RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2023.014

ScienceAsia 49 (2023): **256–265**

The standardized *Centella asiatica* extract ECa 233 regulates the catalytic activities of βAPP-cleaving secretases in human cell lines

Mehbuba Nur Protha[a,†], Therathep Srisutjarit[a,†], Arpita Dey[b], Subhamita Maitra[b,g], Jean-Francois Hernandez[c], Mayuree H. Tantisira[d], Yingrak Boondam[e], Narawut Pakaprot[a,*], Bruno Vincent[b,f,*]

[a] Department of Physiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700 Thailand
[b] Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom 73170 Thailand
[c] Institut des Biomolécules Max Mousseron, UMR5247 CNRS/Université de Montpellier/ENSCM, Faculté de Pharmacie, 34093 Montpellier Cedex 5, France
[d] Faculty of Pharmaceutical Sciences, Burapha University, Chonburi 20131 Thailand
[e] Department of Physiology, Faculty of Pharmacy, Mahidol University, Bangkok 10400 Thailand
[f] Centre National de la Recherche Scientifique, 2 rue Michel Ange, Paris 75016 France
[g] Department of Molecular Biology, Umeå University, Umeå 90736 Sweden

*Corresponding authors, e-mail: narawut.pak@mahidol.ac.th, vincent@ipmc.cnrs.fr
† These authors contributed equally to this work.

Received 20 Jun 2022, Accepted 29 Oct 2022
Available online 9 Feb 2023

ABSTRACT: Alzheimer's disease is a neurodegenerative disorder characterized by the accumulation of amyloid peptides in the brain. While the production of Aβ is dependent on the cleavage of the β-amyloid precursor protein by the β-secretase BACE1, the α-secretase activity, mainly supported by ADAM10, counterbalances this pathway by both preventing Aβ production and triggering the release of the neuroprotective soluble APP alpha (sAPPα metabolite. For this reason, strategies aimed at promoting α-secretase and/or blocking β-secretase seem to be indicated for the purpose of containing the disease. Here we investigated the effects of ECa 233, a standardized extract of the plant *Centella asiatica*, on βAPP levels and sAPPα secretion as well as on the expression and catalytic activity of the α-secretases ADAM10 and ADAM17 and the β-secretase BACE1 in human cells. Our results interestingly demonstrate that the ECa 233 extract is able to significantly stimulate α-secretase activity and to inhibit β-secretase activity in a dose-dependent manner in the human SH-SY5Y neuroblastoma cell line. In conclusion, these results reveal an original doubly beneficial effect of ECa 233, which is both capable of promoting the non-amyloidogenic α-secretase activity and interfering with the amyloidogenic pathway and thereby stands as a promising candidate for the future development of mild, safe and preventive therapeutic treatment of Alzheimer's disease.

KEYWORDS: Alzheimer's disease, soluble APP alpha, *Centella asiatica*, alpha-secretase, beta-secretase

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder and is by far the leading cause of dementia in the elderly, thus representing a major global public health problem. The disease first manifests itself in the appearance of short-term memory impairment followed by a more general alteration in cognitive functions leading irremediably to death. One of the main hallmark of AD is the abnormal load of extracellular β-amyloid peptide (Aβ) leading to the occurrence of senile plaques that first form in the entorhinal cortex and then in the hippocampus before spreading into the cerebral cortex. In addition, the exacerbation of several deleterious pathogenic processes accompanies the development of the disease among which we can cite oxidative stress, neuroinflammation, mitochondrial dysfunction, altered calcium homeostasis and apoptosis.

The production of Aβ peptides is initiated by cleavage of the β-amyloid precursor protein (βAPP) by the β-secretase BACE1 and ends with the hydrolysis of the C99 fragment by the heterotetrameric γ-secretase complex [1]. Concomitantly with this amyloidogenic pathway, βAPP is also, and predominantly, the target of the α-secretase activity, which fulfills a doubly beneficial action by interfering with Aβ production (since it operates in the middle of the Aβ sequence) and giving rise to the secretion of the neuroprotective, neurotrophic and memory-enhancing sAPPα metabolite, thus establishing α-secretase activation as a promising anti-AD therapeutic strategy [2]. However, this approach faces a major problem which is the large number of proteins other than βAPP fulfilling vital physiological functions and which are the target of the two major enzymes responsible for this activity, ADAM10 and ADAM17 [3]. In this context, the use of plant extracts and plant-derived active compounds is increasingly gaining popularity and has been considered in recent years as a credible alternative to anti-AD

pharmacotherapies, principally because the side effects associated with their use are very moderate or even nonexistent [4].

Centella asiatica (Linn.) is a plant of the Apiaceae family that grows in swamps of temperate and tropical areas and holds an important place in the international market of medicinal plants. Chemically, it is mainly composed of the pentacyclic triterpenoids asiaticoside and madecassoside and their respective metabolites asiatic acid and madecassic acid [5]. During the past twenty years, a number of beneficial effects have been attributed to extracts of this plant or to its active ingredients against various pathological conditions [6]. Hence, methanol and water extracts of *C. asiatica* were reported to display anti-hyperlipidemic properties in murine [7]. It has also been shown that *C. asiatica* hydrogel accelerates wound healing in rabbits [8], a property that can reasonably be attributed to asiaticoside as evidenced in rats [9]. In addition, *C. asiatica* water extract was shown to alleviate epilepsy-provoked cognitive impairments in a mouse model of the disease [10] and to reduce adriamycin-induced myocardial failure in rats [11]. Finally, asiatic acid was reported to provide neuroprotection in a mouse model of focal cerebral ischemia [12]. The neuroprotective tonus of *C. asiatica* was further confirmed by several studies. Firstly, a chloroform:methanolic extract of the plant was shown to be neuroprotective in mono sodium glutamate-treated rats [13]. Secondly, asiaticoside, asiatic acid and medecassoside were established as the probable responsible active components mediating neuroprotection as documented *in vivo* following D-galactose-induced cognitive impairments in mice [14].

Further evidence of the beneficial effects of *C. asiatica* on brain function was brought by the demonstration that basal learning and memory performance can be improved following ingestion of aqueous extract of whole plant (200 mg/kg) for 14 days in rats [15], a phenomenon also observed following intraperitoneal injection of asiatic acid (30 mg/kg), suggesting that this triterpenoid group is, at least partly, supporting the memory-enhancing capability of the extracts [16]. Moreover, oral administration of an ethanol extract of the plant (300–1500 mg/kg) was shown to ameliorate learning and memory impairment induced by transient bilateral common carotid artery occlusion in mice [17].

Because learning and memory are severely impaired in AD, the hypothesis that *C. asiatica* could behave as an ameliorating factor with respect to the disease logically appeared and several studies have evidenced some positive effects of aqueous extract of the plant on AD pathology. Thus, it ameliorates behavior in a streptozotocin-injected rat model of AD [18], reduces Aβ-induced neurodegeneration in hippocampal neurons [19], alleviates behavioral deficits in the Tg2576 transgenic mouse model of the disease and conveys neuroprotection in 5×FAD transgenic mice [20].

Although the anti-oxidant and anti-inflammatory properties of *C. asiatica* are likely to contribute to its anti-AD effects [6], whether it can also interfere with βAPP processing via the regulation of βAPP-cleaving secretases remained unexplored to this day. Here we show that the non-toxic [21] and well-characterized [22] standardized *C. asiatica* extract ECa 233 stimulates α-secretase and inhibits β-secretase catalytic activities without modifying the expression of the involved proteins. These results thus established ECa 233 as a beneficial regulator of both the non-amyloidogenic and the amyloidogenic processing of βAPP, altogether making it a possible anti-AD preventive compound.

MATERIALS AND METHODS

Materials

DMEM, fetal bovine serum (FBS) and penicillin-streptomycin mix (Pen/Strep) were from Invitrogen (Carlsbad, CA, USA). Tris buffer and glycine were from VWR Amresco lifesciences (Solon, CA, USA). Polyclonal anti-βAPP antibody (A8717), monoclonal anti-β-actin (A2228), dimethyl sulfoxide (DMSO), SDS and sodium bicarbonate were from Sigma (St. Louis, MO, USA). Polyclonal anti-ADAM10 (AB19026) and polyclonal anti-ADAM17 (AB19027) were from Millipore (Bedford, MA, USA). Monoclonal anti-BACE1 (ab108394) was from Abcam (Cambridge, UK). Skim milk powder was from Bio Basic (Singapore). Monoclonal anti-β-amyloid antibody (2B3), which was used to specifically detect sAPPα was from IBL (Minneapolis, MN, USA). ECL reagent and ammonium persulphate were from GE Health care (Pisataway, NJ, USA). O-Phenanthroline was from Calbiochem (San Diego, CA, USA). Goat anti-mouse (polyclonal 7076) and goat anti-rabbit (polyclonal 7074) peroxidase-conjugated secondary antibodies were from Cell Signaling (Beverly, MA, USA).

Cell lines and treatments

Human HEK293 cells were cultured at 37 °C, 5% CO_2 in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (50 mg/ml). Human SH-SY5Y neuroblastoma cells were grown at 37 °C, 5% CO_2 in high glucose-DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (50 mg/ml). The standardized ECa 233 extract was obtained as previously described by a patent-pending method [23] and contains 53.1% madecassoside and 32.3% asiaticoside as determined by quantification by HPLC and liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously reported [24]. A 100 mg/ml stock solution (in 100% DMSO) was first prepared from which serial dilutions were performed in order to get four 100× intermediate solutions (10 mg/ml, 1 mg/ml, 100 μg/ml and 10 μg/ml) in 10% DMSO. Cells (80% confluence) were treated for

24 h with 10 µl of 100× concentrations in a total volume of 1 ml of complete media (final concentrations: 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml; 0.1% DMSO) before being processed for sAPPα secretion and western blot analysis. For non-treated controls, DMSO was also adjusted to 0.1%.

sAPPα secretion and detection

Secretion and detection of sAPPα in HEK293 and SH-SY5Y cells with the human-specific monoclonal anti-sAPPα antibody (2B3). Briefly, following treatments in complete media, media was removed and cells were incubated with fresh DMEM (1 ml) and allowed to secrete for 5 h. Then, 10% TCA precipitation of the whole medium was performed and the precipitate was subjected to electrophoresis through 10% SDS-PAGE gels, transferred onto nitrocellulose membranes (100 min, 90 volts), incubated in 5% non-fat milk blocking solution for 30 min and incubated overnight at 4 °C with 2B3 (1 µg/ml). After three washes with PBST (PBS containing 0.05% Tween 20), membranes were then incubated with a HRP-conjugated anti-mouse IgG antibody (dilution 1/3000), rinsed three times with PBST incubated with ECL reagent and signals were detected using an Azure c400 (Azure Biosystems, Dublin, CA, USA). Band densities were measured with the Image J software (http://imagej.nih.gov/ij).

Western blot analyses

Cells were collected with phosphate-buffered saline (PBS)-EDTA and resuspended in 70 to 100 µl of lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% triton X-100, 0.5% deoxycholate, 5 mM EDTA). Protein concentrations were determined by the Bradford method and 20–40 µg proteins were loaded onto 10% of SDS-PAGE gels, which were run at 100 volts for 2–2.5 h. Proteins were then transferred onto nitrocellulose membranes for 60–120 min at 90 V. Protein transfer was verified by Ponceau red staining, and nitrocellulose membranes were subsequently incubated in 5% non-fat milk blocking solution for 45 min. Membranes were then incubated with primary antibodies directed against βAPP (dilution 1/2000), ADAM10 (dilution 1/500), BACE1 (dilution 1/1000) or β-actin (dilution 1/5000) on a platform shaker overnight at 4 °C. Bound antibodies were detected using goat anti-mouse (dilution 1/3000, polyclonal 7076, Cell Signaling) or goat anti-rabbit peroxidase-conjugated antibody (dilution 1/3000, polyclonal 7074, Cell Signaling). After 3 washes with PBST, membranes were incubated with a HRP-conjugated anti-rabbit (ADAM10, βAPP and BACE1) or anti-mouse (β-actin) secondary antibody (1/3000) for 2 h, rinsed 3 times with PBST and processed as described above. All protein levels were normalized using β-actin as an internal standard.

α-secretase fluorimetric assay on intact cells

The α-secretase catalytic activity was measured following a procedure previously described [25]. Briefly, SH-SY5Y cells were treated in duplicate without (control) or with ECa 233 for 24 h at 37 °C in 1 ml of DMEM containing 1% FBS. Cells were then incubated for 30 min at 37 °C in the absence or in the presence of the general metalloprotease inhibitor o-phenanthroline (100 µM) in 1.5 ml of PBS. Then, the α-secretase-specific JMV2770 substrate (10 µM) was directly added into the media and cells were kept at 37 °C. Every 15 min, 100 µl of media were collected and the α-secretase-specific activity corresponding to the o-phenanthroline-sensitive fluorescence was recorded in black 96-well plates at 320 nm and 420 nm excitation and emission wavelengths respectively.

β-secretase fluorimetric assay on cell homogenates

SH-SY5Y cells were cultured in 35 mm-dishes until they reach 80% confluence, treated without (control) or with ECa 233 for 24 h at 37 °C in DMEM containing 1% FBS and assayed for their β-secretase activity. Briefly, cells were collected, lysed with Tris 10 mM pH 7.5, homogenized and kept on ice. Samples were assayed for their protein contents with the Bradford method and adjusted to a 3 µg/µl concentration. Thirty µg of each samples (10 µl) diluted in 10 mM sodium acetate buffer pH 4.5 were incubated for 30 min at 37 °C in black 96-well plates (in a final volume of 100 µl) in the absence (triplicate) or in the presence (triplicate) of the β-secretase specific inhibitor JMV1197. Then, the β-secretase-specific JMV2236 substrate (10 µM) was added to all samples and plates were maintained at 37 °C. Every 15 min, the β-secretase-specific activity corresponding to the JMV1197-sensitive fluorescence was recorded at 320 nm and 420 nm excitation and emission wavelengths respectively.

Statistical analysis

Statistical analyses were performed with the Prism software (GraphPad, San Diego, USA) using an unpaired *t*-test for pairwise comparisons. All results were expressed as means ± SEM and the *p* values equal to or less than 0.05 were considered significant.

RESULTS

Effects of ECa 233 on sAPPα production and βAPP, ADAM10, ADAM17 and BACE1 protein levels in cultured human SH-SY5Y cells

We first examined the effect of 24 h-treatments with four different doses of ECa 233 (0.1, 1, 10 and 100 µg/ml) for their ability to promote the secretion of the βAPP-derived sAPPα metabolite produced by α-secretase in cultured SH-SY5Y human neuroblastoma cells. The results showed no significant differences whatever the dose considered when compared

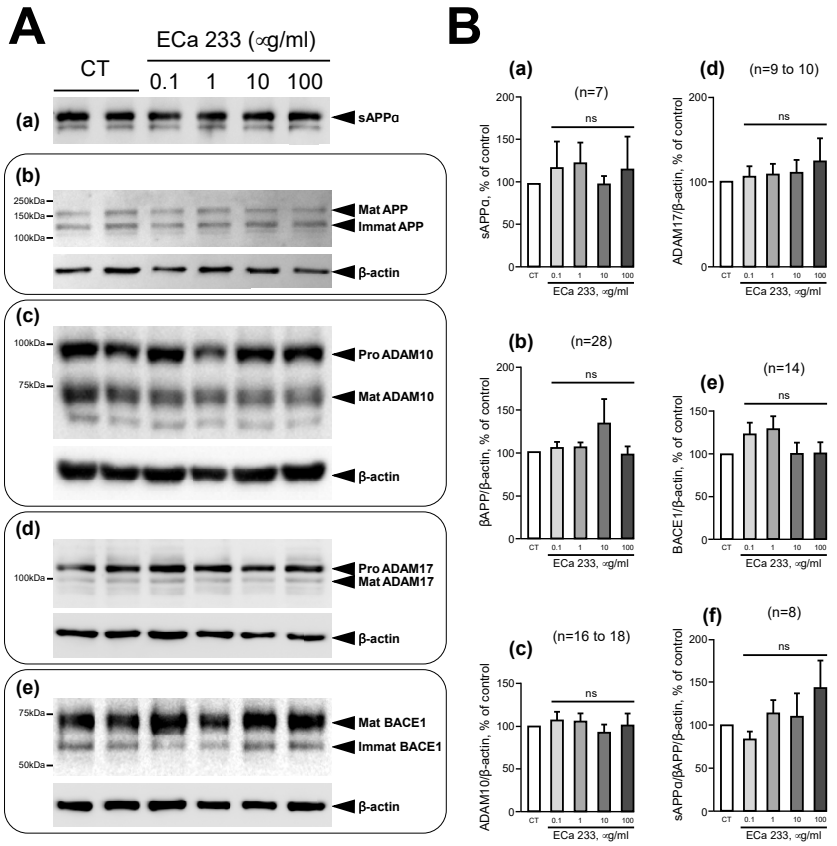

Fig. 1 Effect of ECa 233 on sAPPα secretion, βAPP, ADAM10, ADAM17, BACE1 protein levels and the ratio sAPPα/βAPP in human SH-SY5Y cells. (A) Representative gels of western blot analysis of sAPPα production in media (a) as well as βAPP (b), ADAM10 (c), ADAM17 (d) and BACE1 (e) and their respective β-actin in lysates following treatment of cultured SH-SY5Y cells without (CT) or with the indicated concentrations of ECa 233 for 24 h. (B) Statistical analysis of the data for sAPPα (a), βAPP (b), ADAM10 (c), ADAM17 (d), BACE1 (e) and the ratio sAPPα/βAPP (f). Bars correspond to the densitometric analyses (βAPP, the ratio sAPPα/βAPP, ADAM10, ADAM17 and BACE1 being normalized with β-actin), are expressed as a percent of control taken as 100, and are the means ± SE of the indicated number of independent determinations (*n*); ns, no statistical difference.

to controls (Fig. 1A(a) and B(a)) although a slight upward trend was observed (Fig. 1B(a)). We then wanted to determine whether ECa 233 could modify the protein levels of βAPP, the two α-secretases ADAM10 and ADAM17 and of the β-secretase BACE1. No significant variation in the immunoreactivities of these proteins could be demonstrated by western blot analysis (Fig. 1A(b-e) and B(b-e)), thus showing that there is no impact of ECa 233 on the expression of key proteins of βAPP metabolism. Because the secretion of sAPPα is dependent both on the hydrolysis of βAPP by α-secretase activity and on the expression of βAPP itself, and in order to increase the stringency of our measurements, we also measured the effect of ECa 233 on the sAPPα/βAPP ratio under the same conditions. Despite the fact that it seems that ECa 233 increases this ratio in a dose-dependent manner, the statistical analysis of the results did not allow us to highlight any significant differences (Fig. 1B(f)).

Effects of ECa 233 on sAPPα production and βAPP, ADAM10, ADAM17 and BACE1 protein levels in cultured human HEK293 cells

In order to test the same parameters in a different cell line, we conducted the same experimental protocols with human HEK293 cells. The results show, as observed in SH-SY5Y cells, that ECa 233 did not modify the protein levels of βAPP, ADAM10, ADAM17 and BACE1 (Fig. 2A(b-e) and B(b-e)) and appeared to increase, although not significantly, the production of sAPPα (Fig. 2A(a) and B(a)). Nevertheless, we could establish that treatment with ECa 233 at 100 μg/ml significantly increases the sAPPα/βAPP ratio (Fig. 2B(f)).

Based on these results, we then wanted to determine whether ECa 233 was able to modulate the catalytic activity of α secretase and β-secretase, which compete for βAPP processing and thus control the

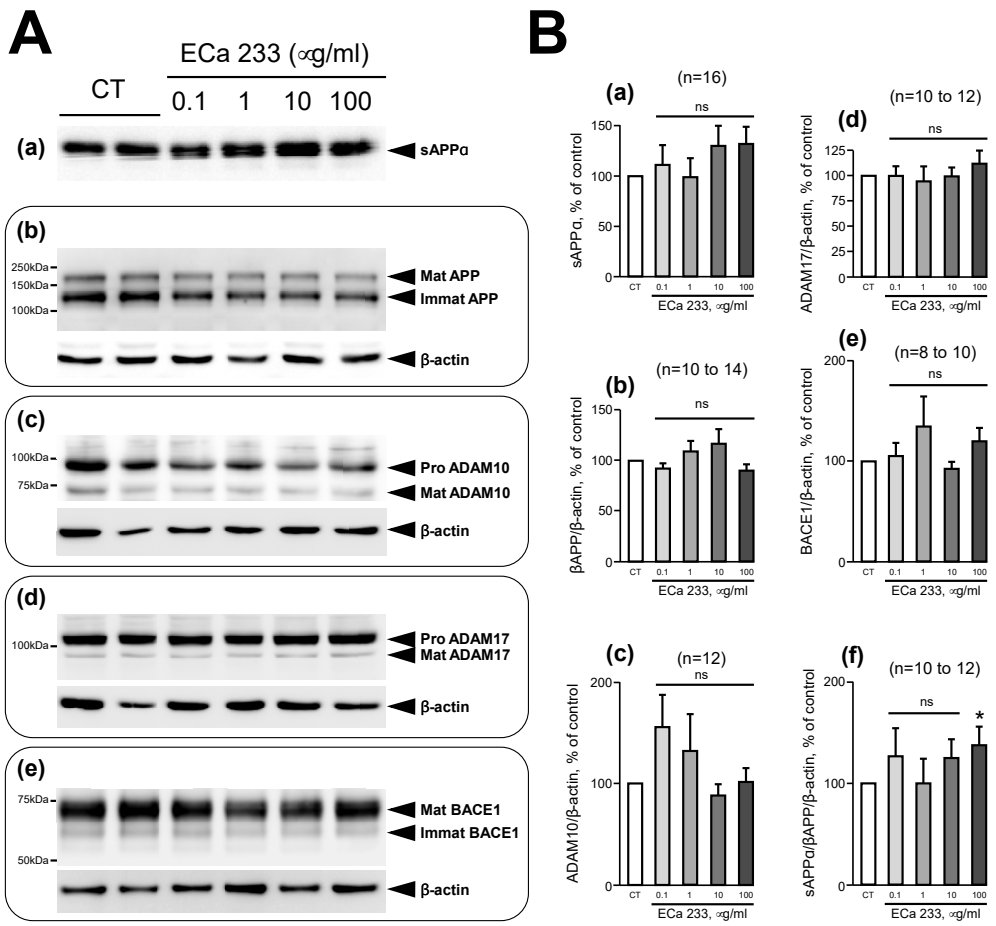

Fig. 2 Effect of ECa 233 on sAPPα secretion, βAPP, ADAM10, ADAM17, BACE1 protein levels and the ratio sAPPα/βAPP in human HEK293 cells. (A) Representative gels of western blot analysis of sAPPα production in media (a) as well as βAPP (b), ADAM10 (c), ADAM17 (d) and BACE1 (e) and their respective β-actin in lysates following treatment of cultured HEK293 cells without (CT) or with the indicated concentrations of ECa 233 for 24 h. (B) Statistical analysis of the data for sAPPα (a), βAPP (b), ADAM10 (c), ADAM17 (d), BACE1 (e) and the ratio sAPPα/βAPP (f). Bars correspond to the densitometric analyses (βAPP, the ratio sAPPα/βAPP, ADAM10, ADAM17 and BACE1 being normalized with β-actin), are expressed as a percent of control taken as 100, and are the means ± SE of the indicated number of independent determinations (n); $*p < 0.05$; ns, no statistical difference.

balancing between the amyloidogenic and the non-amyloidogenic pathways.

Effects of ECa 233 on the α-secretase catalytic activity in cultured human SH-SY5Y neuroblastoma cells

In a first set of experiments, we examined the impact of increasing concentrations (0.1 up to 100 μg/ml) of ECa 233 on the α-secretase activity by measuring the phenanthroline-sensitive hydrolysis of the fluorimetric JMV2770 substrate by cultured SH-SY5Y cells. Despite an increasing trend, no significant difference was noted at 0.1 μg/ml concentration (Fig. 3A(a)). However, our results indicated that ECa 233 dose-dependently enhances the JMV2770-hydrolyzing activity as shown by an increased significance (p value) from 1 to 100 μg/ml concentrations (Fig. 3A(b-d) and B).

Effects of ECa 233 on the β-secretase catalytic activity in cultured human SH-SY5Y neuroblastoma cells

Finally, we undertook to determine if ECa 233 could behave as an inhibitor of the amyloidogenic β-secretase catalytic activity. Taking advantage of a well-characterized BACE1-selective fluorimetric assay, we have measured the impact of the treatment of SH-SY5Y cells with the extract for 24 h at the four previously used concentrations. Indeed, the JMV1197-sensitive hydrolysis of the fluorimetric JMV2236 substrate measured in SH-SY5Y cell extracts at acidic pH was gradually reduced as the concentrations of ECa 233 increased (Fig. 4A and B), thereby evidencing

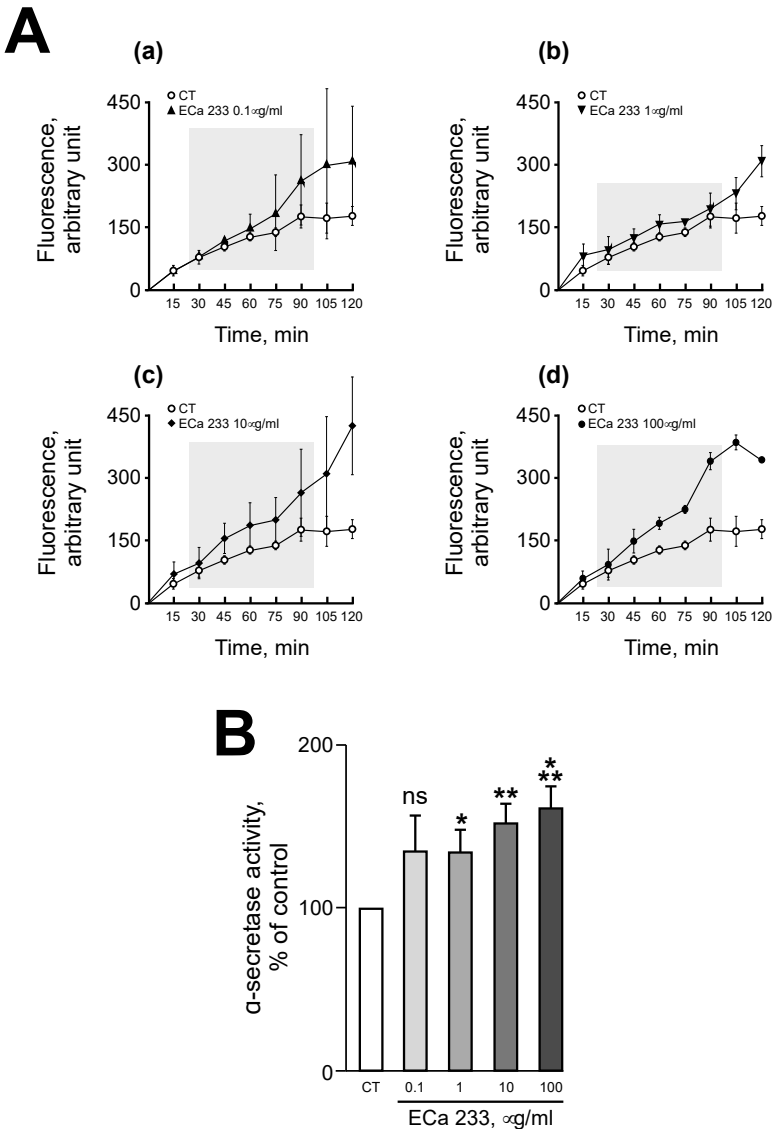

Fig. 3 Effects of ECa 233 on the α-secretase catalytic activity in SH-SY5Y intact cells. (A) The α-secretase catalytic activity (phenanthroline-sensitive hydrolysis of the fluorimetric substrate JMV2770) was measured on cultured SH-SY5Y cells incubated in the absence (control, white circle) or in the presence of 0.1 μg/ml (a), 1 μg/ml (b), 10 μg/ml (c) and 100 μg/ml (d) of ECa 233 for 24 h. The curves represent the mean specific fluorescence ± SE from 2 independent experiments including two controls each. (B) Statistical analysis of the data. Bars are expressed as a percentage of control (white bars, non-treated cells) calculated from the linear parts of the curves (initial velocity, light grey area) and are the means ± SE of 11 independent determinations; $*p < 0.02$; $**p < 0.0003$; $***p < 0.0002$; ns, no statistical difference.

a dose-dependent effect of the extract on the limiting factor of the amyloidogenic processing of βAPP with more than 50% inhibition observed at 100 μg/ml when compared to control untreated cells (Fig. 4A(d) and B).

DISCUSSION

AD is a yet incurable neurodegenerative disorder characterized by a progressive severe loss of memory and cognitive functions. The reason why available medical treatments are still incapable to cure AD symptoms efficiently mostly resides in the fact that AD is a complex and multifactorial disease with multiple pathological processes. Over the past decades, a huge effort, although in vain, has been made to develop novel synthetic drugs with disease-modifying properties and few side effects [26]. As a consequence, compounds extracted from natural sources logically gained popularity and the notion of a preventive rather than a curative approach aimed at combating AD is increasingly taken into account.

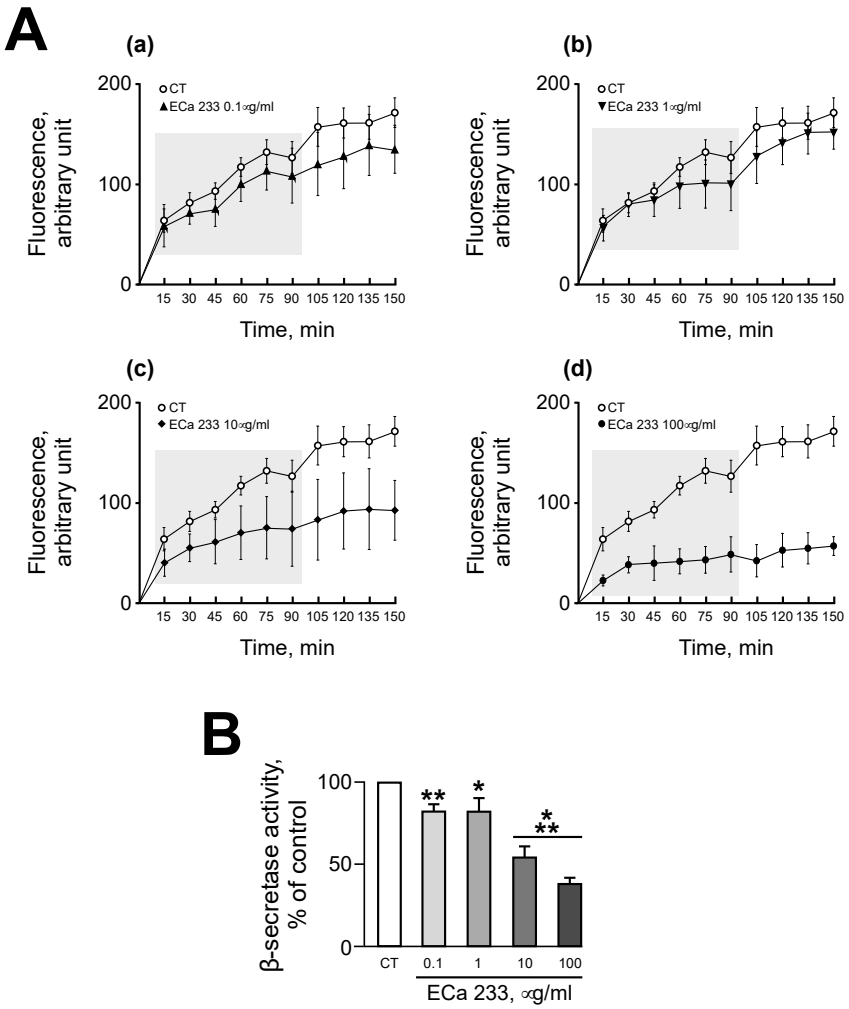

Fig. 4 Effects of ECa 233 on the β-secretase catalytic activity in homogenates of SH-SY5Ycells. (A) The β-secretase catalytic activity (JMV1197-sensitive hydrolysis of the fluorimetric substrate JMV2236) was measured in the homogenates of SH-SY5Y cells beforehand incubated in the absence (control, white circle) or in the presence of 0.1 μg/ml (a), 1 μg/ml (b), 10 μg/ml (c) and 100 μg/ml (d) of ECa 233 for 24 h. The curves represent the mean specific fluorescence ± SE from 5 independent experiments including two controls each. (B) Statistical analysis of the data. Bars are expressed as a percentage of control (white bars, non-treated cells) calculated from the linear parts of the curves (initial velocity, light grey area) and are the means ± SE of 24 independent determinations; $*p < 0.05$; $**p < 0.0002$; $***p < 0.0001$.

The possible use of *C. asiatica* extracts to counteract the development of AD is based on the observation of its beneficial effects on certain pathogenic processes associated with the disease. Firstly, they have been largely reported as antioxidant factors *in vitro* [27] as well as *in vivo* [7, 15] with asiaticoside most likely supporting this function as evidenced by its ability to increase enzymatic and non-enzymatic antioxidants [9]. Secondly, *C. asiatica* extracts were shown to convey anti-inflammatory effects *in vitro* in macrophages [28] and *in vivo* in rats [29], a property shared by the main components asiatic acid, asiaticoside, madecassic acid and madecassoside as evidenced *in vivo* [28]. Thirdly, its widely described neuroprotective properties are arguably based on its ability to promote dendritic arborization of hippocampal neurons [30] and to stimulate nerve regeneration [31]. Finally, some possible underlying molecular mechanisms explaining its positive impact on AD, were evidenced such as an increased phosphorylation of CREB in neuroblastoma cells expressing $A\beta_{42}$ [32] and the anti-acetylcholinesterase activity of asiatic acid [33]. In the end, all of these data strongly argue in favor of a protective action of *C. asiatica* against AD pathogenesis.

Because there exists a fluctuation in the amounts of the biological active constituents in differently prepared crude extracts of *C. asiatica*, well-controlled methods were established to prepare ECa 233 as a

standardized extract of the plant that consistently contains at least 80% of the two triterpenoid glycosides madecassoside and asiaticoside with a ratio between them maintained at 1.5±0.5:1 [23]. Importantly, ECa 233 has been previously shown to trigger a certain number of beneficial effects, both at the periphery on osteoarthritis [34] and in the central nervous system as illustrated by an anxiolytic activity in stressed mice [24], most likely via a fine tuning of neuronal activity [35], as well as a propensity to reduce ischemia-induced neuronal damage and cognitive dysfunction in rodents [36, 37]. Beyond these protective effects under pathological conditions, it is interesting to note that ECa 233 also shows some memory-enhancing properties under physiological conditions in rats that is accompanied by an increase in synaptic plasticity, thereby suggesting that the extract provides memory tonic [38], a function which may be based on the fact that ECa 233 is able to stimulate neurites outgrowth as demonstrated in human neuroblastoma cells [39].

Last but not least, ECa 233 was reported to convey neuroprotection under conditions closely associated with AD pathogenesis. Hence, the extract alleviates neuroinflammation processes in LPS-treated macrophages [40] and reduces memory and learning deficits as well as hippocampal cell loss provoked by intracerebroventricular injection of Aβ in mice [41]. However, whether it could control the metabolism of βAPP via the modulation of βAPP-cleaving secretases remained until this day without answer. The present demonstration that ECa 233 can influence the metabolism of βAPP in two ways, by activating its non-amyloidogenic cleavage by α-secretase and at the same time inhibiting its hydrolysis by the amyloidogenic β-secretase activity, unveil new mechanisms through which the extract can operate and adds additional weight as to its neuroprotective properties with respect to AD.

The fact that we did not evidence some statistically significant differences in sAPPα production under ECa 233 treatment, despite an observed upward trend, is most likely due to a certain lack of sensitivity of the western blot technique that is reflecting sAPPα steady state. Nevertheless, the more stringent α- and β-secretase fluorimetric assays, which specifically measure one single parameter without interfering with off-side biological processes, clearly evidenced some positive effects of the extract as shown by our findings that it displays both pro-α-secretase and anti-β-secretase properties.

It is important to underline here that ECa 233 in the concentrations that modulate secretase activities were previously shown to have no effect on cell viability in human neuroblastoma cells following 24 h treatment [39], thereby indicating harmlessness of this extract under our experimental conditions. Finally, *C. asiatica* is largely consumed as food while ECa 233 capsules does not trigger adverse effects in humans after single and multiple ingestion of 250 or 500 mg of the extract [21]. As a whole and in light of our results, the very favorable safety profile of ECa 233 opens the way to the possible use of this extract in order to prevent the development of AD by intervening on the early mechanisms of the pathology.

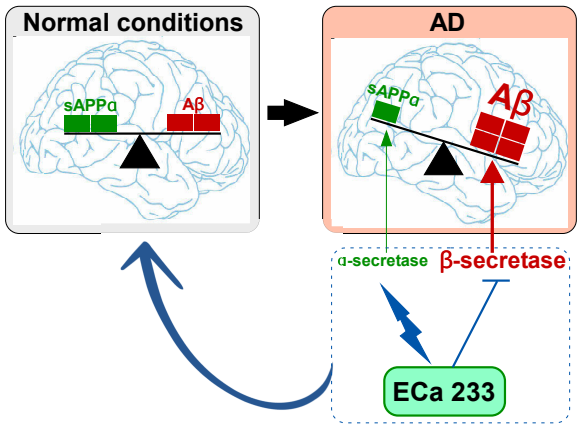

Fig. 5 Schematic outline of the doubly beneficial action of ECa 233 regarding βAPP processing. Alzheimer's disease is characterized by an imbalance of βAPP metabolism in favor of the amyloidogenic pathway leading to an increased Aβ production and a decrease in sAPPα levels. Our results suggest that ECa 233 extract-based treatment could restore this balance by doubly modulating, in an opposite way, the α-secretase activity (activation) and the β-secretase activity (inhibition).

CONCLUSION

Overall, our work described original findings demonstrated that the standardized *C. asiatica* extract ECa 233 conveys both pro-α-secretase and anti-β-secretase effects (Fig. 5). This indeed paves the way for the possible development, so far sparingly explored, of compounds of natural origin able to control in a doubly beneficial manner the metabolism of βAPP by both decreasing the production of Aβ and increasing the production of the neurotrophic, neuroprotective and neurogenic sAPPα metabolite. Thus, ECa 233 is representing a new class of factors to be developed as natural therapeutic tool aimed at preventing the development of Alzheimer's disease.

Acknowledgements: We would like to thank Dr. Narisorn Kitiyanant (Institute of Molecular Biosciences, Mahidol University, Thailand) for providing us with the SH-SY5Y cell line. This work was supported by Mahidol University (NDFR 13/2564) and The Thailand Research Fund (BRG6180002). AD and SM were supported by a Mahidol University postdoctoral research sponsorship.

REFERENCES

1. Schaduangrat N, Prachayasittikul V, Choomwattana S, Wongchitrat P, Phopin K, Suwanjang W, Malik AA, Vincent B, et al (2019) Multi-disciplinary approaches for the targeting of the secretase protein family as a therapeutic route for Alzheimer's disease. *Med Res Rev* **39**, 1730–1778.
2. Vincent B, Govitrapong P (2011) Activation of the α-secretase processing of AβPP as a therapeutic approach in Alzheimer's disease. *J Alzheimers Dis* **24**, 75–94.
3. Vincent B, Checler F (2012) α-secretase in Alzheimer's disease and beyond: Mechanistic, regulation and function in the shedding of membrane proteins. *Curr Alzheimer Res* **9**, 140–156.
4. Wollen KA (2010) Alzheimer's disease: The pros and cons of pharmaceuticals, nutritional, botanical, and stimulatory therapies, with a discussion of treatment strategies from the perspective of patients and practitioners. *Altern Med Rev* **15**, 223–244.
5. Murray MT, Pizzorno JE (2012) *Textbook of Natural Medicine*, 4th edn, Churchill Livingstone, Edinburgh.
6. Sun B, Wu L, Wu Y, Zhang C, Qin L, Hayashi M, Kudo M, Gao M, et al (2020) Therapeutic potential of *Centella asiatica* and its triterpenes: A review. *Front Pharmacol* **11**, 568032.
7. Kumari S, Deori M, Elancheran R, Kotoky J, Devi R (2016) *In vitro* and *in vivo* antioxidant, anti-hyperlipidemic properties and chemical characterization of *Centella asiatica* (L.) extract. *Front Pharmacol* **7**, 400.
8. Ahmed AS, Taher M, Mandal UK, Jaffri JM, Susanti D, Mahmood S, Amiruddin Zakaria Z (2019) Pharmacological properties of *Centella asiatica* hydrogel in accelerating wound healing in rabbits. *BMC Complement Altern Med* **19**, 213.
9. Shukla A, Rasik AM, Dhawan BN (1999) Asiaticoside-induced elevation of antioxidant levels in healing wounds. *Phytother Res* **13**, 50–54.
10. Gupta YK, Veerandra Kumar MH, Srivastava AK (2003) Effect of *Centella asiatica* on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. *Pharmacol Biochem Behav* **74**, 579–585.
11. Gnanapragasam A, Yogeeta S, Subhashini R, Ebenezar KK, Sathish V, Devaki T (2007) Adriamycin induced myocardial failure in rats: Protective role of *Centella asiatica*. *Mol Cell Biochem* **394**, 55–63.
12. Krishnamurthy RG, Senut MC, Zemke D, Min J, Frenkel MB, Greenberg EJ, Yu SW, Ahn N, et al (2009) Asiatic acid, a pentacyclic terpene from *Centella asiatica*, is neuroprotective in a mouse model of focal berebral ischemia. *J Neurosci Res* **87**, 2541–2550.
13. Ramanathan M, Sivakumar S, Anandvijayakumar PR, Saravanababu C, Rathinavel Pandian P (2007) Neuroprotective evaluation of standardized extract of *Centella asiatica* in monosodium glutamate treated rats. *Indian J Exp Biol* **45**, 425–431.
14. Lin X, Zhang S, Huang R, Wei L, Tan S, Liang C, Lv S, Chen Y, et al (2014) Protective effect of madecassoside against cognitive impairment induced by D-galactose in mice. *Pharmacol Biochem Behav* **124**, 434–442.
15. Veeranda Kumar MH, Gupta YK (2002) Effect of different extracts of *Centella asiatica* on cognition and markers of oxidative stress in rats. *J Ethnopharmacol* **79**, 253–260.
16. Nasir MN, Habsah M, Zamzuri I, Rammes G, Hasnan J, Abdullah J (2011) Effects of asiatic acid on passive and active avoidance task in male Sprague-Dawley rats. *J Ethnopharmacol* **134**, 203–209.
17. Doknark S, Mingmalairak S, Vattanajun A, Tantisira B, Tantisira MH (2014) Study of ameliorating effects of ethanolic extract of *Centella asiatica* on learning and memory deficit in animal models. *J Med Assoc Thai* **97S2**, S68–S75.
18. Veerandra Kumar MH, Gupta YK (2003) Effect of *Centella asiatica* on cognition and oxidative stress in an intracerebroventricular streptozotocin model of Alzheimer's disease in rats. *Clin Exp Pharm Physiol* **30**, 336–342.
19. Gray NE, Zweig JA, Murchison C, Caruso M, Matthews DG, Kawamoto C, Harris CJ, Quinn JF, et al (2017) *Centella asiatica* attenuates Aβ-induced neurodegenerative spine loss and dendritic simplification. *Neurosci Lett* **646**, 24–29.
20. Gray NE, Zweig JA, Caruso M, Zhu JY, Wright KM, Quinn JF, Soumyanath A (2018) *Centella asiatica* attenuates hippocampal mitochondrial dysfunction and improves memory and executive function in β-amyloid overexpressing mice. *Mol Cell Neurosci* **93**, 1–9.
21. Songwut P, Chariyavilaskul P, Tantisira MH, Khemawoot P (2019) Safety and pharmacokinetics of standardized extract of *Centella asiatica* (ECa 233) capsules in healthy Thai volunteers. *Planta Med* **85**, 483–490.
22. Hengjumrut P, Anukunwithaya T, Tantisira MH, Tantisira B, Khemawoot P (2018) Comparative pharmacokinetics between madecassoside and asiaticoside presented in a standardized extract of *Centella asiatica*, ECa 233 and their respective pure compound given separately in rats. *Xenobiotica* **48**, 18–27.
23. Saifah E, Suttisri R, Patarapanich C, Laungchonlatan S, Tantisira MH, Tantisira B (2009) Preparation methods of colorless mixture between madesassoside and asiaticoside from *Centella asiatica*. Department of Intellectual Property MoC. Ed. CO7H 1/00, Chulalongkorn University, Bangkok, Thailand.
24. Wanasuntronwong A, Tantisira MH, Tantisira B, Watanabe H (2012) Anxiolytic effects of standardized extract of *Centella asiatica* (ECa 233) after chronic immobilization stress in mice. *J Ethnopharmacol* **143**, 579–585.
25. Maitra S, Sornjai W, Smith DR, Vincent B (2021) Phenanthroline impairs βAPP processing and expression, increases p53 protein levels and induces cell cycle arrest in human neuroblastoma cells. *Brain Res Bull* **170**, 29–38.
26. Reynolds DS (2019) A short perspective on the long road to effective treatments for Alzheimer's disease. *Br J Pharmacol* **176**, 3636–3648.
27. Eze FN, Tola AJ (2020) Protein glycation and oxidation inhibitory activity of *Centella asiatica* phenolics (CAP) in glucose-mediated bovine serum albumin glycoxidation. *Food Chem* **332**, 127302.
28. Cho YC, Vuong HL, Ha J, Lee S, Park J, Wibow AE, Cho S (2020) Inhibition of inflammatory responses by *Centella asiatica* via suppression of IRAK1-TAK1 in mouse macrophages. *Am J Chin Med* **48**, 1103–1120.
29. Guo JS, Cheng CL, Koo MWL (2004) Inhibitory effects of *Centella asiatica* water extract and asiaticoside on inducible nitric oxide synthase during gastric ulcer healing

in rats. *Planta Med* **70**, 1150–1154.
30. Gadahad MRK, Rao M, Rao G (2008) Enhancement of hippocampal CA3 neuronal dendritic arborization by *Centella asiatica* (Linn) fresh leaf extract treatment in adult rats. *J Chin Med Assoc* **71**, 6–13.
31. Hussin HM, Lawi MM, Haflah NHM (2020) *Centella asiatica* (L.)-neurodifferentiated mesenchymal stem cells promote the regeneration of peripheral nerve. *Tissue Eng Regen Med* **17**, 237–251.
32. Xu Y, Cao Z, Khan I, Luo Y (2008) Gotu Kola (*Centella asiatica*) extract enhances phosphorylation of cyclic AMP response binding protein in neuroblastoma cells expressing amyloid beta peptide. *J Alzheimers Dis* **13**, 341–349.
33. Nasir MN, Abdullah J, Habsah M, Ghani RI, Rammes G (2012) Inhibitory effect of asiatic acid on acetylcholinesterase, excitatory post synaptic potential and locomotor activity. *Phytomedicine* **19**, 311–316.
34. Rotpendian N, Wanasuntronwong A, Tapechum S, Care C, Roumwong A, Tilokskulchai K, Tantisira MH, Pakaprot N (2022) Efficacy of a standardized *Centella asiatica* (ECa 233) extract against allodynia in a mouse model of temporomandibular osteoarthritis. *ScienceAsia* **48**, 15–22.
35. Wanasuntronwong A, Wanakhachrnkrai O, Phongphanphanee P, Isa T, Tantisira B, Tantisira MH (2018) Modulation of neuronal activity on intercalated neurons of amygdala might underlie anxiolytic activity of a standardized extract of *Centella asiatica* ECa 233. *Evid Based Complement Alternat Med* **2018**, 3853147.
36. Tantisira MH, Tantisira B, Patarapanich C, Suttisri R, Luangcholatan S, Mingmalailak S, Wanasuntronwong A, Saifah E (2010) Effects of a standardized extract of *Centella asiatica* ECa 233 on learning and memory impairment induced by transient bilateral common carotid artery occlusion in mice. *Thai J Pharmacol* **32**, 22–33.
37. Thong-asa W, Tilokskulchai K, Chompoopong S, Tantisira MH (2018) Effect of *Centella asiatica* on pathophysiology of mild chronic cerebral hypoperfusion in rats. *Avicenna J Phytomed* **8**, 210–226.
38. Boondam Y, Songvut P, Tantisira MH, Tapechum S, Tilokskulchai K, Pakaprot N (2019) Inverted U-shaped response of a standardized extract of *Centella asiatica* (ECa 233) on memory enhancement. *Sci Rep* **9**, 8404.
39. Wanakhachornkrai O, Pongrakhananon V, Chunhacha P, Wanasuntronwong A, Vattanajun A, Tantisira B, Chanvorachote P, Tantisira MH (2013) Neuritogenic effect of standardized extract of *Centella asiatica* ECa 233 on human neuroblastoma cells. *BMC Complement Altern Med* **13**, 204.
40. Sukketsiri W, Tanasawet S, Moolsap F, Tantisira MH, Hutamekalin P, Tipmanee V (2019) ECa 233 suppresses LPS-induced proinflammatory responses in macrophages *via* suppressing ERK1/2, p38 MAPK and Akt pathways. *Biol Pharm Bull* **42**, 1358–1365.
41. Kam-eg A, Tantisira B, Tantisira MH (2009) Preliminary study on effects of a standardized extract of *Centella asiatica*, ECa 233, on deficit of learning and memory induced by an intracerebroventricular injection of β-amyloid peptide in mice. *Thai J Pharmacol* **31**, 9.