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Full Length Research Paper

Effects of extraction techniques on phenolic components and antioxidant activity of Mengkudu (*Morinda citrifolia* L.) leaf extracts

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The effect of four extraction techniques namely; solvent extraction (SE), microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE) and supercritical fluid extraction (SFE) on the phenolic components and antioxidant activity of *Morinda citrifolia* leaf extracts was investigated. Total phenolics compounds (TPC) were quantified spectrophotometrically using Follin-Ciocalteu regent method, while the catechins were analyzed by reverse-phase high performance liquid chromatography (RP-HPLC). Antioxidant activity of the extracts was evaluated by determining 2,2-diphenyl-1-picrylhydrazyl (DPPH) capacity and ferric ion reducing antioxidant potential (FRAP). Overall, MAE produced extract exhibited the maximum amount of TPC and catechin while UAE-extract had the highest antioxidant activity. It could be concluded that, even though MAE extract contained high TPC and catechin, the accelerated temperature used during this extraction technique might have attributed to reduce the antioxidant activity of this extract. Therefore, UAE can be recommended for recovery of potent natural antioxidant components from *M. citrifolia* leaf offering better antioxidant activity.

Key words: *Morinda citrifolia* leaf, antioxidant extraction, total phenolics, ferric reducing potential, 2,2-diphenyl-1-picrylhydrazyl (DPPH), catechin, high performance liquid chromatography (HPLC).

INTRODUCTION

Currently, there is much interest in the functional food uses of plant-based natural antioxidant phenolics, especially derived from fruits, vegetables, wine, tea and cocoa due to their medicinal benefits (Block et al., 1992; Yilmaz, 2006; Cooper et al., 2008; Fernandez-Panchon et al., 2008).

A number of potential health-related biological activities including anti-cancer, antimicrobial and anti-inflammatory and antioxidants have been ascribed to phenolic compounds such as phenolic acids and flavonoids (Valko et al., 2006; Yilmaz, 2006). It is widely accepted that dietary intake of natural antioxidants such as phenolics, vitamin C rand tocopherols is strongly linked with the reduced incidence of oxidative-stress related degenerative diseases namely; cancer, cardiovascular disorders, inflammation and aging (Uttara et al., 2009; Yilmaz, 2006)

One of the potential sources of antioxidants is *Morinda citrifolia*, a tropical herb that has been used as a source of tradition medicine in the folk remedies for over 2000 years. A native of Southeast Asia and Australia, this small evergreen tree or shrub is now widely distributed throughout the tropical world. Commercially known as noni, the plant is known by different names, for example, Indian mulberry (English), “nen nin” (Marshall Islands,

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Chuuk), “non” (Kiribati), canary wood (Australia) and Mengkudu (Malay) (Johansson, 1994; Abbott and Shimazu, 1985; Kuck and Tongg, 1958). All parts of *M. citrifolia* such as fruit, leaf, bark and root have been reported to have medicinal properties including anti-diabetes, anticancer and able to reduce hypertension (Chan-Blanco et al., 2006).

Previous study in our laboratory has revealed that *M. citrifolia* extracts exhibited excellent antioxidant property as compared to that of synthetic antioxidants (Zin et al., 2003). The functionality and medicinal properties of *M. citrifolia* might be attributed to its phytochemicals such as, polyphenols (Deng et al., 2008).

Extraction is a process used to isolate or separate the components from their original matrix. Several factors such as, oxidizing agent, pH and temperature can affect the quality of the extract produced by an extraction process (Tsao and Deng, 2004). Wang and Weller (2006) reviewed different techniques such as solvent extraction (SE), microwave-assisted extraction (MAE), ultrasonic assisted-extraction (UAE) and supercritical fluid extraction (SFE), applicable, for the extraction of nutraceutical compounds. A good extraction technique is expected to be less time consuming and offers high yield of active compounds without sacrificing their functionality.

Since *M. citrifolia* consists of highly complex profile of phytochemicals which are usually sensitive to light, heat and also exist at very low concentrations, utilization of an appropriate extraction technique in isolating such valuable compounds can be quite challenging. Thus, the objective of the present study was to evaluate the efficacy of different extraction techniques namely; SE, SFE, MAE and UAE towards recovery of total phenolic compounds and flavonoids (catechins) as well as the antioxidant activity of the extracts produced from *M. citrifolia* leaf.

MATERIALS AND METHODS

Standards and chemicals

(+)-Catechin and (-)-epicatechin, trifluoroacetic acids (TFA), Follin-Ciocalteu’s phenol reagent, gallic acid and sodium carbonate were obtained from Sigma (St. Louis, USA). Methanol (HPLC grade) and 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) were purchased from Merck (Germany). Hydrochloric acid was purchased from Malinckrodt Baker Inc. (Kentucky, USA). Ethanol was purchased from Fisher (New Jersey, USA).

Sample preparation

Fresh *M. citrifolia* leaves were obtained from the botanical garden of the Institute of Bioscience, Universiti Putra Malaysia, Selangor, Malaysia.

Mature leaves were cleaned and washed under running tap water before being oven-dried (Memmert UFB-50, GmbH, Germany) at 40˚C for 48 h. Dried leaves were then ground to powder and stored at -20ºC for future analysis.

Solvent-extraction

The technique using solvent extraction (SE) was carried out based on modified conditions previously proposed by Chang et al. (1977). Ten gram of *M. citrifolia* leaf powder was weighed and added with 100 mL absolute ethanol in a 250 mL Erlenmeyer flask (GmbH, Germany).

The extraction was carried out in a shaking water bath (Protech, Selangor, Malaysia) at 40ºC, at 100 rpm for 2 h. The supernatant was then filtered using cellulose filters paper (Whatman No. 1) and the residue re-extracted. The supernatants were pooled and solvent was removed under vacuum at 40°C using rotary evaporator (Eyela, Tokyo Rikikai Co. Ltd, Japan).

Microwave-assisted extraction

This extraction was performed based on the modified technique previously proposed by Pan et al. (2003). Ten gram of *M. citrifolia* leaf powder was weighed and added to 100 mL ethanol into a 250 mL Erlenmeyer flask (GmbH, Germany). The samples were then heated using microwave (National 700W, Malaysia) with medium irradiation for three min. The supernatant was then filtered using Whatman cellulose filters paper No. 1 and the residue re-extracted. The supernatant was pooled and solvent removed under vacuum at 40°C using rotary evaporator.

Ultrasound-assisted extraction

In this technique, 10 g of *M. citrifolia* leaf powder was weighed and mixed with 100 mL ethanol in a 250 mL Erlenmeyer flask (GmbH, Germany).

The extraction was carried out using Selecta ultrasonic water bath (Barcelona, Spain) for 30 min. The supernatant was then filtered with Whatman cellulose filters paper No. 1 and the residue re-extracted. The supernatant was pooled and solvent removed under vacuum at 40°C using rotary evaporator.

Supercritical fluid extraction

Fifty g of *M. citrifolia* leaf powder was accurately weighed and placed in extraction vessel. A Thar-50 (Pittsburgh, USA) SFE unit was used for extraction purposes. Carbon dioxide and ethanol were pumped into the extraction vessel at a flow rate of 15 and 2 g/min, respectively. The critical temperature and pressure employed were 50°C and 20 MPa, respectively for more than 3 h, with the collector set at 30°C. The solvent was removed under vacuum using rotary evaporator at 40°C.

Total phenolics compounds

Total phenolics compounds (TPC) were determined using the Folin-Ciocalteu’s phenol reagent according to the method as proposed by Singleton and Rossi (1965). The extract was accurately weighed and dissolved in methanol. An aliquot, 0.5 mL of extract was added to 0.5 mL of Follin-Ciocalteu’s phenol reagent and vortex for 10 s, followed by addition of 10 mL of 7% sodium carbonate to the mixture.

The mixture was then allowed to react at room temperature (28 ± 2°C) for 60 min. The absorbance was then measured at 715 nm using UV-1650 PC UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The amount of total phenolics was expressed as milligrams of gallic acid equivalents (GAE) in 100 g extract.

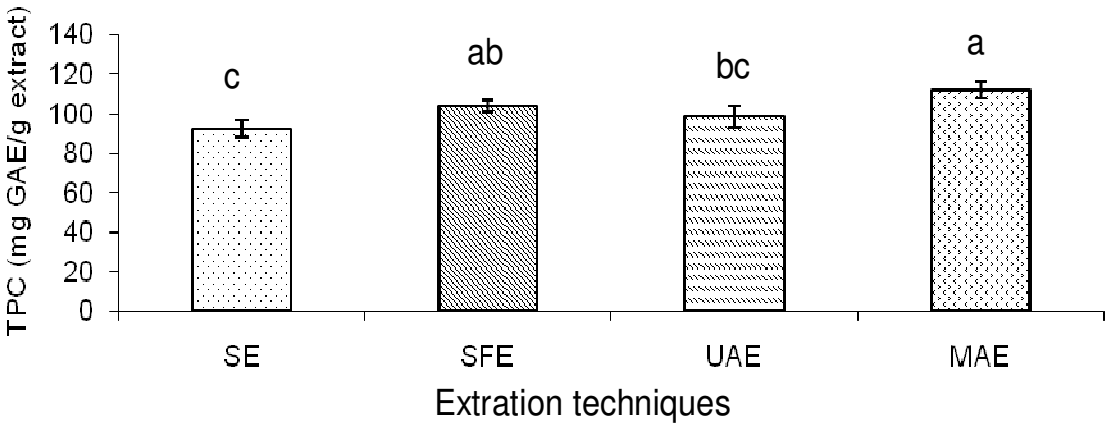

Figure 1. Total phenolic content (TPC) of *M. citrifolia* leaf extracts obtained from various extraction protocols. Data represents mean ± standard deviation. Means followed by different letters are significantly different at ($p < 0.05$) according to Duncan's multiple range test. n = 3. SE, solvent extraction; SFE, supercritical fluid extraction; UAE, ultrasonic-assisted extraction; and MAE, microwave-assisted extraction.

Catechins HPLC analysis

The hydrolysis of the test samples was carried out according to method proposed by Hertog et al., (1992). Briefly, fifty mg of extracts was dissolved in 24 mL methanol and homogenized. Sixteen mL distilled water was then added followed by 10 mL of 6 M HCl. The mixture was then thermostated for 2 h at 95ºC. The final solution was filtered using a 0.45 µm nylon membrane filter prior to high performance liquid chromatography (HPLC) analysis.

The HPLC analysis was conducted using Waters HPLC system equipped with Waters 2487 dual wavelength absorbance detector, Waters 600 Pump and controlled by Waters empower 2 software (Waters, Milford, MA). Separations were carried out using Waters reverse-phase (RP) Symmetry C_{18} column (150 x 3.9 mm, 5 µm) at room temperature. The mobile phase consisted of deionized water with TFA (pH 2.5) as solvent A and absolute methanol (99.99%) as solvent B. The gradient used was as follows: 100 to 50% solvent A (0 to 20 min), 50 to 40% solvent A (20 to 30 min) and 40 to 100% solvent A (30 to 40 min). The mobile phase flow rate was kept at 1.0 mL/min and the detector was set at 280 nm. Peak identification and quantification were carried out based on comparison of retention time and area of standards, respectively.

Antioxidant evaluation

Free radical scavenging capacity

The free radical scavenging capacity was determined using stable free radical, 2,2-diphenyl-1-picrylhdrazyl (DPPH) according to the method proposed previously (Brand-Williams et al., 1995). The extract was dissolved in methanol and 0.5 mL of extract at different concentration was added to 3.5 mL DPPH solution (25 µg/mL). The mixture was left to stand at room temperature (28 ± 2°C) for 30 min. The absorbance was measured at 517 nm using UV-1650 PC UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). Percent free radical scavenging capacity (%I) was calculated as a percentage of DPPH inhibition using the equation:

$$\% I = ((A_{blank} - A_{extract}) / A_{blank}) \times 100 \quad (2)$$

where, A_{blank} is the absorbance of the DPPH solution with methanol and $A_{extract}$ is the absorbance of the solution with extract is expressed as IC_{50}, the concentration of extract required to inhibit fifty percent of DPPH. The IC_{50} value was calculated from the log-concentration of extract against percentage of inhibition.

Ferric reducing antioxidant potential (FRAP*).*

The ability of the extracts to reduce ferric ions was measured using a modified method as previously described by Benzie and Strain (1996). The FRAP reagent was prepared with 300 mM sodium acetate buffer at pH 3.6, 10 mM TPTZ solution and 20 mM $FeCl_3.6H_2O$ solution with ratio 10:1:1, respectively. 200 µl of the extract at 1000 µg/mL was added to 3 mL FRAP reagent and vortexed for 5 s. The mixture was incubated in water bath at 37°C for 30 min. The absorbance was measured at 590 nm using UV-1650 PC UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The reducing capacity was expressed in µM of Trolox equivalent antioxidant capacity (µM TEAC).

Statistical analysis

The data obtained in the study were analyzed using SPSS Version 16. Analysis of variance was performed by ANOVA procedure and significant ($p<0.05$) differences between means were determined using Duncan tests.

RESULTS AND DISCUSSION

Total phenolic content and catechins

In the present study, total phenolic compounds (TPC) and catechins were analysed in *M. citrifolia* leaf extracts produce by different extraction techniques. Figure 1 showed that the extract obtained from MAE (111.81 ± 4.16 mg GAE/g) had the highest TPC followed by that of SFE (103.80 ± 2.74 mg GAE/g), UAE (98.63 ± 5.25 mg GAE/g) and SE (92.39 ± 4.13 mg GAE/g). The highest

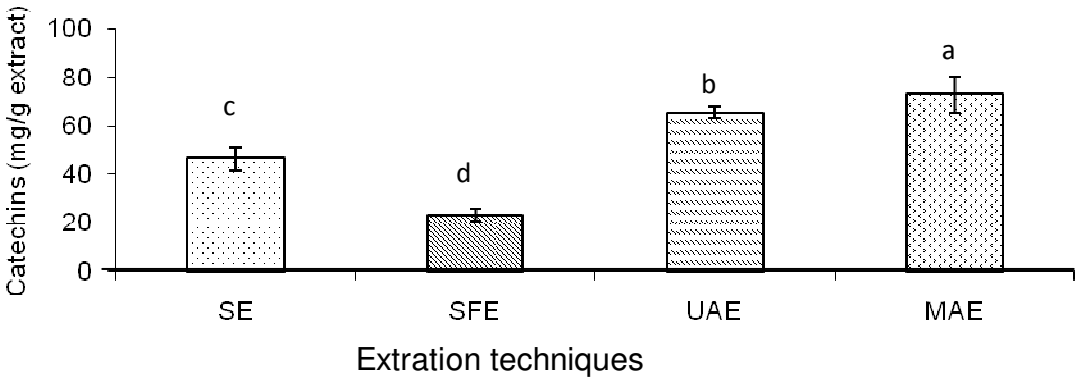

Figure 2. Catechin content of *M. citrifolia* leaf extracts obtained from various extraction protocols. Data represents mean ± standard deviation. Means followed by different letters are significantly different at ($p<0.05$) according to Duncan's multiple range test. n = 3. SE, solvent extraction; SFE, supercritical fluid extraction; UAE, ultrasonic-assisted extraction; and MAE, microwave-assisted extraction

catechin content was also seen in MAE extract (72.86 ± 7.33 mg/g), followed by UAE (65.52 ± 2.21 mg/g), SE (46.54 ± 4.98 mg/g) and SFE (23.35 ± 2.60 mg/g) extracts (Figure 2). The HPLC chromatogram for catechin separation is shown in Figure 3.

There is similarity in the trend for the TPC and catechin content of the extracts, where MAE extract had the highest level of these components, followed by UAE and SE extracts. Phenolic compounds in plants are potent antioxidants and have been shown to exhibit positive health effects towards cancer prevention, decreased hypercholesterolemia and lowering the risk for atherosclerosis (Bravo, 1998). During MAE, microwaves are generated into the system and polar solvent with high dielectric constant and high dissipation factor absorb microwave energy and transform it into heat. In the present study, MAE was conducted at moderate microwave irradiation for 3 min and the final temperature attained was 70°C. The high TPC and catechin content in MAE extract may be attributed to effective recovery of such compounds due to microwave effect as diffusion of bioactive compounds increased with temperature and polarity of the solvent.

Bioactive compounds in leaves are accumulated in vacuoles located inside the plant cell, surrounded by rigid cell wall (Marinova et al., 2007). High temperature and microwave energy may burst the cell wall and release the bioactive compounds into the ethanol. The energy from the microwave dissipates into polar molecule such as phenolic compounds and catechin, generating heat thus leading to enhancing their release from cell walls. As a consequence of heating effect, the components in the cell dissolve into the solvent more effectively, in accordance to the disruption theory (Kaufmann et al., 2001). This explains the highest TPC and catechin content in MAE extract. In SFE, high pressure up to 150 MPa is applied. The results of the study showed that the SFE extract consisted of significantly ($p<0.05$) higher TPC as compared to that of SE. This might be due to the fact that pressurized conditions allowed more phenolic compounds to be extracted from *M. citrifolia* leaf. Similar finding was reported where more vitamin E was extracted from *Silybum marianum* when pressurized liquid extraction was used (Hadolin et al., 2001).

Antioxidant activity

The effect of different extraction techniques on the antioxidant activity was determined using free radical scavenging capacity and ferric reducing antioxidant potential. The result of the study showed that higher DPPH free radical scavenging capacity (IC_{50}) was exhibited by UAE (0.92 ± 0.06 mg/ mL) and SE (0.86 ± 0.11 mg/ mL) extracts. In contrast, MAE (2.12 ± 0.04 mg/ mL) and SFE (2.50 ± 0.24 mg/ mL) extracts inhibited less amount of free radicals (Figure 4).

DPPH is a stable free radical that when reacts with antioxidants changes its color from purple to yellow. The IC_{50} revealed the concentration of extract required to scavenge half of the free radicals present there, a low IC_{50} value indicates that the extracts possess higher antioxidant activity. In this study, UAE and SE extracts showed the lowest IC_{50}, indicating highest antioxidant activity.

Besides, the results of antioxidant activity as measured by using FRAP method showed that most of the extracts demonstrated high antioxidant activity indicating no significant ($p>0.05$) difference among UAE (50.7 ± 5.2 µM TEAC/ mg extract), SFE (41.0 ± 1.3 µM TEAC/ mg extract) and MAE (40.5 ± 7.9 µM TEAC/ mg extract) extracts. Nevertheless, SE (33.2 ± 3.4 µM TEAC/ mg extract) extract revealed significantly ($p<0.05$) lower antioxidant activity as compared with others (Figure 5).

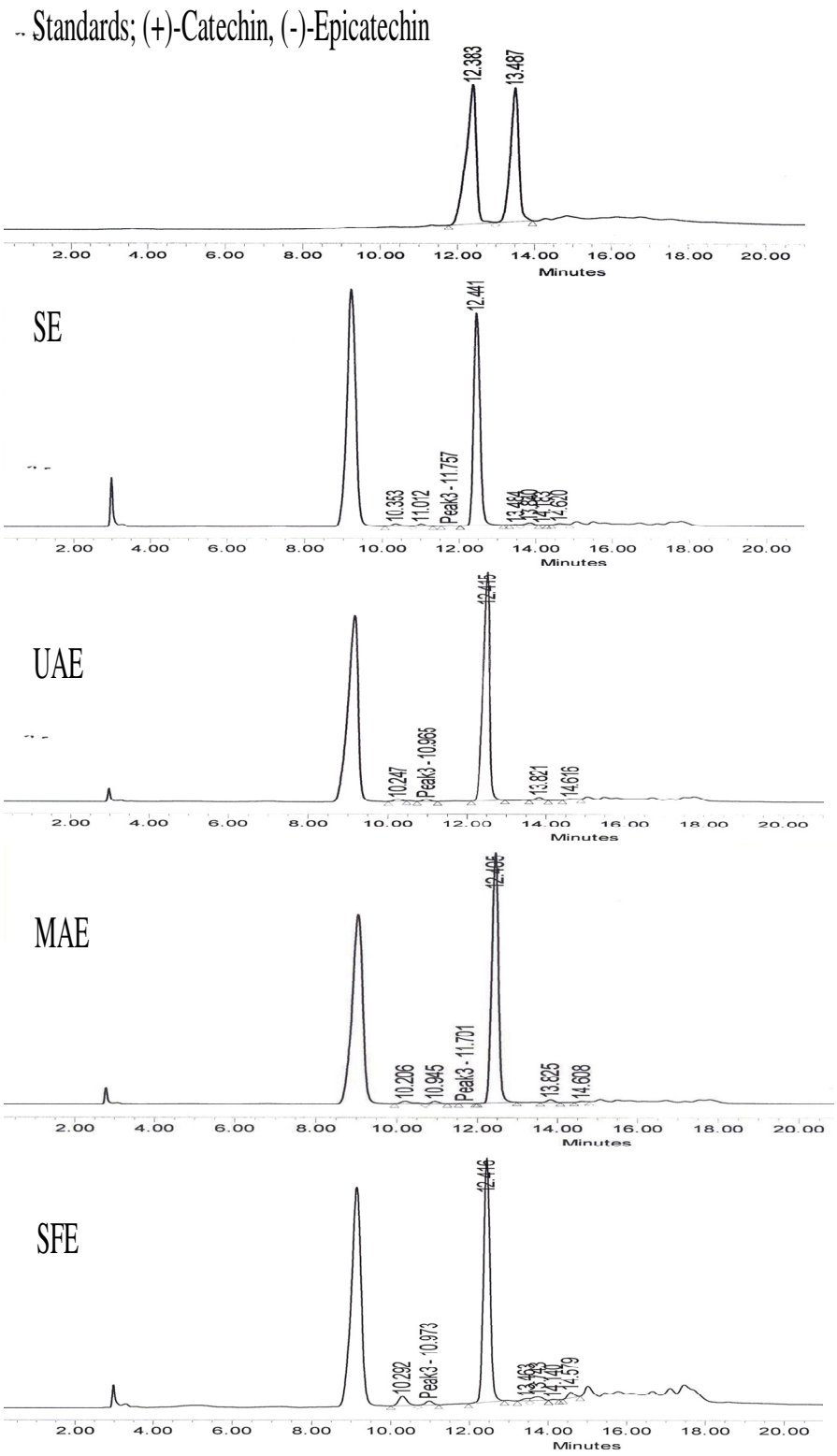

Figure 3. Chromatograms for flavonoids separation using HPLC. SE, solvent extraction; UAE, ultrasonic-assisted extraction; MAE, microwave-assisted extraction and SFE, supercritical fluid extraction. Condition of HPLC: solvent A=deionized water with TFA (pH 2.5), solvent B=absolute methanol (99.99%). The gradient used was as follows 100-50% solvent A, 0-50% solvent B (0-20 min), 50-40% solvent A, 50-60% solvent B (20-30 min) and 40-100% solvent A, 60-0% solvent B (30-40 min).

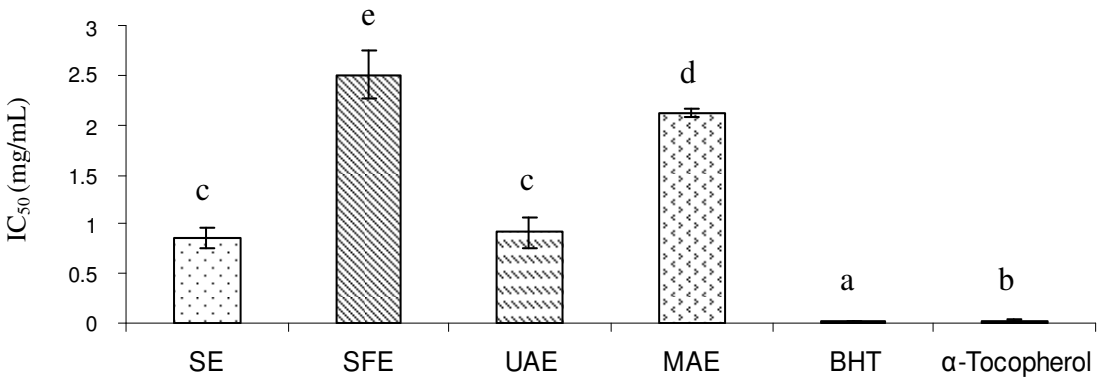

Figure 4. Free radical scavenging activity of *M. citrifolia* leaf extracts obtained from various extraction protocols**.** IC_{50} is the value needed in inhibiting fifty percent of free radical. Data represents mean ± standard deviation. Means followed by different letters are significantly different at ($p < 0.05$) according to Duncan's multiple range test. n = 3. SE, solvent extraction; SFE, supercritical fluid extraction; UAE, ultrasonic-assisted extraction; MAE, microwave-assisted extraction and BHT, Butylated hydroxytoluene.

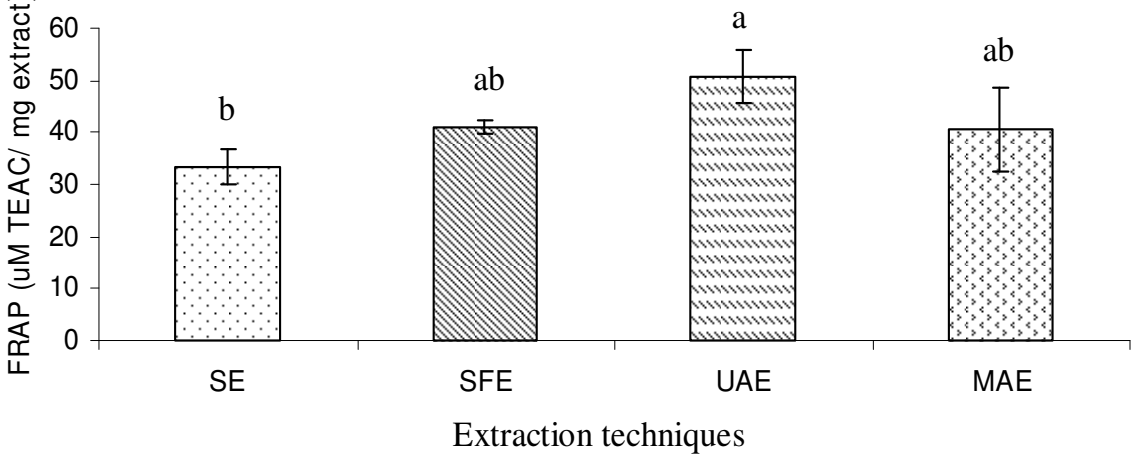

Figure 5. Ferric reducing antioxidant potential (FRAP) of *M. citrifolia* leaf extracts obtained from various extraction protocols. Data represents mean ± standard deviation. Means followed by different letters are significantly different at ($p < 0.05$) according to Duncan's multiple range test. n = 3. SE, solvent extraction; SFE, supercritical fluid extraction; UAE, ultrasonic-assisted extraction; and MAE, microwave-assisted extraction

The ability to chelate metal ion is one of the antioxidant mechanism, which can be used to determine the antioxidant activity of samples. The reducing capacity of extract was determined by evaluating their ability to reduce ferric ion to ferrous ion. In principle, antioxidants are able to donate a single electron to ferric-TPTZ (Fe (III)-TPTZ) complex and reduce the complex into ferrous-TPTZ (Fe (II)-TPTZ) complex (Benzie and Strain, 1996).

The antioxidant property of *M. citrifolia* leaf extracts may be attributed to the presence of bioactive compounds such as phenolics and catechin. Catechin was reported to exhibit antioxidant activity due to its hydrogen donating property, thus stabilizing free radicals (Rice-Evans et al., 1996). Catechin is one of the important flavonoids found abundantly in vegetables and plants and good correlations exist between high intake of this flavonoid compound and health benefits (Hertog et al., 1992). In addition, catechin has been considered as potent bioactive compounds that can be utilized in many food products (Yilmaz, 2006).

UAE extract exhibited significantly ($p<0.05$) higher free radical scavenging capacity as compared with that of MAE and SFE extracts. UAE extract also demonstrated higher FRAP value compared to all other extracts tested. This result is in contradictory since UAE extract consisted of lower concentration of bioactive compound measured

in the present study; TPC and catechin. Although, MAE extract contained higher TPC and catechin content as compared to UAE, exposure to high temperature during MAE extraction might have degraded the functionality of some bioactive compounds in the extract, thus reducing overall antioxidant activity. SE produced extract exhibited higher free radical scavenging capacity than that of MAE, even though it consisted of significantly ($p<0.05$) lower TPC and catechin.

Our present findings can be supported by a previous study where UAE was found to be the best technique for extracting antioxidants from soybean as compared to SE and Soxhlet, extraction due to its better efficiency (Chung et al., 2010).

In the present study, there was no significant correlation observed between antioxidant property of *M. citrifolia* leaf extract and TPC and flavonoid (catechin) content. There is also possibility that the antioxidant activity measured in the present study may partly be attributed to the function of other compounds, apart from TPC and catechin. Studies revealed that the antioxidant activity due to synergistic effect of flavonoids was better than that of individual flavonoid (Pekkarinen et al., 1996). Similarly, green tea exhibited higher total antioxidant activity than pure catechins proportionately combined based on the green tea constituents, which might have been mainly due to the synergistic effect (Rice-Evans et al., 1996).

Other compounds that have been identified in *M. citrifolia* leaf include quercetin, kaempferol, β-sitosterol, leucine, isoleucine, methionine, cystine, phenylalanine, proline, histidine, glycine and ursolic acid (Dittmar, 1993). These compounds may attribute to the synergistic effect with that of TPC and catechin thus contributing better antioxidant activity. These bioactive compounds might be degraded when exposed to high temperatures during extraction technique such as MAE. Previously, it was reported that amount of total phenolics in soybean extracts obtained from different extraction techniques did not show any significant correlation with their antioxidant activity (Chung et al., 2010).

UAE is an extraction technique which uses a wave with frequency higher than 20 kHz. Ultrasonic wave travels into the sample mediums and followed by the expansion and compression cycles in these mediums. The disruption of the samples cell walls by ultrasonic wave facilitates the release of bioactive compounds into the solvent with less temperature overheated. The UAE efficiency is related to cell disruption theory and effective mass transfer (Mason et al., 1996). It is interesting to note that UAE is usually carried out in shorter time as compared to that of SE (Vinatoru et al., 1997; Chemat et al., 2004). The rapidity of UAE has been demonstrated when UAE just needed half of the extraction time as compared with that required by Soxhlet in extracting oil without changing its compositions (Luque-De-Castro and Priego-Capote, 2007).

Similarly, the extraction of saponin from ginseng roots and cultured ginseng cells by using UAE technique was about three times faster than the traditional Soxhelt extraction (Wu et al., 2001). UAE was reported to be more efficient and showed a better effect on the extraction of phytochemicals from tea even at lower temperatures as compared to that of conventional technique (Xia et al., 2006). SFE extract showed a moderate antioxidant activity. It was reported that SFE is better than conventional extraction method due to the absence of light and air during extraction (Wang and Weller, 2006). Likewise, SFE extract from *Physalis peruviana* was reported to have better antioxidant and anti-inflammatory activity than that of SE (Wu et al., 2006).

Conclusion

In summary, the results from this study provided useful data regarding the effect of different extraction techniques on the recovery of phenolics and antioxidant activity of extractable components from *M. citrifolia* leaf. MAE was found to be most effective in extracting phenolic compounds and catechins as compared to other solid-liquid techniques employed. However, UAE technique was noted to be the most efficient for the extraction of antioxidants from *M. citrifolia* leaf with potent antioxidant activity. It can be suggested that temperature plays a significant role in preserving the antioxidant property of *M. citrifolia* leaf extract during extraction.

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