บทความวิจัย

TOTAL PHENOLIC CONTENT, ANTIOXIDANT, AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITIES OF *Vitex trifolia* L. EXTRACTS

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ABSTRACT

Vitex trifolia (family Lamiaceae) known in Thai as Kontee-sor, is a tropical shrub widespread in many areas, especially in northern Thailand such as Chiang Mai. The plant is used commonly in traditional Thai medicine for the treatment of fever, headache, dizziness, cough, high blood pressure, and pain. Total phenolic content, antioxidant, and acetylcholinesterase inhibitory activities of extracts from various parts (flowers, twigs, leaves, and fruits) of *V. trifolia* extracted by some solvents (hexane, dichloromethane, ethyl acetate, and methanol) were determined. Among the extracts used, ethyl acetate extract obtained from the leaves showed the highest phenolic content of 297.26 ± 0.79 mg GAE/g dry extract weight and antioxidant activity against DPPH with IC_{50} 11.16 ± 0.21 µg/mL and 86.12 ± 0.37% DPPH inhibition at 0.1 mg/mL. The properties of extracting solvents affected the measured total phenolic content and antioxidant activity. Their coefficient of determination (R^2 = 0.9155) between total phenolic content and $1/IC_{50}$ suggested that the phenolic compound contributed to the antioxidant activity. Methanol extract of fruits was screened for acetylcholinesterase inhibitory activity by Ellman's microplate colorimetric method and showed IC_{50} values of 30.79 ± 0.11 µg/mL and 59.28 ± 1.48% AChE inhibition at 0.1 mg/mL. Considering this potent AChE inhibition, the extracts could be investigated as new AChE inhibitors with low toxicity as natural drugs.

Keywords: *Vitex trifolia*, Lamiaceae, total phenolic contents, antioxidant activity, acetylcholinesterase inhibitory activity

Introduction

Vitex trifolia L. (Figure 1), belonging to the family Lamiaceae and commonly known as Simpleleaf Chastetree, is a tropical shrub widespread in Southeast Asia, Micronesia, Australia and East Africa (de Kok, 2007). Traditionally, the whole *V. trifolia* is used commonly in Thai medicine for an extensive range of treatments including fever, headache, dizziness, cough, high blood pressure, and pain (Horata et al., 2017; McClatchey, 1996). Previous studies of *V. trifolia* crude extracts have shown interesting biological activities such as cytotoxicity (Gu, & Jiang, 2007; Zheng et al., 2013), insecticidal activity (Kannathasan et al., 2007), and antimicrobial activity (Hossain et al., 2001). The extracts of both aqueous and ethanol solvents have been reported to have hepatoprotective (Manjunatha & Vidya, 2008) activity and methanol extracts have also revealed inhibition of mosquito-borne disease (Kannathasan et al., 2011). The investigation of *V. trifolia* in phytochemical studies have reported flavonoids (Hong-yan et al., 2008; Li et al., 2005; Wang, 2005), terpenoids (Ono et al., 2000; Tiwari et al., 2012), and alkaloids (Döpke, 1962; Luo et al., 2017; Van Luu, 2003) with biological activities as mentioned above.

Figure 1 Flowers, twigs, leaves, and fruits of *V. trifolia*

Free radicals, produced during oxidation process, have been shown to be related to a range of disorders in humans, including central nervous system injury, stroke, traumatic brain injury, spinal cord injury, heart diseases, and cancer (Kumpulainen & Salonen, 1999; Shah et al., 2015). Numerous antioxidant compounds, hydroperoxidase enzymes in human body, synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), propyl gallate, phenolic acid, flavonoids, carotenoids, and vitamins E and C are used as free radical scavengers (Barlow, 1990; Kil et al., 2009). Among the antioxidant compounds, natural phenolic compounds have potential due to the presence of conjugated ring structures and hydroxyl groups for scavenging the free radicals that affect the oxidation process (Shahidi et al., 1992).

In recent research, acetylcholinesterase (AChE) inhibitors were used for treating the neurodegenerative disease as the major therapeutic target based on the cholinergic hypothesis (Davies & Maloney, 1976; Mangialasche et al., 2010). Alzheimer's disease (AD) is a progressive neurological disorder frequently connected with memory impairment, behavioral turbulence, cognitive dysfunction and imperfection in routine life activities (Aisen & Davis, 1997; Bachman et al., 1992). Natural AChE inhibitors have become the important alternatives in treatment of AD and other neurological diseases (Knapp et al., 1994; Nawaz & Choudhary, 2004).

In this study, *V. trifolia* extracts were evaluated for total phenolic content (TPC), antioxidant activity, and their correlation. Moreover, the potent AChE inhibitory activity was also investigated for further isolation and elucidation.

Methods

***Plant material and chemicals*:**

Flowers, twigs, leaves, and fruits of *V. trifolia* were collected from Chiang Mai, Thailand and further identified by Dr.Theerapat Luangsuphabool (Genebank Research and Development Group, Biotechnology Research and Development Office, Department of Agriculture, Ministry of Agriculture and Cooperatives). Commercial and analytical grade solvents including hexane, dichloromethane, ethyl acetate, and methanol were purchased from RCI Labscan. Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), AChE from electric eel (Type V-S lyophilized powder) 480 U/mg solid, 530 U/mg protein, hydrochloric acid (HCl), sodium hydroxide (NaOH), tris(hydroxymethyl) methylamine, bovine serum albumin (BSA), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), sodium chloride (NaCl), magnesium chloride ($MgCl_2$), acetylthiocholine iodide (ATCI), and physostigmine were obtained from TCI, and Sigma-Aldrich.

***Preparation of extracts*:**

Plant materials (10 kg; flowers, twigs, leaves, and fruits) were divided into small pieces and dried at 60 °C for 24 h. The powder of dried plant materials was prepared using an electric grinder and powder samples were used for preparation of the extracts (Figure 2). The methanol extracts of each plant material were obtained by maceration of powder samples (250 g) with methanol (1,000 mL), prepared at room temperature for 24 h, repeated for three times, and evaporated by rotary evaporator. The methanol extracts of each plant material (flowers, twigs, leaves, and fruits), 26.95 g, 28.02 g, 54.62 g, and 31.09 g respectively obtained, were sequentially partitioned with hexane, dichloromethane, ethyl acetate, and methanol to obtain hexane, dichloromethane, ethyl acetate, and methanol extracts, respectively. Each extract was performed in triplicate with equal volumes (500 mL) of solvent. The obtained crude extracts of flowers for the hexane, dichloromethane, ethyl acetate, and methanol extracts were 1.73 g, 1.05 g, 1.52 g, and 9.17 g, respectively. The obtained crude extracts of twigs for the same extracts were 2.44 g, 2.17 g, 2.36 g, and 7.03 g, respectively. The obtained crude extracts of leaves for the same extracts were 2.38 g, 1.21 g, 3.54 g, and 10.17 g, respectively. In addition, the obtained crude extracts of fruits for the same extracts were 2.02 g, 3.47 g, 2.39 g, and 7.65 g, respectively.

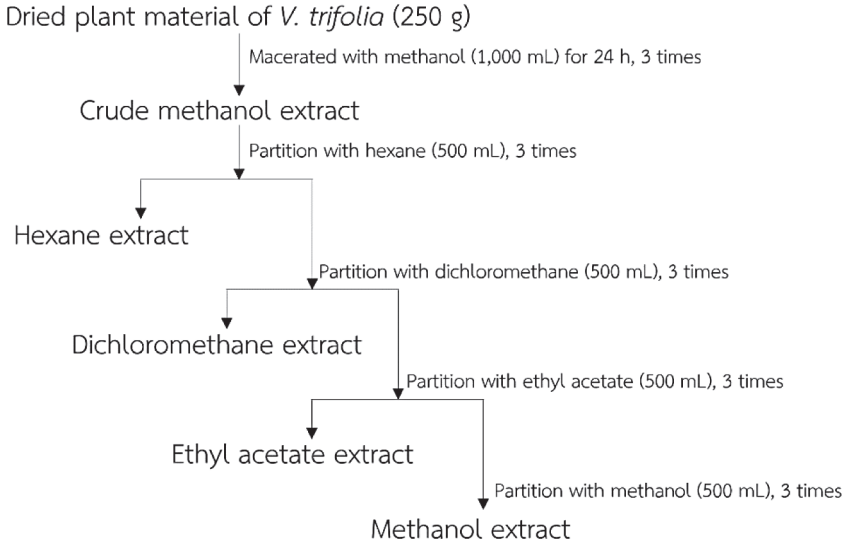

Figure 2 Extraction scheme of *V. trifolia*

***Determination of Phenolic Contents*:**

TPC in the extracts were quantified using modified Folin- Ciocalteu method (Atoui et al., 2005). 20 µL each of the 10 mg/mL extracts in methanol and the serial gallic standard solutions in methanol were loaded on 96-well plates. Then 100 µL of Folin-Ciocalteu reagent was added to each well, mixed well and waited for 5 min. Next, 80 µL of 7.5% Na_2CO_3 solution was added and mixed well. After covering and keeping it in the dark for 2 h, absorbance at 750 nm was measured using microplate reader (VICTOR Nivo Multimode). The total phenolic content was determined using the standard curve obtained for gallic acid. The estimation of the phenolic compounds in each of the extract was performed in triplicate and the results were expressed as mg of gallic acid equivalents (GAE) per g of dry extract weight.

***Determination of Antioxidant Activity by Scavenging*:**

The antioxidant activity of the extracts was determined using modified DPPH free radical scavenging assay in 96-well plates (Tepe et al., 2005). Stock solutions of the extracts were prepared as 1 mg/mL in methanol. Each well was filled with 200 µL extract in methanol starting from 1000 µg/mL down to the lowest 10 µg/mL. Then, 5 µL of the DPPH solution (2.5 mg/mL in methanol) was added to each well. BHT was used as positive control (Yehye et al., 2015). After keeping the plates in the darkness for 30 min, the absorbance was read using microplate reader (VICTOR Nivo Multimode) at 517 nm. The assay was repeated in triplicate and percentage inhibition was calculated using the following formula (Aryal et al., 2019):

$$\% \text{ DPPH radical scavenging} = [(A_0 - A_1)/A_0] \times 100,$$

Where A_0 = absorbance of the control and A_1 = absorbance of the extracts.
A graph of percentage of DPPH radical scavenging was plotted against concentration of the extracts and concentration for 50% inhibition (IC_{50}) was obtained from the graph. The DPPH radical scavenging activity by extracts was examined and compared with BHT as positive control.

Determination of Acetylcholinesterase Inhibitory Activity:

Tris-HCl (50 mM, pH 8) was used as a buffer. The lyophilized AChE enzyme was prepared in the buffer to obtain 1130 U/mL stock solution. The enzyme stock solution was kept at -80 °C until used. The further enzyme dilution was dissolved in 0.1% BSA in buffer. DTNB was dissolved in the buffer containing 0.1 M NaCl and 0.02 M $MgCl_2$. ATCI was dissolved in deionized water. AChE activity was measured using a modified microplate assay based on Ellman's colorimetric method (Ingkaninan et al., 2003). The AChE inhibition of extracts was carried out in 96-well plates. 25 µL of 15 mM ATCI, 125 µL of 3 mM DTNB, 50 µL of 50 mM Tris-HCl (pH 8), and 25 µL of the extract dissolved in buffer containing not more than 10% methanol were added to the wells. Thereafter, 25 µL of AChE solution (0.28 U/mL) was added and the absorbance was measured using microplate

reader (VICTOR Nivo Multimode) at a wavelength of 405 nm and monitored every 15 s for 5 min. Physostigmine was used as positive control (Mathew & Subramanian, 2014). All the reactions were performed in triplicate and inhibitory activity was calculated as follows (Majdoub et al., 2021):

$$\% \text{ AChE inhibition} = [(A_0 - A_1)/A_0] \times 100,$$

Where A_0 = absorbance of the control and A_1 = absorbance of the extracts.

A graph of percentage of AChE inhibition was plotted against concentration of the extracts and concentration for 50% inhibition (IC_{50}) was obtained from the graph. The inhibition of AChE activity by extracts was examined and compared with physostigmine as positive control.

Results and Discussion

The extraction procedures (Figure 2) used commonly used solvents with increasing polarity order (hexane, dichloromethane, ethyl acetate, and methanol) and phenolic compounds are more soluble in polar organic solvents. Phenolic compounds are important phytochemical constituents which have antioxidant activity through redox properties and the hydroxyl groups in their structures (Ayaz et al., 2014). TPC (mg GAE/g dry extract weight) of different *V. trifolia* extracts (Table 1) had high phenolic contents in ethyl acetate extracts of leaves and twigs (297.26 ± 0.79 and 227.89 ± 0.47 mg GAE/g dry extract weight, respectively).

For DPPH assay (Table 1), the results showed high antioxidant activity with IC_{50} values in ethyl acetate extracts of leaves and twigs at 11.16 ± 0.21 and 12.47 ± 0.62 µg/mL, respectively corresponding to percentage of DPPH inhibition at 0.1 mg/mL extract concentration of 86.14 ± 0.87% and 81.14 ± 0.87%, respectively. BHT was used as the positive control for antioxidant which showed IC_{50} value of 6.25 ± 0.12 µg/mL. Moreover, the results expressed the positive correlation between TPC and antioxidant activity as shown in Figures 3 and 4. The relationship of TPC and $1/IC_{50}$ values from DPPH assay was determined with coefficient of determination (R^2) value 0.9155 and this significant correlation suggested that the phenolic compound contributed to the antioxidant activity (Shah et al., 2013). However, phenolic compounds can exhibit different antioxidant activities depending on their structures as well as synergistic or antagonistic effect of other chemical compounds combination and interactions between compounds present in the extract (Freeman et al., 2010).

Table 1 TPC, % DPPH inhibition, and DPPH IC_{50} values of *V. trifolia* extracts

Parts of plant	Extracts	TPC (mg GAE/g dry extract)	% DPPH inhibition at 0.1 mg/mL	IC_{50} for DPPH scavenging assay (µg/mL)
chloro	hexane	9.55 ± 0.52	6.06 ± 0.34	870.51 ± 0.27
	dichloromethane	14.30 ± 0.91	34.18 ± 0.38	232.41 ± 0.33
	ethyl acetate	30.32 ± 0.72	35.27± 0.97	115.02 ± 0.91
	methanol	27.19 ± 0.28	25.09 ± 0.86	293.78 ± 0.78
twigs	hexane	13.02 ± 0.58	10.05 ± 0.81	198.36 ± 0.70
	dichloromethane	59.98 ± 0.22	48.19 ± 0.26	112.16 ± 0.19
	ethyl acetate	227.89 ± 0.47	81.14 ± 0.87	12.47 ± 0.62
	methanol	112.87 ± 0.56	62.03± 0.92	54.49 ± 0.79
leaves	hexane	36.12 ± 0.08	55.86 ± 0.65	71.38 ± 0.42
	dichloromethane	160.08 ± 0.74	61.46 ± 0.29	39.01 ± 0.18
	ethyl acetate	297.26 ± 0.79	86.12 ± 0.37	11.16 ± 0.21
	methanol	172.03 ± 0.83	75.38 ± 0.97	15.97 ± 0.85
fruits	hexane	6.96 ± 0.57	1.46 ± 1.12	>1,000
	dichloromethane	35.21 ± 0.18	11.75 ± 0.30	253.21 ± 0.16
	ethyl acetate	24.20 ± 0.75	3.55 ± 0.95	411.25 ± 0.87
	methanol	16.97 ± 0.26	9.75 ± 0.79	610.42 ± 0.54

Note: Values are expressed as mean ± SD (n=3). IC_{50} for DPPH scavenging assay of BHT (positive controls) was 6.25 ± 0.12 µg/mL.

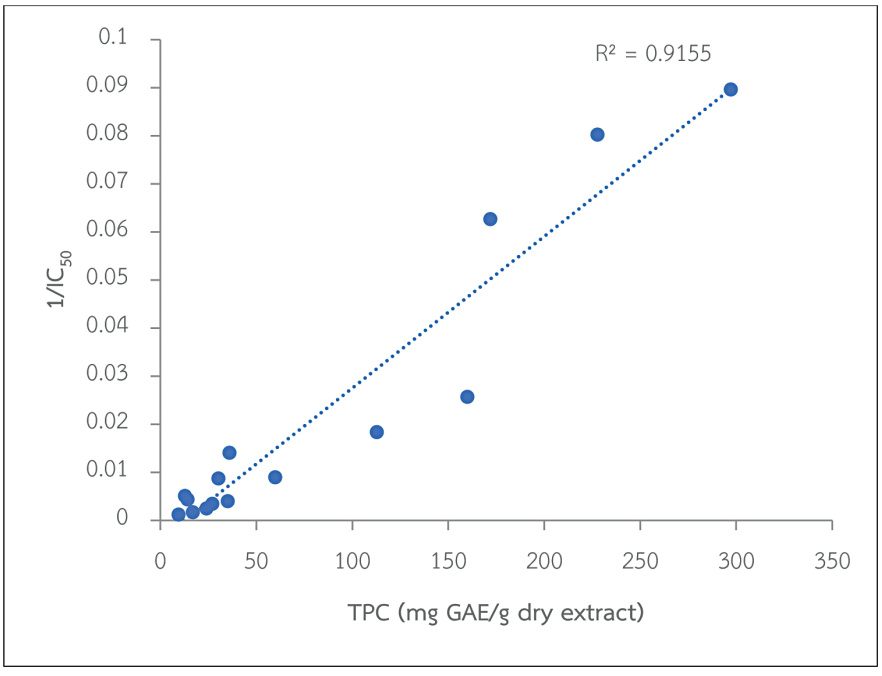

Figure 3 Correlation between TPC and DPPH IC_{50} values of *V. trifolia* extracts

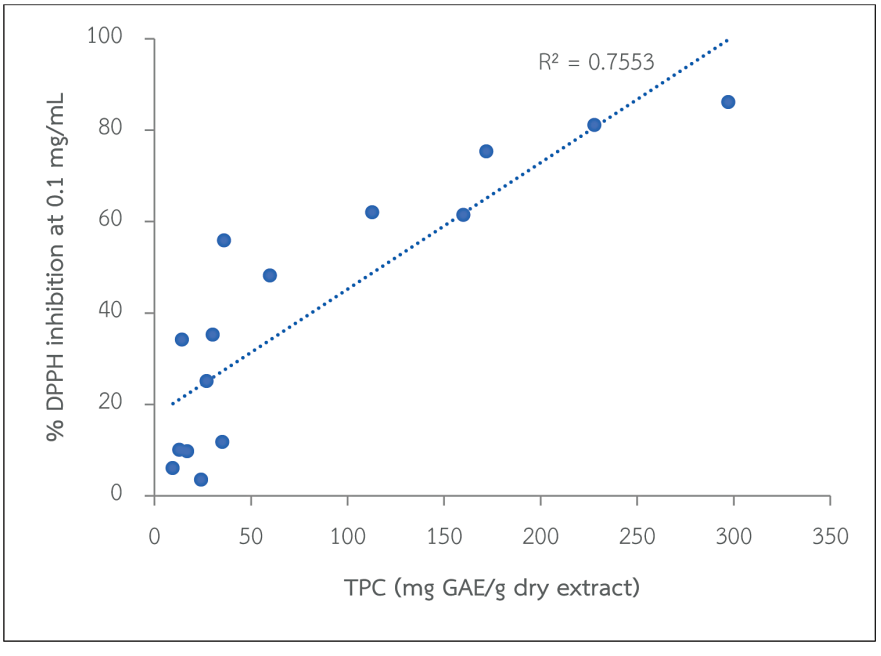

Figure 4 Correlation between TPC and % DPPH inhibition at 0.1 mg/mL of *V. trifolia* extracts

Table 2 % Inhibition and IC_{50} values of *V. trifolia* extracts for AChE inhibition assays

Parts of plant	Extracts	% AChE inhibition at 0.1 mg/mL	IC_{50} for AChE inhibition assay (µg/mL)
leaves	dichloromethane	21.01 ± 7.87	870.48 ± 9.79
	ethyl acetate	36.75 ± 3.03	230.21 ± 2.84
	methanol	42.94 ± 6.29	245.83 ± 5.93
fruits	dichloromethane	13.92 ± 2.94	527.02 ± 1.95
	ethyl acetate	50.40 ± 0.97	77.63 ± 0.97
	methanol	59.28 ± 1.48	30.79 ± 0.11

Note: Values are expressed as mean ± SD (n=3). IC_{50} physostigmine control for AChE inhibition assay was 0.11 ± 0.04 µg/mL.

The AChE inhibitory activity of *V. trifolia* extracts are shown in Table 2 representing the percentage of AChE inhibition at 0.1 mg/mL and IC_{50} for AChE inhibition assay (µg/mL) from dichloromethane, ethyl acetate, and methanol extracts of leaves and fruits. Other extracts are not shown in table 2 due to the IC_{50} values >1,000 µg/mL. Physostigmine was used as the positive control for AChE inhibitor which showed IC_{50} value of 0.11 ± 0.04 µg/mL. The only methanol extract of fruits showed the potent AChE inhibitory activity with IC_{50} value 30.79 ± 0.11 µg/mL corresponding to percentage of AChE inhibition at 0.1 mg/mL expressed to be 59.28 ± 1.48%. From approved drugs for AD, galantamine and rivastigmine are cholinesterase inhibitors derived from plant alkaloids (Nordberg & Svensson, 1998) and some alkaloids from dried fruits and leaves of *V. trifolia* were reported (Döpke, 1962; Luo et al., 2017; Van Luu, 2003). These results suggested that the extracts have the potential as AChE inhibitor. Further isolations for new AChE inhibitors with low toxicity from these extracts are necessary for the investigations of natural drugs.

Conclusions

TPC, free radical scavenging activity, and AChE inhibitory activity of *V. trifolia* extracts varied according to plant parts and solvent polarity used. The ethyl acetate extract of leaves had the most phenolic content (297.26 ± 0.79 mg GAE/g dry extract weight) as well as the best antioxidant properties (IC_{50} 11.16 ± 0.21 µg/mL and 86.12 ± 0.37% DPPH inhibition at 0.1 mg/mL). There was a significant linear correlation between total phenolic content and $1/IC_{50}$ values from DPPH assay due to the phenolic compounds were a major component of antioxidants in *V. trifolia*. Additionally, the

methanol extract of fruits also showed inhibitory activity against AChE enzymes (IC_{50} 30.79 ± 0.11 µg/mL and 59.28 ± 1.48% AChE inhibition at 0.1 mg/mL).

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