2017

Effects of Asiatic Acid on Neurite Outgrowth in Neuro-2a Cells

Aishah Asiri
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Effects of Asiatic Acid on Neurite Outgrowth in Neuro-2a Cells

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BY

Aishah Asiri

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Abstract

Recently, medicinal plants from ancient Ayurvedic medicine have provided clues to the discovery of novel therapeutics for various diseases. In Ayurvedic medicine, a common Indian plant, *Centella asiatica* is highly regarded as a "rasayana" or nerve tonic. The *Centella* extract is used to ward off age-related dementia and to increase memory and intelligence. The mechanism by which *Centella* improves memory and learning and reduces the risk of dementia is unclear.

We recently tested the effects of asiatic acid, the main active component of *Centella*, on neuronal growth. We hypothesized that asiatic acid will promote neuronal growth and neurite network formation. To test this hypothesis, we examined the effects of asiatic acid on neuronal growth in murine neuroblastoma cells, Neuro2a. Neuro2a cells were cultured for 24 hours in DMEM medium containing10 mM glucose and 10% FBS in six-well plates at a concentration of 200,000 cells/well. The cells were further cultured for 72 hours in DMEM containing 10 mM glucose and with either 1 μM asiatic acid in ethanol or ethanol alone (vehicle). Cells were photographed, and neurite outgrowth quantified using NeuronJ software. The results revealed that asiatic acid treatment significantly increased the percentage of cells bearing neurites as compared to neurons grown in medium alone. In addition, asiatic acid treatment increased neurite extension and combined length of neurites. To investigate the impact of asiatic acid on bioenergetics in Neuro2a cells, we first analyzed the electron transport chain of the mitochondria via respirometry. The respiration rates of Neuro2a cells cultured in medium containing asiatic acid was significantly ($p < 0.05$) higher than cells grown in

medium containing vehicle alone. Also, western blot analyses were used to examine if asiatic acid could increase the mitochondrial complex. The result showed that Asiatic acid increased complex 1, 2, 3, and 4. In addition, we examined if asiatic acid would increase oxidative phosphorylation instead of glycolysis that results in lactate production. The results indicated that Neuro2a cells treated for 24 hours with 1 μM of asiatic acid induced less lactate as compared to Neuro2a with ethanol alone (vehicle). Also, the MTT assay was used to detect the viable cells in Neuro2a cells treated with either1μM asiatic acid or ethanol alone (vehicle) for two days. The result shows that Neuro2a cells treated with asiatic acid showed increased cell viability as compared to Neuro2a exposed to ethanol alone. Finally, the effect of asiatic acid on cell proliferation was examined using standard trypan blue staining. The data revealed that doubling time was significantly slower in cells cultured in presence of asiatic acid as compared to cells grown in vehicle (ethanol) alone ($p < 0.05$).

Together these results suggest that asiatic acid is neurotrophic. This effect may explain the beneficial role of *Centella asiatica* extract on learning and memory and in preventing neurological disorders.

Introduction

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

Traditional herbal medicine is 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, notably ginseng (*Panax quinquefolius* L.) Another member of this family is Centella *asiatica* L. Urban (syn. *Hydrocotyle asiatica* L.), (CA, herein). *Centella asiatica* is a herb known by various names such as Indian Pennywort (English) and Gotu kola (Chinese medicine).

CA is an herb that can be found in moist areas of countries with tropical and subtropical climate such as India, Pakistan, Sri Lanka, Madagascar, South Africa, Venezuela, and Columbia as well as area of the South pacific and Eastern Europe. It proliferates in abundance in wet, sandy or clay soils where it forms wide clumps that exhibit a dense green carpet landscape. *Centella asiatica* is characterized by small fan-shaped green leaves with flowers that can be of various colors (white, light purple-to-

pink) and oval shaped fruits (Gohil *et al.*, 2010). The plant is reported to be slender, tender, faintly aromatic and tasteless (Gohil *et al.*, 2010; Jamil *et al.,* 2007).

CA is a tropical herb that has been widely used for many centuries in both Indian Ayurvedic and Traditional Chinese medicines to improve intelligence, learning, memory, and cognitive performance. It is also used as a brain tonic for promoting brain growth and prevents mental retardation. One medicinal practice in which CA plays an important role is the Ayurveda medicine where the plant constitutes one of the main herbs used for revitalizing nerve and brain cells (Chaitanya *et al.,* 2011). Ayurveda is a system of traditional medicine native to India. Originating in prehistoric times, Ayurveda is based on two foundational textbooks dated to the period of 900 BCE – 600 BCE. Interestingly, in a chapter devoted to curing mental illnesses, including dementia-like Alzheimer's disease, extract of a common Indian plant includes CA and is highly regarded as a "rasayana" or nerve tonic.

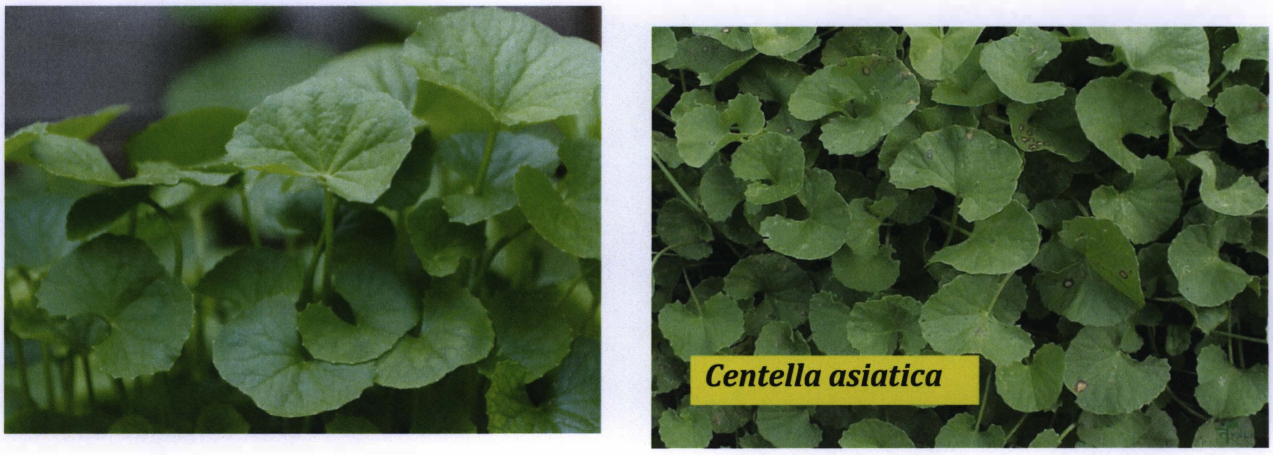

Figure 1 – Photographs of *Centella asiatica*

Studies on cell culture and animal models 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 vivo*, CA extracts enhanced dendrititc arborization in the hippocampus and amygdala (Rao *et al* . , 2006; Rao *et al*., 2005), 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, 2011). Aqueous extracts of CA ameliorated 3-nitroprorionic acid-induced oxidative stress and mitochondrial dysfunctions in brains of mice. 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 extract is beneficial to neuronal structure and function, and may alleviate neurological diseases and conditions in humans.

Few human studies have examined the effects of CA in placebo-controlled setting. CA treatment for 12 weeks in mentally retarded children improved general mental ability and behavioral problems (Appa Rao in Soumyanath *et al.,* 2005). A single 12-g oral administration of CA significantly reduced acoustic startle response in healthy subjects as compared with placebo group, suggesting that CA has anxiolytic activity in humans (Bradwejn 2000 in Soumyanath *et al*., 2015). A randomized, placebo-controlled double-blind study found treatment of healthy individuals with CA extract for 2 months enhanced working memory and self-rated mood. CA has also been used to treat a variety of non-neurological diseases and conditions including ulcers, cancer, hypertension, atherosclerosis, eczema, wounds, and leprosy (G.K. *et al*.,

2011). In recent years CA's popularity has soared, and is now used worldwide as an herbal dietary supplement called *Gottu kola.*

Chemical analysis found a variety of polyphenols and tirterpernes (G.K. *et al.,* 2011) in CA extracts. The most common triterpenoids in CA extracts include asiatic acid (AA), madecassic acid, asiaticoside, and madecassoside (Nataraj *et al*., 2016). Three of the 28 asiaticoside derivatives, AA, Asiaticoside 6, and SM2, tested in cell cultures studies showed neuroprotective effects against □-amyloid induced neurotoxicity (Soumyanath *et al.,* 2005). All three asiaticoside derivatives reduced H_2O_2-induced cell death and lowered intracellular free radical concentration. Similarly, derivatives of asiatic acid protected cultured cortical neurons against glutamate-induced excitotoxicity by potentiating cellular oxidative defense mechanism (Lee, MK in Soumyanath *et al.,* 2005). The exact component of CA extract and the molecular mechanism whereby it confers neuroprotection is still unclear.

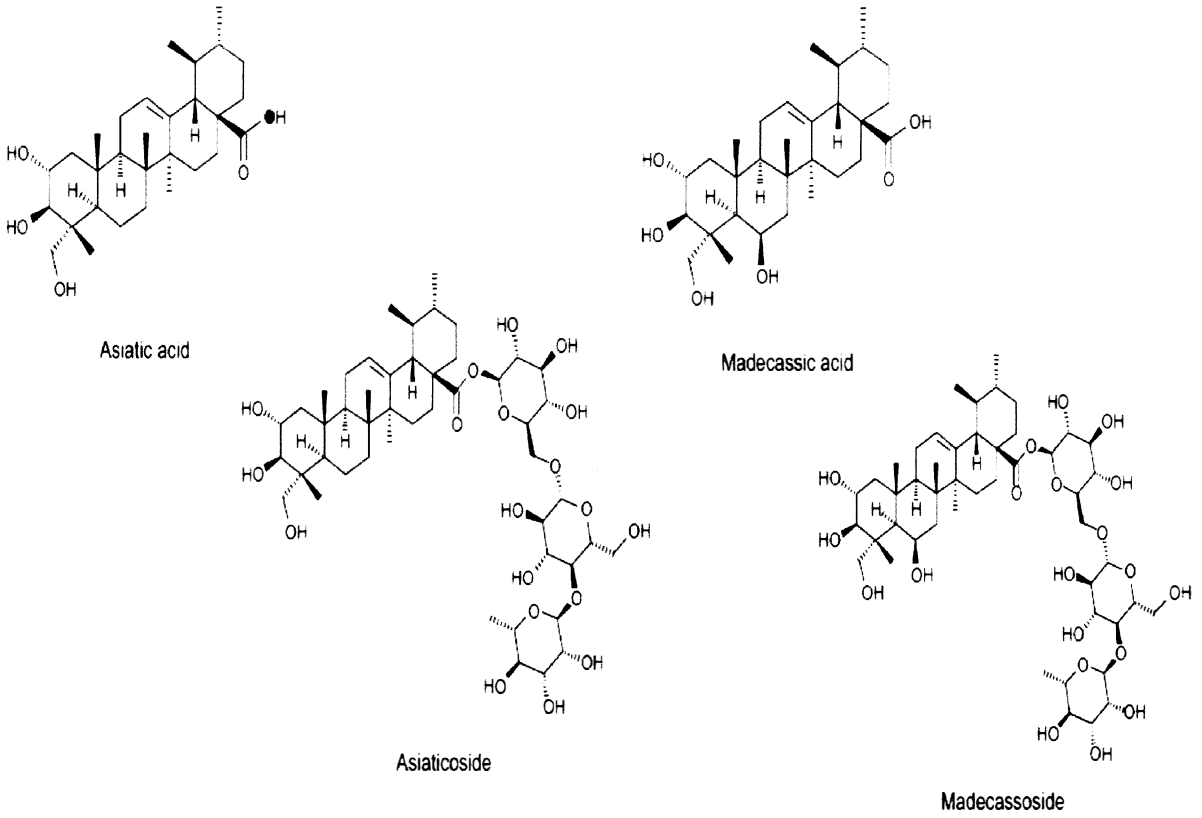

Figure 2 - Chemical structure of the main active components in *Centella asiatica* with medicinal properties. Asiatic acid, madecassoside, asiaticoside, and madecassic (Maramaldi *et al.,* 2013) are shown.

Table 1: Main components found in *Centella asiatica* (Gohil, *et al.,* 2010 ; R. Priya & K. Shaival, 2012)

Category	Example of compounds
Triterpene acids	Asiatic, madecassic, terminolic, centic, centellic, centoic acid , indocentoic acid, brahmic, and madasiatic acids
Glycosides	Asiaticoside A, asiaticoside B, madecassoside, centelloside, indocentelloside, brahmoside, brahminoside, thankuniside and iso thankuniside
Fatty oil	glycerides of palmitic, stearic, lignoceric, oleic, linoleic and linolenic acids
Flavonoids	Flavonoids, 3-glucosylquercetin, 3-glucosylkaempferol and 7-glucosylkaempferol

Other compounds	Mesoinositol, centellose (oligosaccharide) centellose, kaempferol, quercetin, stigmasterol, sitosterol, campesterol, polyacetylenes, carotenoids, vitamin B1 and vitamin C1, an alkaloid (hydrochotine), a bitter component (vellarine), tannins, sugars, inorganic acids, resin, amino acids (aspartic acid, glycine, glutamic acid, α-alanine and phenylalanine), chloride, sulphate, phosphate, iron, calcium, magnesium, sodium and potassium

In this project, we examined the effects of asiatic acid on cell proliferation, neurite outgrowth, and mitochondrial structure and function in Neuro-2a neuroblastoma cell line. Since CA is extensively used as an herbal medicine to promote neurological health, we hypothesized that asiatic acid, the main component of CA, will have beneficial effects on neurons. We tested this hypothesis by examining the effects of asiatic acid on neurite outgrowth and mitochondrial structure and function in neuroblastoma cell line, Neuro2a.

We found that

1. Asiatic acid treatment significantly increased the percentage of neurite bearing cells as compared to neurons grown in vehicle (ethanol) alone.
2. Asiatic acid treatment significantly increased neurite extension and combined length of neurites as compared to neurons grown in vehicle alone.
3. Asiatic acid treatment increased cells proliferations and the number of viable cells as compared to neurons grown in vehicle alone.
4. Asiatic acid treatment increased levels of mitochondria complexes, respiration rates, and decreased lactate production as compared to neurons grown in vehicle alone.

Materials and Methods

Neuro-2a (N2a) culture

Neuro-2a (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, 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 was purchased from Sigma Aldrich (St. Louis, MO). Asiatic acid stock solution (10 mM) was prepared in ethanol due to its poor solubility in water. The stock solution was diluted to 1 µM using ethanol.

Neuro2a cells were grown in DMEM containing 1X L-glutamine, 1X PSA), 1X sodium pyruvate, 10 mM of glucose, and 10% of FBS. Cultures were maintained at 37 °C and 6.5% CO_2. Cells were treated with either 10 µl of 1 mM asiatic acid (1 µM final concentration) of asiatic acid in ethanol or 10 µl of ethanol alone (vehicle) in 10 ml media. Medium was replaced every three days with re-addition of asiatic acid or ethanol. Cells were cultured in the respective medium for 6 weeks to ensure all metabolic changes were complete.

Measurement of neurite outgrowth

To examine the effects of the asiatic acid on neurite outgrowth, Neuro2a cells at a concentration of 200,000 cells/well were plated in DMEM medium containing10 mM glucose and 10% FBS in a six-well plate. The cells were further incubated for 72 hours in DMEM containing 10 mM of glucose and with either 1 µM asiatic acid in ethanol or ethanol alone (vehicle). The cells were photographed using Olympus BX 50 fluorescent microscope. Neurite outgrowth was quantified using NeuronJ, an ImageJ add-on. Each

neurite was traced and length was recorded in pixels and converted to µm. 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. Minimums 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 was measured. In addition, the researcher was unaware of the treatment condition (asiatic acid versus ethanol).

Cell proliferation

Neuro2a cells were plated in six-well plates at a concentration of 250,000 cells per well in three ml of DMEM containing 10 mM glucose and either 1 µM asiatic acid in ethanol or ethanol alone (vehicle). Cells were incubated for 48 hours, then stained with Trypan blue, and counted using a hemocytometer. Doubling time was calculated using the formula: *Doubling Time = [Duration * log(2)] / [log(Final Concentration) – log(Initial Concentration)]*.

MTT assay

Neuro2a cells were cultured for 48 hours at 37 °C in 6.5 % $CO_{2\ in}$ a ninety-six-well plate at a concentration of 5000 cells per well in 100 µl of DMEM containing 10 mM glucose with either 1 µM asiatic acid in ethanol or ethanol alone (vehicle). The medium was replaced with 100 µL of DMEM medium with 10 µl of MTT stock solution (12 mM) Thermo-Fisher Scientific, Chicago, IL). Cells were further incubated for four hours at 37 °C in 6.5 % CO_2. The medium was aspirated, and cells were incubated for 10 minutes in 10 µL of DMSO. The absorbance at 540 nm was measured using a microplate reader

L-Lactate assay

Neuro2a cells were cultured in 10 ml of DMEM at a concentration of 2000,000 cells with either asiatic acid or ethanol (vehicle). Cells were allowed to incubate for 24 hours at 37 °C in 6.5 % CO_2 and 93.5% humidified air. The cells were harvested, rinsed in PBS, and harvested in 500 µL of lactate assay buffer. Cells were centrifuged and 15 µL of cold TCA solution was added to 100 µL of samples in microcentrifuge tubes. Samples were incubated on ice for 15 minutes and centrifuged for 5 minutes at 12000 g. This latter procedure was repeated once more, and the TCA in the supernatant was neutralized by adding 10 µL of cold neutralization solution. 50 µl of reaction mix was added to 50 ul of sample in a 96 well microplate, and absorbance was measured at OD 450 nm using a microplate reader.

Western blot

Neuro2a cells cultured in DMEM with either asiatic acid or ethanol were lysed and proteins isolated using a RIPA buffer cocktail, including 1x protease inhibitor and 1x EDTA. The protein concentration of the samples was quantified using BCA quantification, and denaturized using beta-mercaptoethanol. Western blot was performed as per usual protocol. Levels of mitochondrial complex protein were assessed using the Abcam total OXPHOS Rodent Western Blot Antibody cocktail, at a concentration of 1.5 uL/mL in blocking buffer. This is a cocktail of five mouse antibodies used to detect CI subunit NDUFB8, CII-30kDa, CIII-Core protein 2, CIV subunit I, and CV alpha subunit as an optimized premixed cocktail.

Respirometry

Respiration was measured at 37 °C using 0.5×10^6 cells per mL in each chamber of the Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria). Routine respiration of intact cells was measured in either DMEM supplemented with glucose and asiatic acid, or DMEM supplemented with glucose and ethanol (vehicle control). The media compositions in these experiments were identical to the media used to culture cells. Cellular respiration was uncoupled by successive titrations of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 0.5 µM steps). LEAK respiration was measured in the presence of the F_0-F_1 ATPase inhibitor, oligomycin (2 µg/mL). DATLAB software (OROBOROS Instruments, Innsbruck, Austria) was used for data analysis and acquisition.

Statistical analysis

All experiments were repeated at least four times using different Neuro2a cultures and reagents. The data in individual experiments were presented as mean ± standard error, and statistical analysis (One way ANOVA, Post-hoc Bonferroni Corrected t-tests) was performed using Excel software.

Results

Asiatic acid treatment increases neurite outgrowth in Neuro2a cells

Previous studies have shown that extracts from *Centella asiatica* (CA) induces neurite outgrowth. We examined if asiatic acid, the main component of the CA, promotes neurite outgrowth in Neuro2a cells (N2a). The cells were incubated for 3 days in medium containing 1 □M asiatic acid in ethanol or ethanol alone (vehicle). Following incubation, the cells were photographed (Figure 1). We found that the asiatic acid increased neurite outgrowth, as compared to Neuro2a cells grown in ethanol alone (vehicle). Furthermore, various parameters of neurite outgrowth were measured using NeuronJ software. Our measurements revealed that the percentage of neurite bearing cells were significantly ($p<0.05$) higher in cells incubated with asiatic acid as compared to vehicle (Figure 2). Asiatic acid incubation also significantly increased the length of neurites. Incubation of Neuro2a cells with asiatic acid significantly ($p<0.05$) increased neurite extension as compared to vehicle (Figure 3). In addition, the combined length of all neurites in cells incubated with asiatic acid was significantly higher than that in cells incubated with vehicle (Figure 4).

Asiatic acid treatment increases mitochondrial number and function in Neuro2a cells

Since neurite outgrowth is an energy consuming process, we examined if asiatic acid could increase mitochondrial structure and function. We used western blot analysis to examine if asiatic acid increases mitochondrial complexes. The results showed that asiatic acid increased the levels of mitochondrial complexes 2, 3, 4, and 5 as compared to levels in cells incubated with vehicle (Figure 5).

To examine the effects of asiatic acid on mitochondrial function, high-resolution respirometry was performed (Figure 6). Culturing Neuro2a cells in medium containing asiatic acid for two weeks increased routine oxygen consumption by about 10% ($n = 4$, $p < 0.05$, Fig. 6). Maximum uncoupled respiration rates were significantly elevated by 31% for cells cultured in medium supplemented with asiatic acid in presence of the potent chemical uncoupler, FCCP ($n = 4$, $p < 0.05$, Fig. 6).

Asiatic acid treatment decreases lactate production in Neuro2a cells

Given the increase in levels of mitochondrial complexes and associated increase in respiratory function, we then examined if asiatic acid would increase oxidative phosphorylation instead of glycolysis that results in lactate production. Neuro2a cells were treated for 24 hours with either 1 □M of asiatic acid or ethanol alone (vehicle). Following incubation lactate levels in the medium was quantified as described in Methods section. The results revealed that lactate levels were significantly ($p<0.05$) lower in Neuro2a cells incubated with asiatic acid as compared to levels in Neuro2a cells grown in ethanol alone (Figure 7).

Asiatic acid increases proliferation in Neuro2a cells

We next examined the effects of asiatic acid on cell proliferation in Neuro2a cells. The cells were incubated for 2 days in medium containing 1 □M asiatic acid in ethanol or ethanol alone (vehicle). Doubling time was measured as described in the Methods section. The results showed that incubation of Neuro2a cells with asiatic acid significantly ($P<0.05$) decreased doubling time as compared cells incubated with vehicle alone (Figure 8).

Asiatic acid treatment increases the number of viable cells in Neuro2a cells

We used MTT assay to examine if asiatic acid can regulate cell death in Neuro2a cells. MTT assay was performed in cells cultured in medium containing either 1 □M of asiatic acid in ethanol or ethanol alone (vehicle) for two days. The results revealed that asiatic acid treatment significantly ($p<0.05$) increased the number of viable cells as compared to cells grown in ethanol alone (Figure 9).

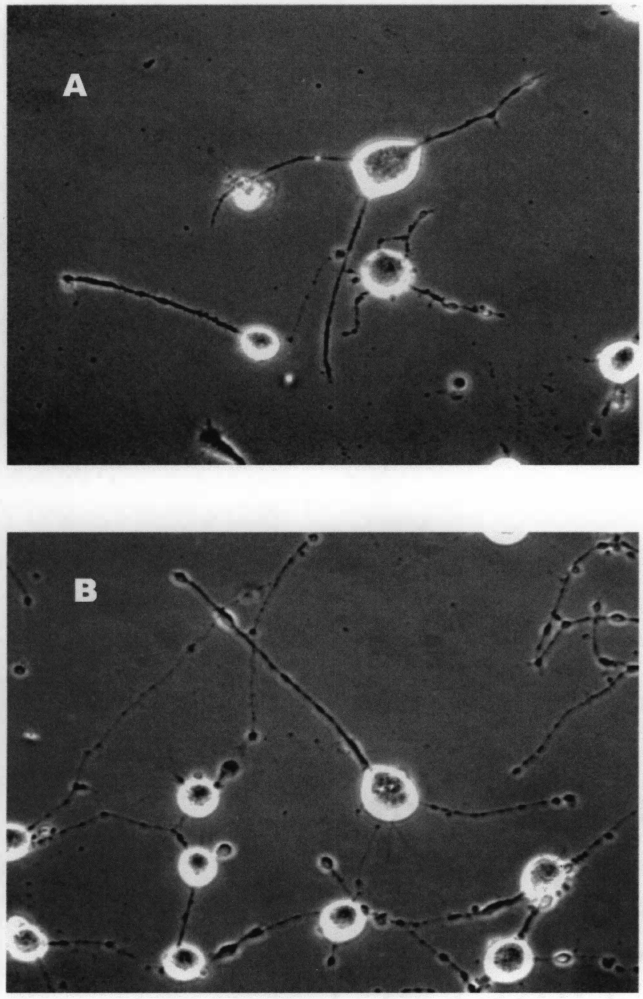

Figure 1 – Asiatic acid increased neurite outgrowth in Neuro2a cells. Phase contrast photographs of representative neurons in cultures incubated with ethanol (vehicle, A) or with asiatic acid (B). Scale bar = 20 mM

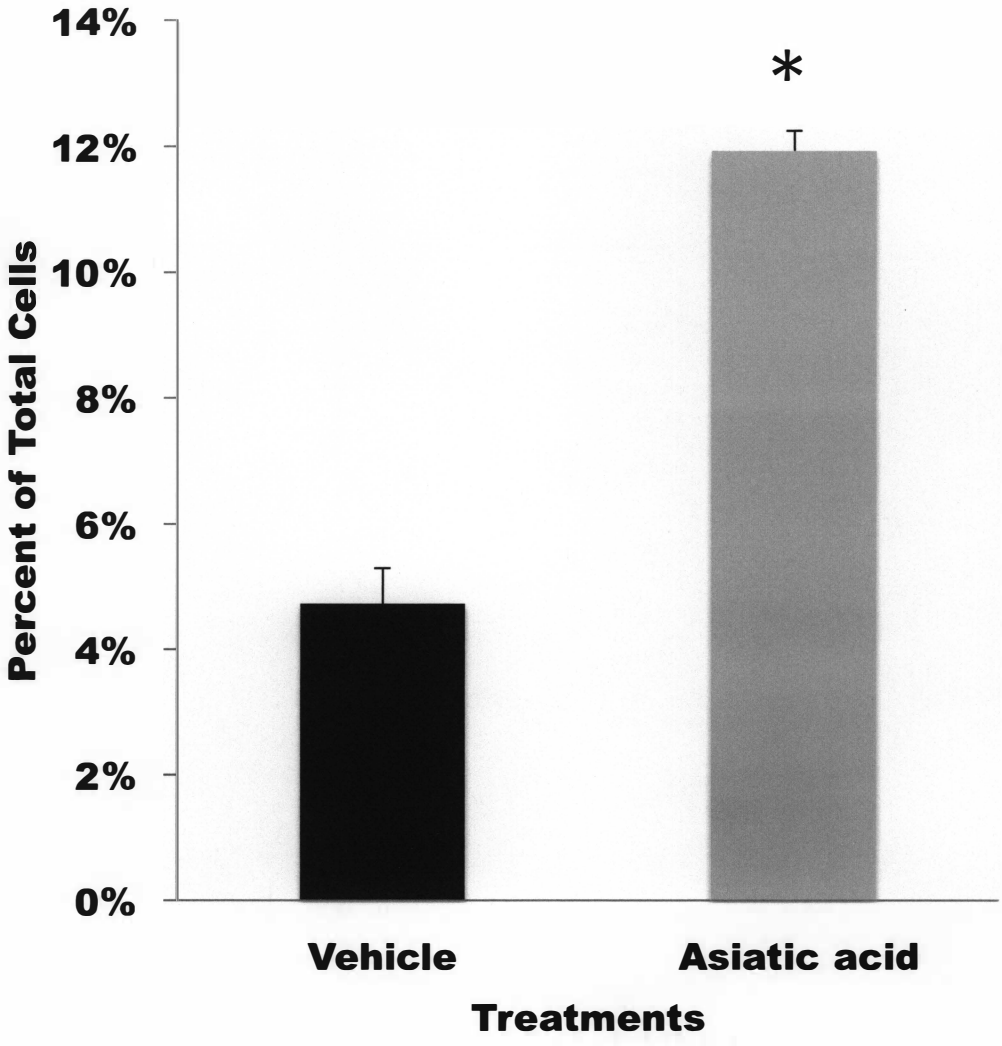

Figure 2 – Quantification of the effects of asiatic acid on the number of neurite-bearing cells in Neuro2a cultures. Incubation of Neuro-2a cells with asiatic acid significantly ($p<0.05$) increased the number of neurite bearing cells as compared those in cultures incubated with ethanol alone (vehicle).

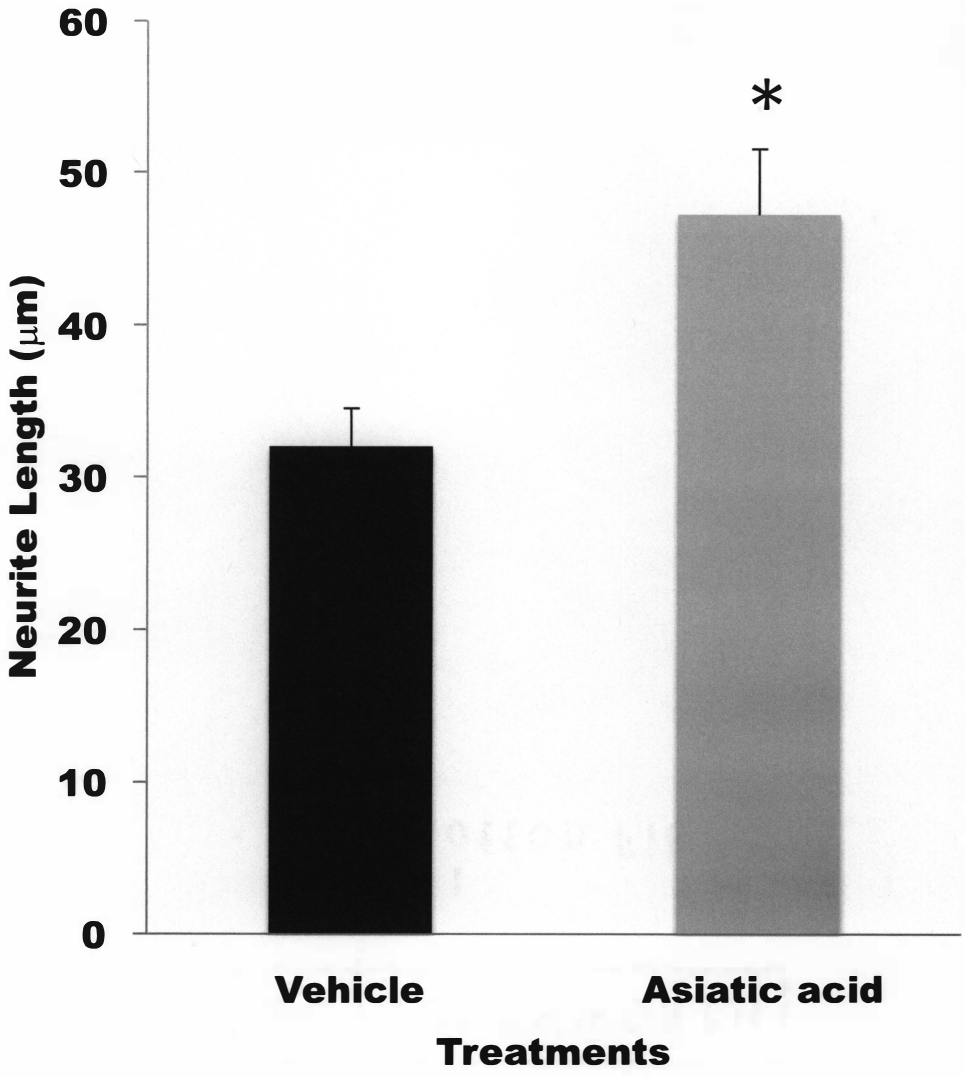

Figure 3 – Quantification of the effects of asiatic acid on the neurite length Neuro2a cultures. Incubation of Neuro-2a cells with asiatic acid significantly ($p<0.05$) increased the length of longest neurite as compared to cells incubated with ethanol alone (vehicle).

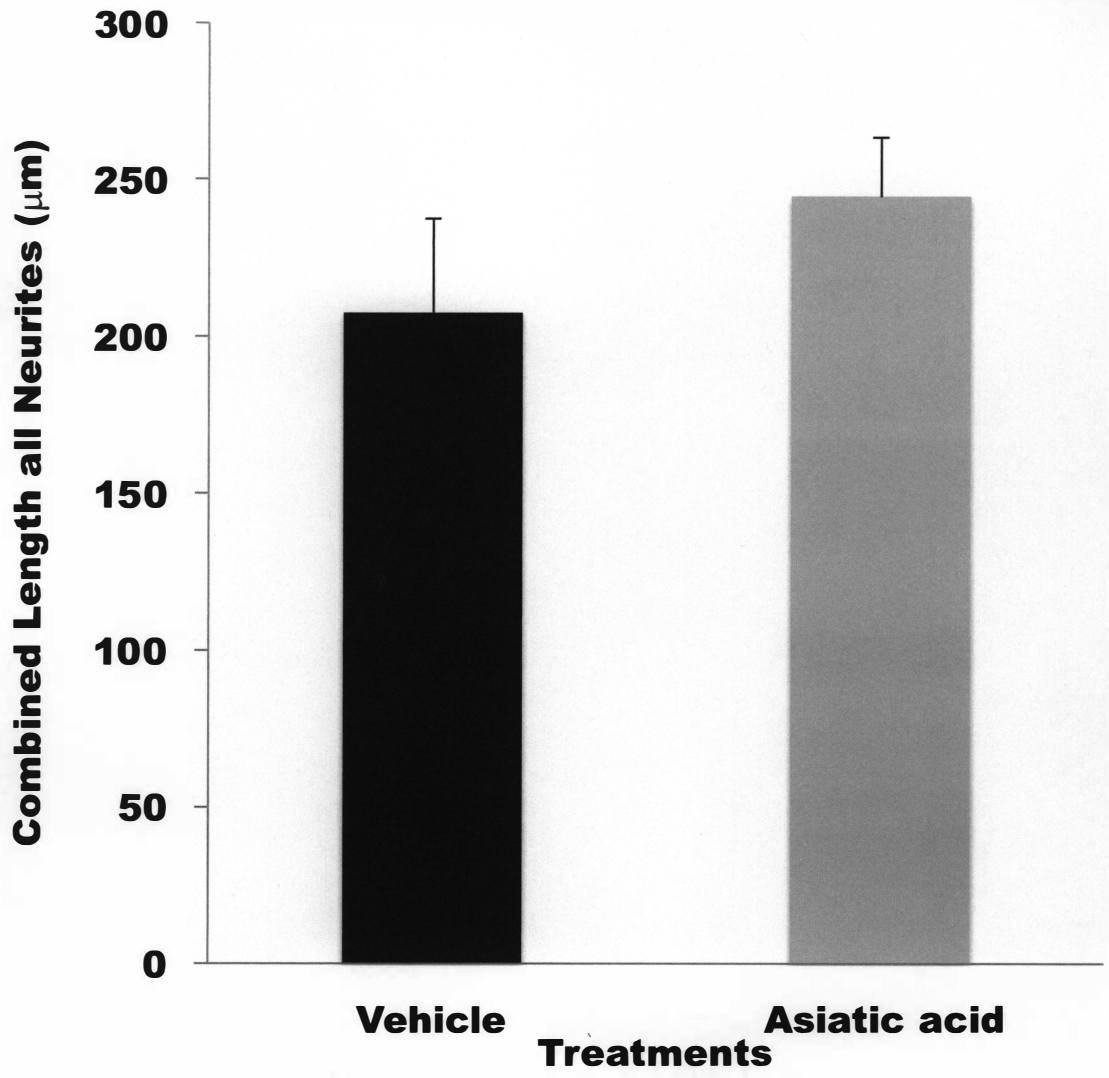

Figure 4 – Quantification of the effects of asiatic acid on the combined length of neurite in Neuro2a cultures. Incubation of Neuro-2a cells with asiatic acid increased the combined length of neurite as compared to cells incubated with ethanol alone (vehicle). However, this difference did not reach statistical significance ($p > 0.05$).

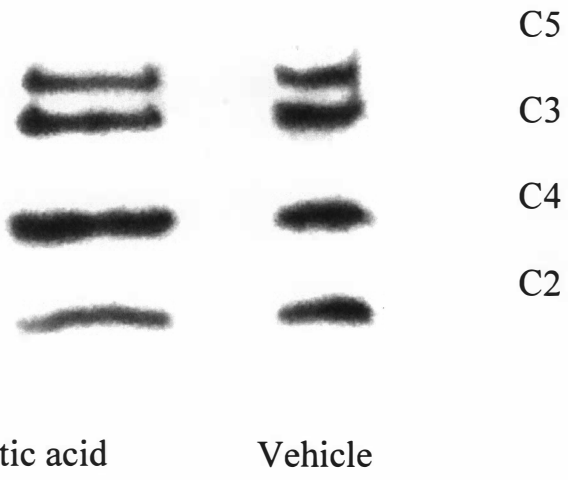

Figure 5 – A representative immunoblot of mitochondrial complexes in Neuro2a cells cultured in DMEM containing either l μM of asiatic acid or ethanol alone (vehicle).

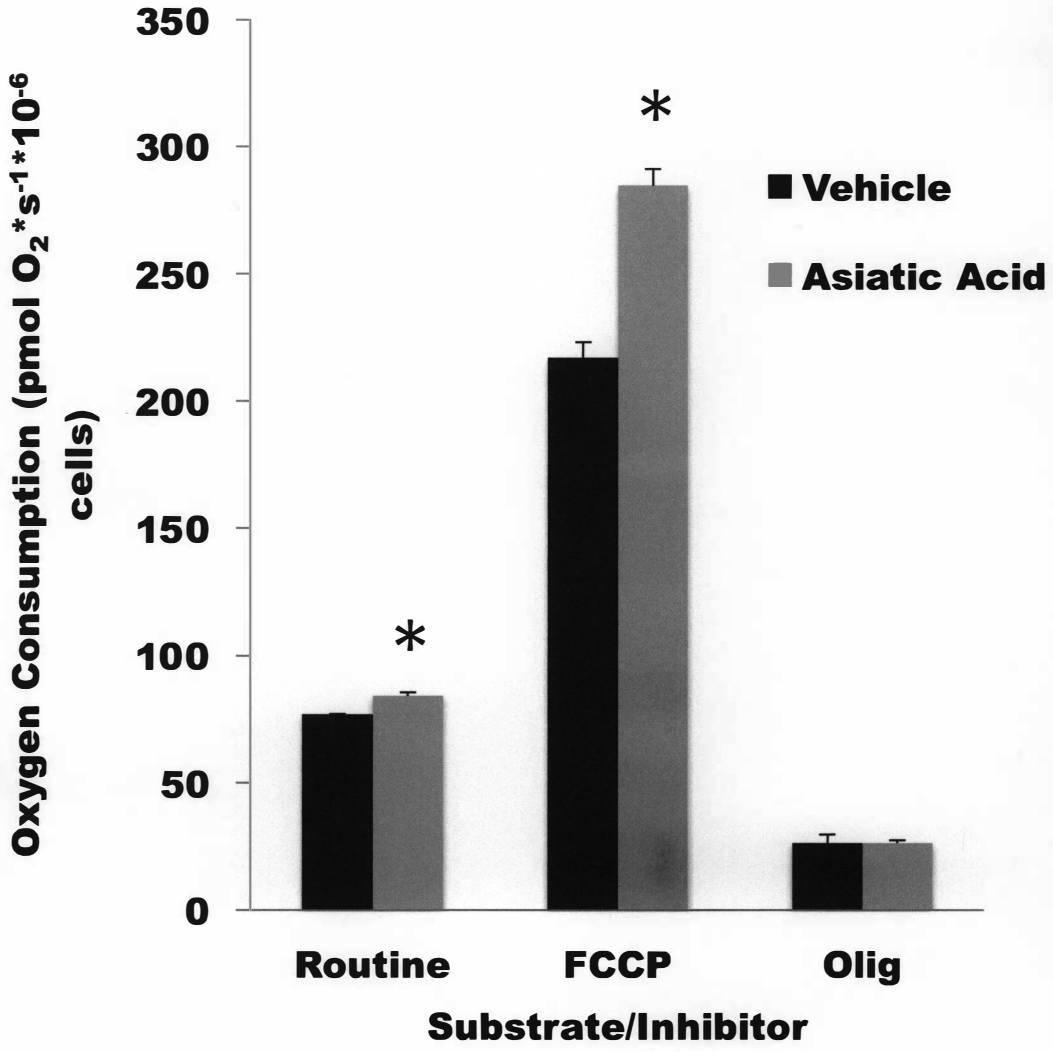

Figure 6- Effects of asiatic acid on maximum uncoupled respiration in Neuro2a cells. Asiatic acid treatment significantly ($p>0.05$) increased both routine and uncoupled respiration as compared to vehicle-treated cells.

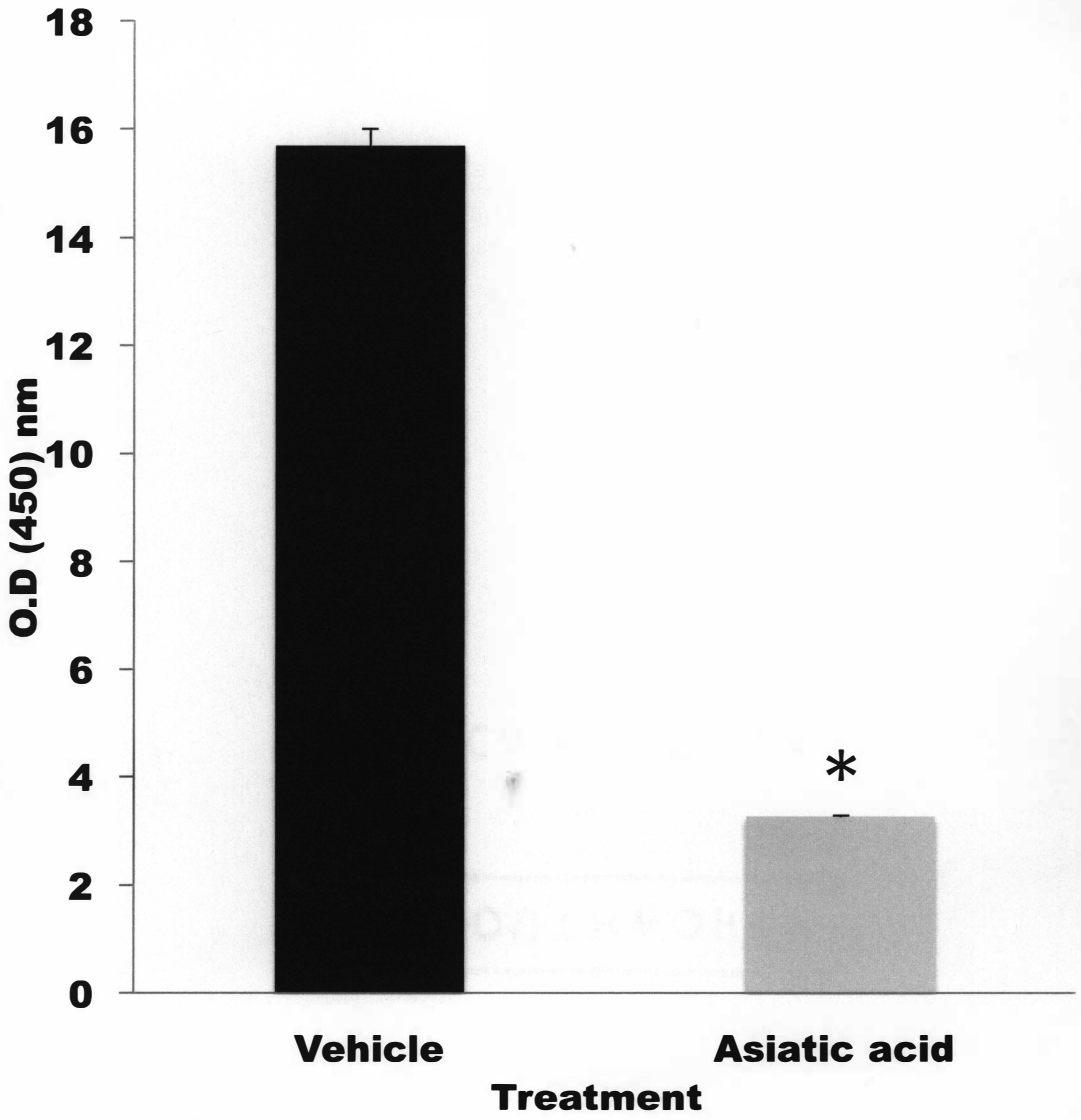

Figure 7- Asiatic acid treated Neuro2a cells produced significantly ($p < 0.05$) lower levels of lactate as compared to Neuro2a cells treated with ethanol.

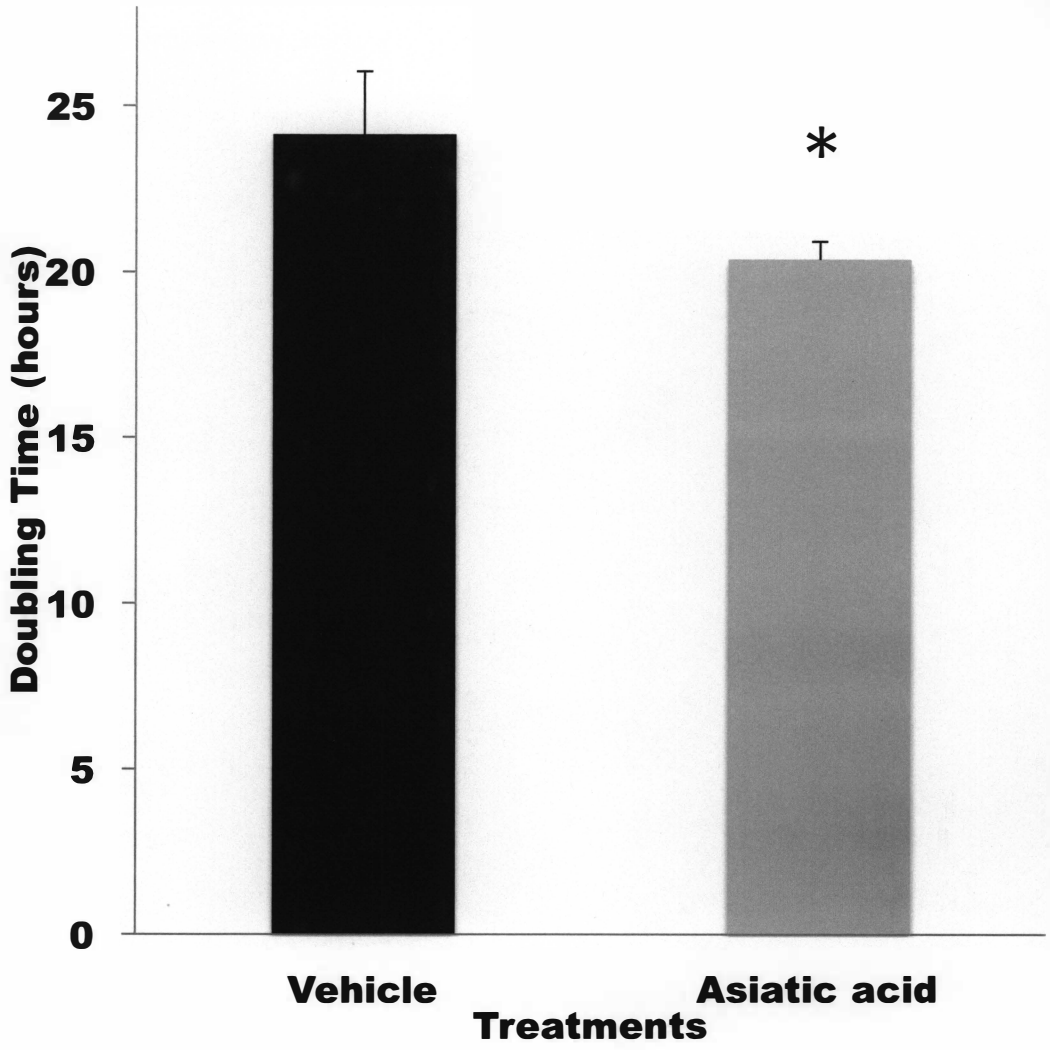

Figure 8 – Treatment of Neuro2a cells with asiatic acid significantly ($p < .05$) decreased the doubling time as compared to vehicle (ethanol) treatment.

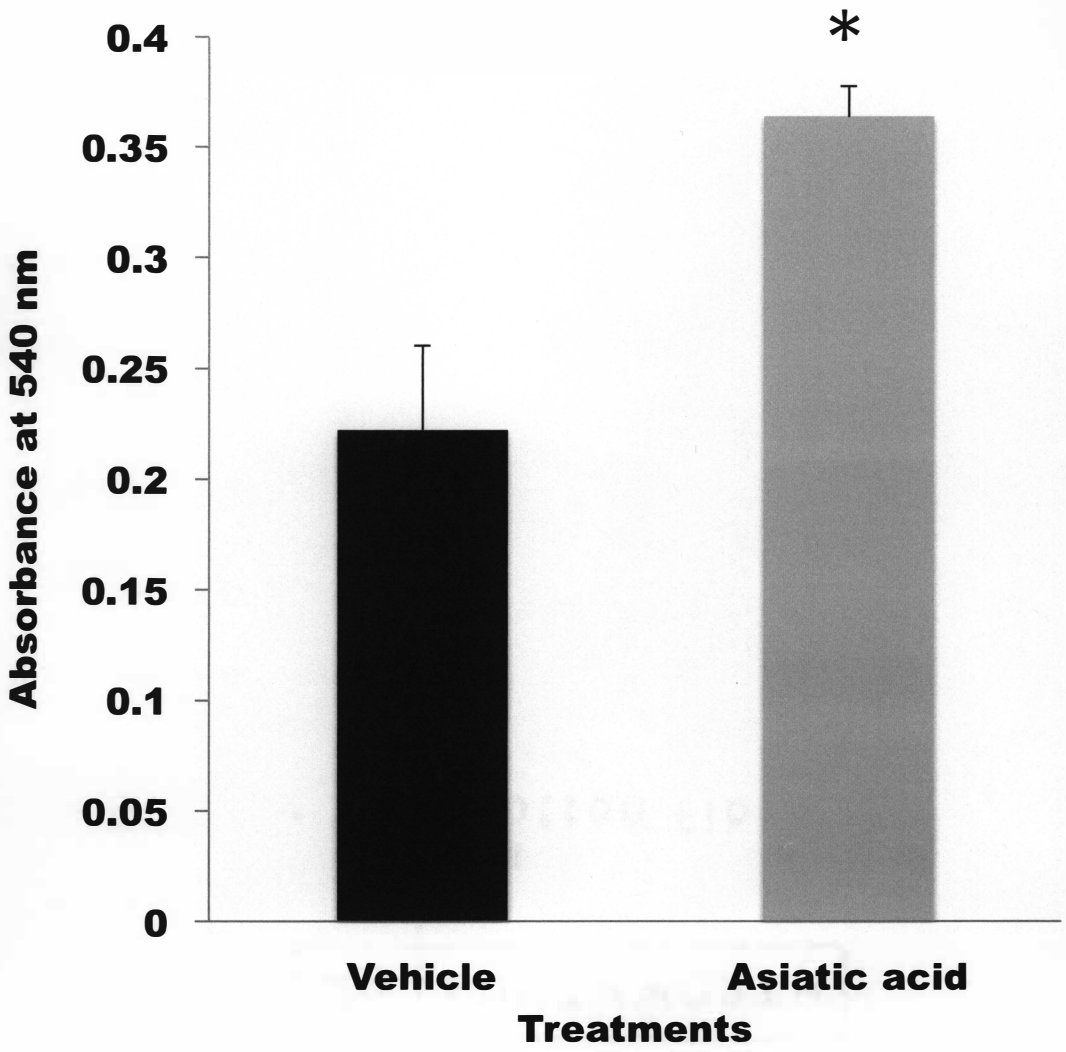

Figure 9- Asiatic acid treatment significantly ($p<0.05$) increased cell viability as compared to vehicle-treated cells in MTT assay.

Discussion

Effects of asiatic acid on neurite outgrowth in Neuro2a cells

The neurite outgrowth is a crucial step in the process of formation of neural network. Our results indicate that asiatic acid facilitates neurite outgrowth. Asiatic acid significantly increased the number of neurite-bearing cells, neurite extension, and combined length of neurites.

Our results are consistent with a previous study that showed increased neuronal differentiation in PC12 cells incubated with ethanol extract of *Centella asiatica* (CA) (Jiang *et al.,* 2016). Another study showed that the ethenolic extracts of *Centella asiatica* significantly increased the neurite outgrowth in SH-SY5Y human neuroblastoma cells .The same study further observed that asiatic acid enhanced the stimulation of the neurite elongation in SH-SY5Y neuroblastoma cells (Soumyanath *et al.,* 2005) . Additional experiments showed that asiatic acid and madecassic acid are the main neurite outgrowth promoting factors in the CA extract. However, these previous studies used a high concentration (14.4 □□) of purified asiatic acid to induce neurite outgrowth as compared to 1 □M asiatic acid 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 asiatic acid increases neurite outgrowth is unclear. Several cellular pathways have been implicated in regulating neuronal growth. Signaling pathways, including MEK/ERK and P13K/Akt, are shown to play critical roles in facilitating neurite outgrowth. A previous study indicated that inhibitors of ERK/RSK signaling pathway abolished the neurite outgrowth promoting effect of CA extract in

neuroblastoma cells (Xu *et al.,* 2008). Consistently, 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.

Furthermore, Neurite outgrowth is regulated by mechanisms involving the MEK/ERK and PI3K/Akt signaling pathways and various neurotrophic factors (such as BDNF, NT-3), and neuron growth factors (such as NGF) (Wanakhachornkrai, *et al.,* 2013). Presently we are testing the effects of asiatic acid on signal transduction pathways known to regulate neurite outgrowth.

Effects of asiatic acid on cell proliferation in Neuro2a cells

Asiatic acid treatment significantly increased cell proliferation, which is evident by the decrease in doubling time observed in our studies. Our results are consistent with previous studies that demonstrated that asiatic acid treatment increases proliferation of hippocampal cells in rodents (Welbat *et al.,* 2016; Sirichoat *et al.,* 2015). Interestingly, several studies have shown that asiatic acid inhibits cell proliferation in a variety of cancer cells, including HepG2 hepatoma, non-small cell lung cancer cells, and ovarian cancer cells (Ren *et al.,* 2016; Chen *et al*., 2014; Wang *et al.,* 2013). The reasons behind these discrepancies are not known, but differences in cell type and treatment conditions could have played a role. Many research has showed that asiatic acid causes programmed cell death (apoptosis) depending on a dose and time dependent processes .It elevates expression of microtubules -associated protein 1 light chain and decreases the expression level of P62. Asiatic acid also significantly alters mitochondrial structure and function. Asiatic acid, at higher doses, leads to the breakdown of mitochondrial membrane and also

leads to the production of reactive oxygen species (ROS) (Daniel , 2005). In mouse xenograft models, it has been shown that administration of asiatic acid results in tumor inhibition, both volume and weight. Additionally, it decreases the expression of PCNA (proliferating cell nuclear antigen). This is usually done by inactivating P13K/Akt/mTOR pathway. Asiatic acid has shown to induce apoptosis in human hepatoma cells, and also in breast cancer (Daniel, 2005). Asiastic acid participates in up regulation of p21WAF1/CIP1 protein expression but it has very limited effects on the expression of p21WAF1/CIP mRNA in the HepG2 hepatoma cells of human. Based on these findings it is suggested that asiatic acid treatment is beneficial in curing cancer.

Effects of asiatic acid on mitochondrial number and function

Our results indicate that asiatic acid increased the mitochondrial complexes in Neuro2a cells. The increase in mitochondrial number can be attributed to the pentacyclic tripertene compound of asiatic acid that can be derived from the species Centella asiatica. This compound displays neuroprotective characteristics for the cell (Krishnamurthy *et al.,* 2009) that then contribute to the increase of mitochondrial number. Moreover, according to the studies of Mook-Jung et al. in 1999 and Jew *et al*. in 2000, the mitochondrial function that has been induced by asiatic acid in cellular systems include the protection against β-amyloid-induced cell death in the neuroblastoma B103 cell line (as cited in Krishnamurthy *et al.,* 2009). Additionally, the reduction of H2O2-related cell death and decreased intracellular free radical concentration has been seen on the study of Lee et al. in 2000 (as cited in Krishnamurthy *et al.,* 2009).

Since mitochondria regulate energy metabolism especially in apoptotic pathways (Gray et al., 2015), it is very critical for the survival of Neuro2a cells. The improvement

of neuronal growth and differentiation has been determined as the cause of mitochondrial increase (Wanet *et al.,* 2015). According to the study of Lee et al. in 2009, the derivatives of asiatic acid have been effective at releasing primary rat cortical cells from glutamate-induced toxicity via stimulation of cellular oxidative defense pathway (as cited in Sarumathi and Saravanan, 2015). Also, with the use of western blot analysis, asiatic acid promoted the increase of phosphorylation of glycogen in the cell as supported by the reduction of reactive oxygen species (ROS) accumulation (Huang *et al.,* 2016).

In our study, we also found that Asiatic acid increased routine oxygen consumption, which indicates that asiatic acid enhanced the mitochondrial function. Asiatic acid consequently participates in stimulation of succinate, which support respiration rate and promotes cytochrome C release. These factors contribute to increased biogenesis of mitochondrion to drive energy processes. Asiatic acid prevented decrease in mitochondrial membrane potential (MMP). In addition, both of Sirt1 and PGC-1 genes that are responsible for mitochondrial biogenesis and function, increased in cells treated with asiatic acid (Lokanathan *et al.,* 2015). Furthermore, *Centella asiatica* extracts prevented mitochondrial dysfunctions in D-galactose-treated mice, which suggests that CA extracts regulated NADH dehydrogenase, succinate dehydrogenase activity, and MTT ability in D-galactose-treated mice (Kumar *et al.,* 2011).

Effect of asiatic acid on lactate production in Neuro2a cells

Asiatic acid treatment decreased lactate production in Neuro2a cells. A mechanism for such decrease is increased oxidative phosphorylation instead of glycolysis that results in lactate production. Oxidative phosphorylation is defined as the process where Adenosine Triphosphate (ATP) is formed as a result of the transfer of electrons from NADH or FADH2 to O2 by a series of electron carriers (Berg *et al.,* 2002).

Asiatic acid treatment regulate lactate signaling cascade in previous studies. One of theses studies showed that mitochondrial energy metabolism and cardiomyocyte apoptosis in numerous cardiovascular diseases are linked. Asiatic acid treatment is cytoprotective in Neonatal rat cardiomyocytes By reducing lactate-induced apoptosis signaling cascade. In addition, asiatic acid also inhibits oxidative stress and mitochondria-dependent caspase activation, together with the increase of mitochondrial number (Gao *et al.,* 2016)

Our results showed that asiatic acid significantly improves cell viability in lactate dehydrogenase (LDH). Further studies regarding the effect of asiatic acid on cells such as H9c2 cardiomyocytes showed that treating these cells with asiatic acid could significantly improve cell viability which prevents lactate dehydrogenase (LDH) release depending on the intensity of concentration exposure. The greater the exposure of the cells with Asiatic acid, the greater the inhibition of apoptotic cell death and the greater suppressing of activities of caspase-3 and caspase-9 (Huang *et al.,* 2016).

Conclusion

Medicinal plants from ancient Ayurvedic medicine have provided clues to the discovery of novel therapeutics for various diseases. The *Centella asiatica* (CA) extract is used to ward of age-related dementia and to increase memory and intelligence. Studies in humans, and numerous rodent models lend support to CA's ability to improve memory and learning. Given this literature, dried CA is sold as a dietary supplement called "Gotu kola" in Western countries. The mechanism by which CA improves memory and learning and prevents dementia is unclear. Our findings demonstrate that asiatic acid, which is the main active component of CA, increases neurite outgrowth and mitochondrial function in neuroblastoma cells line. Essentially, asiatic acid treatment enhanced the growth of the neurons. Thus, asiatic acid promotes the formation of the neuronal network, which may contribute to preventing neurological diseases, such as Alzheimer's diseases. Furthermore, results from our study showed that asiatic acid increased mitochondrial proteins and improved mitochondrial function. Given that mitochondrial dysfunction is the cause of many neurological diseases, our results suggest that asiatic acid treatment would ameliorate such mitochondrial dysfunction, and improve cell viability. Future studies on asiatic acid will help develop new treatment to slow or cure neurological diseases like Alzheimer's disease.

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