Transactions of the Illinois State Academy of Science
(2019) Volume 112, pp. 1-4

received 12/2/18
accepted 6/7/19

Asiatic Acid and Madecassic Acid Promote Neurite Outgrowth in Neuro2a Cell

Melissa A. Nathan and Gordon C. Tucker*
Eastern Illinois University, Department of Biological Sciences
600 Lincoln Ave., Charleston IL 61920
*corresponding author: gctucker@eiu.edu

ABSTRACT

Centella asiatica (CA) has been used for many centuries in herbal medicines to improve intelligence, learning, memory, and cognitive performance. The exact component of CA extract and the molecular mechanism whereby it confers neuroprotection is still unclear. In this study, we examined the effects of asiatic acid (AA) and madecassic acid (MA) on neurite outgrowth in Neuro-2a (N2a) neuroblastoma cell line. Treatment of N2a cultures with AA decreased the percentage of neurite bearing cells, but increased neurite extension and combined length of neurites per cell in a dose-dependent manner. Treatment of N2a with MA did not significantly alter the percentage of neurite bearing cells, but increased neurite extension and combined length of neurites per cell in a dose-dependent manner. These findings suggest that AA and MA have positive effects on neuronal structure in N2a cultures. Future studies to examine the beneficial effects of AA and MA in animal models of neurological diseases would help elucidate the therapeutic potential of AA and MA in neurodegenerative diseases involving mitochondrial dysfunction and compromised nerve regenerations.

Key words: Asiatic acid, madecassic acid, neurite outgrowth, *Centella asiatica*, herbal medicine, Neuro2a, nerve regeneration, mitochondria, lactate, cell proliferation, and cell viability.

INTRODUCTION

The nervous system of adult mammals has limited capacity to repair and regenerate following injury caused by physical, chemical, or disease-related processes. Although pathways and molecular mechanisms leading to central nervous system (CNS) repair and subsequent nerve regeneration have been extensively studied, presently there are no approved treatments to facilitate nerve regeneration in the human CNS. Given this, there is a dire need for compounds that promote CNS regeneration to treat patients with a variety of CNS injury including, spinal injury, stroke, and neurodegenerative disorders.

Traditional herbal medicines are well established as a source of novel compounds to treat a wide range of medical conditions. Numerous species of plants from many families offer promising leads in identifying potential compounds to promote repair and regeneration in the nervous system. The family Araliaceae, sister family to the Apiaceae, is rich in species used in traditional medicine in many parts of the world, most notably ginseng (*Panax quinquefolius* L.). Another member of this family, *Centella asiatica* (L.) Urban (syn. *Hydrocotyle asiatica* L.), (CA, herein) has been used for many centuries in both Indian Ayurvedic and traditional Chinese medicines to improve intelligence, learning, memory, and cognitive performance (Farooqui et al., 2018). Studies on cell culture and animal models have supported the beneficial effects of CA on the nervous system. CA leaf extracts increased neuronal differentiation and neurite elongation in PC12 cells and SH-SY5Y cells respectively (Jiang et al., 2016; Soumyanath et al., 2005). In addition, these studies showed that asiatic acid (AA) and madecassic acid (MA) are the dominant components in fractions of CA extract with higher neurite outgrowth promoting property. In vivo, CA extracts enhanced dendritic arborization in the hippocampus and amygdala (Mohandas Rao et al., 2006; Rao et al., 2012), and accelerated nerve regeneration and functional recovery following sciatic nerve crush injury (Soumyanath et al., 2005). In addition, CA treatment during postnatal period improved learning and memory in rats (Rao et al., 2005). Also, long-term treatment with CA extract ameliorated colchicine-induced memory impairment in rats (Kumar et al., 2009). Aqueous extracts of CA ameliorated 3-nitroprorionic acid-induced oxidative stress and mitochondrial dysfunctions in mice brains. In addition, a water extract of CA increased the expression of antioxidant and mitochondrial genes in mice, and also improved their cognitive function (Gray et al., 2016). These studies on animal models suggest that CA extracts are beneficial to neuronal structure and function and may be used to alleviate neurological diseases and conditions in humans.

Few human studies have examined the effects of CA in placebo-controlled settings. Three months of treatment with a herbal mixture containing CA were shown to improve short-term memory in children (Sarokte and Rao, 2013). A single 12-g oral administration of dried CA herb significantly reduced acoustic startle response in healthy subjects as compared with placebo group, suggesting that CA has anxiolytic activity in humans (Bradwejn et al., 2000). A randomized, placebo-controlled double-blinded study found treatment of healthy individuals with CA extract for 2 months enhanced working memory and self-rated mood (Wattanathorn et al., 2008). CA has also been used to treat a variety of non-neurological diseases and conditions including ulcers, cancer, hypertension, atherosclerosis, eczema, wounds, and leprosy (Babu et al., 1995; De Sanctis et al., 2001; Hausen, 1993). In recent years, CA's popularity has soared, and is now used worldwide as an herbal dietary supplement called Gottu kola.

Chemical analysis of CA extracts found a variety of polyphenols and triterpenes (Rumalla et al., 2010). The most common

triterpenoids in CA extracts include AA, MA, asiaticoside, and madecassoside (Jew et al., 2000; Mook-Jung et al., 1999). Three of the 28 asiaticoside derivatives, AA, asiaticoside-6, and SM2 tested in cell cultures studies showed neuroprotective effects against β-amyloid induced neurotoxicity (Mook-Jung et al., 1999). All three asiaticoside derivatives reduced H2O2-induced cell death and lowered intracellular free radical concentration. Similarly, derivatives of AA protected cultured cortical neurons against glutamate-induced excitotoxicity by potentiating cellular oxidative defense mechanism (Lee et al., 2000). However, the exact component of CA extract and the molecular mechanism whereby it confers neuroprotection is still unclear. In this study, we examined the effects AA and MA on neurite outgrowth in Neuro-2a (N2a) neuroblastoma cell line.

METHODS

Neuro-2a (N2a) culture. Neuro-2a (N2a, murine neuroblastoma cells) were obtained from the American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM), sodium pyruvate, L-glutamine, PBS, trypsin, penicillin-streptomycin-amphotericin (PSA), and tissue culture plates were purchased from Thermo-Fisher Scientific (Chicago, IL). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Asiatic acid (AA) and madecassic acid (MA) were purchased from Sigma Chemicals (St Louis, MO) and stock solution (1 mM) was prepared in ethanol due to its poor solubility in water. N2a cells were grown in DMEM containing 1X L-glutamine, 1X PSA, and 1X sodium pyruvate, 10 mM of glucose, and 10% of FBS. Medium was replaced every three days and cultures were maintained at 37 °C and 6.5% CO_2.

Measurement of neurite outgrowth. To examine the effects of AA on neurite outgrowth, N2a cells were plated in a six-well plate at a concentration of 200,000 cells/well in DMEM medium containing 10 mM glucose, 10% FBS, and 1X PSA for 24 hours. The cells were further incubated for 24 hours in DMEM containing 10 mM of glucose, 1X PSA, and with various concentrations of either AA or MA in ethanol or ethanol alone (vehicle). The cells were photographed using an Amscope MU 1400-CK microscope camera. Neurite outgrowth was quantified using NeuronJ, an ImageJ add-on. Each neurite was traced and length was recorded. Only neurites measuring at least 30 μm were considered in the calculation of percent neurite bearing cells, but all measurements were used for longest neurite and combined length of neurites calculations. To calculate percentage of neurite bearing cells, the number of neurons with a neurite length measuring at least 30 μm was divided by the total number of neurons in the visual field. The resulting decimal was multiplied by 100 to derive the percentage of neurite bearing cells. For measuring the longest neurite and combined length of neurites, a minimum of 60 neurons were measured for each treatment condition. To avoid bias in measurements, all neurons in the visual fields located at 5 quadrants (center, northeast, northwest, southeast, and southwest) of the well were measured. In addition, the researcher making the measurement was unaware of the treatment conditions (medium alone, ethanol, AA, or MA).

Statistical analysis. All experiments were repeated at least four times using different N2a cultures and reagents. The data in individual experiments were presented as mean ± standard error, and statistical analyses (one-way ANOVA, Post-hoc corrected t-tests) were performed using Excel software.

RESULTS AND DISCUSSION

Asiatic acid affects neurite outgrowth in N2a cells. Previous studies have shown that extracts from CA induces neurite outgrowth (Jiang et al., 2016; Soumyanath et al., 2005). We examined whether AA and

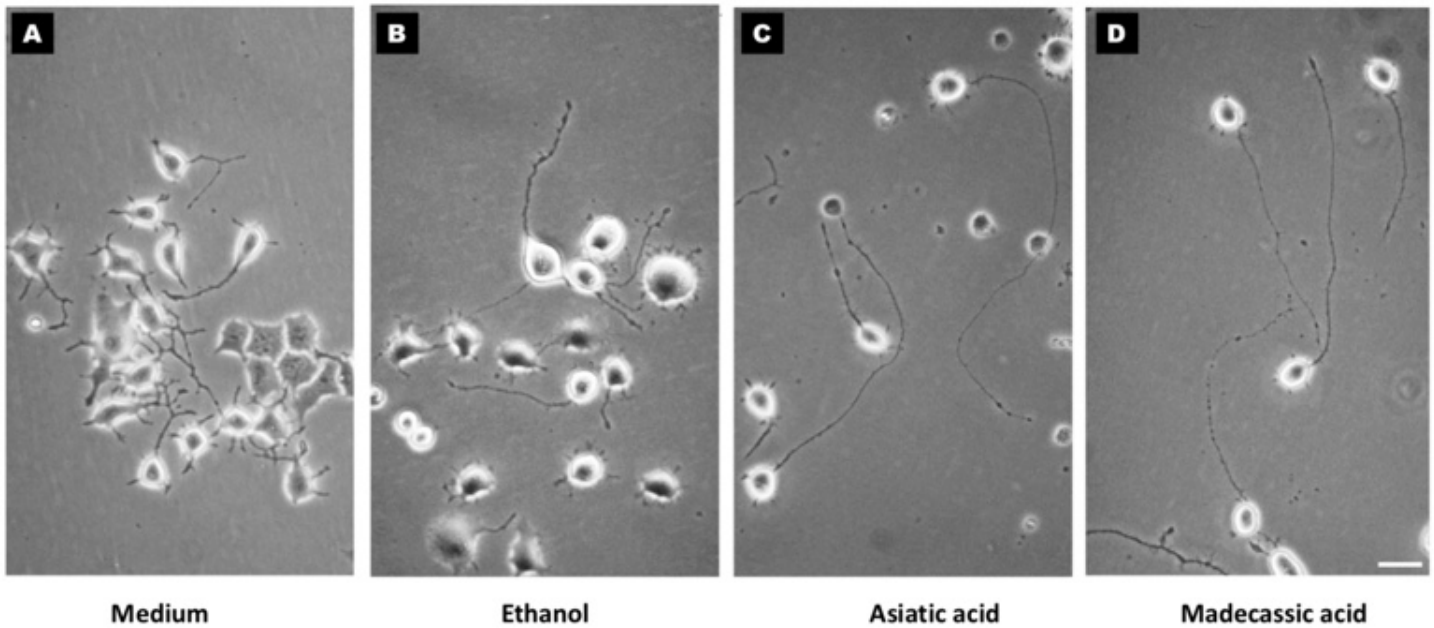

Figure 1. Phase contrast photographs of representative neurons in N2a cells incubated in medium alone (A), in medium containing ethanol (vehicle, B), asiatic acid (5 μM, C), or madecassic acid (5 μM, D). Scale bar = 20 μm.

MA, the main component of the CA, promotes neurite outgrowth in N2a cells. The cells were incubated for 2 days in medium containing 0.5, 1, 2, 5, and 10 μM AA or MA in ethanol or ethanol alone (vehicle). Following incubation, the cells were photographed and various parameters of neurite outgrowth were measured using NeuronJ software.

Incubation of N2a cells with ethanol (vehicle) had no effect on the percentage of neurite bearing cells in the culture as compared to cells grown in medium alone (Figure 1). Treatment of N2a cells with AA significantly decreased the percentage of neurite bearing cells as compared to cells grown in vehicle (Figure 2A). The magnitude of decrease in the percentage of neurite bearing cells was higher in lower concentrations (0.5 and 1 μM) as compared to higher concentrations (2 and 5 μM). In addition, AA incubation significantly ($p<0.05$) increased neurite extension as compared to cells incubated with vehicle alone (Figure 2B). Furthermore, the combined length of all neurites in cells incubated with AA was significantly ($p<0.05$) higher than that in cells incubated with vehicle alone (Figure 2C). The greater increase in neurite extension and combined length in higher concentration (5 μM) of AA may have contributed to the increase in the percentage of neurite bearing cells as compared to lower concentration (0.5 μM).

Incubation of N2a cells with vehicle or MA had no significant ($p>0.005$) effect on the percentage of neurite bearing cells as compared to cells grown in medium alone (Figure 3A). Similar to AA, MA significantly ($p<0.05$) increased neurite extension as compared to cells incubated with vehicle alone (Figure 3B). In addition, the combined length of all neurites in cells incubated with MA was significantly ($p<0.05$) higher than that in cells incubated with vehicle alone (Figure 3C).

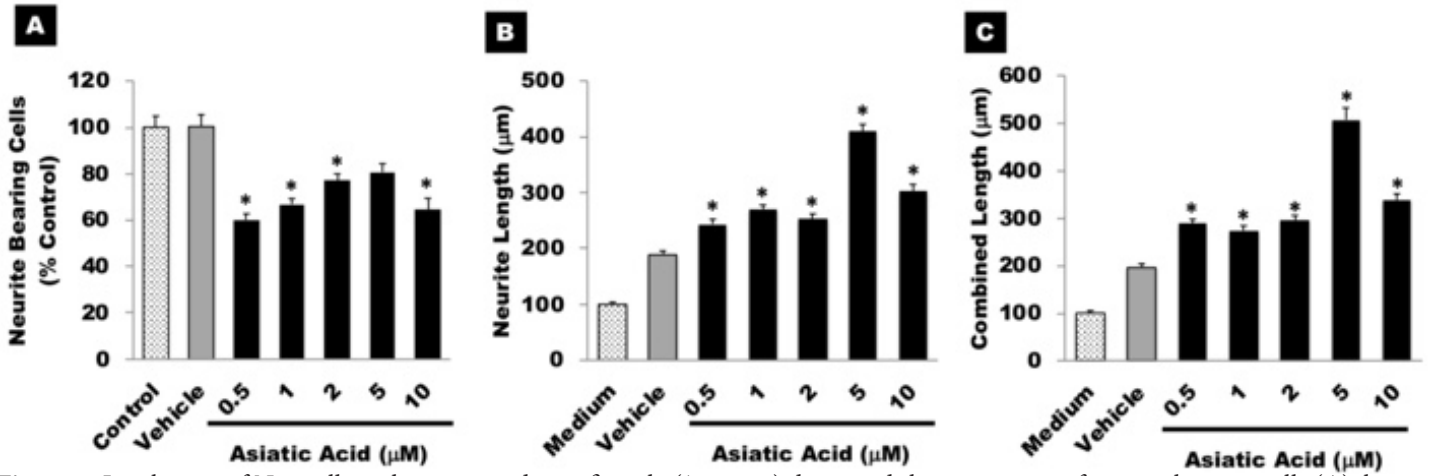

Figure 2. Incubation of N2a cells with asiatic acid significantly (* $p<0.05$) decreased the percentage of neurite bearing cells (A), but increased neurite extension (B), and combined length of neurites (C) as compared to cells incubated with ethanol alone (vehicle). Data are mean+/- SE from 4 different experiments.

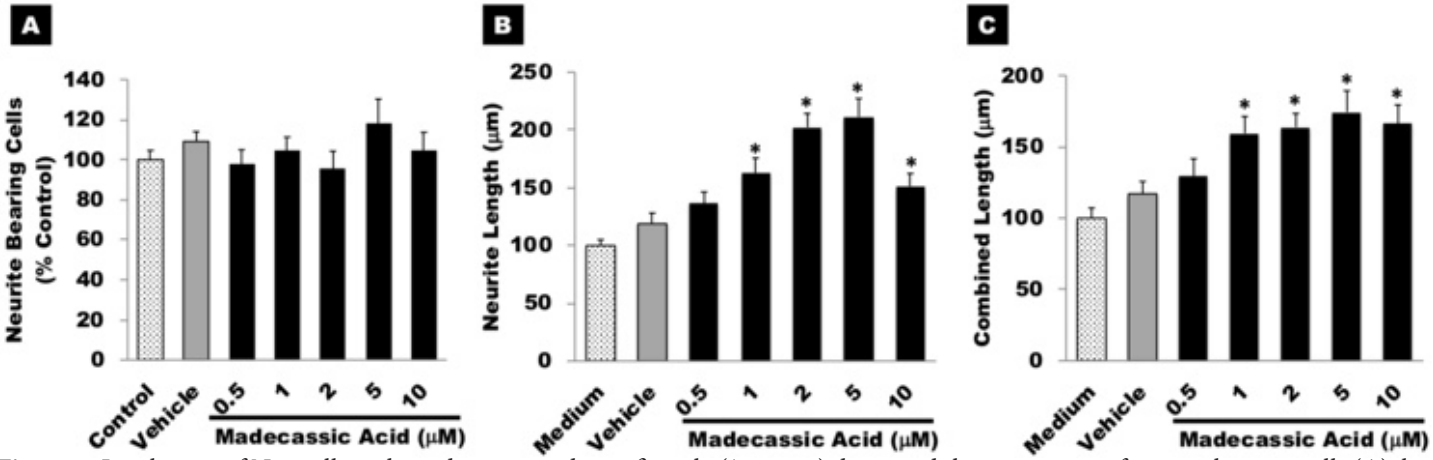

Figure 3. Incubation of N2a cells with madecassic acid significantly (* $p<0.05$) decreased the percentage of neurite bearing cells (A), but increased neurite extension (B), and combined length of neurites (C) as compared to cells incubated with ethanol alone (vehicle). Data are mean+/- SE from 4 different experiments.

These results are consistent with a previous study that showed increased neuronal differentiation in PC12 cells incubated with ethanol extract of CA (Jiang et al., 2016). In this study CA extract was fractionated using the microporous resin method to yield 45 fractions. LC-MS fingerprint analysis of these fractions revealed that AA and madecassic acid are the main neurite outgrowth promoting factors in the CA extract. However, this previous study used a high concentration (14.4 μM) of purified AA to induce neurite outgrowth as compared to 1 μM AA used in this study. The reason behind this discrepancy is unclear, but difference in cell type could be a contributing factor.

The underlying mechanism whereby AA increases neurite outgrowth is unclear. Several cellular pathways have been implicated in regulating neuronal growth. A previous study showed that inhibitors of ERK/RSK signaling pathway abolished the neurite outgrowth promoting effects of CA extract in neuroblastoma cells (Xu et al., 2008). Similarly, another study showed that CA extracts significantly upregulated the level of activated ERK1/2 and Akt in neuroblastoma cells, suggesting their involvement in the neurite promoting effects of CA extracts (Wanakhachornkrai et al., 2013).

Conclusions. The results from this study showed that treatment of N2a cultures with AA decreased the percentage of neurite bearing cells, but increased neurite extension and combined length of neurites per cell in a dose-dependent manner. In addition, treatment of N2a cultures with MA did not significantly alter the percentage of neurite bearing cells, but increased neurite extension and combined length of neurites per cell in a dose-dependent manner. These findings demonstrate that AA and MA facilitate neuronal extension in N2a cultures. Future studies to examine the beneficial effects of AA and MA in animal models of neurological diseases would help elucidate its therapeutic potential in neurodegenerative disease involving mitochondrial dysfunction and compromised nerve regeneration.

List of abbreviations. CA, *Centella asiatica* (L.) Urban; AA, asiatic acid; AA, madecassic acid; N2a, Neuro2a murine neuroblastoma; and CNS, central nervous system.

Funding. Department of Biological Sciences, Eastern Illinois University and Pine Honors College, Eastern Illinois University.

LITERATURE CITED

Babu, T.D., Kuttan, G., Padikkala, J., 1995. Cytotoxic and anti-tumour properties of certain taxa of Umbelliferae with special reference to *Centella asiatica* (L.) Urban. J Ethnopharmacol. 48, 53-7.

Bradwejn, J., Zhou, Y., Koszycki, D., Shlik, J., 2000. A double-blind, placebo-controlled study on the effects of Gotu Kola (*Centella asiatica*) on acoustic startle response in healthy subjects. J Clin Psychopharmacol. 20, 680-4.

De Sanctis, M.T., Belcaro, G., Incandela, L., Cesarone, M.R., Griffin, M., Ippolito, E., Cacchio, M., 2001. Treatment of edema and increased capillary filtration in venous hypertension with total triterpenic fraction of *Centella asiatica*: a clinical, prospective, placebo-controlled, randomized, dose-ranging trial. Angiology. 52 Suppl 2, S55-9.

Farooqui, A.A., Farooqui, T., Madan, A., Ong, J.H., Ong, W.Y., 2018. Ayurvedic Medicine for the Treatment of Dementia: Mechanistic Aspects. Evid Based Complement Alternat Med. 2018, 2481076.

Gray, N.E., Harris, C.J., Quinn, J.F., Soumyanath, A., 2016. *Centella asiatica* modulates antioxidant and mitochondrial pathways and improves cognitive function in mice. J Ethnopharmacol. 180, 78-86.

Hausen, B.M., 1993. *Centella asiatica* (Indian pennywort), an effective therapeutic but a weak sensitizer. Contact Dermatitis. 29, 175-9.

Jew, S.S., Yoo, C.H., Lim, D.Y., Kim, H., Mook-Jung, I., Jung, M.W., Choi, H., Jung, Y.H., Kim, H., Park, H.G., 2000. Structure-activity relationship study of asiatic acid derivatives against beta amyloid (A beta)-induced neurotoxicity. Bioorg Med Chem Lett. 10, 119-21.

Jiang, H., Zheng, G., Lv, J., Chen, H., Lin, J., Li, Y., Fan, G., Ding, X., 2016. Identification of *Centella asiatica*'s Effective Ingredients for Inducing the Neuronal Differentiation. Evid Based Complement Alternat Med. 2016, 9634750.

Kumar, A., Dogra, S., Prakash, A., 2009. Neuroprotective Effects of *Centella asiatica* against Intracerebroventricular Colchicine-Induced Cognitive Impairment and Oxidative Stress. Int J Alzheimers Dis. 2009.

Lee, M.K., Kim, S.R., Sung, S.H., Lim, D., Kim, H., Choi, H., Park, H.K., Je, S., Ki, Y.C., 2000. Asiatic acid derivatives protect cultured cortical neurons from glutamate-induced excitotoxicity. Res Commun Mol Pathol Pharmacol. 108, 75-86.

Mohandas Rao, K.G., Muddanna Rao, S., Gurumadhva Rao, S., 2006. *Centella asiatica* (L.) leaf extract treatment during the growth spurt period enhances hippocampal CA3 neuronal dendritic arborization in rats. Evid Based Complement Alternat Med. 3, 349-57.

Mook-Jung, I., Shin, J.E., Yun, S.H., Huh, K., Koh, J.Y., Park, H.K., Jew, S.S., Jung, M.W., 1999. Protective effects of asiaticoside derivatives against beta-amyloid neurotoxicity. J Neurosci Res. 58, 417-25.

Rao, R.V., Descamps, O., John, V., Bredesen, D.E., 2012. Ayurvedic medicinal plants for Alzheimer's disease: a review. Alzheimers Res Ther. 4, 22.

Rao, S.B., Chetana, M., Uma Devi, P., 2005. *Centella asiatica* treatment during postnatal period enhances learning and memory in mice. Physiol Behav. 86, 449-57.

Rumalla, C.S., Ali, Z., Weerasooriya, A.D., Smillie, T.J., Khan, I.A., 2010. Two new triterpene glycosides from *Centella asiatica*. Planta Med. 76, 1018-21.

Sarokte, A.S., Rao, M.V., 2013. Effects of Medhya Rasayana and Yogic practices in improvement of short-term memory among school-going children. Ayu. 34, 383-9.

Soumyanath, A., Zhong, Y.P., Gold, S.A., Yu, X., Koop, D.R., Bourdette, D., Gold, B.G., 2005. *Centella asiatica* accelerates nerve regeneration upon oral administration and contains multiple active fractions increasing neurite elongation in-vitro. J Pharm Pharmacol. 57, 1221-9.

Wanakhachornkrai, O., Pongrakhananon, V., Chunhacha, P., Wanasuntronwong, A., Vattanajun, A., Tantisira, B., Chanvorachote, P., Tantisira, M.H., 2013. Neuritogenic effect of standardized extract of *Centella asiatica* ECa233 on human neuroblastoma cells. BMC Complement Altern Med. 13, 204.

Wattanathorn, J., Mator, L., Muchimapura, S., Tongun, T., Pasuriwong, O., Piyawatkul, N., Yimtae, K., Sripanidkulchai, B., Singkhoraard, J., 2008. Positive modulation of cognition and mood in the healthy elderly volunteer following the administration of *Centella asiatica*. J Ethnopharmacol. 116, 325-32.

Xu, Y., Cao, Z., Khan, I., Luo, Y., 2008. Gotu Kola (*Centella asiatica*) extract enhances phosphorylation of cyclic AMP response element binding protein in neuroblastoma cells expressing amyloid beta peptide. J Alzheimers Dis. 13, 341-9.