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Original Article

Total Phenolic Contents, Quercetin Determination and Anti Elastase Activity of *Melastoma malabathricum* L. Leaves Extract from Different Method of Extractions

Thia Amalia[1], Fadlina Chany Saputri[2*], Silvia Surini[3]

ABSTRACT

Objective: Leaves of *M. malabathricum* has been detected to contain quercetin and other phenolic contents. Quercetin has been proven to have elastase inhibitory activity. The aim of this study was to evaluate the effects of extraction method on total phenolic and quercetin contents as well as elastase inhibitory activity of *M. malabathricum* leaves extracts. **Methods**: Leaves powder was extracted by two conventional methods, maceration and reflux. Two different concentrations of ethanol were used as a solvent, 70 and 96% ethanol. Leaves were also defatted with chloroform before further extraction. The total phenolic content was determined by the Folin-Ciocalteu method and quercetin content was determined by using the high-performance liquid chromatography method. Elastase inhibitor activity of leaves extract was determined on human neutrophil elastase *in vitro*. **Results**: Generally, 96% ethanol obtained higher total phenolic and quercetin content than 70% ethanol. However, defatted extract of 70% ethanol contained higher total phenolic content than defatted 96% ethanol. The highest elastase inhibitory activity of the sample was obtained from 70% ethanol extract with the value of 89.50% at 200 ppm, which is no significant difference compared to quercetin with the value of 93.86%. **Conclusion**: Extraction methods and different concentration of solvents affect the total phenolic and quercetin contents of the extracts. *M. malabathricum* leaves have potential effect as anti-elastase as well as quercetin, where the anti-elastase activity of *M. malabathricum* leaves is not only due to quercetin.

Key words: *Melastoma malabathricum* L., Total phenolic content, Quercetin, Anti-Elastase, Extraction methods.

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INTRODUCTION

Melastoma malabathricum L. (Melastomataceae) is a native plant in the tropical and subtropical region, especially Southeast Asian. *M. malabathricum* can be found throughout South and South-East Asia, China, Taiwan, Australia and the South Pacific Ocean. The plant is a small shrub that commonly growth wildly in roadside and waste land.[1]

Phytochemically the leaves of *M. malabathricum* contained amides, flavonoid, triterpenes, saponins, tannins, steroids, glycosides and phenolic compounds.[1-3] Leaves extract of *M. malabathricum* contained quercetin, quercitrin and kaempferol-3-O-(2′,6′-di-O-p-trans-kumaroyl)-β-glucoside.[4] Besides that, other components also found in the leaves extracts such as 5-hydroxymethylfurfural, palmitic acid, stearic acid methyl ester, trans-squalene and tocopherol.[5]

M. malabathricum is an important ethnomedicine and used by tribes in Malaysia, China, India and Indonesia as traditional medicine.[1] Leaves and roots were used as wound healing and prevent scarring from smallpox by Dayak Tribe in Kalimantan, Indonesia.[6] Roots were also used as a cough and asthma medicine.[7] Leaves extract of *M. malabathricum* reported has activity as an antioxidant, hepatoprotective agent,[3] antiinflammation,[8,9] antinociceptive,[10] antimicrobial,[11] and wound healing.[12]

Extraction method can affect the quality and constituents of extracts.[13] Thus, the extraction method and appropriate solvents need to be optimized. The aim of this study was to evaluate total phenolic contents and quercetin level of *M. malabathricum* leaves extracts with different extraction methods. As the plant used traditionally as wound healing, this study also evaluated elastase inhibitory activity on human neutrophil. Elastase is one of neutrophil-derived protease that plays role in wound healing, but excessive production of this protease caused damage to tissue in wound healing process.[14] Thus, an inhibitor of elastase is needed to restore elastase-anti elastase imbalance.[15]

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MATERIALS AND METHODS

Chemicals

Ethanol and chloroform used for extraction were purchased from Brataco, Indonesia. Methanol, acetonitrile and acetic acid (HPLC grade) and also ethanol (analytical grade) were purchased from Merck, Germany. Quercetin (certified reference material) and gallic acid were purchased from Sigma Aldrich, Singapore. Elastase inhibitor screening kit was obtained from Abcam, Cambridge, UK.

Plant materials and Preparations of Leaves Extracts

Melastoma malabathricum L. leaves were collected from Samboja, Kutai Kertanegara, East Kalimantan, Indonesia. The leaves were identified in Indonesian Institute of Science, Bogor, West Java, Indonesia. The leaves were air dried under a black cloth to prevent direct sunlight. The dried leaves were grounded with an electric grinder. The powder was then extracted with 70% and 96% ethanol by maceration and reflux method according to methods below.

Maceration

Four portions of powdered leaves (25 g each) were prepared. Two portions were pre-extracted with chloroform (1:10 w/v, 3x24h) to obtain chloroform extracts (MCh). These two portions were next separately macerated with 70% and 96% (v/v) ethanol (1:10 w/v, 3x24h) to obtain defatted macerated ethanol extracts (70MChE and 96MChE, respectively). Two another portions were macerated separately with the same proportions solvent to obtained crude macerated ethanol extract (70ME and 96ME).

Reflux

Powdered leaves were sampled duplo (25 g each). Two samples were separately refluxed triply with 70% and 96% (v/v) aqueous ethanol (1:10 w/v, 2h) to give crude refluxed ethanol extract (70RE and 96RE).

Determination of percentage yield (%)

The organic solvent was evaporated from the extracts using a vacuum rotary evaporator (Buchi Rotavapor R-100, Japan). The extracts dried in the oven under vacuum (50°C). The dry weight of each extract and sample powders were used to calculate percentage yield of the extract by the formula:

$$\text{Yield of extraction}\left(\%\frac{w}{w}\right)=\frac{(\text{weight of dried extract})}{(\text{weight of sample powder}}\times 100$$

Determination of total phenolic content (TPC)

The total phenolic contents (TPC) was determined according to the Folin-Ciocalteu method using microplate reader.[16] Sample (25 µL) and 25% Folin-Ciocalteu (100 µL) were pipetted to flat bottom 96-well microplate (Nunc, Denmark) and were homogenized with a shaker for 60 sec and left for 4 min at room temperature in the dark. Then 75 µL sodium carbonate solutions were added and shaken for 60 sec. The mixture then incubated for 2 h at room temperature in the dark. Absorbance was measured at 750 nm wavelength using microplate reader (Versamax™ ELISA Microplate Reader, USA). Gallic acid stock solution with concentration 100, 50, 25, 12.5 and 6.25 ppm was used to the obtained calibration curve. The TPCs of the extracts were calculated from the absorbance of the sample and calibration curve. The TPCs were expressed as mg gallic acid equivalents (GAE) per g dry weight of the extracts (mg GAE/g DW extracts).

Determination of quercetin content

Quercetin contents of the extracts determined by high performance liquid chromatography (HPLC) method adapted from Ang *et al.* (2014) with slight modification.[17] HPLC analysis was performed using Shimadzu LC-20AD (Japan) and reversed phase column packed Hypersil Gold C18, 150x4.6 (mm) with 5 µm diameter particles. The mobile phase consists of acetonitrile-2% acetate acid (40:60, v/v) was filtered through a 0,45 µm nylon membrane filter (Whatmann) and degassed ultrasonically for 10 min prior to use. The dry weights extracts (20 mg) were diluted accurately in 5 mL methanol HPLC. The samples were filtered through a PVDF syringe filter (25 mm, 0.45 µm, Agilent) before injection. An injection volume of the samples was 20 µL, the flow rate was 1 mL/min and detection was done at 370 nm using a diode array detector. Quercetin content of the extract was determined by plotting the area of the peaks with a calibration curve of the standards. Calibration curves were established by plotting of the areas of peaks against eight concentration of quercetin standards (500-3.90625 µg/mL).

Anti elastase activity *in vitro* assay

Neutrophil elastase (NE) inhibitor activity assay was performed according to the protocol provided in the kit. Briefly, 200 ppm of each extract were prepared as sample tests and quercetin standard was prepared as a control. A 50 µL diluted NE solution and a 25 µL sample tests were mixed in 96-well black microplate (Thermo) and incubated at 37°C for 5 min. A 25 µL assay buffer was used in enzyme control well. After that, 25 µL substrate was mixed to each sample. The plate was measured in a fluorometric microplate reader (Glomax) at Ex/Em 400/505 nm (R1). Then, the plate was incubated at 37°C for 30 mins protected from light and measured again at Ex/Em 400/505 nm (R2). Relative activity of each sample test was calculated as:

$$\text{Inhibition activity}\,(\%)=\frac{(\Delta R\ \text{sample test})}{(\Delta R\ \text{enzyme control})}\times 100$$

Statistical analysis

Results were reported as means ± standard deviation (SD). The statistics (one-way analysis of variances, HSD Tukey's tests and Pearson correlation) were performed using SPSS (IBM Statistics) with α 0.05.

RESULTS

Percentage yield of extract

The results showed that yield of extraction with 70% ethanol was higher than extraction with 96% ethanol. The refluxed extract was also showed a higher percentage of yield than macerated extract in both solvents. The highest percentage of yield was 70RE, followed by 70MChE, 70ME, 96RE, 96ME and 96MChE. The result of the percentage yield is given in Figure 1.

Total Phenolic Content

The results of TPC are shown in Table 1. The crude extract showed that 96% ethanol resulted in higher TPC value than 70% ethanol in two extraction method. However, after defatted, 70MChE gave the highest TPC with the value of 222.85 mg GAE/g.

Determination of Quercetin

The chromatogram of quercetin standard and *M. malabathricum* leave extract are shown in Figure 2. The result of the quercetin content of the extracts is given in Table 1. Extraction methods (maceration and reflux) did not affect quercetin content in the extracts. However, the 96% ethanol

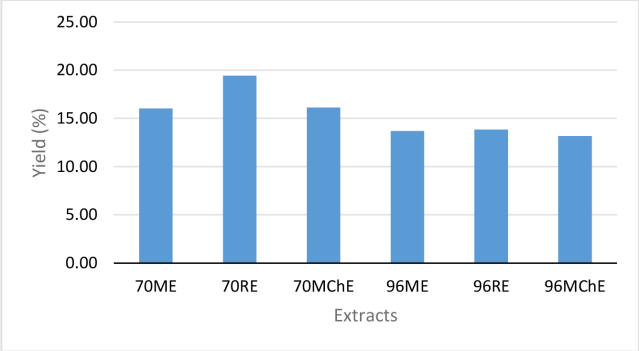

Figure 1: Percentage of yield [Extract code: crude ethanol extract from maceration (70ME and 96ME), defatted ethanol extracts from maceration method (70MChE and 96MChE), crude ethanol extract from reflux (70RE and 96RE)].

Table 1: Total Phenolic and quercetin contents of *M. malabathricum* leave extracts from different extraction methods.

Sample	Total Phenolic Content (mg GAE/g)	% Quercetin (w/w)
70ME	167.43 ± 15.34	0,430 ± 0,041
70RE	145.80 ± 8.68	0,439 ± 0,070
70MChE	222.85 ± 10.81	0,358 ± 0,010
96ME	171.09 ± 3.19	0,877 ± 0,034
96RE	212.95 ± 4.37	0,861 ± 0,036
96MChE	216.50 ± 1.80	0,873 ± 0,027

extract from both extraction method (96ME and 96RE) is contained quercetin higher than 70% ethanol extract (70ME and 70RE). Beside that, defatted process did not influence the quercetin content in the extract.

Anti-elastase activity

Each extract at a concentration of 200 ppm was used as a sample test. The result of the anti-elastase activity of the extracts are given in Table 2. The 70% ethanol extracts have slightly less activity than 96% ethanol extracts from both methods except reflux, but not significantly different (p>0.05). The result showed that the highest inhibition was from 70RE which also significantly different with 70ME and 70MChE. Inhibition of elastase activity of quercetin was higher than the extracts. Statistical analysis showed that anti-elastase activity of 70ME, 70MChE, 96ME, 96RE and 96MChE are significantly different from quercetin (p<0.05).

DISCUSSION

The quality and phytoconstituents of extracts are influenced by factors such as extraction methods, the solvent used for extraction and solvent ratio. Therefore this study used two conventional extraction methods, maceration and reflux. The yield of extraction from the reflux method was higher than maceration in this study. This is similar to the previous study in which heating increase the yield of *Raphanus sativus* leaves extract.[13]

The solvent used in this study was 70% and 96% aqueous ethanol because ethanol was a universal solvent which can extract many constituents, such as polar compounds (amino acid, glycoside compounds), polar phenolic

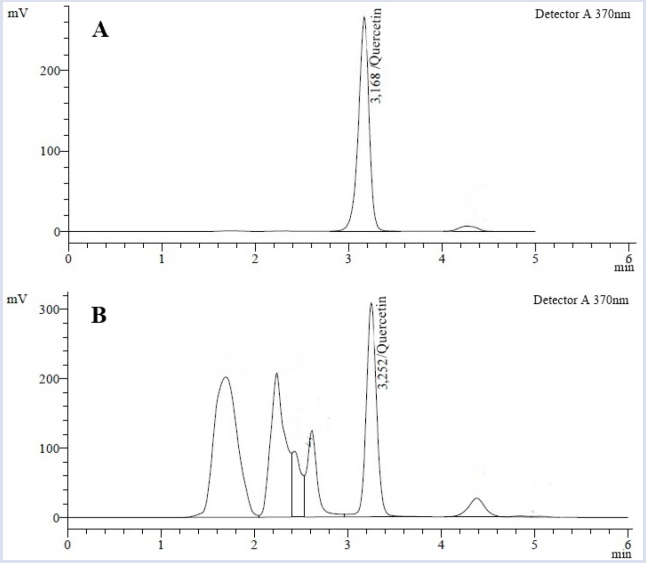

Figure 2: Chromatogram of quercetin standard (A) and *M. malabathricum* leave extract (B).

Table 2: Anti-elastase activity of *M. malabathricum* leaves extracts from different extraction methods.

Samples	Elastase inhibition (%)
Quercetin	93.86 ± 0.79
70ME	81.98 ± 0.21*
70RE	89.50 ± 1.53
70MChE	82.18 ± 0.38*
96ME	84.79 ± 0.63*
96RE	84.27 ± 2.50*
96MChE	85.29 ± 1.14*

*Significantly difference compare to quercetin (p<0.05).

compounds, aglycon flavonoids, anthocyanin, terpenoid, saponin, tannin and polyphenol.[18] Ethanol was also can extract quercetin with a three-fold yield higher than ethyl acetate and hexane.[19]

Phenolic is semi-polar compound. The total phenolic content of *M. malabathricum* in this study was tending to soluble in the semi-polar solvent, which 96% ethanol extract has higher TPC than 70% ethanol extract. Other study showed that aqueous semi-polar organic solvents are best to extracted flavonoid and phenolic contents of clove bud.[20] Phenol contents of tropical seagrass were also soluble in polar and semi-polar solvent.[21] Aqueous methanol (50% and 75% v/v) also had the highest TPC of *Gaultheria procumbens* leaves extracts.[22]

Generally, 96% ethanol can extract the quercetin stronger than 70% ethanol. Where, the highest quercetin level of *M. malabathricum* leaves resulted from 96MChE. Quercetin is a hydrophobic compound and soluble in the aqueous alcoholic solvent. The solubility of quercetin increase with increasing alcoholic contents.[23] The result of this study was also similar to the previous study which quercetin level of the extract was optimum with 80% ethanol or higher.[24]

Crude extracts contained ballast contents which can interfere with physical characteristics and biological activity. Therefore, a few study

defatted leaves and fruits powders before further extraction.[22,25] In this study, defatted extract (70MChE) shows the highest TPC and the highest quercetin (90MChE). This result was similar to another study which after pre-extraction with chloroform, methanolic extracts of eastern teaberry leaves give increasing TPC level with slightly decreasing yield.[22]

The highest inhibitory activity on elastase is shown by the 70RE sample. The result showed that the quercetin is not the only one responsible for the anti-elastase activity. This phenomenon is strengthened by the results of the correlation analysis between quercetin and anti-elastase activity that show weak positive correlation (r=0.110). Previous studies showed that rutin and kaempferol contents in the leaves extracts were higher than quercetin content.[26,27] There is a possibility the activity is due to these compound. The extracts also contained many compounds which can affect its biological activity including anti-elastase activity. Melzig, Pertz and Krenn (2001) stated that flavonoid, such as quercetin, hyperoside and isoquercetin, contributed to the elastase inhibition activity of *Drosera madagascariensis* extract.[28] Quercetin has the anti-elastase activity,[29] which is also shown in this study. There is needs further study to screening the responsible compounds which affect the anti-elastase activity of *M. malabathricum* leaves extracts.

CONCLUSION

Extraction methods and different concentration of solvents affect the total phenolic and quercetin contents of the extracts. The highest total phenolic content was resulted from defatted process of extraction, while the highest quercetin content was resulted from extraction with 96% alcohol. *M. malabathricum* leaves have potential effect as anti-elastase as well as quercetin, but the anti-elastase activity of *M. malabathricum* leaves is not only due to quercetin.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

70MChE: Defatted macerated 70% ethanol extract; **70ME**: Macerated 70% ethanol extract; **70RE**: Refluxed 70% ethanol extract; **96MChE**: Defatted macerated 96% ethanol extract; **96ME**: Macerated 96% extract; **96RE**: Refluxed 96% ethanol extract; **GAE**: Gallic Acid Equivalents; **HPLC**: High Performance Liquid Chromatography; **MCh**: Chloroform extact; **NE**: Neutrophil Elastase; **TPC**: Total Phenolic Contents.

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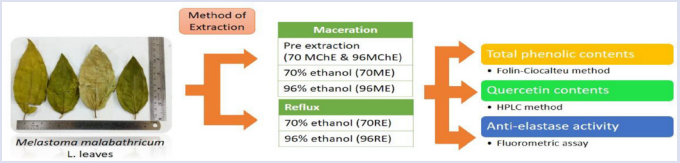
GRAPHICAL ABSTRACT



SUMMARY

- *M. malabathricum* leaves were extracted with two conventional methods (maceration and reflux) and different ethanol concentration (70% and 96%). Leaves were also defatted with chloroform. The highest total phenolic content resulted from the defatted extract, 70MChE (222.85±4.37 mg GAE/g) and the highest quercetin content resulted from maceration with 96% ethanol (96ME, 0.877±0.034 % w/w). The highest anti-elastase activity resulted from 70RE with 89.50±1.53. There is a weak positive correlation between quercetin and anti-elastase activity, that can be concluded that the activity of *M. malabathricum* is not only due to quercetin.

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