the calibration curve.

Determination of Antioxidant Activity

The radical scavenging activity of *Moringa oleifera* ethanolic extracts against the DPPH radical was determined according to Blois.[19] Determination procedures were as follow: 3 mL of 0.1 mM DPPH radical solution was mixed with 3 mL of methanolic solutions of *M. oleifera* extracts (concentration series of 75 – 175 µg/mL). After 30 min incubation at the dark room, absorbance decrease of the mixture was monitored at 515 nm (*A sample*). During reduction by the antioxidant, the solution colour changed from violet to yellow pale. Blank samples with 3 mL of methanol and 3 mL of 0.1 mM DPPH radical solution were prepared and measured daily at same wavelength (*A blank*). Quercetin was used as positive control. The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following formula.

$$\% \text{ inhibition} = \left(\frac{\text{A blank} - \text{A sample}}{\text{A blank}} \right) \times 100$$

The 50% inhibitory concentration (IC_{50}) was expressed as the quantity of the extracts to react with a half of DPPH radicals.

Statistical Analysis

One-way analyses of variance (ANOVA) followed by a Duncan post hoc test were used to estimate the significance of the growing regions on the levels of total phenolic, total flavonoid and quercetin content. Calculations were performed using SPSS 17.0 (SPSS. Inc, Chicago IL, USA).

RESULTS

Physicochemical Properties and Phytochemical Screening

The result of physicochemical analysis such as water content, water and ethanol soluble extractive matter, were given in Table 1. It is found that extracts of *M. oleifera* from regions with medium elevation (Sigi regency, ±70 m) showed higher content of water and ethanol soluble extractive matters than other regions.

Further identification on ethanolic extracts by thin layer chromatography using mobile phase *n*-hexane: ethyl acetate (5:2) showed that all extracts contained flavonoids (two yellow spot at Rf 0.64 and 0.51), triterpenoids (one brown spot at Rf 0.81), steroid (one green spot at Rf 0.94), tannin (one blackish green spot at Rf 0.72), saponin (one blue spot at Rf 0.92) and phenolics (one blackish blue spot at Rf 0.77).Alkaloids is not found in all extracts identification (Table 2)

Table 1: Physicochemical analysis of ethanolic extract of *Moringa oleifera* leaf.

Parameters	Values obtained w/w on dry weight extract		
	Low Altitude (Parigi Regency)	Medium Altitude (Sigi Regency)	High Altitude (Palu City)
Organoleptic	Blackish green dry powder, bitter taste and characteristic odor	Blackish green dry powder, bitter taste and characteristic odor	Blackish green dry powder, bitter taste and characteristic odor
Water soluble extractive matters	19.6%±0.9	36.3%±0.7	19.8%±0.5
Ethanol soluble extractive matter	32.9%±0.4	50.1%±1.9	33.4%±0.7
Water content	6.4% ± 0.8	7.4% ± 1.0	6.8% ± 2.4
Total ash content	3.1% ± 0.7	3.3% ± 0.5	4.3% ± 0.3
Acid Insoluble ash content	1.7% ± 0.6	2.1% ± 0.4	2.3% ± 0.4

Table 2: Thin layer chromatography (TLC) identification.

No	Type of Compounds	Retention Factors (Rf)		
		Low Altitude (Parigi Regency)	Medium Altitude (Sigi Regency)	High Altitude (Palu City)
1	Flavonoids	0.65	0.74	0.64;0.51
2	Triterpenoids	0.82	0.94	0.81
3	Steroid	0.82	0.94	0.81
4	Tannin	0.71	0.77	0.72
5	Saponin	0.82	0.92	0.85
6	Alkaloids	-	-	-
7	Phenolic	0.71	0.77	0.72

Total Phenolic, Total Flavonoid, Quercetin Content and Antioxidant Activity

The result of total phenolic, total flavonoid and quercetin concentration showed that the extract of *M. oleifera* from regions with medium altitude (Sigi regency, ± 70 m) is higher than other regions. It is also showed highest ability to scavenge free radicals compared to others (Table 3). Determination of total phenolic and total flavonoid use gallic acid and quercetin as standards where the calibration curve equations obtained were y=0.005x + 0.161 (R^2 = 0.992) and y=0.004x + 0.157 (R^2 = 0.948), respectively

DISCUSSION

Moringa oleifera is a famous plant that has been used as medicinal plant for diverse pharmacological activity. To evaluate the effect of regional variations on total phenolic, total flavonoid, quercetin content and antioxidant activity of *M. oleifera* leaf extract, samples were taken together at the same time (10.00 o'clock on the morning) and the same maturity (plant age more than 3 months) from three regions with different elevation that are Parigi regency (low altitude), Sigi regency (medium altitude) and Palu city (high altitude). After drying, the dried leaves directly extracted by maceration using ethanol solvent. Ethanol was chosen because it has been reported as the best solvent for *M. oleifera* extractions.[12]

Table 3: Total phenolic, total flavonoid, quercetin concentration and IC_{50} of *M. oleifera* leaf extract from three regions on Central Sulawesi.

Analysis	Low Altitude (Parigi Regency)	Medium Altitude (Sigi Regency)	High Altitude (Palu City)
Total phenolics (mg/100 mg) in GAE	2.6 ± 0,03[a]	3.0 ± 0,2[a]	2.5 ± 0,7[a]
Total flavonoids (mg/100 mg) in QE	8.9 ± 0.7[a]	9.6 ± 0.5[b]	8.1 ± 0.4[a,b]
Quercetin concentration (mg/g)	0.0644 ± 0.0001[a]	0.0648 ± 0.0001[b]	0.0638 ± 0.0002[c]
IC_{50} (µg/ml)	138.9	134.5	146.7

To ensure the quality and purity of *M. oleifera* ethanol extract on antioxidant activity associated with the amounts of bioactive compounds in the extracts, the physicochemical analysis has been conducted. The result can be seen in Table 1. All extracts showed values that fulfil the requirements for extracts quality and purity. The water-soluble extractive matters were lower than ethanol-soluble extractive matters, indicated the content of polar compounds is higher than content of sugar, acid and any inorganic compounds that dissolved in water. The value of water content showed values less than 10% that means the extract will not allow for microbial growth and deterioration because of hydrolysis. The mean of ash contents was in range of 3.1% to 4.3% (total) and 1.7 to 2.3% (acid insoluble ash) that mean the extract contains low content for carbonates, phosphates, silicates and silica.

Total phenolic and total flavonoid represent the total amounts for phenolic and flavonoid compounds in the extracts. As reported, *M. oleifera* extract was rich with phenolic compounds, such as quercetin, kaempferol and rutin which are flavonoid compounds. These constituents always associated with some factors such as environmental factors and growing regions. Siddhurajuand Becker reported the effect on growing regions on total phenolics of *M. oleifera* in Nicaragua (4.25%) was higher than India and Niger (2.94% and 3.66%, respectively) as well as for total flavonoid.[12] This study also confirms the effect of growing regions on total phenolic and total flavonoid where the total phenolic and total flavonoid of *M. oleifera* ethanol extract from medium altitude (Sigi regency) was higher (3.0 ± 0.2 mg/100 mg GAE and 9.6 ± 0.5 mg/100 mg QE, respectively) than other regions (Table 2). It is also found that the difference between regions are not significantly different for total phenolic, but significantly different ($p < 0.05$) for total flavonoid between Sigi regency and Parigi regency.

Measurement of quercetin concentration in *M. oleifera* leaf extract was performed by using high performance liquid chromatography (HPLC) method with quercetin as standard. Figure 1 represent the separation of quercetin on ethanolic extracts of *M. oleifera* possessed adequate peak resolution that indicated the used method is proper for quercetin identification. The calibration curve of five concentration used was represented in Figure 2. It has linearity with a coefficient correlation (R^2) of 0.993. The quercetin content was in range of 0.0638 to 0.0648 mg/g. Statistical analysis showed that quercetin concentration was significantly different between regions ($p < 0.05$).

Antioxidant ability of *M. oleifera* leaves collected from three regions with different elevation did not show difference significantly. However, the antioxidant activity of *M. oleifera* extracts from regions with medium altitude (Sigi regency) was higher (IC_{50} of 134.5 ug/mL) than other

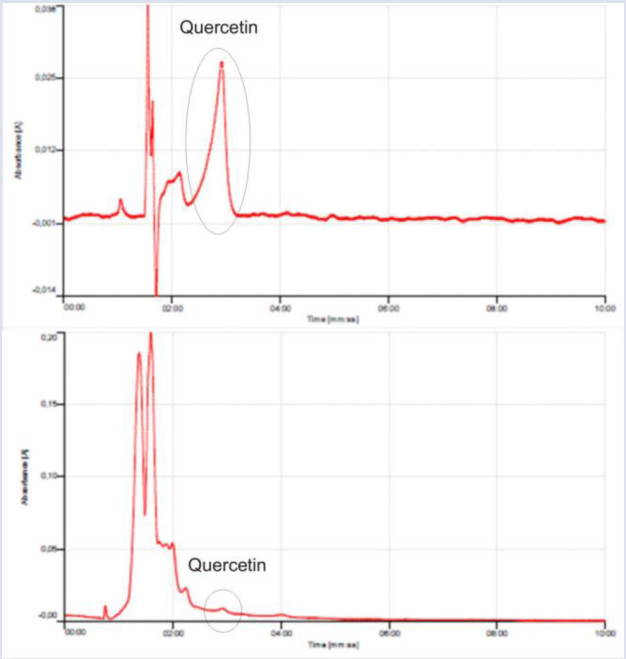

Figure 1: HPLC chromatogram profile of quercetin in ethanolic extract of *M. oleifera* leaf. Standard peak of quercetin (above), Peak of quercetin present in ethanolic extract (below)

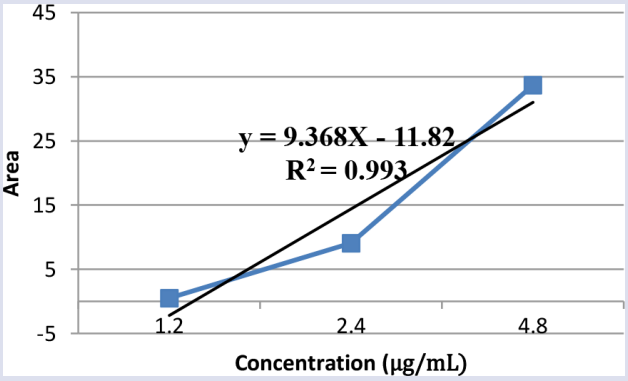

Figure 2: Curve calibration of quercetin standard.

regions (Figure 3). This study confirmed that the antioxidant activity of *M. oleifera* extract were attributed to the values of total phenolic, total flavonoid and quercetin concentration. Thus, the development of *M. oleifera* to be source of herbal medicine was suggested to be cultivated in the regions with medium altitude.

CONCLUSION

The standardized ethanol extract of *M. oleifera* leaves from regions with medium altitude (Sigi regency) has higher total phenolic, total flavonoid and quercetin content than other regions affording higher antioxidant activity as well

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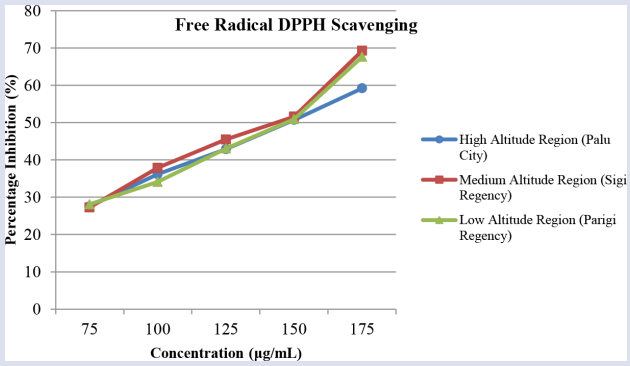

Figure 3: Antioxidant activity of *M. oleifera* extract from regions with different elevation

CONFLICT OF INTEREST

No conflict of interest

ABBREVIATIONS

$AlCl_3$: Aluminium Chloride; $FeCl_3$: Ferric Chloride; SPSS: Statistical Package for the Social Sciences; DPPH: 2,2-diphenyl-1-picrylhydrazyl.

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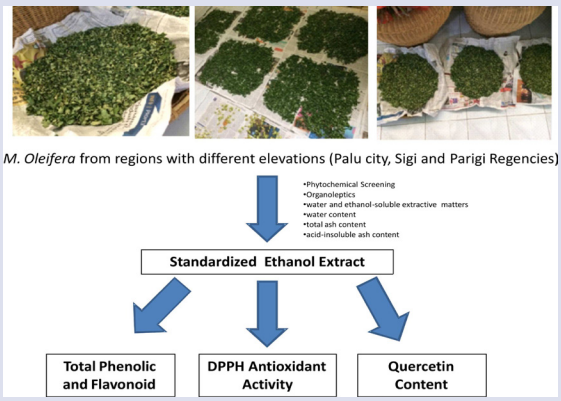
GRAPHICAL ABSTRACT



SUMMARY

- *M. oleifera* leaf ethanolic extract from regions with different evaluation was evaluated for organoleptic, water and ethanol-soluble extractive matter, water content, total ash and acid insoluble ash content
- *M. oleifera* leaf ethanolic extract from region with medium altitude (Sigi regency) has higher total phenolic, total flavonoid and quercetin content than other regions
- *M. oleifera* leaf ethanolic extract from region with medium altitude (Sigi regency) is also possessing higher antioxidant activity (IC_{50} of 134.5 µg/mL)

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