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Identification of Antioxidants and Acetylcholinesterase Inhibitors from *Hapaline benthamiana* Schott. Using at Line LC-ESI-QTOF MS/MS

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Abstract

In this work, total phenolics, total flavonoids, antioxidant activity, and acetylcholinesterase inhibition potency of *Hapaline benthamiana* schott are reported. In addition, the investigation of antioxidant and anti-acetylcholinesterase compounds of this plant coupled to the at line-LC-ESI-QTOF-MS/MS technique is introduced for the first time. The results show that the methanolic extract contains the highest amount of total phenolics, total flavonoids, antioxidant activity, and acetylcholinesterase inhibition activity. The at line-LC-ESI-QTOF-MS/MS technique has provided information on the biological activity of each compound while mass spectrometry provided information on their molecular weights. Based on their mass spectra, 53 compounds were identified with most of them being phenolic, flavonoid, and glycolipid compounds. Seventeen compounds exhibited a strong antioxidant activity ranging between 51.52 - 63.58 % inhibition whereas one compound, namely septentriodine, showed a strong acetylcholinesterase inhibitory effect of more than 50 %. This study indicates that *Hapaline benthamiana* schott is a source of active compounds containing antioxidant and anti-acetylcholinesterase activities with health benefits. In addition, at line-LC-ESI-QTOF-MS/MS has been shown to be an effective technique for the rapid and simultaneous analyses of antioxidant and anti-acetylcholinesterase compounds in plant extracts.

Keywords: *Hapaline benthamiana* schott, Acetylcholinesterase, At line-LC-ESI-QTOF-MS/MS, Antioxidant, Phenolics, Flavonoids, Glycolipid

Introduction

Free radicals are the main cause of many diseases in humans such as diabetes mellitus, neurodegenerative disorders (Parkinson's disease-PD, Alzheimer's disease-AD), and various cancers. Therefore, people have used various plants to treat many diseases and pay attention to the study of the active compounds in natural products that are related to mitigating the effects of free radicals. Studies on herbal plants, vegetables, and fruits have indicated the presence of many antioxidant compounds such as phenolics, flavonoids, tannins, and anthocyanidins [1-3]. Such compounds are secondary metabolite products found in natural products, which have anti-oxidation potential and can scavenge free radicals. They are active compounds that play an important role in many physiological processes affecting human health and can also reduce the risk of several diseases including cancer, diabetes, heart disease, and neurodegenerative diseases, especially Alzheimer's disease (AD) [4-7]. AD is the most common disease in the dementia group (neurodegenerative diseases), which is one of the top 7 diseases found among the elderly in Thailand. In recent years, there has been growing interest in finding active compounds from plants that act as antioxidants because of their free radical scavenging abilities, oxidative damage inhibition, and potential to prevent neurodegenerative diseases [8-10]. Plants usually contain many complex compounds. Therefore, various methods for isolation, structure elucidation, and bioactivity evaluation of active compounds from plants have been established. Liquid chromatography (LC) coupled with mass spectrometry (LC-MS) is one of the techniques widely used in natural product research to identify compounds in complex mixtures. Several researchers have successfully combined this technique with online bioassays for the analyses of biologically active compounds in natural products [11-15]. However, the method is not suitable for some assays, such as enzymatic assays, that require a longer reaction time. At-line assays are a new method for bioactivity screening with the assay being done in combination with a

fractionation method. This technique has proved to be a beneficial method capable of providing biological activity information for each compound while mass spectrometry provides information of the molecular weights. In addition, the molecular structure can be obtained from fragmentation data of the compounds of interest. Therefore, this technique is convenient, uses a small amount of sample, and is useful for bioassays that need a long reaction time [14,16,17].

Several plants from the Araceae family have been used for a long time in food and in traditional Thai medicine remedies with a wide range of medicinal properties such as blood tonic, nourishing the heart and brain, and cancer protection. The studies on the edible parts of the plants in the Araceae family have mostly surveyed the chemical constituents and investigated the antioxidant, antitumor, and cytotoxic properties of these plants. Many active compounds such as phenolics, flavonoids, anthocyanins, and alkaloids were obtained from the members of the Araceae family [18-30]. Previously, the total phenolic content and antioxidant activity in leaves and petioles of *A. farisii*, which is a species of the Araceae family, have been determined. The methanol crude extract exhibited the highest values for both tests [18]. In addition, the antioxidant activity and concentrations of bioactive compounds such as flavonoids and triterpenoids of the aerial part and rhizome of *Homalomena pierreana*, one of the members of Araceae family, have been reported. The data revealed that the antioxidant activities of *H. Pierreana* aerial part were higher than those of the rhizome. Furthermore, the aerial part also exhibited higher concentrations of flavonoids and triterpenoids in comparison to those of the rhizome [26]. Pohlit reported that the extracts of the Araceae species inhibit the *in vitro* growth of the human malaria parasite, and that they can be used to treat malaria and its symptoms [27]. Furthermore, the presence of flavonoids, including C-glycosylflavones and proanthocyanidins, as the main group of chemical constituents in the Araceae family species has been reported by Iwashina [28]. In addition, Fatma reported that 4 flavonoids luteolin, isoorientin, vitexin, and β-sitosterol have been isolated from *Arum hygrophilum* Boiss., one of the members of the Araceae family, and that the ethanol extract showed good antioxidative capacities [29].

Hapaline benthamiana schott is a traditional Thai plant from the genus Hapaline, Araceae family, commonly known as Bon Tao. Almost all parts of this plant are used as food due to their richness in starch. The antioxidant activity, inhibitory activities of α-amylase, α-glycosidase, AGEs formation, and various phytochemical compounds from ethanolic crude extracts of *Hapaline benthamiana* schott were previously studied. The extract showed good values for both activities and content of phenolic compounds including caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, and protocatechuic acid [30]. However, the chemical composition and bioactivities of *H. benthamiana* have appeared in only a few literature reports. In order to get a more complete information and to provide benefit to consumers, this research focuses on the study of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant and anti-acetylcholinesterase activities. The analysis of active compounds from the *H. benthamiana* was also conducted. The active compound profiles related to biological activities were determined using at line-LC-ESI-QTOF-MS/MS analysis for antioxidant and acetylcholinesterase inhibition assays. This work is expected to be beneficial to consumers and could provide useful information for the development of natural antioxidants or alternative anticholinesterase drugs for treating Alzheimer's disease in the future.

Materials and methods

Chemicals and reagents

Folin-Ciocalteu's reagent, quercetin, gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE) type VI-S from electric eel, 5,5′-dithiobis[2-nitrobenzoicacid] (DTNB), galantamine, sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), and sodium nitrite ($NaNO_2$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All organic solvents (analytical grade) were purchased from Merck Co. (Darmstadt, Germany).

Preparation of dried plant materials

H. benthamiana used in this study was collected from Uttaradit province, Thailand, in November 2021. The plant materials were chopped into small pieces and freeze-dried for 3 days. The freeze-dried samples were ground into a fine powder using a high-speed blender. The resulting powder was stored at 4 °C until analysis.

Preparation of the crude extracts

The preparation of the crude extracts was performed according to the method of Carter *et al.* [31] with slight modification. Briefly, dried samples were extracted with 3 different solvents including hexane, ethyl acetate, and methanol by sequential extraction. A weighed sample (5.00 g) of the dried plant material was first extracted with 50 mL of hexane. The extract was shaken and centrifuged at room temperature and then the organic layer was collected. The extraction of the remaining powder was repeated twice. Then, the filtrates were pooled and concentrated using a rotary evaporator. The residues were dried further in vacuo at ambient temperature to yield the hexane crude extract. The procedure was repeated with ethyl acetate and methanol as solvents to obtain the ethyl acetate and methanol crude extracts, respectively. All dried extracts were weighed and stored at 4 °C for further use.

Determination of antioxidant activity

The antioxidant activity of the crude extracts was investigated based on the radical scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH)-free radical according to the method of Alías *et al.* [3] with slight modification. Briefly, dried extracts were dissolved in methanol to obtain stock solutions with the concentrations of 200 mg/mL. 1.5 mL DPPH solution (3×10^{-4} M) was mixed with 1.5 mL of extract solutions of various concentrations (20 - 120 mg/mL). The solution mixtures were placed in dark for 30 min at room temperature and subsequently the absorbance of the solution was measured at 517 nm using Perkin Elmer 554 UV-VIS spectrophotometer. A mixture of methanol (1.5 mL) and DPPH solution (3×10^{-4} M, 1.5 mL) was used as blank and BHT was used as a positive control. All test analyses were repeated in triplicate. The inhibition percentage of DPPH activities of crude extracts was calculated using Eq. (1). The IC_{50} value was determined by nonlinear regression analysis of the dose-response curve plotting % free radical scavenging versus sample concentration using GraphPad Prism 5;

$$\text{Inhibition percentage} = [(A_c - A_s)/A_c]\times100 \quad (1)$$

where A_c is the absorbance of the control reaction (containing all reagents except the extract), and A_s is the absorbance of the reactions containing the crude extracts.

Measurement of total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu colorimetric method, which is a simple, sensitive, and precise method, using gallic acid as a standard phenolic compound [3,32]. Briefly, 0.5 mL of each crude extract (1 mg/mL) was diluted up to 1 mL with methanol, mixed thoroughly with 1.5 mL of the Folin-Ciocalteu reagent, and then the reaction was neutralized with 2 mL of 7.5 % (w/v) sodium carbonate. The mixture was left for 30 min at room temperature for color development. The absorbance of the solutions was measured at 765 nm on a Perkin Elmer 554 UV-VIS spectrophotometer. Total phenolics were calculated using a calibration curve prepared with gallic acid standard solutions (5 - 80 µg/mL) and expressed as mg gallic acid equivalents (GAE) per g of crude extract. All determinations were carried out in triplicate.

Determination of total flavonoid content

The flavonoid content of crude extracts was determined using an aluminum chloride colorimetric assay [28] with quercetin being used to make the standard calibration. The quercetin standard solutions were prepared by serial dilutions using methanol (0 - 60 μg/mL). An amount of 0.3 mL of diluted standard quercetin solution or extract, 3.4 mL of 30 % methanol, 0.15 mL of $NaNO_2$ (0.5 M), and 0.15 mL of $AlCl_3.6H_2O$ (0.3 M) were mixed. After 5 min, 1 mL of NaOH (1 M) was added. The solution was mixed and incubated for 60 min at room temperature. The absorbance of the reaction mixtures was measured against blank at 420 nm using a Perkin Elmer 554 UV-VIS spectrophotometer. The total flavonoid content in the samples was calculated from the calibration plot and expressed as mg quercetin equivalent (QE) per of g of crude extract. All analyses were performed in triplicate.

Determination of acetylcholinesterase (AChE) inhibitory activity

The inhibition of AChE activity was measured using the spectrophotometric Ellman's method with minor modifications using acetyl-thiocholine (ATCI) as a substrate [33,34]. The acetylcholinesterase enzyme hydrolyses the acetylthiocholine to thiocholine and acetic acid as products. The thiocholine then reacts with Ellman's reagent (DTNB) resulting in the formation of 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate. The yellow color of the product can be measured at 405 nm on a microplate reader (Synergy H1 Hybrid Reader). The assay of crude extracts was performed in 96-well plates at various

concentrations of sample. In detail, 25 µL of 15 mM ATCI, 125 µL of 3 mM DTNB, 50 µL of Tris-HCl pH 8.0, and 25 µL of crude extract dissolved in buffer were added to the wells of a 96-well microplate. Then 25 µL of AChE (0.28 U/mL) was added and the absorbance of the solution was measured by using a microplate reader at a wavelength of 405 nm and monitored every 10 s for 2 min. Galantamine was used as a positive control in this experiment and all reactions were performed in triplicate. The percentage inhibition was calculated using Eq. (2);

$$\text{Inhibition (\%)} = [(V_{control}-V_{sample})/V_{control}]\times 100 \quad (2)$$

where $V_{control}$ is the mean velocity of the control reaction (containing all reactants except crude extract) and V_{sample} is the mean velocity of the reaction containing crude extract.

At line-LC-ESI-QTOF-MS/MS analysis

The active subfraction of *H. benthamiana* extract was prepared at a concentration of 20 mg/mL and injected into an Agilent 1,260 infinity Series HPLC system (Agilent Technologies, Inc., Waldbronn, Germany) coupled to a 6,540 QTOF-MS spectrometer (Agilent Technologies, Inc., Singapore) with an electrospray ionization (ESI) source. The operating parameters for the MS detection were as follows: Drying gas (N_2) flow rate, 10.0 L/min; drying gas temperature 350 °C; nebulizer pressure, 30 psig; capillary, 3,500 V; skimmer, 65 V; octapole RFV, 750 V; and fragmentor voltage, 100 V in negative mode. The mass range was set at m/z 100 - 1,000 am with a 250 ms/spectrum scan rate. The mass fragmentation was operated in an auto MS/MS mode with 3 collision energies of 10, 20 and 40 V, respectively. All acquisition and analysis of data was controlled by Agilent MassHunter Qualitative Analysis Software B.05.01 and Agilent MassHunter Qualitative Analysis Software B.06.0, respectively. The sample was separated on a Luna C18 (2) 100°A, 4.6×150 mm^2, 5 µm (serial no. 728946-40) column (Phenomenex, USA) at a flow rate of 0.5 mL/min. The mobile phase, was composed from 0.1 % formic acid in water (v/v) (A) and 0.1 % formic acid in acetonitrile (v/v) (B), and was delivered using a linear gradient elution from 5 to 95 % Solvent B over 30 min, followed by holding the composition constant for 10 min. The elution was followed by a post-run sequence for 5 min. The injection volume was 10 microliters, and the column temperature was set to 35 °C. The eluent was split into 2 flows using a 9:1 ratio. The major part was collected in a 96-well plate with a 30 s per well collection time, while the minor part was delivered to the ESI-QTOF-MS system. The micro-fractions in the 96-well plate were dried using a sample concentrator (Techne, Staffordshire, UK) and all micro-fractions were tested for bioactivity.

Antioxidant activities and acetylcholinesterase (AChE) inhibitory assay for at-line micro-fractions

The at-line micro-fractions in a 96-well plate were tested for bioactivity. For the antioxidant activity, the dried micro-fractions were dissolved with 50 µl of MeOH and 150 µl of 0.100 mM DPPH was then added. The reaction mixture was shaken and placed in dark at room temperature for 30 min. Afterwards, the solution's absorbance (Abs) was measured at 517 nm using a microplate reader (Synergy H1 Hybrid Reader). Methanol was used as a blank. The percentage of inhibition was calculated by using the Eq. (1).

The AChE inhibitory activity of the at-line micro-fractions was also determined using Ellman's colorimetric method with minor modifications [34]. First, 50 µL of Tris-HCl buffer (pH 8.0) was added to the dried micro-fractions and the mixture was shaken for 5 min. Afterwards, 25 µL of 15 mM ATCI, 125 µL of 3 mM DTNB, and 25 µL of AChE (0.28 U/mL) were added into the micro-fraction solution. The absorbance of the solution was monitored every 10 s for 2 min by using microplate reader (Synergy H1 Hybrid Reader) at the wavelength of 405 nm. The percentage inhibition was calculated using Eq. (2).

Identification of active compounds

The samples from the 96 well plate that showed bioactivity were linked to the LC-ESI-QTOF/MS chromatogram based on the retention time. The active compounds with MS and MS/MS data were tentatively identified. The mass data was compared with previous reports and with the help of a public databases; the Human Metabolomics Database (http://www.hmdb.ca; accessed on 15 April 2022), METLIN Metabolite Personal Compound Database (PCD, Agilent Technologies), and lipid database (lipidmaps.org; accessed on 15 April 2022).

Statistical analysis

Statistical analyses of percent DPPH radical scavenging activity, IC_{50} value, and total phenolic and flavonoid contents from crude extracts obtained using different types of solvent were conducted by one-

way ANOVA at 95 % confidence level using the general linear model of SPSS 14.0 for Windows. Statistical significance was defined as $p < 0.05$.

Results and discussion

Investigation of total extraction yield (%) and antioxidant activity of *H. benthamiana* crude extracts

The total extraction yields (%) of *H.benthamiana* with hexane, ethyl acetate, and methanol were approximately 2.86, 3.80 and 8.63 %, respectively. It was found that the methanolic extract exhibited the highest extraction yield whereas the hexane extract produced the lowest yield (**Table 1**). As can be seen in **Table 1**, the extraction yield increases with increasing polarity of the solvent used in the extraction. This result indicates that the chemical components of *H. benthamiana* are mostly soluble in polar solvents. Subsequently, the antioxidant activity of the crude extracts was studied by the DPPH radical-scavenging method, which is frequently used to assess the antioxidant capacity of many plants [3]. Percent DPPH radical scavenging activity was calculated using Eq. (1). Antioxidant activities of different concentrations of the crude extracts (20 to 120 µg/mL) were determined and the results are shown in **Figure 1**. The IC_{50} value of for the crude extracts was determined because the IC_{50} value represents the concentration of extract that can scavenge 50 % of the DPPH free radical. The results of this determination are shown in **Table 1**.

Table 1 Extraction yield and *in vitro* antioxidant activity of *H. benthamiana* crude extract.

Samples	Extraction yield (%)	% Inhibition at 120 µg/mL	IC_{50} µg/mL (Mean ± SD)
Hexane extract	2.86	58.85	102.2 ± 0.05
Ethyl acetate extract	3.80	72.82	45.7 ± 0.03
Methanolic extract	8.63	90.52	12.2 ± 0.02
Butylated hydroxytoluene (BHT)	-	-	10.6 ± 0.10

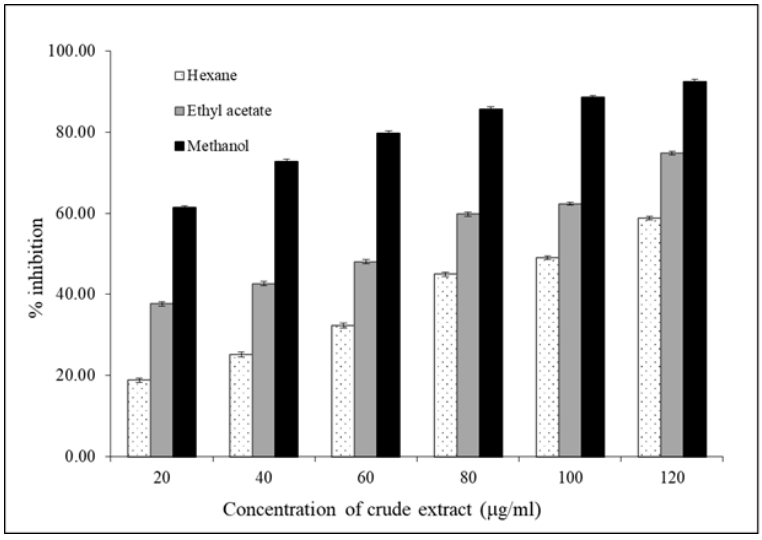

Figure 1 Antioxidant activity of the crude extracts at various concentrations determined by the DPPH method; data represent mean ± SD, $n = 3$.

Data shown in **Figure 1** indicates that all crude extracts exhibited concentration-dependent increases in radical scavenging capacity. As the concentration of the crude extract increases, the antioxidant activity increases as well. There was significant difference ($p < 0.05$) in percent DPPH radical scavenging activity and IC_{50} values for crude extracts obtained using different types of solvent. It was found that the crude methanolic extract exhibited the best antioxidant activity among the 3 crude extracts, with maximum

inhibition of 90.52 % at 120 µg/mL and lowest IC_{50} value of 12.2 µg/mL (**Table 1**). It can be assumed that active compounds such as phenolics, flavonoids, alkaloids, and tannins known to possess potent antioxidant activity are mainly found in the methanolic fraction. Similar findings were reported by previous research works, which indicate that most of active compounds were obtained in crude methanolic extracts of many plants. Thus, the crude methanolic extract is endowed with the greatest antioxidant activity [32,34]. Butylated hydroxytoluene (BHT) was used as the positive control and presented an IC_{50} value of 10.6 ± 0.1 µg/mL as shown in **Table 1**. It is interesting to note that the antioxidant activity of the isolated crude extract is close to that of BHT. The results show that the methanolic extract could serve as free radical inhibitor or scavenger, possibly acting as an antioxidant, which might be beneficial in illustrating the significant sources of natural antioxidants.

Investigation of total phenolic contents and flavonoid contents in *H. benthamiana* crude extracts

Phenolic and flavonoid compounds are usually the most abundant secondary metabolites in plants and act as antioxidants by chelating ions and scavenging free radicals. In this study, the values of total phenolic content of the extracts were derived from a calibration curve of gallic acid made with concentrations ranging from 5 to 80 µg/mL ($y = 0.0043x+0.004$, $R^2 = 0.9980$, where Y is the absorbance at various concentrations of gallic acid and X is concentration of gallic acid) and expressed in gallic acid equivalents (GAE) in mg per g of crude extract. The values of total flavonoid contents were derived from a calibration curve of quercetin made with concentrations ranging from 5 to 60 µg/mL ($y = 0.0239x+0.0887$, $R^2 = 0.9980$, where Y is the absorbance at various concentrations of quercetin and X is concentration of quercetin) and expressed in quercetin equivalents (QE) in mg per g of crude extract. Gallic acid and quercetin were used to determine the standard curves. The choice of these compounds as standards is based on their availability as pure and stable substances. In addition, the response of gallic acid and quercetin has been shown to be representative of most other phenolic and flavonoid compounds in vegetables on a mass basis [28]. **Table 2** shows that the total phenolic and flavonoid contents in crude extracts varied widely, ranging from 42.06 ± 0.40 to 94.28 ± 0.30 GAE (mg/g crude extract) and from 38.85 ± 0.80 to 75.08 ± 0.4 mg QE (mg/g crude extract), respectively. There was significant difference ($p < 0.05$) in total phenolic contents and flavonoid contents between the different crude extracts. It can be seen that the methanolic crude extract exhibited the highest total phenolic and flavonoid contents followed by the ethyl acetate and hexane crude extracts. This can be explained by the fact that phenolic and flavonoid compounds usually have high polarity due to the presence of hydroxyl groups resulting in these compounds being more readily soluble in methanol. In addition, these results positively correlated with the inhibition percentage and good agreement could be found between these results and those obtained by the DPPH scavenging method. In general, the observations show that higher total phenolic and flavonoid contents of the crude extract result in higher total antioxidant capacity. Several literature reports have studied the correlation coefficients between antioxidant activity and total phenolic and flavonoid content of the plants. It was found that the total phenolic and flavonoid contents of plants show strong correlation to their antioxidant properties where increases in phenolic and flavonoid contents result in increases in antioxidant activity [32,35,36].

Table 2 Total phenolic and flavonoid contents found in *H. benthamiana* crude extracts.

Crude samples	Total phenolic content GAE in mg/g crude extract	Total flavonoid content QE in mg/g crude extract
Hexane extract	42.06 ± 0.4	38.85± 0.8
Ethyl acetate extract	65.36 ± 0.6	51.22± 0.4
Methanolic extract	94.28 ± 0.3	75.08± 0.4

Investigation of acetylcholinesterase (AChE) inhibitory activity in *H. benthamiana* crude extracts

Acetylcholinesterase (AChE) is the most important enzyme regulating the level of acetylcholine (Ach) in the brain. Therefore, the screening of the inhibitory activity of various compounds for this enzyme should provide information for the development of efficient plants for the treatment of diseases exhibiting abnormal activity of this enzyme. In this study, crude extracts were tested for their AChE inhibitory activity using Ellman's colorimetric method in 96-welled microplate and galantamine was used as positive control. The AChE inhibitory activity of various concentrations of crude extracts (20 - 200 μg/mL) was investigated

and the results are shown in **Figure 2**. Methanolic extract showed inhibition of AChE ranging between 21.38 and 88.42 %, whereas it ranged from 19.65 to 64.62 % for the ethyl acetate extract, and from 8.83 to 59.43 % for the hexane extract. Among all samples tested, the methanolic extract showed maximum AChE inhibitory activity with 88.42 % of inhibition at 200 µg/mL followed by ethyl acetate (64.62 %) and hexane (59.43 %) at the same concentration. From the determination of the IC_{50} values, it was found that the methanolic extract has the lowest IC_{50} value of 48.38 µg/mL, which indicates the greatest AChE inhibitory activity. The ethyl acetate and hexane crude extracts showed high IC_{50} values of 121.12 and 163.16 µg/mL, respectively. Galantamine as positive control completely inhibited the activity of the enzyme at the concentration of 100 µg/mL and presented the percentage inhibition of 95.26 % at 20 µg/mL and an IC50 value of 5.76 µg/mL. Although the AChE inhibitory activity of the methanolic extract was lower than that of galantamine, it might possess a relatively high potency for inhibitory activity against acetylcholinesterase.

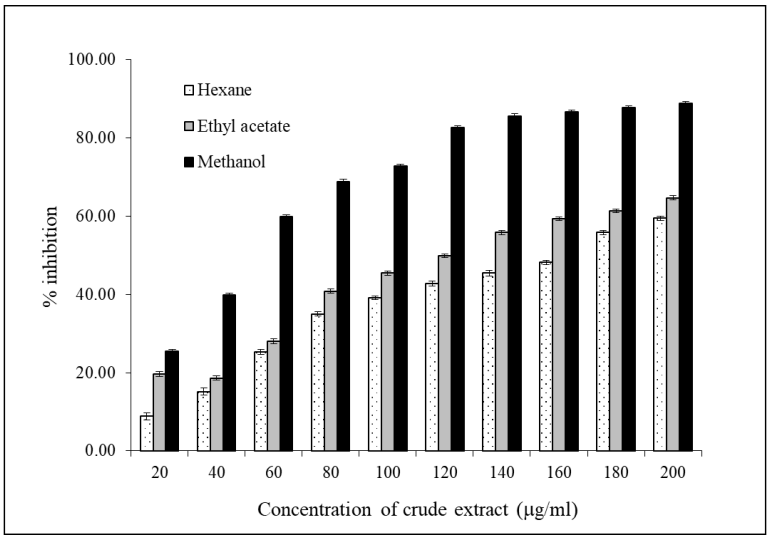

Figure 2 Acetylcholinesterase inhibition efficiency of the 3 crude extracts at various concentrations determined by the Ellman's colorimetric method; data represent mean ± SD, $n = 3$.

Structural characterization of chemical constituents in *H. benthamiana* methanolic extract

Since the methanolic extract of *H. benthamiana* showed strong antioxidant activity and AChE inhibition, it was fractionated using HPLC and the fractions were collected in a 96 well plate. The micro fractions from the 96 well plate that showed bioactivity were linked to the LC-ESI-QTOF/MS chromatogram by retention time. The extract was analyzed in 2 MS ionization modes, positive and negative. Mass spectrometers convert molecules into a charged ionized state. The formation of positive or negative ions (depending on the sign of the applied electrical field) occurs in high intensity. The mass spectrum appears as a plot of intensity versus m/z. High intensity of signal improves sensitivity of the analysis. It was found that the negative mode exhibited high signal intensity and the number of chemical compounds found in this way was higher than for the positive mode. In addition, the pattern of chemical composition in negative mode showed the retention time values ranging from 3 to 32 min as shown in **Figure 3**. Therefore, the negative mode was selected for chemical constituent analysis. The extract was separated, fractions were collected in a 96 well plate, and the collected micro-fractions were dried and tested for antioxidant and AChE inhibitory activities. In the study, the active compounds were tentatively identified using MS and MS/MS data. Fifty-three out of 56 compounds were identified and most of them were phenolic compounds especially from the groups of hydroxycinnamic acid, flavonoids, and glycolipids. The glycolipids present in peaks 42 - 55 in this plant extract contained mono-, di-, and tri-galactose conjugates with glycerol and mono acyl group of fatty acids C16:0, C18:1, C18:2 and C18:3. The characteristic acyl anions of these compounds in negative ion mode are observed at m/z 255.23, 277.21, 279.23, 281.25 and 283.26. Besides glycolipids, fatty acids with medium chains and long chains were also found (peak no.34 - 41, 56). Two fructosamine compounds, found in peak numbers 5 and 6, have previously been reported to have relevance to pathologies in diabetes and aging [37,38]. The list of compounds and their bioactivities

are shown in **Table 3** and **Figure 4**. The data in **Figure 4** shows that each compound presented different levels of antioxidant and AChE inhibitory activities. It was found that phenolic and flavonoid compounds showed more than 50 % inhibition in antioxidant activity whereas the remaining compounds such as glycolipids, fatty acids, and amino acids exhibited low antioxidant activities. Compounds from the hydroxycinnamic acid group, such as caffeic acid (peak no.23), ferulic acid (peak no.33), caffeoylquinic acid (peak no.7, 11 and 15), feruloylquinic acid (peak no.25), and coumaroylquinic acid (peak no.16, 22, 26) showed good antioxidant activities in the range 51.52 - 63.58 % inhibition with caffeic acid (peak no.23) showing the highest inhibitory activity. Small phenolic acids including quinic acid (peak no.3), protocatechuic acid (peak no.9), and gentistic acid (peak no.28) also exhibited good antioxidant activities of 51.53, 65.15 and 61.32 % inhibition, respectively. Five different flavonoid compounds (peak no.10, 14, 19, 27 and 30) also demonstrated good antioxidant activities in the range 52.02 - 55.82 % inhibition with kaempferol 3-neohesperidoside (peak no.30) having the highest inhibitory activity. In the case of AChE inhibitory activity, septentriodine (peak no.29), was the only compound exhibiting inhibitory activity of more than 50 %. This is an expected result because this compound is from the norditerpenoid alkaloid class. Several studies have reported that alkaloids can be a source of potential acetylcholinesterase inhibitors due to their complex nitrogen-containing structures, which, once positively charged, bind to the "anionic" active site of AChE [8,39]. On the other hand, non-alkaloid compounds, which include phenolics, flavonoids, and other glycoside compounds, exhibited lower anti-AChE activities than that of alkaloid compounds. Three compounds including pueraria glycoside (peak no.27), kaempferol 3-neohesperidoside (peak no.30), and gingerglycolipid C (peak no.52) presented low inhibitory activity in the range of 32.67 - 39.18 % whereas the rest of the compounds have inhibition activity below 30 %. It can be postulated that such non-alkaloid compounds might be bound to other binding sites of AChE resulting in the observed moderate to low anti-AChE activity. It should be noted that the compounds showing lower activity may low amounts of the corresponding components due to the micro fractions being collected for only 30 s.

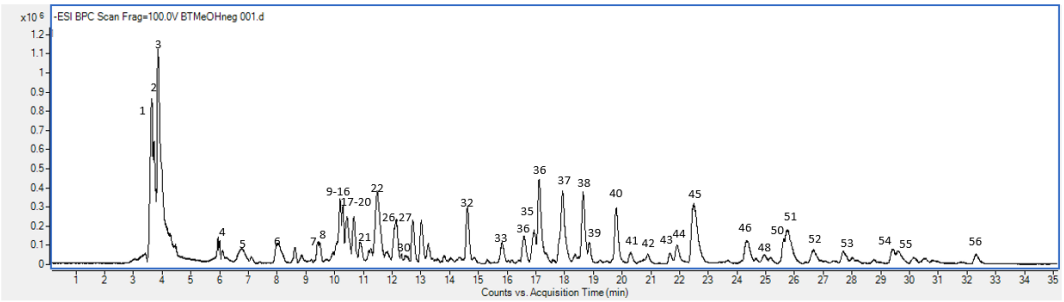

Figure 3 Chromatogram of the methanolic extract from *H. benthamiana* at a concentration of 20 mg/mL in negative ionization mode. The peak numbers correspond to the peak numbers of the identified compounds listed in **Table 3**.

Table 3 Metabolites identified in aerial part of *H. benthamiana* by LC- ESI(-)-QTOF-MS/MS.

Peak no.	RT (min)	m/z	Adduct	MS/MS fragmentation	Tentative identification	Formula	Error (ppm)
1	3.652	215.0311	$[M + Cl]^-$	89.0236, 71.0134, 59.0137	Hexose	$C_6H_{12}O_6$	7.86
2	3.716	377.0833	$[M + Cl]^-$	341.1071, 179.0544, 89.0240, 59.0135	Sucrose	$C_{12}H_{22}O_{11}$	6.13
3	3.871	191.0546	$[M - H]^-$	85.0288	Quinic acid	$C_7H_{12}O_6$	7.91
4	6.001	290.0859	$[M - H]^-$	200.0550, 128.0337	2-Deoxy-2,3-dehydro-N-acetylneuraminic acid	$C_{11}H_{17}NO_8$	7.72
5	6.771	292.1424	$[M - H]^-$	130.0861, 59.0126	N-(1-Deoxy-1-fructosyl)leucine	$C_{12}H_{23}NO_7$	−7.61

Peak no.	RT (min)	m/z	Adduct	MS/MS fragmentation	Tentative identification	Formula	Error (ppm)
6	8.039	326.1271	$[M-H]^-$	164.0707, 147.0442, 101.0235	N-(1-Deoxy-1-fructosyl)phenylalanine	$C_{15}H_{21}NO_7$	−7.89
7	9.423	353.0909	$[M-H]^-$	191.0549, 179.0340, 135.0439, 111.0434	Caffeoylquinic acid	$C_{16}H_{18}O_9$	−8.76
8	9.477	273.1366		229.1424, 211.1329, 193.1223, 181.1204, 163.1114, 73.0291	unidentified		
9	9.934	153.02	$[M-H]^-$	109.0283	Protocatechuic acid	$C_7H_6O_4$	−4.36
10	10.096	593.1571	$[M-H]^-$	549.2582, 503.1161, 473.1067, 431.0940, 383.0763, 353.0648, 311.0543, 282.0522	Apigenin-6,8-di-C-glycopyranoside	$C_{27}H_{30}O_{15}$	−9.96
11	10.105	353.0908	$[M-H]^-$	191.0547, 173.0434, 135.0437	Caffeoylquinic acid	$C_{16}H_{18}O_9$	−8.48
12	10.192	257.1422		213.1495, 195.1378, 155.1063, 57.0348	unidentified		
14	10.284	593.1573	$[M-H]^-$	473.1068, 353.0649, 297.0707, 117.0367	Isovitexin 2''-O-glucoside	$C_{27}H_{30}O_{15}$	−10.29
15	10.433	353.0916	$[M-H]^-$	191.0547, 173.0443, 135.0436, 111.0433, 93.0331	Caffeoylquinic acid	$C_{16}H_{18}O_9$	−10.75
16	10.483	337.0959	$[M-H]^-$	191.0553, 163.0390, 119.0490	Coumaroylquinic acid (p-)	$C_{16}H_{18}O_8$	−8.93
17	10.668	447.1557	$[M+HCOO]^-$	401.1442, 269.1018, 161.0442, 101.0231, 59.0136	Benzyl beta-primeveroside	$C_{18}H_{26}O_{10}$	−10.96
18	10.763	431.1956	$[M+HCOO]^-$	179.0547, 89.0234	Corchoionol C 9-glucoside	$C_{19}H_{30}O_8$	−7.72
19	10.897	593.1563	$[M-H]^-$	549.2818, 503.1199, 431.0950, 383.0705, 311.0546, 297.0388, 282.0519	Isovitexin 7-glucoside	$C_{27}H_{30}O_{15}$	−8.61
20	11.192	509.2283	$[M+HCOO]^-$	463.2160, 331.1755	Linalool oxide D 3-[apiosyl-(1->6)-glucoside]	$C_{21}H_{36}O_{11}$	−8.51
21	11.259	491.145	$[M+HCOO]^-$	445.1325, 293.0864, 233.0666, 151.0385, 89.0237	Monotropeoside	$C_{19}H_{26}O_{12}$	−8.9
22	11.494	337.0965	$[M-H]^-$	191.0548, 173.044	Coumaroylquinic acid (p-)	$C_{16}H_{18}O_8$	−10.71
23	11.786	179.036	$[M-H]^-$	135.0439, 79.0533	Caffeic acid	$C_9H_8O_4$	−5.68
24	11.815	177.0201	$[M-H]^-$	135.0439, 105.0325, 89.0389	Caffeoquinone	$C_9H_6O_4$	−4.34
25	11.832	367.1066	$[M-H]^-$	191.0546, 173.0440, 134.0362, 93.0334, 67.0185	Feruloylquinic acid	$C_{17}H_{20}O_9$	−8.56
26	12.091	337.096	$[M-H]^-$	191.0549, 173.0443, 93.0336	Coumaroylquinic acid (p-)	$C_{16}H_{18}O_8$	−9.22
27	12.139	431.1023	$[M-H]^-$	341.0651, 311.0549, 283.0603, 161.0229, 117.0328	Pueraria glycoside	$C_{21}H_{20}O_{10}$	−9.12
28	12.166	153.0197	$[M-H]^-$	109.0277, 81.0325	Gentisic acid	$C_7H_6O_4$	−2.4
29	12.454	699.3457	$[M-H]^-$	549.2692, 399.2023, 149.0585	Septentriodine	$C_{37}H_{52}N_2O_{11}$	5.91

Peak no.	RT (min)	m/z	Adduct	MS/MS fragmentation	Tentative identification	Formula	Error (ppm)
30	12.468	593.1558	$[M-H]^-$	285.0396, 255.0238	Kaempferol 3-neohesperidoside	$C_{27}H_{30}O_{15}$	−7.77
31	12.535	537.2149	$[M+Cl]^-$	311.0963, 221.0653, 149.0440, 89.0230	Eriojaposide A	$C_{24}H_{38}O_{11}$	−7.61
32	14.623	187.0983	$[M-H]^-$	125.0961, 97.0648, 57.0343	3-Methylsuberic acid	$C_9H_{16}O_4$	−3.83
33	15.829	193.052	$[M-H]^-$	161.0232, 134.0360	Ferulic acid	$C_{10}H_{10}O_4$	−7.08
34	16.588	185.0834	$[M-H]^-$	141.0912, 113.0230, 69.0344	Methylcyclohexane-1,2-dicarboxylic acid	$C_9H_{14}O_4$	−7.93
35	16.944	183.0678	$[M-H]^-$	139.0754	4-Formyl-3-(formylmethyl)-4-hexenoic acid	$C_9H_{12}O_4$	−8.29
36	17.12	327.2216	$[M-H]^-$	309.2064, 291.1967, 269.1737, 229.1432, 211.1322, 171.1009, 137.0953, 85.0285	Corchorifatty acid F	$C_{18}H_{32}O_5$	−11.93
37	17.932	329.2368	$[M-H]^-$	311.2235, 293.2097, 229.1441, 211.1331, 171.1017, 139.1114, 99.0807, 57.0337	9,10,13-Trihydroxy-11-octadecenoic acid	$C_{18}H_{34}O_5$	−10.49
38	18.649	327.2214	$[M-H]^-$	309.2075, 291.1907, 273.1869, 201.1132, 171.1016, 137.0966	Corchorifatty acid F	$C_{18}H_{32}O_5$	−11.31
39	18.862	371.2476	$[M-H]^-$	327.2527, 87.0446	13,14-dihydro-19(R)-hydroxyPGE1	$C_{20}H_{36}O_6$	−9.93
40	19.799	329.2372	$[M-H]^-$	311.2216, 293.2104, 201.1127, 171.1017, 139.1119	5,8,12-Trihydroxy-9-octadecenoic acid	$C_{18}H_{34}O_5$	−11.7
41	20.301	307.1942	$[M-H]^-$	235.1335, 185.1171, 97.0649	Corchorifatty acid A	$C_{18}H_{28}O_4$	−8.84
42	20.885	837.4214	$[M-H]^-$	559.1862, 397.1322, 277.2153, 179.0523, 89.0241	Tri- glycosyl monoacylglycerol	$C_{39}H_{66}O_{19}$	−10.56
43	21.671	721.373	$[M+HCOO]^-$	675.3522, 577.2651, 397.1357, 277.2160	Gingerglycolipid A	$C_{33}H_{56}O_{14}$	−10.8
44	21.923	721.3735	$[M+HCOO]^-$	675.3618, 577.2663, 397.1339, 277.2173	Gingerglycolipid A	$C_{33}H_{56}O_{14}$	−11.49
45	22.509	721.3661	$[M+HCOO]^-$	675.3587, 577.2566, 397.1338, 277.2156	Gingerglycolipid A	$C_{33}H_{56}O_{14}$	−1.23
46	24.35	723.389	$[M+HCOO]^-$	677.3733, 397.1330, 279.2321	Gingerglycolipid B	$C_{33}H_{58}O_{14}$	−11.25
47	24.657	699.3875	$[M+HCOO]^-$	653.3747, 415.1450, 397.1346, 255.2322	Di- glycosyl monoacylglycerols C16:0	$C_{31}H_{58}O_{14}$	−9.49
48	24.945	699.391	$[M+HCOO]^-$	653.3721, 397.1337	Di- glycosyl monoacylglycerols C16:0	$C_{31}H_{58}O_{14}$	−14.5
49	25.166	559.3178	$[M+HCOO]^-$	513.3070, 277.2177, 253.0919	Di- glycosyl monoacylglycerols C18:3	$C_{27}H_{46}O_9$	−9.68
50	25.643	559.317	$[M+HCOO]^-$	513.3073, 277.2176, 253.0921	Di- glycosyl monoacylglycerols C18:3	$C_{27}H_{46}O_9$	−8.25

Peak no.	RT (min)	m/z	Adduct	MS/MS fragmentation	Tentative identification	Formula	Error (ppm)
51	25.767	699.3859	$[M + HCOO]^-$	653.3758, 397.1351, 255.2323, 179.0547, 89.0242	Di- glycosyl monoacylglycerols C16:0	$C_{31}H_{58}O_{14}$	−7.21
52	26.669	725.4059	$[M + HCOO]^-$	679.3914, 397.1346, 281.2483, 235.0832, 161.0452	Gingerglycolipid C	$C_{33}H_{60}O_{14}$	−12.95
53	27.722	561.334	$[M + HCOO]^-$	515.3223, 311.1621, 279.2322, 253.0916	1-O-Linoleoyl-3-O-beta-D-galactopyranosyl-L-glycerol	$C_{27}H_{48}O_9$	−10.62
54	29.428	537.3271	$[M + HCOO]^-$	491.3235, 255.2321	glucosyl-2-palmitoyl glycerol	$C_{25}H_{48}O_9$	1.74
55	29.605	727.4085	$[M + HCOO]^-$	681.4061, 415.1450, 397.1352, 283.2623	glucosylglycerol lipid	$C_{33}H_{62}O_{14}$	5.03
56	32.322	271.2254	$[M + HCOO]^-$	225.2215, 197.1937, 57.0347	Pentadecanal	$C_{15}H_{30}O$	9.1

(Note: Peaks at RT 12.7 - 13.3 min correspond to contaminants from Nylon membrane)

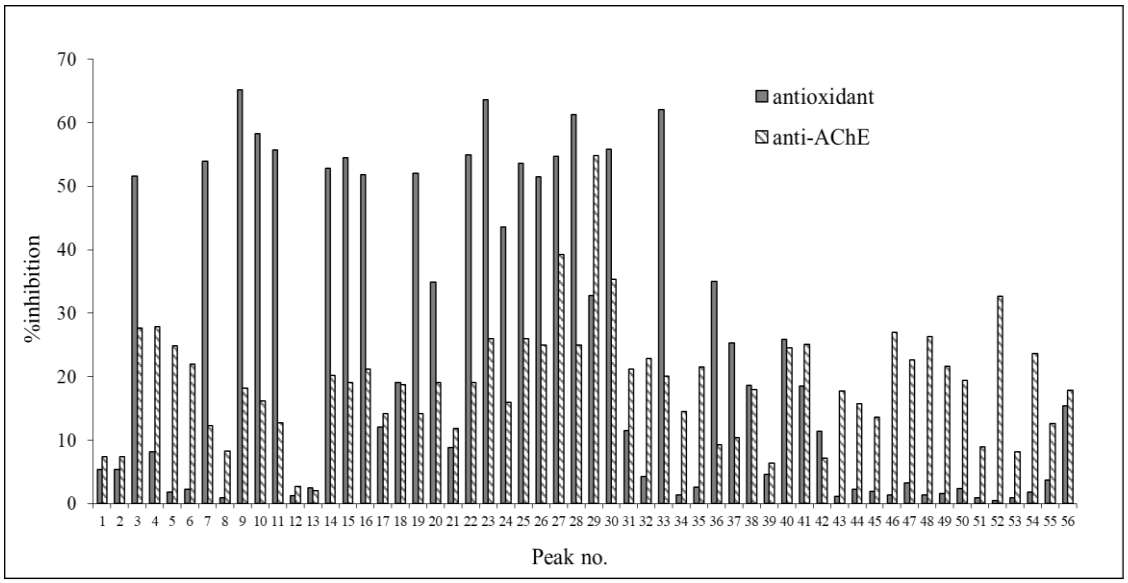

Figure 4 Antioxidant and anti-acetylcholinesterase (AChE) efficiency observed in the collected At-line micro-fractions of *H. benthamiana*.

Conclusions

In this study, the investigation of antioxidant and acetylcholinesterase inhibitory properties in *H. benthamiana* crude extract are reported for the first time. Our study indicates that the methanolic extract possessed high antioxidant and acetylcholinesterase inhibitory activities. This extract was separated and the collected micro fractions that showed bioactivity were linked to an LC-ESI-QTOF/MS chromatogram using retention time values. This technique presents a rapid option for lead identification in a group of known compounds and for the elucidation of their bioactivities. The at-line study led to the tentative identification of 53 compounds, most of which were phenolic, flavonoid, and glycolipid compounds. Seventeen phenolic and flavonoid compounds showed high antioxidant activity. In addition, only one alkaloid compound was found, and it exhibited a strong acetylcholinesterase inhibitory activity. This study indicates that *H. benthamiana* is an important source of active compounds containing antioxidant and anti-acetylcholinesterase activities with potential health benefits. This finding provides useful information about

the bioactivity of *H. benthamiana* and applications of this plant in the future. However, further studies investigating other biological activities and quantitative analyses of active compounds in *H. benthamiana* extract are required to fully appreciate the potential of this plant.

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