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Research article

Antioxidant and acetylcholinesterase inhibitory activities of *Morinda citrifolia* L. extracts

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Abstract

Extractions of roots, leaves and stems of *Morinda citrifolia* L. were carried out by maceration using ethanol, 40% ethanol/water and water, respectively. The aqueous leaf extract, ethanolic stem extract and aqueous root extract were obtained with highest percentage yields of 10.92%, 3.92% and 6.37% dry weight, respectively. The bioactivities of all the crude extracts were evaluated regarding their antioxidant and anti-acetylcholinesterase (AChE) activities. The crude ethanolic extract from roots had the relatively highest antioxidant activity with a half maximal inhibitory concentration (IC_{50}) value of 5.97 mg/mL and total phenolic contents of 13.33 mg gallic acid equivalent/g crude extract. Moreover, the ethanolic root extract also had the most potent anti-AChE inhibitory activity with an IC_{50} value of 454 µg/mL. This study indicated that ethanolic *M. citrifolia* root extract should be a good candidate for AChE inhibitors which may be applied as herbal supplements for the prevention of Alzheimer's disease.

Introduction

Nowadays, there is a notable global increase in aging societies, including in Thailand, due to the increasing numbers entering this cohort (Chunharas, 2002). Alzheimer's disease (AD) is one of the most commonly prevalent neurodegenerative diseases, causing dementia in people over the age of 65 years (Moya-Alvarado et al., 2016). When nerve cells in the brain are destroyed, the destroyed part cannot function properly and this leads to a gradual reduction in the brain volume which worsens dementia symptoms (Thummayot et al., 2014). AD is a progressive disease which cannot be cured or stopped its progression; however, medical treatment can relieve the symptoms, but these can worsen in the long run (Watkins et al., 1994).

Several studies have found that an important factor in the pathology is the formation of amyloid plaques and neurofibrillary tangles in the brain caused by the accumulation of amyloid-beta (Aβ) as the main component of amyloid plaques, resulting in toxication of neurons (Su et al., 2016). The certain cause of AD is unclear, but there are two main hypotheses. The non-cholinergic hypothesis is associated with the formation of amyloid, resulting in hormone secretion and oxidative stress from free radical secretion (Holmes et al., 2008) Free radicals potentially cause damage to both lipid and protein tissues, in particular at cellular membranes (Terry and Buccafusco, 2003). Although, the human immune system can control and prevent

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free radicals, they can still harm either cells or tissues in the body (Sun et al., 2010). However, various factors including increasing age, stress, and lifestyle result in the production of a decreasing amount of antioxidants which may then be insufficient to prevent free radicals daily (Selkoe, 1991). In the cholinergic hypothesis of AD, acetylcholine (ACh) is a major neurotransmitter in the brain which has an important role in memory (Giacobini, 2002). In dementia patients, the amount of ACh decreases in the hippocampus, leading to the loss of memory (Haam and Yakel, 2017). Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as hydrolytic enzymes can catalyze the breakdown of acetylcholine (Kitphati et al., 2011). The levels of AChE and BChE increased in dementia patients by 10–15% and 40–90%, respectively (Tarawneh et al., 2012).

Currently the drugs available for treatment of AD are ChE inhibitors such as Galantamine, Donepezil, Rivastigmine and Tacrine; these drugs increase the level of ACh at nerve cells by preventing its breakdown in the brain (Grossberg, 2003). The effectiveness of these drugs has been used in the treatment of forgetful patients by improving their emotional conditions and providing better recognition (Doraiswamy et al., 2002). However, there are some reports of these drugs having multiple side effects such as nausea, vomiting and diarrhea (Kano et al., 2013). The drugs are only effective in patients at an early stage of AD and are useless when the symptoms indicate severe dementia (Roseiro et al., 2012).

Thai herbs can be used as an alternative for the prevention and treatment of some common diseases. The current study investigated *M. citrifolia* extracts regarding their antioxidant and anti-acetylcholinesterase inhibitory properties. The *M. citrifolia* plant is also known in Thai as 'yo-ban' and is used in folk medicine for treatments of various common diseases such as cold, influenza, high blood pressure and headache (Whistler, 1985). In addition, the leaves and fruits of this plant are often used to prepare some healthy drinks and foods (Krishnaiah et al., 2015). Nowadays, juice products from fruits enriched with various phenolic compounds are useful to reduce blood pressure and to prevent allergies (Su et al., 2005). Many biological activities of *M. citrifolia* extracts have been reported, including antifungal, antimicrobial, anti-inflammatory, antiviral and antioxidant activities as well as nourishment of the brain and nervous system (Torres et al., 2017). Recently, Surangkul et al. (2018) reported that crude ethanolic extract from the roots of *M. citrifolia* inhibited AChE. Hence, the objectives of the current study were to investigate the effect of solvents in the preparation of bioactive extracts from different parts of *M. citrifolia* (leaves, stems and roots) using maceration with different solvents (ethanol, 40% ethanol/water and water) according to the local wisdom of Thai traditional medicine. In addition, the total phenolic contents (TPCs) of the crude extracts were determined and both the antioxidant and AChE inhibitory activities were evaluated.

Materials and Methods

Plant materials

Roots, stems and leaves of *M. citrifolia* were collected during January 2017 in an area of Nong Mae Taeng sub-district, Sai Ngam district, Kamphaeng Phet province, Thailand. A voucher specimen (QBG No.105894) was deposited at the Queen Sirikit Botanic Garden Herbarium (QBG; Chiang Mai, Thailand). The roots, stems and leaves were air-dried at ambient temperature and then ground into fine powder.

Preparation of crude extracts of M. citrifolia using maceration

The air-dried *M. citrifolia* roots, stems and leaves were finely ground, and each powder plant sample was individually extracted by maceration for 3 d using three different solvents (EtOH, 40%EtOH/H_2O and H_2O). Each solution of plant fragments was filtered using muslin cotton wool and Whatman® No. 1 filter paper, respectively. Each residue was re-extracted with fresh solvents until the extract solutions became colorless. Each extract solution was evaporated using a rotary evaporator at 40°C to give corresponding crude extracts (EtOH, 40%EtOH/H_2O and H_2O). All crude extracts were evaluated based on the determination of total phenolic contents and investigation of antioxidant and acetylcholinesterase (AChE) inhibitory activities.

In vitro antioxidant inhibition assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay of the crude extracts of *M. citrifolia* was accessed by the method described by Yen and Hsieh, 1997. Each stock solution of all crude extracts (20 mg/mL) was prepared in the solvent used for extraction. Each stock solution (50 µL) was transferred into each well of the 96-well plates followed by serial dilution. A solution of 0.3 mM DPPH in EtOH (200 µL) was added to each sample (the final volume was 250 µL) and the mixture was mixed and shaken well. The reaction mixture was then incubated at room temperature in the dark for 60 min. The absorbance of each reaction mixture was measured using an ultraviolet (UV)-visible spectrophotometer at a wavelength of 517 nm. Butylated hydroxyl toluene (BHT) and ascorbic acid were used as standard drugs and the pure DPPH solution served as a control. The percentage of radical scavenging activity was calculated using Equation 1:

$$\% \text{ Radical scavenging activity} = [(Abs_{control} - Abs_{sample})] / Abs_{control}] \times 100 \quad (1)$$

where $Abs_{control}$ is the absorbance of the control and Abs_{sample} is the absorbance of each crude extract solution.

The half maximal inhibitory concentration (IC_{50}) value was defined as the concentration of each test sample that inhibited DPPH free radicals at 50% which can be determined by a plot of sample concentrations (x axis) versus percentages of radical scavenging activity (y axis). The experiment was performed in triplicate and represented as $IC_{50} \pm SD$.

Total phenolic contents

The total phenolic contents (TPCs) of all crude extracts were measured using a UV-visible spectrophotometer based on the Folin-Ciocalteu colorimetric method which was slightly modified from the method described by Folin and Ciocalteu (1927). A 500 µL amount of each (10 mg/mL) was added to 2.5 mL of freshly prepared Folin-Ciocalteu reagent. The mixture was diluted in deionized water at a ratio of 1:10 volume per volume. The solution mixture was shaken well and set aside for 6 min, and subsequently followed by the addition of 2 mL of Na_2CO_3 (7.5% weight per volume). The sample was then stored in the dark for 90 min. The absorbance of the sample was measured using a UV-visible spectrophotometer at a wavelength of 765 nm. Gallic acid was used as the standard for determining the TPCs which were expressed as milligrams of gallic acid equivalent (GAE) per gram of crude extract. Each sample was analyzed in triplicate.

In vitro acetylcholinesterase inhibition assay

The AChE inhibition assay was slightly modified from the method described by Ellman et al. (1961, and determined using a colorimetric assay. The activity of AChE was measured by the reaction of acetylthiocholine iodide as a substrate and 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB), giving 5-thio-2-nitrobenzoate (TNB) as a yellow product. The inhibitory activity of the crude extracts against AChE was measured using a UV-visible spectrophotometer at a wavelength of 412 nm, in terms of TNB. Briefly, the standard drug (Tacrine at a concentration of 1.0 µg/mL) was used as a positive control. All crude extracts were dissolved in 0.5%DMSO over a range of concentrations (100–2,000 µg/mL). AChE (0.3 U/mL) and DTNB (3 mM) were dissolved in 20 mM Tris hydrochloride buffer (pH 7.5) and 10 mM phosphate buffer (pH 7.4), respectively. Each crude extract solution (25 µL) was mixed with AChE (25 µL) and incubated at 37°C for 15 min. Subsequently, a mixed solution (150 µL) containing 1 mM acetylthiocholine iodide (25 µL) and 3 mM DTNB (125 µL) was added to the reaction mixture which was incubated at 37°C for 30 min. The enzymatic activity was quantified by immediately measuring the absorbance at a wavelength 412 nm.

Data analysis

All data in the experiments (conducted in three replications) were tested using analysis of variance (ANOVA) with the SPSS software package (version 16.0; SPSS Inc.; Chicago, IL, USA) to determine any significant differences for the measured properties. Significance in the ANOVA was tested at $p < 0.05$.

Results and Discussion

Percentage yield of crude extracts from M. citrifolia (leaves, stems and roots) using different extraction solvents

Ground air-dried leaves, stems and roots of *M. citrifolia* from Kamphaeng Phet Province were extracted using maceration and three seperate solvents according to the increasing polarity of the solvent. The three solvents (EtOH, 40%EtOH/H_2O and H_2O) provided the crude extracts from different parts of plant in each solvent. The current study focused on the extraction of phenolic compounds from *M. citrifolia*. All phenolic compounds are quite polar (Khoddami, 2013), so the three polar solvents were selected for extraction. The percentage yields of all crude extracts are provided in Table 1. All crude leaf extracts were dark-green, viscous liquids and yielded the highest percentages of 7.32% dry weight (dry wt), 10.63% dry wt and 10.92% dry wt in EtOH, 40%EtOH/H_2O and H_2O, respectively. The percentage yields of all leaf extracts tended to be higher than both the percentage yields of the crude root extracts (dark-brown, viscous liquids with yields of 4.76% dry wt, 7.69% dry wt and 6.26% dry wt, respectively) and the crude stem extracts (dark-brown, viscous liquid with yields of 2.17% dry wt, 3.30% dry wt and 2.76% dry wt, respectively). The higher percentage yields from the leaf extracts may have been due to the high solubility of the considerable chlorophyll content stored in the leaves that was present in the polar solvents.

Table 1 Percentage yields of *M. citrifolia* extracts from air-dried leaves, stems and roots using different solvents

Solvent	Weight of crude extract (g)			% Crude extract/dry weight		
	Leaves	Stems	Roots	Leaves	Stems	Roots
EtOH	1.73	0.65	5.25	7.32	3.92	5.42
40% EtOH/H_2O	2.72	1.00	5.89	10.63	3.25	6.08
H_2O	2.80	1.17	6.09	10.92	3.78	6.37

All crude extracts from leaves, stems and roots of *M. citrifolia* in the three different extraction solvents were tested for DPPH free radical scavenging activity. The IC_{50} values of *M. citrifolia* extracts versus standard drugs (ascorbic acid and BHT) are shown in Table 2. The crude ethanolic extracts from all selected parts (roots, leaves and stems) of *M. citrifolia* had relatively high antioxidant activity with IC_{50} values of 5.98 mg/mL, 6.10 mg/mL and 6.19 mg/mL, respectively. These IC_{50} values were slightly higher than those of the crude 40%EtOH/H_2O and aqueous extracts. This suggested that phenolic compounds such as anthraquinones, flavonoids and coumarins from *M. citrifolia* were the major and active components (Kamiya et al., 2005; Wang et al., 2016). For example, damnacanthal (Fig. 1A), 1,3,6-trihydroxy-2-methylanthraquinone (Fig. 1B), alizarin-1-methyl ether (Fig. 1C), morindone-5-methylether (Fig. 1D) and rubiadin (Fig. 1E) (Fig. 1) were reported as sources of bioactive anthraquinones isolated from the roots of *M. citrifolia.* (Deng et al., 2007; Lv et al., 2011; Sang and Ho, 2006). These compounds are composed of one or more hydroxyl groups which are directly attached to an aromatic ring, resulting in enhancement of their solubility in EtOH (Phumyai and Supha, 2015). It is suggested that this is associated with the antioxidant activities results identified in the current study.

Table 2 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activities (mean IC_{50} value ± SD; n = 3) of all crude extracts of *M. citrifolia* and positive controls.

M. citrifolia extract	IC_{50} value (mg/mL)
Crude ethanolic root extract	5.97 ± 0.08[a]
Crude ethanolic leaf extract	6.10 ± 0.27[a]
Crude ethanolic stem extract	6.19 ± 0.15[a]
Crude 40% ethanolic/water root extract	10.49 ± 0.08[b]
Crude 40% ethanolic/water leaf extract	7.41 ± 0.16[c]
Crude 40% ethanolic/water stem extract	8.22 ± 0.27[d]
Crude aqueous root extract	15.90 ± 0.42[e]
Crude aqueous leaf extract	10.01 ± 0.11[f]
Crude aqueous stem extract	15.38 ± 0.19[g]
Ascorbic acid (positive control)	0.033 ± 0.01
Butylated hydroxyl toluene (positive control)	0.062 ± 0.03

IC_{50} = half maximal inhibitory concentration.
Values with the same letter are not significantly different (P = 0.05) from each other

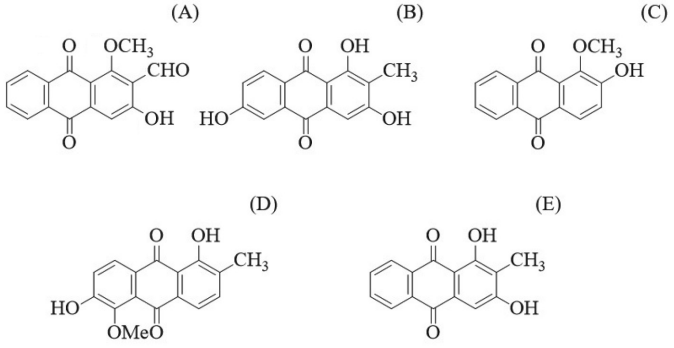

Fig. 1 Structures of major bioactive constitutents (Fig. 1A–E) isolated from *M. citrifolia* roots

Total phenolic contents

Some polyphenolic compounds (such as flavonoids) possessing antioxidant activity (Andarwulan et al., 2010; Sowndhararajan and Kang, 2013) were quantified in the crude extracts and were compared among the three extraction solvents (Table 3). The TPCs of the leaf extracts in the three different solvents were higher than for both root and stem extracts. These results indicated that a number of major components from the *M. citrifolia* leaves contained water-soluble polyphenolic species, such as flavonol glycosides and iridoid glycosides (Fig. 2), in greater amounts than in the extracts from the root and stem parts (Sang et al., 2011). The amount of polar substances is higher when extracting with a polar solvent (such as water), resulting in an increase in the TPCs (Wang et al., 2002). However, all ethanolic leaf, root and stem extracts had the highest values of TPCs among all crude extracts which was consistent with the antioxidant properties identified in the current study.

In vitro acetylcholinesterase inhibitory activity

Screening of the AChE inhibitory activity of *M. citrifolia* root, leaf and stem extracts was determined using Ellman's method which is a diagnostic technique to indicate the level of Ach (Ellman et al., 1961). All crude extracts of *M. citrifolia* produced inhibition of AChE, and the ethanolic root extract also produced relatively strong inhibition of AChE with the percentage inhibition of 75.66% at a concentration of 2,000 µg/mL, while the other extracts showed AChE inhibitory activity of less than 50% at the same concentration (Fig. 3). The ethanolic root extract exhibited potency in inhibiting AChE with an IC_{50} value of 454 ± 33.0 µg/mL, but this extract was less active than tacrine used as a positive control drug which had an IC_{50} value of 0.044 ± 0.002 µg/mL (Table 4).

Recently, research related to searching for potential AChE inhibitors has been popular in the area of medicinal chemistry and natural product chemistry. For example, the methanolic fruit extract of *Phyllanthus acidus* had AChE inhibitory activity with an IC_{50} value of 1,009.87 ± 19.27 µg/mL (Moniruzzaman et al., 2015). In addition, the aqueous extract of *Ganoderma lucidum* as fungi which were grown on germinated brown rice showed inhibition of AChE with an IC_{50} value of 1.01 mg/mL (Hasnat et al., 2013). Oxidative stress is one of the disease causes corresponding to AD (Sun et al., 2010) because it has a direct impact on the function of AChE (Melo et al., 2003). Controlling the level of ACh by an AChE-inhibition process and preventing oxidative stress are the best solutions to deal with symptoms of AD (Lahiri et al., 2002). The results of the current study indicated that the ethanolic root extract had relatively good antioxidant and AChE inhibitory properties which were higher than for the other extracts reported. This indicated that it has potential as a source of cholinergic inhibitors. Moreover, it was shown to have no cytotoxic activity when treated with SK-N-SH neuroblastoma cells (Srisawad et al., 2018).

Table 3 Effect of different solvents on total phenolic contents (mean ± SD; n = 3) of the crude *M. citrifolia* extracts

M. citrifolia extract	Total phenolic contents (mg gallic acid equivalent/g of extract)		
	Ethanol extract	40% Ethanol/water extract	Aqueous extract
Roots	13.33 ± 0.03[a]	7.16 ± 0.05[d]	7.10 ± 0.06[d]
Leaves	12.61 ± 0.38[b]	12.53 ± 0.07[b]	13.41 ± 0.04[a]
Stems	12.88 ± 0.01[c]	10.90 ± 0.02[e]	7.79 ± 0.04[f]

Values with the same letter are not significantly different (p = 0.05) from each other

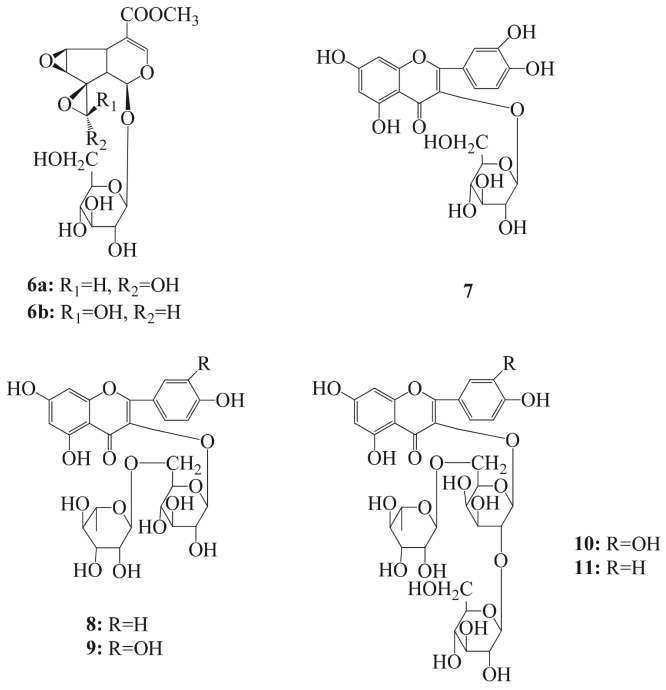

Fig. 2 Structures of iridoid glycosides (6a-b) and flavonol glycosides (7-11) isolated from *M. citrifolia* leaves

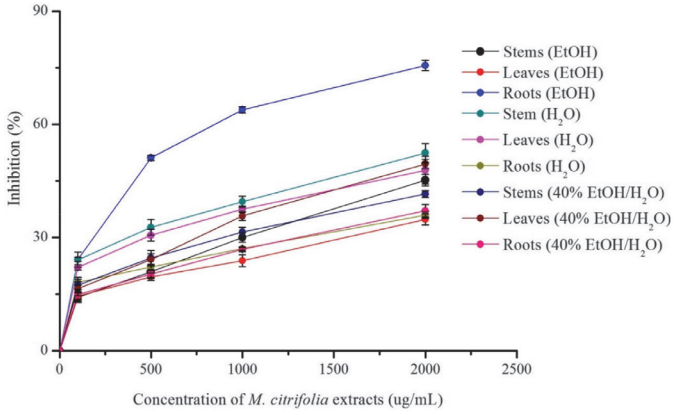

Fig. 3 AChE inhibitory activity of *M. citrifolia* extracts from different parts by using different Solvents, where error bars indicate ± SD

Table 4 Anti-acetylcholinesterase activities (mean IC_{50} value ± SD; n = 3) of crude ethanolic root extract and pure tacrine (positive control).

Extract	IC_{50} value (µg/mL)
Crude ethanolic root extract	454 ± 33.0
Tacrine (positive control)	0.044 ± 0.002

In conclusion, the crude ethanolic root extract of *M. citrifolia* containing secondary metabolites such as phenolic compounds provided the best antioxidant activity with an IC_{50} value of 5.98 mg/mL, as well as having the greatest quantity of TPCs (13.33 mg GAE/g crude extract). Thus, some vital secondary metabolites found in this crude extract may potentially protect the immune system from the damage caused by oxidative stress. Interestingly, the extract also showed good AChE inhibitory activity with an IC_{50} value of 454 µg/mL. Although the extract had a higher IC_{50} value than tarcrine (IC_{50} value of 0.044 µg/mL), it produced less side effects. These results indicated that the ethanolic extract from the roots of *M. citrifolia* has the potential for further isolation and purification of the bioactive constituents for AChE inhibitory activity assay in order to be used as a herbal supplement for the prevention of AD.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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