

## SUPPLEMENTARY MATERIAL

### **Normal phase HPLC-based activity profiling of non-polar crude plant extracts – acetylcholinesterase inhibiting guttiferones from *Montrouziera cauliflora* as a case study**

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## **Normal phase HPLC-based activity profiling of non-polar crude plant extracts – acetylcholinesterase inhibiting guttiferones from *Montrouziera cauliflora* as a case study**

The study describes bioactive compounds as inhibitors of AChE, from the stem bark extract of *Montrouziera cauliflora*, selected among nineteen dichloromethane extracts from Clusiaceae species. Our work focused on the development of an original normal phase HPLC microfractionation strategy to rapidly assess highly active zones from this crude active nonpolar plant extract. Two different microfractionation collection methods were evaluated for the assessment of the AChE inhibition. Two guttiferones and a tocotrienol were directly isolated among five compounds identified off-line by NMR after upscaling the fractionation and their AChE inhibition was evaluated. The strengths and weaknesses of the two microfractionation collection methods for HPLC-AChE activity-based profiling are discussed.

**Keywords:** Guttiferone; acetylcholinesterase; NP HPLC-HPLC microfractionation; Clusiaceae; *Montrouziera cauliflora*.

### **Experimental**

#### ***Chemical and reagents***

Solvents used for plant extractions were of analytical grade (Carlo Erba reactif, Val de Reuil, France). HPLC grade solvents were purchased from Sigma-Aldrich. AChE from *Electrophorus electricus*, acetylthiocholine iodide (ATCI), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), tacrine (9-amino-1,2,3,4-tetrahydrotacridine HCL), galanthamine, huperzine-A, naphthyl acetate, Fast Blue B salt,  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  were purchased from Sigma-Aldrich.

#### ***Plant material and extraction***

Plants were originating from Malaysia, New Caledonia or Vietnam and belonging to the Clusiaceae or Calophyllaceae families. These plants were collected thanks to the partnership with the Institut de Chimie des Substances Naturelles (ICSN, Gif/Yvette, France) and listed as previously described (Ferchichi et al. 2012). *Montrouzeria caulifflora* was collected in the dense rainforest of ‘Aoupinié North Province in ‘Réserve spéciale de faune du Col d'Amieu et Table Unic’ (New Caledonia, November 2000). The plant material was dried in an air dryer (DIÉMO's hairdryer, the temperature was of 45°C approximately and the time of drying 8 hours). A voucher specimen LIT-0412, identified by Marc Litaudon, was deposited in the

herbarium of the Botanical and Tropical Ecology Department of the IRD Center, Noumea, New Caledonia.

As described in our previous work (Ferchichi et al. 2012), extracts were performed at 100°C with DCM and MeOH, at a constant pressure of 100 bars, with a DIONEX ASE 200 instrument equipped with a solvent controller (dionex S.A., Voisins le Bretonneux, France). The static extraction time was 10 min and the purge time was 120 s. Only dichloromethane extracts (10-20% of yield) of 10 g of dried and ground bark, leaf, fruit or roots of each species were selected for this study.

### ***NP HPLC-UV-VIS-ELSD analysis***

The apparatus consisted of an HP-1050 liquid chromatographic system (Hewlett Packard) equipped with quaternary pumps, an in-line degasser, an auto-sampler and an photodiode array detector (PAD), coupled to an electrospray light scattering detector (500 ELSD, Alltech) by an HP interface (35900, Hewlett Packard). An analytical Si column (Cosmosil 5Si-II, 4.6x250mm, 5µm) was used. Elution was achieved at a flow rate of 1 ml/min using *n*-hexane (HPLC grade, solvent A) and ethyl acetate (HPLC grade, solvent B). In addition to the ELSD detection (60°C; Standard litre per minute 0.75), UV detection was also obtained at 280 nm using a photodiode array detector. Usually, 10 µl was injected for the extract at 10 mg/ml. For the extract profiling, a gradient elution was used and comprised the following steps: initial isocratic step at 16 %B for 5 min, a gradient from 16%B to 19%B from 5 to 11 min, isocratic step at 19%B from 11 to 39 min, and a gradient from 19%B to 100%B between 39 min and 60 min (Figure S1).

### ***Microfractionation by semi-preparative NP HPLC-UV-VIS***

Extracts (50 mg/5ml) were microfractionated at room temperature (25°C) by NP HPLC with an apparatus composed of a binary pump (System Gold High Performance Liquid Chromatograph P127, Beckman) and a UV-Visible detector (HP1050, Hewlet Packard) using a semi-preparative silica column (Cosmosil 5Si-II, 10x250 mm, 5 µm) with a pre-column (Cosmosil 5Si-II, 10x20 mm, 5 µm). Elution was achieved at a flow rate of 4.7 ml/min using *n*-hexane (HPLC grade, solvent A) and ethyl acetate (HPLC grade, solvent B). The preparative scale procedure, as well as flow rate, were obtained by direct geometric upscale from the analytical procedure (Guillarme et al. 2008, 2009). The microfractionation was done using a multistep gradient composed of 84% of B reaching 100% of A. Microfractions were

collected by a fraction collector (FC205, Gilson) from 2 to 33 min with a fraction every 0.33 min. Each fraction had a fixed volume of 1.5 ml.

#### ***Microfraction collection method 1 (fixed concentration)***

Each 1.5 ml microfractions were collected into separate vials. Fractions were then evaporated to dryness. Each microfraction was weighed and DMSO was added to provide stock solutions at 5, 1 and 0.1 mg/ml to be used in the AChE microplate assay upon 100-fold dilution.

#### ***Microfraction collection method 2 (extract composition dependent concentrations)***

1.5 ml microfractions were all collected into a single 96-well reservoir. The reservoir was evaporated to dryness and the fractions were all dissolved in fixed volume of MeOH (1 ml) in a single step, using 96-well pipette. Daughter 96-well plates were prepared by the collection of 20  $\mu$ l diluted fractions. Daughter plates were evaporated to dryness and the fractions were diluted again using 60  $\mu$ l/well of either MeOH (for the TLC enzymatic assay) or DMSO (for the microplate enzymatic assay). These later plate correspond to the stock plate used for the AChE microplate assay upon 100-fold dilution. In this conditions the fraction content transferred to the measured plate correspond to 0.09% of the collected fractions. Considering that each microfraction from method 1 weighed an average of 300  $\mu$ g (50 mg / 96 microfractions), 0.09% of 300  $\mu$ g corresponds to 0.27  $\mu$ g, which were then diluted in 0.270 ml; thus correspond to an average tested concentration of 1  $\mu$ g/ml.

#### ***AChE assay using microplate Ellman's method***

The enzymatic assay was carried out in 96-well plates as described before (Passos et al. 2013), adapted from Ellman et al. (Brühlmann et al. 2004). Each well was filled with 227.3  $\mu$ l of Ellman's reagent in 0.1 M phosphate buffer (pH 7.4), 20  $\mu$ L of a solution of acetylcholinesterase (1 U/ml), and 2.7  $\mu$ L of test compound ( $10^{-5}$  M) or extract solution (5 mg/ml) in DMSO. For 100% activity controls, test compound solutions were replaced by the corresponding volume of DMSO. Tacrine and galanthamine were used as inhibitory positive controls at  $10^{-7}$  M and  $10^{-5}$  M, respectively. The enzymatic reaction was initiated by addition of 20  $\mu$ L of acetylthiocholine iodide (ATCI) solution in demineralised water (final concentration equal to the  $K_m$  value). The final assay volume was 270  $\mu$ l. The plate was shaken for 3 s and the increase in absorbance at 412 nm was monitored using a microplate spectrophotometer. Data are means of three independent experiments, each performed in triplicate.

## **NMR**

<sup>1</sup>H spectra were recorded on a Varian Inova 500 MHz spectrometer (Palo Alto, CA, USA). The chemical shifts are given in  $\delta$  (ppm) with use of the residual CD<sub>3</sub>OD signal as internal reference ( $\delta_{\text{H}}$  3.31 and  $\delta_{\text{C}}$  49.0). The coupling constants ( $J$ ) are given in Hz. Data have been obtained from HMBC, HMQC and COSY spectra. Low amount of isolated compounds did not allow us to have a whole <sup>13</sup>C spectrum.

## **Infusion ESI-HRMS**

High-resolution MS spectra were recorded on a Q-TOF Micromass (Waters). Samples solution dissolved in chloroform / methanol / water 2:7:1 with 5 mM ammonium acetate were mixed with leucine-enkephalin (for internal calibration of the MS spectra; Sigma-Aldrich, Steinheim, Germany) dissolved in acetonitrile (100  $\mu\text{g}/\text{ml}$ ) in 1:3 proportions and infused using a TriVersa NanoMate<sup>®</sup> (Advion) (Zhang et al. 2004). It contains microfabricated nanoESI nozzles, which operated at solution flow rates of 83 nL/min. The nanoESI was initiated by applying 1.6 kV to the pipette tip and 0.5 psi (nitrogen) gas pressure on the liquid. The  $m/z$  range was set to be 100-1000 Da in centroid mode with a scan time of 0.25 s and an inter-scan delay of 0.01 s.

**Guttiferone I** <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD): 1.02 (3H, s, H-23), 1.24 (3H, s, H-22), 1.49 (3H, s, H-28), 1.51 (1H, m, H-6), 1.54 (3H, s, H-37), 1.59 (3H, s, H-36), 1.65 (6H, s, H-20, 27), 1.68 (3H, s, H-21), 1.69 (3H, s, H-38), 2.01 (2H, m, H-32), 2.05 (2H, m, H-33), 2.08 (1H, m, H-24"), 2.15 (1H, m, H-24'), 2.11 (1H, m, H-7"), 2.18 (1H, m, H-7'), 2.51 (2H, d,  $J = 6.7$  Hz, H-29), 2.56 (1H, m, 17"), 2.72 (1H, dd,  $J = 13.4, 8.8$  Hz, 17'), 4.88 (1H, br t,  $J = 6.9$  Hz, H-25), 4.96 (1H, br t,  $J = 8.8$  Hz, H-18), 5.07 (1H, br t,  $J = 6.4$  Hz, H-34), 5.18 (1H, br t,  $J = 6.7$  Hz, H-30), 6.67 (1H, d,  $J = 8.3$  Hz, H-15), 6.98 (1H, dd,  $J = 8.3, 2.1$  Hz, H-16), 7.20 (1H, d,  $J = 2.1$  Hz, H-12). <sup>13</sup>C NMR (126 MHz, CD<sub>3</sub>OD): 16.8 (C-38), 17.8 (C-37), 18.1(C-28), 18.3 (C-21), 23.2 (C-22), 25.9 (C-36), 26.0 (C-27), 26.4 (C-20), 27.0 (C-17), 27.3 (C-23), 27.7 (C-33), 30.2 (C-24), 31.8 (C-29), 40.6 (C-7), 41.0 (C-32), 47.9 (C-6), 49.5 (C-5), 68.1 (C-4), 115.1 (C-15), 117.1 (C-2), 117.3 (C-12), 120.6 (C-30), 121.0 (C-18), 125.2 (C-34), 125.3 (C-16), 125.5 (C-25), 129.4 (C-11), 132.1 (C-35), 133.3 (C-26), 135.5 (C-19), 139.0 (C-31), 145.9 (C-13), 152.3 (C-14), 194.9 (C-1), 195.4 (C-3), 195.6 (C-10), 210.0 (C-9). ESI-HRMS:  $m/z = 601.3351$  [M-H]<sup>-</sup> calculated for C<sub>38</sub>H<sub>49</sub>O<sub>6</sub><sup>-</sup>: 601.3535.

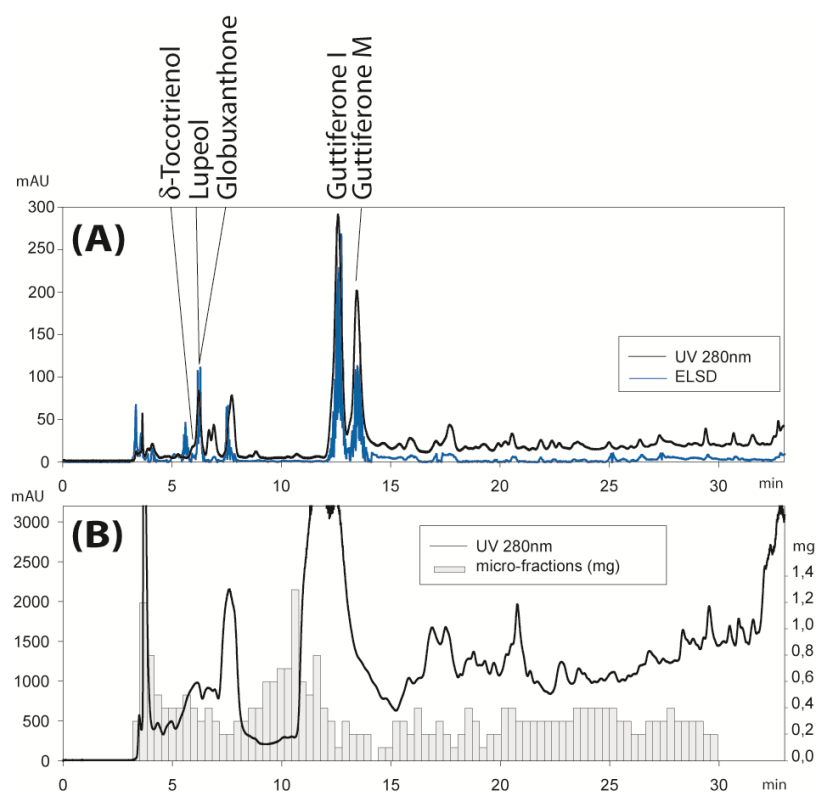
**Guttiferone M**  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ): 0.79 (3H, s, H-23), 1.17 (3H, s, H-22), 1.49 (1H, t,  $J = 13.0$  Hz, 7"), 1.55 (3H, s, H-37), 1.57 (3H, s, H-28), 1.59 (3H, s, H-36), 1.63 (3H, s, H-20), 1.68 (9H, s, H-21, 27, 38), 1.70 (1H, m, H-6), 1.74 (1H, m, H-24"), 1.99 (2H, m, H-32), 2.03 (2H, m, H-7', 33"), 2.07 (1H, m, H-33'), 2.14 (1H, m, H-24'), 2.50 (2H, m, H-29), 2.60 (1H, dd,  $J = 14.7, 7.1$  Hz, H-17"), 2.69 (1H, dd,  $J = 14.7, 7.1$  Hz, H-17'), 4.92 (1H, br t,  $J = 7.1$  Hz, H-18), 5.02 (1H, br t,  $J = 6.1$  Hz, H-25), 5.08 (1H, br t,  $J = 5.2$  Hz, H-34), 5.17 (1H, br t,  $J = 6.7$  Hz, H-30), 6.67 (1H, d,  $J = 8.4$  Hz, H-15), 7.00 (1H, dd,  $J = 8.4, 2.0$  Hz, H-16), 7.24 (1H, d,  $J = 2.0$  Hz, H-12).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CD}_3\text{OD}$ ): 16.3 (C-23), 16.7 (C-38), 17.6 (C-37), 17.8 (C-28), 18.1 (C-21), 23.6 (C-22), 25.7 (C-36), 25.8 (C-17, 27), 26.1 (C-20), 27.5 (C-33), 29.1 (C-24), 30.9 (C-29), 40.8 (C-32), 42.7 (C-7), 43.8 (C-6), 48.0 (C-5), 69.4 (C-4), 114.8 (C-15), 117.0 (C-12), 120.7 (C-30), 121.3 (C-18), 123.7 (C-25), 124.9 (C-16), 125.1 (C-34), 130.2 (C-11), 132.0 (C-35), 134.0 (C-26), 134.8 (C-19), 138.7 (C-31), 146.0 (C-13), 152.0 (C-14). ESI-HRMS:  $m/z = 601.3507$  [ $\text{M}-\text{H}$ ] $^-$  calculated for  $\text{C}_{38}\text{H}_{49}\text{O}_6^-$ : 601.3535.

**$\delta$ tocotrienol**  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ): 1.25 (3H, s, H-11), 1.53 (1H, m, H-1'b), 1.57 (3H, s, H-15'), 1.58 (3H, s, H-16'), 1.59 (3H, s, H-14'), 1.61 (1H, m, H-1'a), 1.66 (3H, s, H-13'), 1.73 (1H, dt,  $J = 13.7, 6.9$  Hz, H-3b), 1.79 (1H, dt,  $J = 13.7, 6.9$  Hz, H-3a), 1.95 (2H, t,  $J = 7.2$  Hz, H-9'), 1.98 (2H, t,  $J = 7.3$  Hz, H-5'), 2.05 (4H, m, H-5', 6'), 2.07 (3H, s, H-12), 2.12 (2H, q,  $J = 8.0$  Hz, H-2'), 2.68 (2H, m, H-4), 5.08 (2H, m, H-7', 11'), 5.14 (1H, t,  $J = 8.0$  Hz, H-3'), 6.32 (1H, d,  $J = 2.6$  Hz, H-5), 6.41 (1H, d,  $J = 2.6$  Hz, H-7).  $^{13}\text{C}$  NMR (126 MHz,  $\text{CD}_3\text{OD}$ ): 15.9 (C-16'), 16.1 (C-15'), 16.3 (C-12), 17.8 (C-14'), 23.2 (C-2'), 23.5 (C-4), 24.5 (C-11), 25.9 (C-13'), 27.5 (C-6'), 27.8 (C-10'), 32.8 (C-3), 40.6 (C-1'), 40.8 (C-5', 9'), 76.2 (C-2), 113.6 (C-5), 116.6 (C-7), 122.3 (C-9), 125.4 (C-7'), 125.5 (C-11'), 125.8 (C-3'), 127.8 (C-8), 132.0 (C-12'), 135.8 (C-4'), 135.9 (C-8'), 146.4 (C-10), 150.4 (C-6). ESI-HRMS:  $m/z = 397.3272$  [ $\text{M}+\text{H}$ ] $^+$  calculated for  $\text{C}_{27}\text{H}_{41}\text{O}_2^+$ : 397.3101.

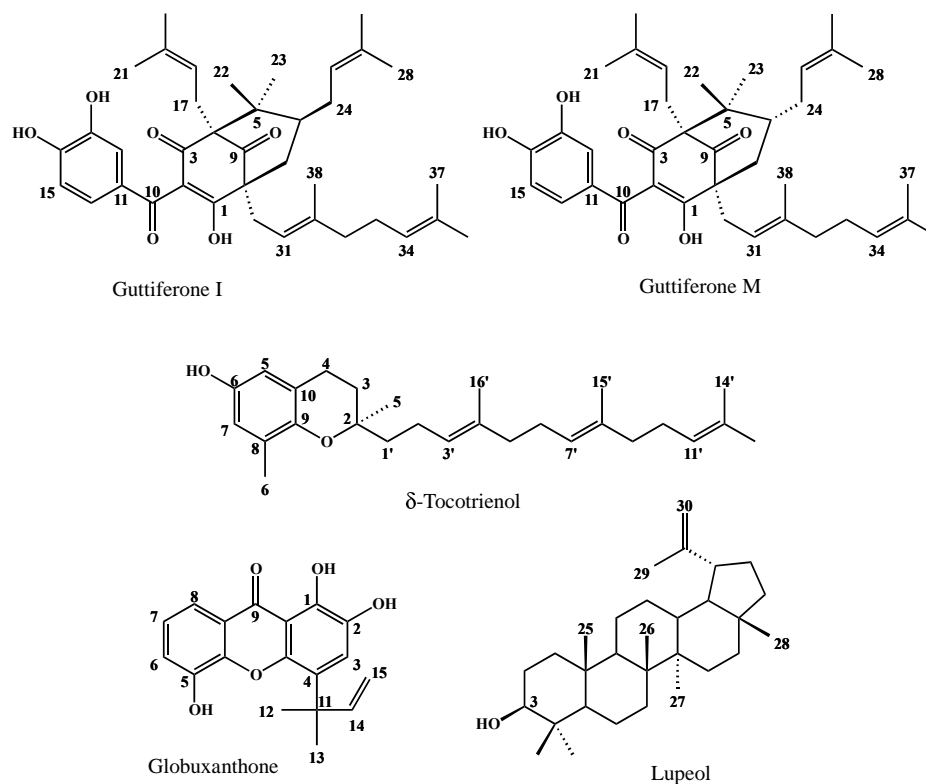
**Lupeol**  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ): 0.70 (1H, d,  $J = 9.1$  Hz, H-5), 0.76 (3H, s, H-24), 0.82 (3H, s, H-28), 0.87 (3H, s, H-25), 0.93 (1H, m, H-1"), 0.95 (3H, s, H-23), 0.98 (3H, s, H-27), 1.02 (1H, m, H-15"), 1.07 (3H, s, H-26), 1.09 (1H, m, H-12"), 1.21 (1H, m, H-22"), 1.24 (1H, m, H-11"), 1.31 (1H, m, H-9), 1.34 (1H, m, H-21"), 1.39 (1H, m, H-22'), 1.41 (1H, m, H-16"), 1.42 (2H, m, H-7, 11'), 1.43 (1H, m, H-18), 1.44 (1H, m, H-6"), 1.46 (1H, m, H-16'), 1.54 (1H, m, H-6'), 1.57 (1H, m, H-2"), 1.61 (1H, m, H-2'), 1.68 (1H, m, H-1'), 1.69 (3H, s, H-29), 1.70 (1H, m, H-12'), 1.71 (1H, m, H-13), 1.74 (1H, m, H-15'), 1.94 (1H, m, H-21'), 2.41 (1H, td,  $J = 11.1, 5.8$  Hz, H-19), 3.13 (1H, dd,  $J = 11.5, 4.8$  Hz, H-3), 4.56 (1H, dq,  $J = 2.5, 1.2$  Hz, H-30"), 4.69 (1H, d,  $J = 2.5$  Hz, H-30').  $^{13}\text{C}$  NMR (126 MHz,  $\text{CD}_3\text{OD}$ ): 15.0 (C-27), 16.1 (C-24), 16.6 (C-26), 16.7 (C-25), 18.4 (C-28), 19.5 (C-6, 29), 22.1 (C-11), 26.5 (C-12), 28.1 (C-

2), 28.5 (C-15), 28.6 (C-23), 30.9 (C-21), 35.5 (C-7), 36.7 (C-16), 38.3 (C-10), 39.5 (C-13), 40.0 (C-4), 40.1 (C-1), 41.0 (C-22), 42.1 (C-8), 44.0 (C-14), 44.1 (C-17), 49.5 (C-18, 19), 51.9 (C-9), 56.8 (C-5), 79.7 (C-3), 110.1 (C-30), 151.9 (C-20). ESI-HRMS:  $m/z = 427.3834$   $[M+H]^+$  calculated for  $C_{30}H_{51}O^+$ : 427.3934.

**Globuxanthone**  $^1H$  NMR (500 MHz,  $CD_3OD$ ): 1.65 (6H, s, H-12, 13), 5.04 (1H, dd,  $J = 10.7$ , 1.3 Hz, H-15"), 5.14 (1H, dd,  $J = 17.5$ , 1.3 Hz, H-15'), 6.35 (1H, dd,  $J = 17.5$ , 10.7 Hz, H-14), 7.21 (1H, t,  $J = 7.8$  Hz, H-7), 7.27 (1H, dd,  $J = 7.8$ , 1.6 Hz, H-6), 7.36 (1H, s, H-3), 7.67 (1H, dd,  $J = 7.8$ , 1.6 Hz, H-8).  $^{13}C$  NMR (126 MHz,  $CD_3OD$ ): 27.7 (C-12, 13), 41.0 (C-11), 111.5 (C-15), 115.9 (C-8), 120.8 (C-6), 121.8 (C-8a), 123.9 (C-3), 124.6 (C-7), 127.5 (C-4), 140.0 (C-2), 146.9 (C-1), 147.4 (C-10a), 148.3 (C-14), 148.4 (C-5), 148.6 (C-4a), 185.0 (C-9). DI-HRMS:  $m/z = 311.0915$   $[M-H]^-$  calculated for  $C_{18}H_{15}O_5^+$ : 311.0914.



**Figure S1:** Analytical (A) and geometrically upscaled (Guillarme et al. 2008, 2009) preparative (B) profiles on dichloromethane stem bark crude extract of *M. cauliflora*. The histograms corresponds to the quantity of each fraction collected in mg every 0.33 min.



**Figure S2:** Chemical structures of isolated compounds.

**Table S1:** AChE inhibitory activity of isolated compounds.

Compounds	% of inhibition <sup>1</sup>
Guttiferone M ( $10^{-3}$ M)	19.1 ± 2.5
Guttiferone I ( $10^{-3}$ M)	27.6 ± 11.2
δ-Tocotrienol ( $10^{-3}$ M)	22.0 ± ND
Globuxanthone + lupeol (0.7:1.0 mol) (500 µg/ml)	18.9 ± 8.7
Tacrine ( $10^{-7}$ M) <sup>2</sup>	43.1 ± 4.7
Galanthamine ( $10^{-3}$ M) <sup>2</sup>	99.6 ± 0.1
Galanthamine ( $10^{-7}$ M) <sup>2</sup>	12.3 ± 2.6

<sup>1</sup>Average ± SD of three independent experiments in triplicate.

<sup>2</sup>Positive controls.



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