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Phytochemical Constituents and Cholinesterase Inhibitory Activities of *Millingtonia hortensis*

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Abstract

Acetylcholine (ACh) is an important neurotransmitter in the human brain and nervous system. Acetylcholinesterase inhibitors (AChEIs) are commonly used to improve cognitive function and exist in many plants, including *Millingtonia hortensis*. *M. hortensis*, a Thai medicinal plant, has been used as a smoke delivered bronchodilator. The aims of this study were to identify phytochemical constituents and evaluate the cholinesterase inhibitory activity of *M. hortensis* leaves and flower extracts. The phytochemical identifications were performed by gas chromatography-mass spectrometry. The inhibitory activities of acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) were measured through kinetic enzyme analysis by Ellman's method. The results showed that all the extracts exhibited specific AChEIs less than 30 % inhibition. While only the chloroform leaf extract inhibited BChE below 30 % inhibitory activities at 5.0 mg/mL. The GC-MS fingerprints revealed 15 main phytochemical constituents in the crude extracts. Additionally, all plant extracts showed antioxidant activity. The leaf extracts were non-poisonous when AChE activity is decreased by 20 - 30 % compared to normal AChE activity.

Keywords: Acetylcholinesterase, cholinesterase inhibitors, *Millingtonia hortensis*, phytochemical constituents, GC-MS

Introduction

Acetylcholine is an important neurotransmitter in the human nervous system. It activates skeletal muscles via the peripheral nervous system, exerts various effects on the autonomic nerve system (ANS), specifically by sympathetic and parasympathetic neurons. Acetylcholine (ACh) is synthesized in the presynaptic neuron from choline and the acetyl coenzyme. After binding with its receptors, ACh is hydrolyzed by acetylcholinesterase (AChE) in order to terminate synaptic transmission [1-3]. The AChE ensures that the nervous system functions properly by preventing the accumulation of acetylcholine and the over stimulation of muscles and nerves [4,5]. Reduction of the enzyme activity may lead to nervous system dysfunction. Analysis of cholinesterase activity has become a useful tool for drug design and discovery both in medicine and toxicology.

Acetylcholinesterase inhibitors (AChEIs) inhibit hydrolysis of ACh by the cholinesterase enzyme, and therefore enhance both the level and the action period of the neurotransmitter. AChEIs drugs were developed to improve cognitive function in memory disorders, dementia syndrome and Alzheimer's disease [6,7]. AChEIs are found in many medicinal plants. They preferentially act on muscarinic receptors, however, at high concentration they may show some activity at nicotinic receptors. The adverse effects of indirect-acting cholinergic agonists on the central nervous system (CNS) may lead to

convulsions when high doses are used. Inhibition of AChE at the skeletal neuromuscular junction causes the accumulation of acetylcholine and, ultimately, results in paralysis of skeletal muscle [8].

Millingtonia hortensis is an ancient medicinal plant found in Southern Asia, including Thailand, Myanmar, India and South China. It has been used as smoked inhaled bronchodilator agent in patients with asthma and chronic obstructive pulmonary disease (COPD). The main phytocomponent isolated from the flower of *M. hortensis* was a bioactive flavonoid named hispidulin. It was reported to exhibit the bronchodilator effect on rat's trachea at the local muscarinic receptor [9] and exert an inhibitory activity on the 5-lipoxygenase pathway [10]. Additionally, hispidulin from *Cirsium rivulare* has been reported to contain anxiolytic and pro-cognitive effects. Many studies of *M. Hortensis* phytochemical constituents suggest that they possess anti-microbial properties. Pharmacological activities of different extracts of *M. hortensis* [11] were investigated against various fungal pathogens [12], larva [13,14], microbial [15], mutagenicity [16], and cell proliferation [17].

Due to the phytochemical component of *M. hortensis* as mentioned above, especially, the effects on the nervous system including antiepileptic, anticonvulsant, anxiolytic and pro-cognitive effects seem like the cholinesterase inhibitory activities. Therefore, this study aimed to determine the total phenolic content, antioxidant, and cholinesterase inhibitory activities of different solvent extract of *M. hortensis* leaves and flowers. Moreover, the phytochemical components of the extracts were identified using GC-MS analysis.

Materials and methods

Extract preparation

The dried leaves and flowers of *M. hortensis* were coarse powders and macerated for 7 days in different solvents including methanol, chloroform, *n*-butanol and diethyl ether. The extracts were centrifuged at 3,000 rpm for 10 min. The supernatants were concentrated on a rotary evaporator. Five milligrams/milliters (mg/ml) of each component was reconstituted in methanol, and then was analyzed by GC-MS. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were assessed by a GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, Germany).

Blood collection and preparation

The EDTA blood samples were collected from 10 healthy volunteers. Plasma from each sample was separated by centrifuging at 3,000 rpm for 10 min using a Universal 320R centrifuge (Hettich, Germany). The study was approved by Western university ethic committee.

Determination of total phenolic content

The total phenolic content of the different solvent extracts were determined by the Folin-Ciocalteu method with some modifications [18]. Ten microliters of the sample was added to 50 µL of Folin-Ciocalteu reagent and 790 µL of distilled water. The solution was allowed to stand for 8 min. Then 150 µL of 2 g% Na_2CO_3 was added. The above solution was then incubated at room temperature for 2 h. Absorbance was measured at 765 nm on a GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, Germany). Gallic acid (0 - 1,000 µg/mL) was used to produce the standard calibration curve. The total phenolic content was expressed in mg of Gallic acid equivalent per g of dry weight extract (mgGAE/gDWE) and all samples were analyzed in triplicate.

DPPH free radical scavenging assay

The measurement of the DPPH assay was performed according to a modified methodology described by Brand-Williams *et al*. [19]. Four concentrations (125, 250, 500, and 1,000 µg/mL) of the samples were reacted with the stable DPPH radical in methanol solution. The reaction mixture consisted of 50 µL of sample, 1.5 mL of 0.04 mM DPPH radical solutions in methanol. When the antioxidant compound reacts with DPPH, which can donate hydrogen, it is reduced. The absorbance of the yellow color was read at 517 nm after 30 min of reaction using a GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, Germany). The methanol mixture and sample served as a blank. The inhibition

percentage of free radical scavenging was calculated with the following formula [20]. Ascorbic acid was used as a positive control.

$$\text{DPPH scavenging activity (\%)} = \frac{Acontrol - Asample}{Acontrol} \times 100 \quad (1)$$

GC-MS analysis

GC-MS analysis of the herb extracts composition were performed on a Agilent 7890 GC system instrument equipped with HP-5MS (5 % diphenyl 95 % dimethylpolysiloxane) column (30 m × 0.25 mm × 0.25 µm) and interfaced to a 5975C inert XL MSD with Triple-Axis Detector. An injection volume of 2 µL was employed (split ratio of 10:1) and an injector temperature 250 ºC. The column temperature was increased from 60 to 250 ºC at a rate 5 ºC/min. The outlet temperature was 280 ºC. Mass spectra were taken at 70 eV and fragments from 40 to 700 Da. The MS transfer line temperature was 250 ºC. Identification of the compounds was conducted using the database of the National Institute of Standards and Technology (NIST) library. The name, molecular weight, molecular formula, and area under the peak of the test material components were ascertained.

Cholinesterase activity assay

The activity of AChE or BChE in packed red blood cell (PRBC) or plasma was determined according to the methods developed from Ellman's method as previously described [21-23]. The mixture was prepared by mixing 10 µL in each aliquot of 1:10 PRBC/plasma and extracts added in 1.0 mL of 0.25 mM DTNB buffer. This was pre-incubated for 5 min at room temperature and the reaction was started with the addition 25 µL of acetylthiocholine iodide/butyrylthiocholine iodide. The absorbance per minute (ΔA/min) of thiocholine product was determined at 405 nm. The AChE/BChE activity was calculated and expressed as U/L (AChE factor = 76838, BChE factor = 7684). The experiment was run in triplicate.

$$\text{ChE activity} = \left(\frac{\Delta A}{min}\right) \times \text{Factor} \quad (2)$$

$$\text{\% Inhibition} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100 \quad (3)$$

Statistical analysis

All values were expressed as the mean±standard deviation for 3 replicates. Data were analyzed by using GraphPad Prism 5 version 5.01 (GraphPad Software Inc. La Jolla, CA. USA). Data were analyzed by one-way ANOVA and the statistically significant differences were analyzed using the student paired t-test. $p < 0.05$ was considered statistically significant.

Results and discussion

Extract yield of difference solvent of *M. hortensis*

The percentage yields obtained from the different solvent extracts of *M. hortensis* leaves and flowers are shown in **Table 1**. The methanolic flower extract of *M. hortensis* showed the highest yield (31.70 %) while the diethyl ether flower extract of *M. hortensis* showed the lowest yield (2.17 %). The recovery of extractable constituents from *M. hortensis* leaves and flowers in different solvents showed that the highest recovery was the methanol extraction, following by butanol, chloroform, and diethyl ether, respectively.

Total phenolic content

The total phenolic content of the *M. hortensis* extract was determined using Folin-Ciocalteu reagent. Total phenolic content of the sample was calculated on the basis of the standard curve for Gallic acid and the results were expressed as mg of Gallic acid equivalent (mgGAE)/g of dry weight extract (gDWE). The results were given in **Table 1**. The amount of total phenolic content varied for the different solvents ranging from 10.55±0.08 to 45.47±0.59 mgGAE/gDWE. The total phenolic content of chloroform

($LCHCl_3$), methanol (LMeOH), butanol (LBuOH) and diethyl ether (LEt_2O) leaf extract of *M. hortensis* were 45.47±0.59, 10.55±0.08, 32.27±0.28 and 74.65±0.45 mgGAE/gDWE, respectively. While, the total phenolic content of chloroform ($FCHCl_3$), methanol (FMeOH), butanol (FBuOH) and diethyl ether (FEt_2O) flower extract of *M. hortensis* were 36.04±0.00, 6.57±0.91, 12.78±3.57 and 47.54±0.78 mgGAE/gDWE, respectively. The results of the total phenolic content in *M. hortensis* leave and flowers are statistically different ($p < 0.05$) (**Figure 1**). The highest total phenolic content was found in the diethyl ether leaf extract of *M. hortensis* (74.65±0.45 mg GAE/g DWE) and the lowest was in the methanolic flower extract of *M. hortensis* (6.57±0.91 mgGAE/gDWE).

DPPH free radical scavenging activity

The DPPH scavenging activity of the *M. hortensis* leaves and flowers extract in comparison to ascorbic acid as the positive control is shown in **Figure 2**. In all the solvent extracts, the free radical scavenging activity was found to be solvent dependent. The IC_{50} values of DPPH free radical scavenging activity of the *M. hortensis* leaves extract was found to decrease in the following order to ascorbic acid (1.35 mg/mL), diethyl ether (11.83 mg/mL), butanol (13.65 mg/mL), methanol (26.27 mg/mL), and chloroform (89.43 mg/mL). The IC_{50} values of the DPPH activity in chloroform, methanol, butanol, and diethyl ether flower extract of *M. hortensis* were 45.08, 11.49, 24.72, and 23.16 mg/mL, respectively (**Table 1**).

Table 1 Percentage yield, total phenolic content and DPPH free radical scavenging of different solvent extracts of *M. hortensis*.

Solvent	Weight of the crude extract (g)		Extract yield (%)		Total phenolic content (mgGAE/gDWE)		IC_{50} DPPH free radical scavenging (mg/mL)	
	Leaves	Flowers	Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
Chloroform	2.47	1.54	4.95	3.08	45.47±0.59	36.04±0.00	89.43	45.08
Methanol	10.58	15.85	21.16	31.70	10.55±0.08	6.57±0.91	26.27	11.49
Butanol	2.97	3.54	5.95	7.09	32.27±0.28	12.78±3.57	13.65	24.72
Diethyl ether	1.87	1.08	3.75	2.17	74.65±0.45	47.54±0.78	11.83	23.16

IC_{50} of ascorbic acid = 1.35 mg/mL

Acetylcholinesterase activities and Acetylcholinesterase inhibitory activities

The results obtained by Ellman's method indicating the extracts acetylcholinesterase activity is shown in **Figure 3**. The baseline AChE activity in dilution 1:10 packed red blood cell (PRBC) was 18,902.15±855.63 U/L. At the 5 mg/mL concentration of chloroform, methanol, butanol, and diethyl ether leaf extracts of *M. hortensis* showed mild AChE inhibition (14.36 - 27.64 %) while the flower extract showed no symptoms of intoxication on AChE inhibition (10.57 - 28.86 %) as shown in **Figure 4**. The AChE activity decreased approximately to the one third of the baseline activity (less than 33 %), consistent with no symptoms of intoxication.

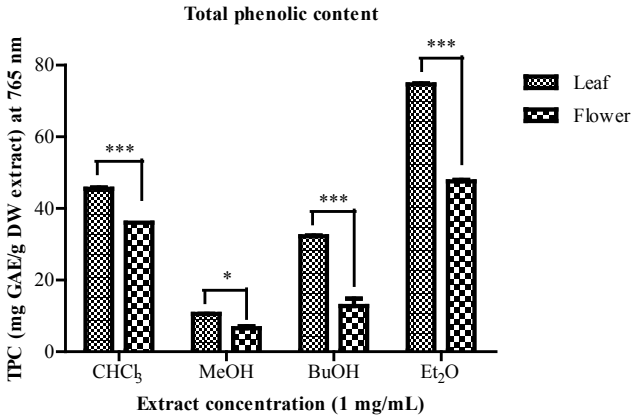

Figure 1 Total phenolic content of different solvent extracts of *Millingtonia hortensis* leaves and flowers extract, * $p < 0.05$, *** $p < 0.001$.

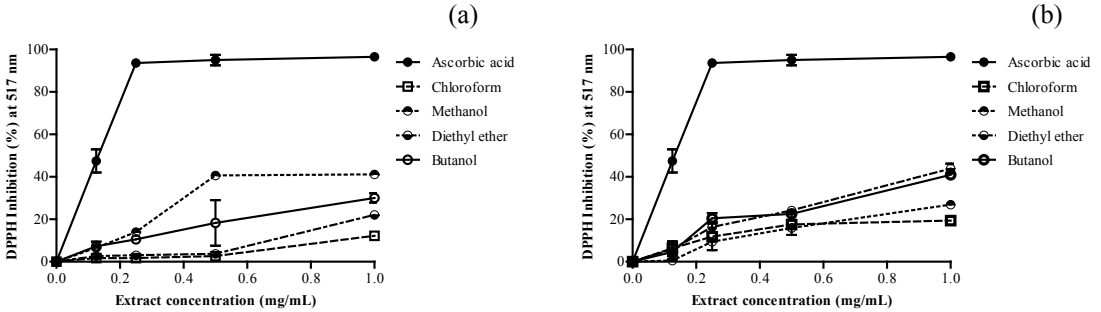

Figure 2 DPPH scavenging activities of various solvent extracts of *Millingtonia hortensis* leaves and flowers; (a) Leaves extract, and (b) flowers extract.

Butyrylcholinesterase activities and Butyrylcholinesterase inhibitory activities

The baseline BChE activity in plasma was 4,587.35±254.73 U/L. The BChE activity showed a mild decrease in the presence of the extract as shown in **Figure 3**. The BChE inhibitory activities of chloroform, methanol, butanol, and diethyl ether of *M. hortensis* leaf extract were 29.31, 41.60, 41.09, and 45.28, respectively. While the BChE inhibitory activities of chloroform, methanol, butanol, and diethyl ether of *M. hortensis* flower extract were 36.80, 40.34, 34.23, and 38.47, respectively. Summarized results are shown in **Figure 4**. At 5 mg/mL concentration the chloroform leaf extract showed no symptoms of intoxication on BChE inhibition (29.31 %), while methanol, butanol, and diethyl ether leaves extracts of *M. hortensis* showed mild BChE inhibition (41.60 - 45.28 %). All of the flower extracts in different solvents showed a mild BChE inhibition (34.23 - 46.34 %). The depression of about 30 - 50 % is accompanied by muscarinic symptoms of intoxication.

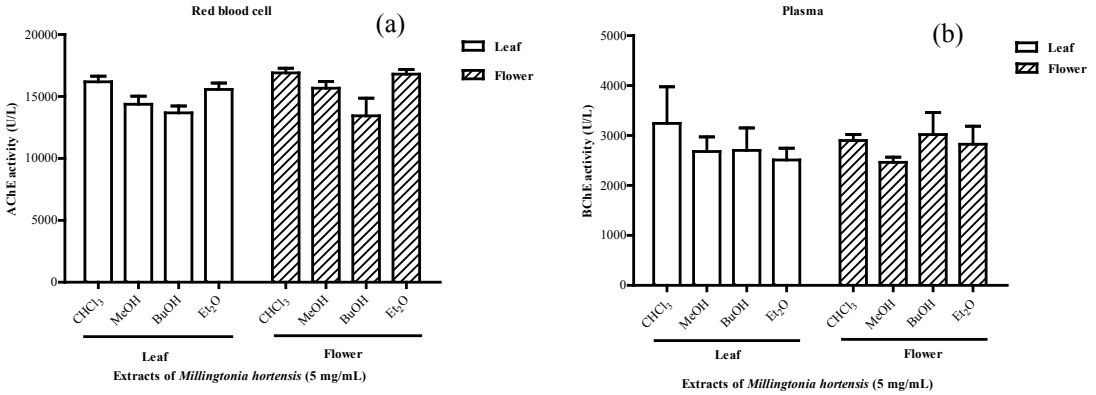

Figure 3 (a) Acetylcholinesterase activities on red blood cell, and (b) Bytyrylcholinesterase activities on plasma. AChE and BChE activities of the leaves and flowers extracts of *Millingtonia hortensis* in packed red blood cell and plasma. Determinations were done in triplicate. Each extract represents the mean±SD.

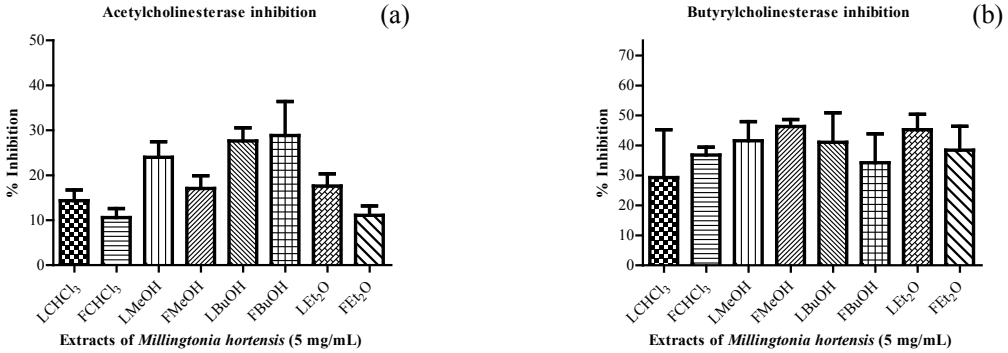

Figure 4 (a) Acetylcholinesterase inhibition, and (b) Butyrylcholinesterase inhibition. AChE and BChE inhibitory activities of *Millingtonia hortensis* extracts in packed red blood cell and plasma of the extracts at concentration of 5 mg/mL. Experimental were done in triplicate and represented mean±SD.

The GC-MS chromatogram analysis of different solvent extracts of *M. hortensis* showed the fifteen main constituents in **Figure 5**. In comparison, the mass spectra of the constituents with the NIST library, the fifteen phytochemical compounds were characterized and identified and their retention time, molecular formula and molecular weight (**Table 2**). The principle components were 2,3-dihydro benzofuran, (Coumaran), (E)-2-methoxy-4-(prop-1-enyl)phenol (*trans*-isoeugenol), 9-oxa-bicyclo[3.3.1]nonane-2,7-diol, phytol acetate, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol), hexadecanoic acid methyl ester (Methyl palmitate), n-Hexadecanoic acid (Palmitic acid), 9,12,15-octadecatrienoic acid methyl ester (Z,Z,Z)-(Methyl linolenate), 9,12,15-octadecatrienoic acid, (Z,Z,Z)-(Linolenic acid), heneicosane, α-tocopherol (Vitamin E), dl-α-tocopherol, Z-12-pentacosene, β-sitosterol, and 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol,(3β5Z,7E)- (Vitamin D).

Therefore, we assume that the bioactivities exhibited by *M. hortensis* in this study are correlated to the occurrence of these bioactive compounds in the different solvent extract for cholinesterase inhibitory activities. However, further studies on the isolation, characterization, and biological evaluation of these identified compounds are necessary to confirm their potential benefits.

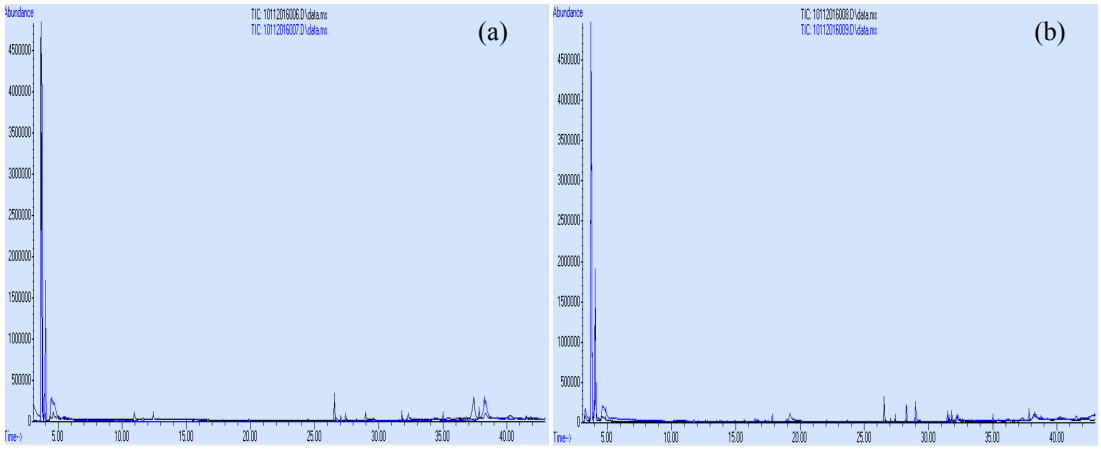

Figure 5 The chromatogram of the compositions of the chloroform and methanolic extracts from the leaves and flowers of *M. hortensis* by GC-MS analysis; (a) chloroform extract (b) methanol extract.

Table 2 Composition of extract from the leaves and flower of *M. hortensis* by GC-MS analysis.

Peak No.	RT	Compound	MF	MW
1	11.771	2,3-Dihydrobenzofuran (Coumaran)	C_8H_8O	120
2	17.857	(E)-2-Methoxy-4-(prop-1-enyl)phenol (*trans*-isoeugenol)	$C_{10}H_{12}O_2$	164
3	19.231	9-Oxa-bicyclo[3.3.1]nonane-2,7-diol	$C_8H_{14}O_3$	158
4	26.532	Phytol, acetate (Phytol acetate)	$C_{22}H_{42}O_2$	338
5	27.044	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	$C_{20}H_{40}O$	296
6	28.266	Hexadecanoic acid methyl ester (Methyl palmitate)	$C_{17}H_{34}O_2$	270
7	28.956	n-Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	256
8	31.574	9,12,15-Octadecatrienoic acid methyl ester (Z,Z,Z)-(Methyl Linolenate)	$C_{19}H_{32}O_2$	292
9	32.285	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-(Linolenic acid)	$C_{18}H_{30}O_2$	278
10	34.995	Heneicosane	$C_{21}H_{44}$	296
11	37.292	α-Tocopherol (Vitamin E)	$C_{29}H_{50}O_2$	430
12	37.396	dl-α-Tocopherol	$C_{29}H_{50}O_2$	430
13	37.821	Z-12-Pentacosene	$C_{25}H_{50}$	350
14	38.225	β-Sitosterol	$C_{29}H_{50}O$	414
15	38.350	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3β5Z,7E)- (Vitamin D)	$C_{27}H_{44}O_3$	416

RT = Retention time, MF = Molecular formula, MW = Molecular weight

Conclusions

AChE inhibitors are used as anti-Alzheimer disease medicines. The various side effects of these agents are toxicity, tolerability, and loss of efficiency. It is interesting for researchers to consider alternative natural substances to synthetic medicines. In the current study, 7 extracts have been identified, that effectively inhibit BChE, which is related to the mechanism of memory dysfunction. From these findings, we can conclude that most of the extracts contain inhibitory activities against AChE and could be considered worthwhile in the future studies for the treatment of dementia dysfunctional. There is no symptom intoxication that activates muscarinic receptors in smooth muscle.

According to our results, it could be concluded that the leaves and flowers of *M. hortensis* have various bioactive compounds and further studies will be needed to isolate phytocomponents of *M. hortensis* and investigate their biological activities. Therefore, it is recommended as a plant of pharmaceutical importance.

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