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Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method

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ABSTRACT

Moringa oleifera L. (Moringaceae) has been used as traditional medicines in many tropical and subtropical countries. Having phenolics and flavonoids as major constituents, the leaf extract has been reported to exhibit antioxidant activity both *in vitro* and *in vivo*. To obtain the maximum yields of these compounds, which consequently influence the antioxidant activity, varying extraction methods were examined. Squeezing, decoction, maceration, percolation and soxhlet extraction were used to extract fresh and dried leaves of *M. oleifera*. Distilled water was used in squeezing and decoction, while 50 and 70% ethanol were used in the other methods. The contents of total phenolics and total flavonoids, free radical scavenging activity and ferric reducing power (FRP) of each extract were quantitatively determined. Quantitative analysis of active compounds was accomplished through high performance liquid chromatography (HPLC). Extract from the most effective extraction method was then selected for reactive oxygen species assay (ROS) in HEK-293 cells. Maceration with 70% ethanol of dried leaves promoted the extract with maximum amounts of total phenolics (13.23 g chlorogenic acid equivalents/100 g extract) and total flavonoids (6.20 g isoquercetin equivalents/100 g extract). This extract also exhibited high DPPH-scavenging activity (EC_{50} 62.94 μg/mL) and the highest FRP value (51.50 mmol $FeSO_4$ equivalents/100 g extract). At the concentration of 100 μg/mL, the extract could significantly reduce relative amount of intracellular ROS. The contents of major active components, crypto-chlorogenic acid and isoquercetin, in the dried plant powder were 0.05 and 0.09% (w/w), respectively. Considering various factors involved in the extraction process, maceration with 70% ethanol was advantageous to other methods with regards to simplicity, convenience, economy, and providence of the extract containing maximum contents of total phenolics and total flavonoids with the highest antioxidant activity. Maceration and 70% ethanol were recommended as the extraction method and solvent for high quality antioxidant raw material extract of *M. oleifera* leaves for pharmaceutical and nutraceutical development.

1. Introduction

Extraction of plant materials depends on various factors such as solvents, methods, and extraction time to separate different quality and quantity of bioactive components in the crude extracts (Hayat et al., 2009). In addition, the nature of the sample matrix and the compounds to be extracted also substantially affect the efficiency of extraction (Mustafa and Turner, 2011). Theoretically, the optimal extraction method should be simple, rapid, economical and applicable to a large scale industry (Pothitirat et al., 2010).

Various parts of *Moringa oleifera* Lam. (moringa) of the Family Moringaceae have been used as herbal medicines in tropical and subtropical countries such as India, Pakistan, the Philippines, Thailand and Africa. The leaves, flowers and immature pods of this plant are used as high nutritive supplement with various pharmacological properties (Anwar et al., 2007; Chumark et al., 2008; Anjula et al., 2011). Moreover, moringa have long been recognized in the Ayurvedic and Unani systems of medicine for prevention and treatment of several diseases, *e.g.*, gastric ulcers, skin diseases, hay fever, fatigue and bronchitis (Anwar et al., 2007). The leaf extracts of *M. oleifera* have been reported to exhibit antioxidant activity both *in vitro* and *in vivo* due to abundant phenolic acids and flavonoids (Chumark et al., 2008; Verma et al., 2009). Some

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researchers claimed that moringa leaves were rich in chlorogenic acid, gallic acid, kaempferol and quercetin glycosides (Bennett et al., 2003; Brahma et al., 2009).

Recently, moringa leaves have become popular as a dietary supplement and herbal medicine. Therefore, it would be of nutritional benefit to determine the appropriate extraction method and solvent that promote the extract with high biological activities and high yield of active compounds for nutraceutical production. Thus, this work aimed to probe the appropriate extraction method and solvent that would promote *M. oleifera* leaf extract with the highest contents of total phenolics, total flavonoids among other major active compounds, and the highest antioxidant activity. The results from this experiment could be used as the guidance for further standardization and application of moringa leaf extract in pharmaceutical/nutraceutical production.

2. Materials and methods

2.1. Chemicals

Isoquercetin, crypto-chlorogenic acid, chlorogenic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), penicillin, streptomycin, dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dichlorfluorescein-diacetate (DCFH-DA), phosphate buffered saline pH 7.4 (PBS) and triton X-100 were obtained from Sigma–Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent, potassium ferricyanide and ferric chloride were obtained from Fluka Biochemika (Steinheim, Germany). Aluminum chloride, trichloroacetic acid, potassium dihydrogen phosphate and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). Sodium bicarbonate, and ferrous sulfate were purchased from Ajax Finechem (NSW, Australia), and Carlo Erba (Val de Reuil, France), respectively. Other chemicals and solvents were of analytical grade and purchased from Labscan Asia (Bangkok, Thailand), except for 95% ethanol which was obtained from the Excise Department, Bangkok, Thailand and was distilled before use.

2.2. Plant materials

The mature leaves of *M. oleifera* were collected from Baan Klang Sub-District, Muang District, Pathum Thani Province, Thailand in October 2010. The samples were identified by Dr. W. Gritsanapan and the voucher specimens (BVMO011010) were deposited at Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The leaves were cleaned by tab water and a portion was dried in a hot oven at 60 °C for 24 h. The dried samples were ground and passed through a sieve (20 mesh). The powdered drugs were kept in sealed containers and protected from light until used. Another portion of fresh sample was used for squeezing, decoction, and maceration.

2.3. Investigation of suitable method for extracting of M. oleifera leaves

Several extraction methods were performed using 50 and 70% (v/v) ethanol as solvents, except for squeezing and decoction in which distilled water was used. Each extraction was repeated many times until exhaustion. Each method was done in triplicate.

2.3.1. Squeezing (SZ)

The fresh leaves of *M. oleifera* were extracted by mincing with distilled water (1:10, w/v) and the mixture was squeezed and filtered through muslin cloth and Whatman No. 1 filter paper. The filtrate was lyophilized to yield a freeze-dried squeezing leaf extract (SZ).

2.3.2. Decoction of fresh leaves (DF)

The fresh leaves were minced into small pieces, boiled with distilled water (1:10, w/v) at 100 °C for 30 min and filtered through a Whatman No. 1 filter paper. The marc was repeatedly extracted until exhaustion.

2.3.3. Decoction of dried leaves (DD)

The dried powdered leaves was boiled with distilled water (1:10, w/v) at 100 °C for 30 min and then filtered. The marc was re-extracted until exhaustion.

2.3.4. Maceration of fresh leaves (MF70)

The fresh leaves were minced into small pieces and macerated with 70% ethanol (1:20, w/v) for 72 h at room temperature (28 ± 2 °C) with occasional shaking. The extract was filtered and the marc was re-macerated with the same solvent until the extraction was exhausted.

2.3.5. Maceration of dried leaves (MD50 and MD70)

The dried powdered leaves were separately macerated with 50 and 70% ethanol (1:40, w/v) for 72 h at room temperature (28 ± 2 °C) with occasional shaking. The extract was filtered and the marc was re-extracted by the same process and solvent until the extraction was exhausted.

2.3.6. Percolation of dried leaves (PD50 and PD70)

The dried powdered leaves were separately mixed with 50 and 70% ethanol (1:5, w/v) and the mixture was allowed to stand for 1 h. Then the mixture was transferred to a percolator, and 50 and 70% ethanol was added (final proportion of 1:10, w/v). The extraction was done at room temperature with a flow rate of 1 mL/min until the percolation was exhausted.

2.3.7. Soxhlet extraction of dried leaves (SD50 and SD70)

The dried powdered leaves were separately placed into a thimble and were extracted with 50 and 70% ethanol (1:50, w/v) in a soxhlet apparatus. Extraction was carried out at five cycles/h until exhaustion (20 h).

The combined extract from each extraction method (except squeezing) was separately filtered through a Whatman No. 1 filter paper. The filtrate was dried under reduced pressured at 50 °C using a rotary vacuum evaporator. The crude extract was weighed and kept in a tight container protected from light.

2.4. Determination of total phenolic compounds content

The content of total phenolic compounds was determined using Folin-Ciocalteu procedure (Pothitirat et al., 2009). Each sample (1000 μg/mL), 200 μL was mixed with 500 μL of the Folin-Ciocalteu reagent (diluted 1:10 with deionized water) and 800 μL of sodium bicarbonate solution (7.5%, w/v). The mixture was allowed to stand at room temperature for 30 min with intermittent shaking. The absorbance was measured at 765 nm using a UV–Visible (UV–VIS) spectrophotometer (PerkinElmer, USA). The content of total phenolic compounds was calculated as mean ± SD ($n = 3$) and expressed as grams of chlorogenic acid equivalents (CAE) in 100 g of the extract and dried powder.

2.5. Determination of total flavonoid compounds content

Total flavonoids were analyzed using aluminum chloride colorimetric method (Pothitirat et al., 2009). Sample (1000 μg/mL)