SUPPLEMENTARY MATERIAL

β-secretase 1 Inhibitory Activity and AMP-Activated Protein Kinase Activation of Callyspongia samarensis Extracts

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Abstract The methanolic extract of *Callyspongia samarensis* (MCS) significantly inhibited β-secretase 1 (IC_{50} 99.82 μg/mL) in a dose-dependent manner and demonstrated a noncompetitive type of inhibition. Furthermore, it exhibited the highest AMPK activation (EC_{50} 14.47 μg/mL) as compared with the standard, Aspirin (EC_{50} >100 μg/mL). HPLC/ESI-MS analysis of MCS extract revealed 15 peaks, in which 9 peaks demonstrated similar fragmentation pattern with the known compounds in literature and in database library: 5-aminopentanoic acid **(1)**, 4-aminobutanoic acid **(3)**, Luotonin A **(4)**, (E)-3-(1H-imidazol-5-yl) prop-2-enoic acid **(8)**, Galactosphingosine **(10)**, D-sphingosine **(11)**, 5,7,4'-trihydroxy-3',5'-dimethoxyflavone **(12),** hydroxydihydrovolide **(13)**, and 3,5-dibromo-4-methoxyphenylpyruvic acid **(14)**; and 6 peaks are not identified **(2**, **5-7, 9,** and **15)**. Acute oral toxicity test of MCS extract revealed that it is nontoxic, with an LD_{50} of >2000 mg/kg. Assessment of BBB permeability of MCS extract showed that Compound 15 was able to cross the BBB making it a suitable candidate for developing CNS drugs.

Keywords: Alzheimer's disease; AMPK; blood brain barrier; β-secretase 1; *Callyspongia samarensis*; marine sponge

Experimental

Biological material

The marine sponge *Callyspongia samarensis* was collected in Agdangan, Quezon Province by hand at a depth of 6 to 10 ft. (13°51'8"N, 121°55'1"E). The sponge has a shape of thin finger-like projections. It is colour blue to dark green in situ. It is soft and compressible to touch. The surface ornamentation is conulose. The sample was identified and verified by Dave Anthony P. Valles. A voucher specimen was deposited at the Biological Museum, University of Santo Carlos, Cebu, Philippines (Voucher number USCBM2018.0001). It was identified as *Callyspongia samarensis* (Order Haplosclerida, Family Callyspongiidae). The megascleres are oxeas with both ends pointed and there are no microscleres. The ectosomal skeleton is characterized by multispicular reticulation. It has choanosomal skeleton with multispicular primary fibres.

Extraction

The freeze-dried sample was ground using an Osterizer blender and was sequentially extracted at room temperature with series of solvents of increasing polarity from hexane (3L x 4), dichloromethane (4L x 3), and methanol (4L x 5). Each extracts were concentrated under reduced pressure at 40^{o}C.

β-secretase 1 inhibitory activity assay

Analysis of β-secretase 1 inhibitory activity of *Callyspongia samarensis* extracts was performed using SensoLyte® 520 β-Secretase Assay Kit *Fluorimetric* and was carried out based on the procedure provided by the manufacturer. Control wells were established by mixing the assay components to a final volume of 100 µL. The microplate was incubated for 10 mins. at assay temperature prior to addition of β-secretase 1 substrate solution. The fluorescence was recorded immediately on a microplate spectrofluorometer (GloMax® Discover system) set at excitation 490 nm, emission 520 nm. The fluorescence was continuously recorded every 5 mins. for 2 hours. The percent (%) β-secretase 1 inhibition was calculated using the following equation:

$$\% \text{ β-secretase 1 inhibition} = \{1 - [(S_2-S_0)/(C_2-C_0)]\} \times 100$$

where S_2 was the fluorescence of the tested sample containing the enzyme, sample solution, and substrate after 2 hours of incubation, S_0 was the Initial fluorescence of the

tested sample, C_2 was the fluorescence of the control containing the enzyme, buffer, and substrate after 2 hours of incubation, and C_0 was the Initial fluorescence of the control.

Enzyme kinetics

The most active extract was subjected to enzyme kinetics analysis to determine the type of β-secretase 1 inhibition. The same procedure as β-secretase 1 inhibitory activity assay was employed for enzyme kinetics analysis but using different concentrations of the substrate. Lineweaver-Burk plot was used to analyse enzyme kinetics.

AMPK activation assay

Analysis of 5'adenosine monophosphate-activated protein kinase (AMPK) activation of *Callyspongia samarensis* was performed using CycLex AMPK kinase assay kit and was carried out based on the procedure provided by the manufacturer. The percent (%) AMPK activation was computed using the following formula:

$$\% \text{ AMPK Activation} = \{[(A_{sample} - A_{blank})/ A_{blank}]\} \times 100$$

where A_{sample} was the absorbance of the sample and A_{blank} was the absorbance of the control.

Acute oral toxicity test

Approval of the animal study protocol was obtained from the University of Santo Tomas - Institutional Animal Care and Use Committee (UST-IACUC) prior to the conduct of the experiments. Estimation of the lethal dose of the most active extract was performed following the Organization for Economic Cooperation and Development (OECD) guideline 425 limit test. Five (5) female Sprague-Dawley rats were utilized and dosed with 2000 mg/kg of the sample and was observed for 14 days. All surviving animals were humanely sacrificed and subjected to gross necropsy. A licensed veterinarian performed the gross necropsy of all test animals. After the gross necropsy, the liver and kidney of the animals were subjected to histopathological examination for further toxicity profiling.

Blood brain barrier permeability assay

The blood brain barrier permeability assay was performed based on the procedure of Vareed et al. (2014) but with some modifications.

Animals and treatment

Twenty-two (22) male Sprague-Dawley rats (8 - 12 weeks old and weighing 150 - 200

grams) were utilized. A concentration of 50 mg/mL of MCS extract was prepared by dissolving in 0.9% normal saline solution (NSS). The rats were given a dose of 250 mg/kg and 500 mg/kg of the extract via intra-peritoneal injection and were observed for any signs of discomfort and toxicity.

Extraction and Preparation of test compound from blood and brain

Blood collection and brain extraction were conducted every 30 minutes, 1 hour, and 2 hours after administration. Test animals were euthanized by CO_2 inhalation chamber prior to blood collection and brain extraction. One milliliter (1 mL) of blood was immediately collected from each rat by cardiac puncture. After blood collection, the brains were quickly extracted, excess blood were removed from the brains by blotting dry with filter paper and were homogenized using glass pestle. The blood and brain samples were diluted in 20 mL of methanol, vortex mixed for 1 min. and kept for 3 hours at 4^oC. After 3 hours, samples were centrifuge at 10,000 rpm and the supernatants were collected, this process was repeated thrice. The collected supernatants were concentrated under reduced pressure at 30^oC. The concentrated brain and blood extracts were re-suspended in methanol, vortex for 1 minute and ultrasonicate for another 1 minute. The resulting solution was analysed through high-performance liquid chromatography coupled with mass spectrometry (HPLC/ESI-MS) detection.

High Performance Liquid Chromatography – Mass Spectrometry (HPLC/ESI-MS)

The HPLC analysis was performed in Agilent 1100 HPLC system using Shim-pack GIST C_{18} column (5μm, 4.6 x 250 mm). Separation of the compounds was carried out in isocratic mode using methanol: water (95:5) with 0.1%formic acid. The flow rate was 0.3 mL/min and the injection volume was 10 μL through manual injection. MS analysis was performed in Bruker Esquire 2000 mass spectrometer with electrospray ionization source (ESI). The parameters for MS analysis were as follows: operated in positive ion mode, nebulizer pressure 2.0 psi, dry gas 11.5 L/min, gas temperature 180^oC, Octopole RF Amplitude 150.0 Vpp, octopole delta 2.40 Volt, octopole 2.35 Volt, Skim 1 24.5 Volt, and Skim 2 6.0 Volt.

Table S1. Positive ionization HPLC/ESI-MS data of methanolic extract of *Callyspongia samarensis.*

Peak #	RT (mins.)	Precursor ion (*m/z*)	Precursor type	Compound	% Match[a] / Match Score[b]	Formula	Monoisotopic mass (Da)
1	5.5	118.0	$[M+H]^+$	5-aminopentanoic acid	97.1%[a]	$C_5H_{11}NO_2$	117.01
2	5.6	130.0	$[M+H]^+$	Unknown	-	-	-
3	6.5	103.9	$[M+H]^+$	4-aminobutanoic acid	87.7%[a]	$C_4H_9NO_2$	103.06
4	7.2	286.0	$[M+H]^+$	Luotonin A	80.8%[a]	$C_{18}H_{11}N_3O$	285.09
5	7.3	165.9	$[M+H]^+$	Unknown	-	-	-
6	7.5	114.9	$[M+H]^+$	Unknown	-	-	-
7	7.8	129.8	$[M+H]^+$	Unknown	-	-	-
8	8.1	138.9	$[M+H]^+$	(E)-3-(1H-imidazol-5-yl) prop-2-enoic acid	81.0%[a]	$C_8H_{10}O_2$	138.04
9	8.2	153.9	$[M+H]^+$	Unknown	-	-	-
10	8.5	282.1	$[M+H]^+$	Galactosylsphingosine	0.8410[b]	$C_{24}H_{47}NO_7$	461.34
11	8.8	300.0	$[M+H]^+$	D-spingosine	74.2%[a]	$C_{18}H_{37}NO_2$	299.28
12	9.0	331.1	$[M+H]^+$	5,7,4'-trihydroxy-3',5'-dimethoxyflavone	0.7408[b]	$C_{17}H_{14}O_7$	330.07
13	9.1	198.9	$[M+H]^+$	Hydroxydihydrobovolide		$C_{11}H_{18}O_3$	198.12
14	25.2	351.4	$[M+H]^+$	3,5-dibromo-4-methoxyphenylpyruvic acid		$C_{10}H_8O_4Br_2$	350.0
15	39.1	337.9	$[M+H]^+$	Unknown		-	-

Note: [a]Percentage match based on database search using mzcloud; [b]Match score based on database search using Massbank, perfect score = 1.0000

Figure S1. *Callyspongia samarensis* in its natural habitat

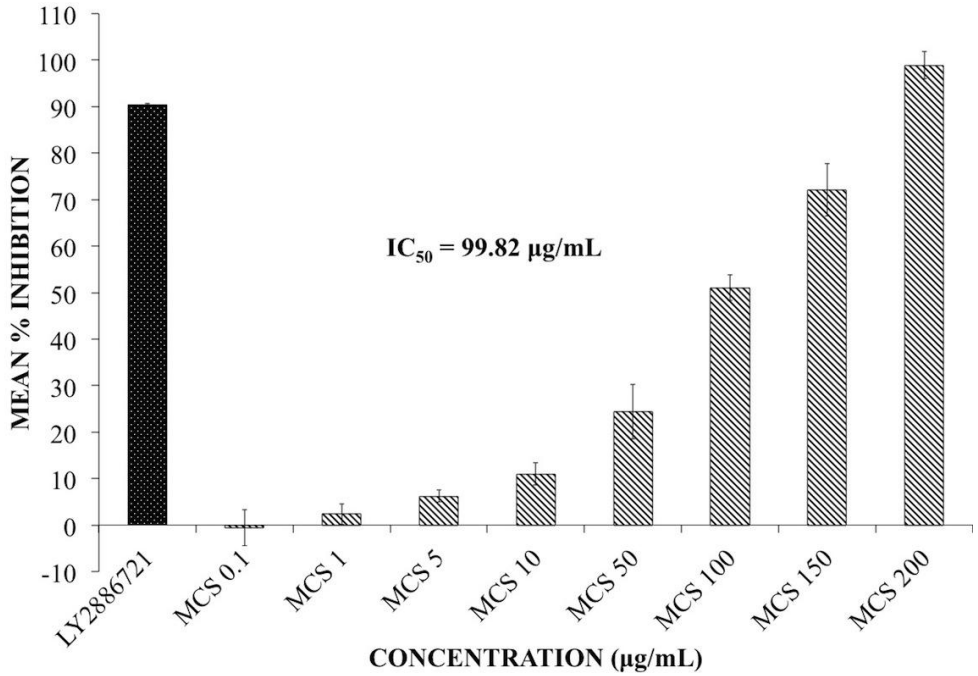

Figure S2. β-secretase 1 inhibitory activity of the methanolic extract of *Callyspongia samarenesis*

Note: LY2886721 - name of the standard β-secretase 1 inhibitor; **MCS –** methanol extract of *Callyspongia samarensis*

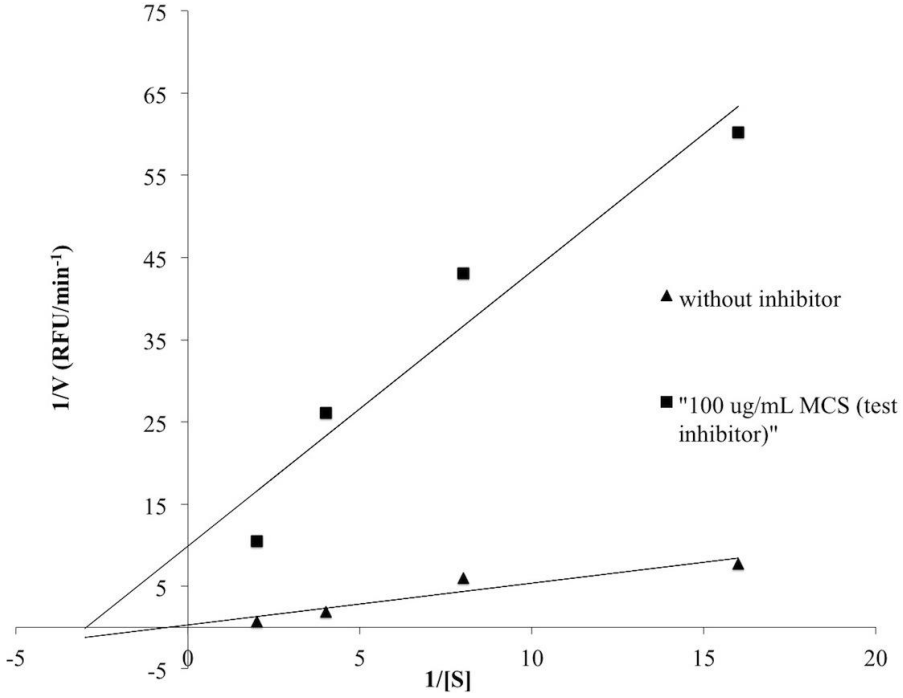

Figure S3. Lineweaver-Burk plot of β-secretase 1 inhibition by MCS extract

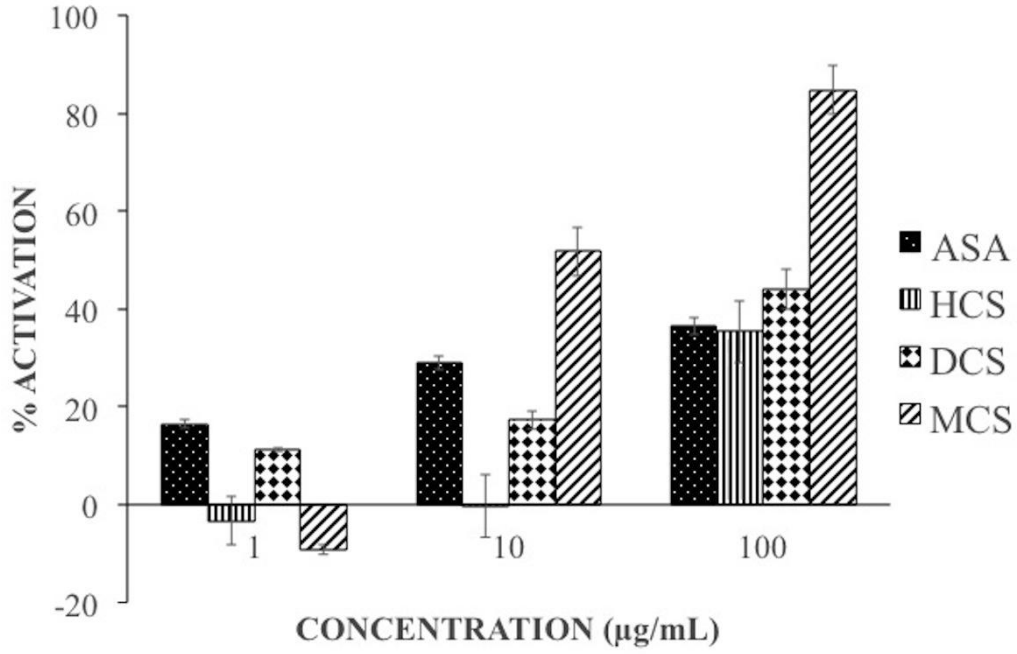

Figure S4. AMPK Activation of *Callyspongia samarensis* crude extracts

Note: ASA – standard AMPK activator Acetylsalicylic acid (Aspirin); **HCS –** hexane extract of *Callyspongia samarensis;* **DCS –** dichloromethane extract of *Callyspongia samarensis;* **MCS –** methanol extract of *Callyspongia samarensis*

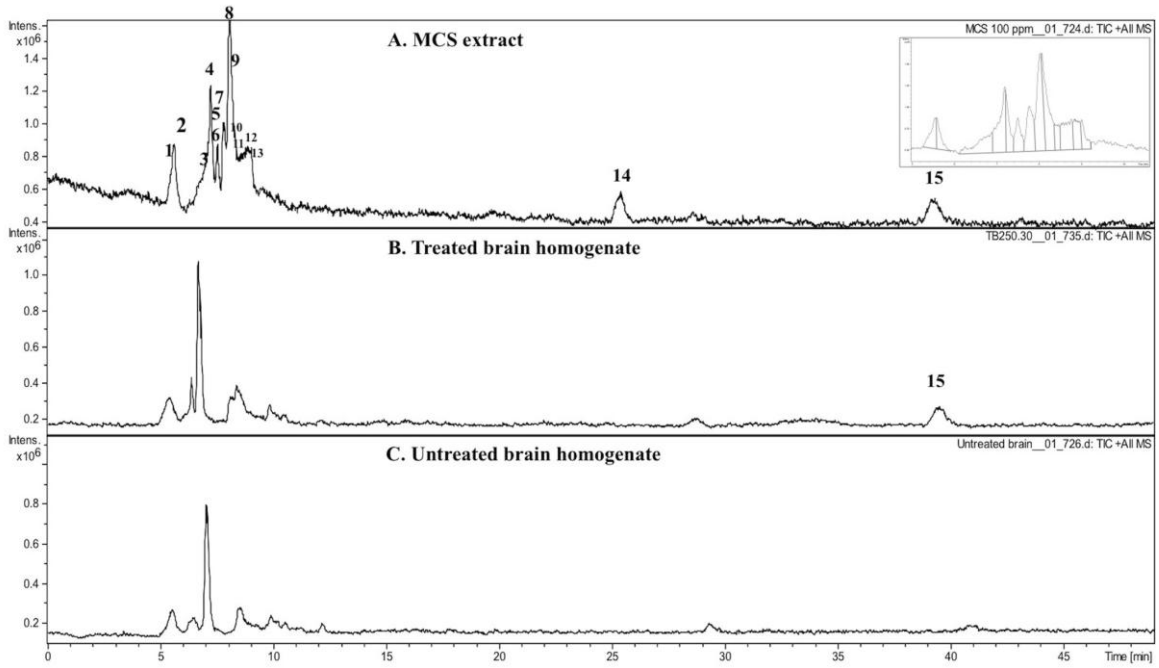

Figure S5. Total ion chromatogram of (A) MCS extract, (B) treated brain homogenate (C) untreated brain homogenate

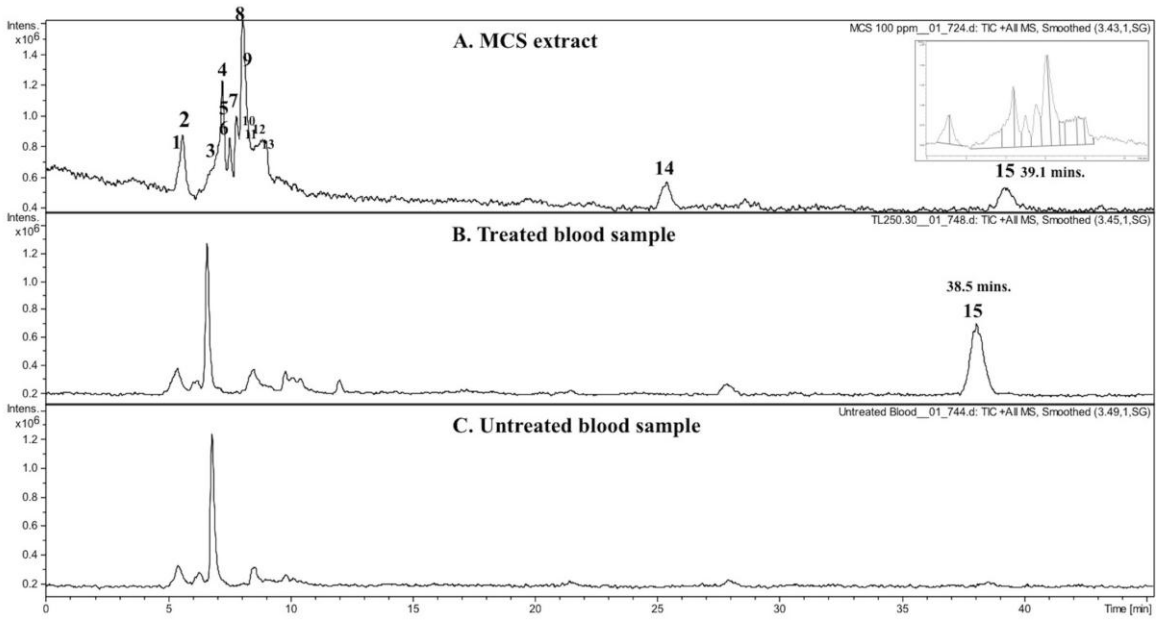

Figure S6. Total ion chromatogram of (A) MCS extract, (B) treated blood sample, (C) untreated blood sample

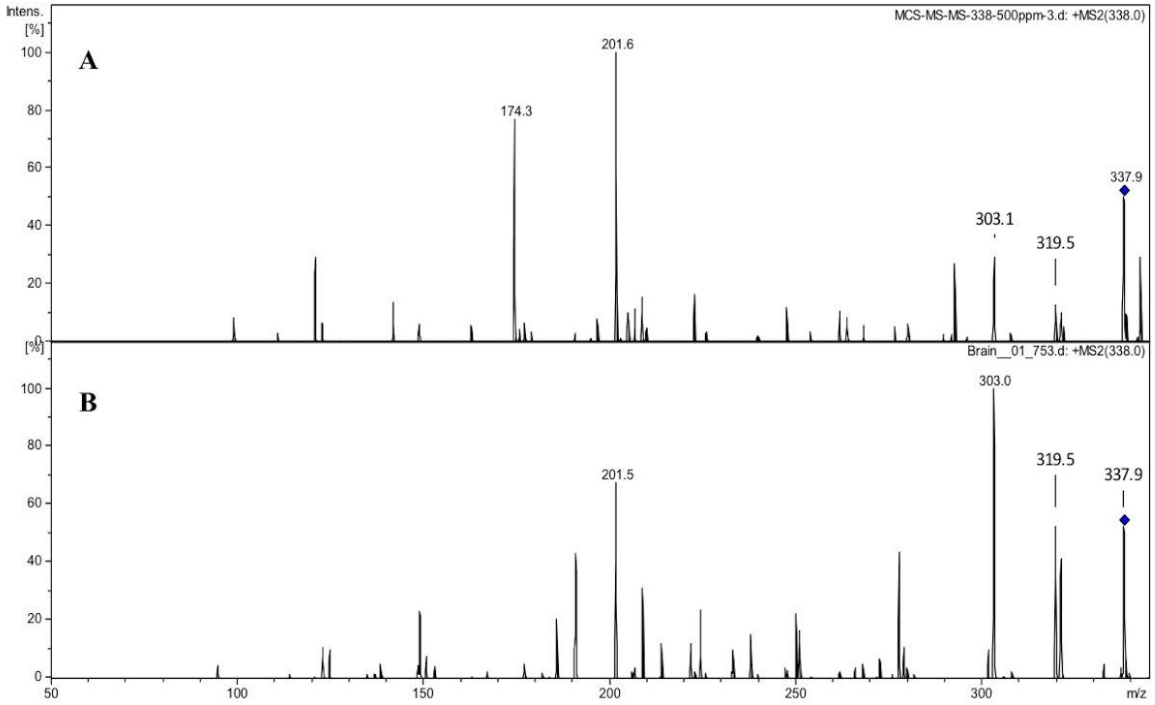

Figure S7. Tandem mass spectra of compound 15 [*m/z* 337.9 (M+H)] in (A) MCS extract and (B) treated brain homogenate

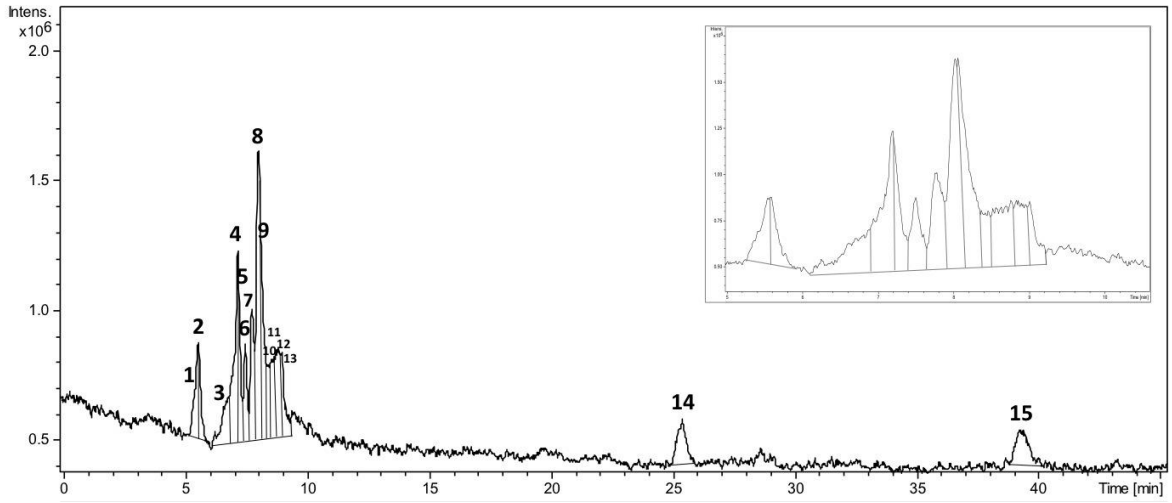

Figure S8. Total ion chromatogram of MCS extract (Agilent 1100 HPLC system, Bruker Esquire 2000 mass spectrometer, positive ion mode, 95% methanol and 5% water with 0.1% formic acid, 0.3 mL/min flow rate, Shim-pack GIST C_{18} column)

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School of Arts and Sciences
Department of Biology

16 May 2018

CERTIFICATION

TO WHOM IT MAY CONCERN:

This is to CERTIFY that the sponge specimen from Agdangan, Quezon Province, tentatively identified by Miss Danica L. Resuello for her study entitled "*B-secretase 1 Inhibitory Activity and AMP-Activated protein kinase Activation of Callyspongia samarensis Extracts*" has been verified as;

***Callyspongia (Cladochalina) samarensis* (Wilson, 1925)**
Class Demospongiae
Subclass Heteroscleromorpha
Order Haplosclerida
Family Callyspongiidae

Taxonomic Citation:
Van Soest, R.W.M; Boury-Esnault, N.; Hooper, J.N.A.; Rützler, K.; de Voogd, N.J.; Alvarez, B.; Hajdu, E.; Pisera, A.B.; Manconi, R.; Schönberg, C.; Klautau, M.; Picton, B.; Kelly, M.; Vacelet, J.; Dohrmann, M.; Díaz, M.-C.; Cárdenas, P.; Carballo, J. L.; Ríos, P.; Downey, R. (2018). World Porifera database. *Callyspongia (Cladochalina) samarensis* (Wilson, 1925). Accessed at: http://www.marinespecies.org/porifera/porifera.php?p=taxdetails&id=166225 on 2018-05-16

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Figure S9. Certificate of Authentication

References

Vareed S, Bauer A, Nair K, Liu Y, Jayaprakasam B, Nair M. 2014. Blood-Brain Barrier Permeability of Bioactive Withanamides Present in *Withania somnifera* Fruit Extract. Phytother Res. 28:1260-1264.