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Transcriptome Analysis of Neuro-2a Cells Treated with Asiatic and Madecassic Acid

Fatimah M. Alqam
Eastern Illinois University

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By

Fatimah M. Alqam

THESIS

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Thesis Advisors

Dr. Thomas Canam

Dr. Britto Nathan

Committee Member

Dr. Gordon Tucker

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"Don't let the sun go down without saying thank you to someone, and without admitting to yourself that absolutely no one gets this far alone."

- Stephen King

First and foremost, thanks to God for showering me with countless blessings during my research work to complete the research successfully.

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Abstract

Traditional herbal medicine is ingrained as a source of therapeutic compounds to medicate various diseases. The family Araliaceae (Ginseng family) is rich in traditional medicine species, such as *Centella asiatica* (CA). For many centuries, CA has been used by the indigenous Indian and Chinese in Ayurvedic and traditional medicine, respectively, to improve intelligence, learning, memory, and cognitive performance. Previous studies on cell culture and animal models supported the beneficial effects of CA on the nervous system. However, the exact composition of CA extract and its molecular mechanism that leads to neuroprotection is still unclear. We examined the effect of asiatic acid (AA) and madecassic acid (MA) on neuronal growth, and hypothesized that AA and MA, major neurite promoting factors in CA extract, would induce expression of genes involved in neurite outgrowth and survival. We tested this hypothesis by examining the effects of AA and MA on murine neuroblastoma cells, Neuro-2a (N2a). N2a cells were incubated for two days in a medium containing either 5 µM AA or MA in ethanol or ethanol alone (vehicle). Following incubation, various parameters of neurite outgrowth were measured using NeuronJ software. To explore the impact of AA, MA, and ethanol (vehicle) on neuronal gene expression, transcriptome analysis was used. The results demonstrated that AA and MA facilitate neuronal extension in N2a cultures, and that AA and MA samples had unique transcriptomes compared to the ethanol (vehicle). Furthermore, several of the predicted proteins from the core transcriptome of cells treated with AA and MA have the potential to be involved in neurite outgrowth and related functions.

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1. Introduction

The aging population in recent years has led to a surge in neurodegenerative diseases, including Alzheimer's disease (AD) (Heemels, 2016). AD is the most common form of dementing illness. AD is characterized by loss of memory and higher mental function (Palmer, 2002), with about 5.4 million people affected in the USA alone, and this number is expected to triple over the next 20 years. It is estimated that AD and other dementias will cost the nation $305 billion and is expected to surge to as high as $1.1 trillion by 2050 (Alz.org). Presently, there is no cure or treatment for AD. In addition, there is no known strategy or treatment to slow the progression of the disease. The present treatments for AD are simply aimed at treating symptoms. Given this, there is a dire need to identify novel treatments to slow the disease progression, or even better, to cure AD.

Recent studies have shown that medicinal herbs used in traditional medicine could provide novel compounds to neurological diseases. Many species of the family Araliaceae have been used in traditional medicine. For example, red ginseng (*Panax quinquefolius* L.) has been shown to delay the progression of AD (Lee et al., 2008). *Centella asiatica* L. Urban (syn. *Hydrocotyle asiatica* L.), another species from the Araliaceae family, has been used for many centuries to treat neurological diseases and conditions in India and China. *Centella asiatica* (CA, herein; Figure 1) is a tropical herb that thrives in moist areas of India, Sri Lanka, Indonesia, China, Malaysia, and Madagascar (Jamil et al., 2007).

Figure 1. Photograph of *Centella asiatica* (Shutterstock image by ONGUSHI).

A few studies have examined the effects of CA on neurons in cell culture paradigms and animal models. For example, CA extract treatment in PC12 cells and SH-SY5Y increased neuronal differentiation and neurite elongation (Jiang et al., 2016; Soumyanath et al., 2005). Rao et al. demonstrated that fresh leaf extract of CA stimulated dendritic growth in the hippocampal region in neonatal rat pups (Rao et al., 2006). A later study showed a similar increase in dendritic arborization in rat amygdala (Rao et al., 2009). CA extract has also been shown to facilitate nerve regeneration and repair following sciatic nerve crush injury (Soumyanath et al., 2005). In addition, a few studies that examined the effects of CA extract on neuronal function revealed that CA improved learning and memory in rats in the presence and absence of memory impairing experimentally induced damage to the nervous system (Rao et al., 2005; Kumar et al., 2009). Very few studies have examined the molecular mechanism underlying CA's beneficial effects. Even though preliminary, the results from these studies suggest that CA serves as an antioxidant to ameliorate mitochondrial dysfunctions (Gray et al., 2016).

There have been a few studies examining the CA effects in humans. A study that examined the effects of CA extract on mentally retarded children showed that 12 weeks of CA treatment improved the cognitive performance and behavior of the children (Rao et al., 1977). A double-blind, placebo-controlled study in healthy subjects showed that a single 12-g dose of CA reduced acoustic startle response 30 to 60 minutes following oral administration (Bradwejn et al., 2000). In contrast to these positive effects of CA on human neurological function, a recent meta-analysis revealed that CA treatment has no

significant improvement in cognitive function when compared to placebo treatment (Puttarak et al., 2017).

CA extracts contain a variety of polyphenols and triterpenes (Shinamol et al., 2011). Asiatic acid (AA), madecassic acid (MA), asiaticoside, and madecassoside are the most common phytoactive substances (Nataraj et al., 2017; Figure 2). The exact components of CA extract and the molecular mechanism whereby they confer neuroprotection is still unclear. Since CA is extensively used as an herbal medicine to promote neurological health, we hypothesized that AA and MA, the main components of CA, will promote expression of genes that are involved in neuronal growth and survival. We tested this hypothesis by RNA sequencing in Neuro-2a (N2a) cells incubated with ethanolic solutions of AA and MA. We found that asiatic acid and madecassic acid significantly promote neurite outgrowth *in vitro*, and both treatments led to unique transcriptome responses compared to the ethanol vehicle, suggesting that AA and MA significantly influence gene expression in N2a cells.

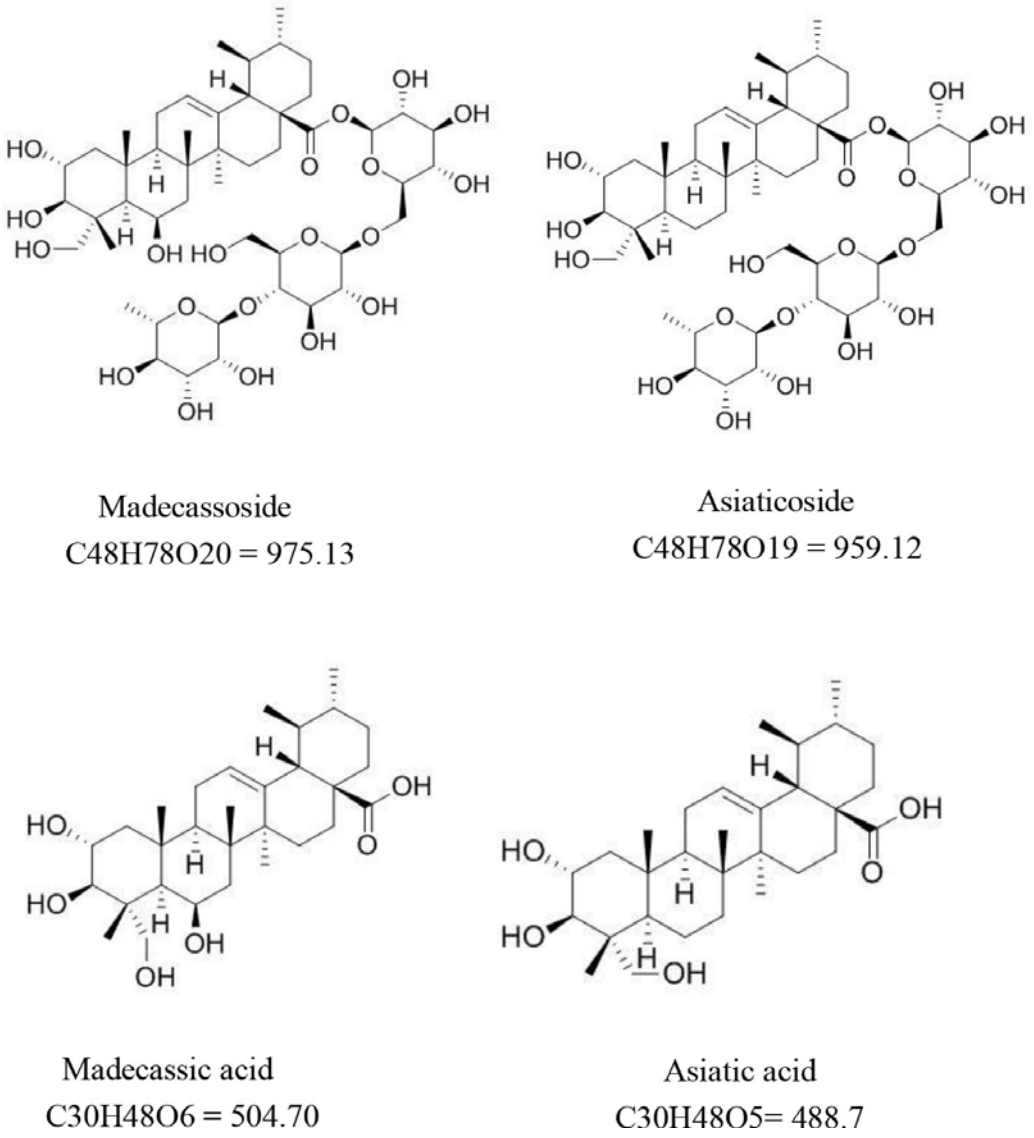

Figure 2. Molecular structures of triterpenoids in CA extracts (Sun et al., 2020).

2. Materials and Methods

2.1. Neuro-2a (N2a) culture

Murine neuroblastoma cells (Neuro-2a, N2a) were purchased 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). Stock solutions (1 mM) of AA and MA were prepared in ethanol. The cells were grown in DMEM containing 1X L-glutamine, 1X PSA, 1X sodium pyruvate, 10 mM glucose, and 10% of FBS The cultures were maintained at 37°C and 6.5% CO_2, and the medium was replaced every three days.

2.2. Measurement of neurite outgrowth

The N2a cells were plated 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 5 µM 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. 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).

2.3. Statistical analysis

The experiments were repeated 4-5 times. Excel software was used to create graphs and to perform statistical analyses (one-way ANOVA, post-hoc corrected t-tests).

2.4. RNA extraction and quantification

The growth medium from each plate was carefully removed using a serological pipet and the cells were rinsed with warm (approximately 35°C) phosphate buffered saline (PBS). After removing the PBS solution from the cells, 1 mL of TRIzol reagent (Invitrogen) was added to the culture plate. The cells were gently resuspended in the TRIzol reagent using a micropipettor before transferring the suspension to a sterile 1.5 mL microcentrifuge tube. Total RNA was purified using the TRIzol instructions provided by the manufacturer. Briefly, 0.2 mL of chloroform was added to the TRIzol solution and the mixture was incubated at room temperature for 3 minutes. The samples were then centrifuged for 15 min at 13,400 rpm and 4°C. The top phase containing the RNA was transferred to a new 1.5 mL microcentrifuge tube and then mixed with 0.5 mL of ice-cold isopropanol, followed by incubation at room temperature for 10 min. The samples were then centrifuged for 10 min at 13,400 rpm and 4°C. The resulting RNA pellet was carefully rinsed with 1 mL of 75% ethanol, followed by

centrifugation for 5 min at 8,000 rpm and 4°C. After removing the ethanol, the RNA pellet was allowed to air dry for 10 min. The pellet was then resuspended in 50 µL of RNase-free water and incubated at 55°C for 10 min.

The RNA samples were quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific), diluted to 100 ng/µL using RNase-free water, and then analyzed using an Experion Automated Electrophoresis System (Bio-Rad) with an Experion RNA StdSens chip (Bio-Rad) according to the manufacturer's instructions. RNA samples with RQI (quality) scores of greater than 7/10 were considered suitable for subsequent transcriptome analysis.

2.5. Transcriptome sequencing and processing

The RNA samples (2.5 µg each) were submitted to the Roy J. Carver Biotechnology Center at the University of Illinois for sequencing using RNA-Seq technology with HiSeq 4000 instrumentation (Illumina). The reads were 150 nucleotides in length, and were demultiplexed with the bcl2fastq v2.20 conversion software (Illumina). The subsequent fastq files were imported to ArrayStar software (DNAStar Lasergene v.15.2.0.130) and processed using the *Mus.musculus.GRCm38.cdna.all.fasta* transcript file (uswest.ensembl.org) and the *gencode.vM20.annotation.gff3* annotation file (gencodegenes.org; Frankish et al., 2018). The data were transformed to reads per kilobase million (RPKM) and subjected to the F-test (ANOVA) feature of ArrayStar with FDR (Benjamini Hochberg) multiple testing correction. The data were exported as an Excel document for further processing. To minimize background noise, only transcripts with 12.0 RPKM or greater across the 12 samples (i.e. averaging 1 RPKM per

sample and above) were maintained for analysis. In addition, only those transcripts with F-test (ANOVA) p-values of 0.05 and below were included in subsequent analyses (4859 transcripts).

3. Results and Discussion

3.1. Asiatic acid and madecassic acid increases neurite outgrowth in N2a cells

We examined whether AA and MA, main components of CA, promote neurite outgrowth in N2a cells. The cells were incubated for 2 days in medium containing 5 µM AA or MA in ethanol or ethanol alone (vehicle). We have earlier reported that 5 µM AA or MA promotes maximum neurite outgrowth (Tucker & Nathan, 2019). The cells were photographed and neurite outgrowth was 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 (Figures 3 and 4). Treatment of N2a cells with AA significantly decreased the percentage of neurite bearing cells as compared to cells grown in vehicle (Figure 4). In addition, AA incubation significantly ($p<0.05$) increased neurite extension as compared to cells incubated with vehicle alone (Figure 5). 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 6).

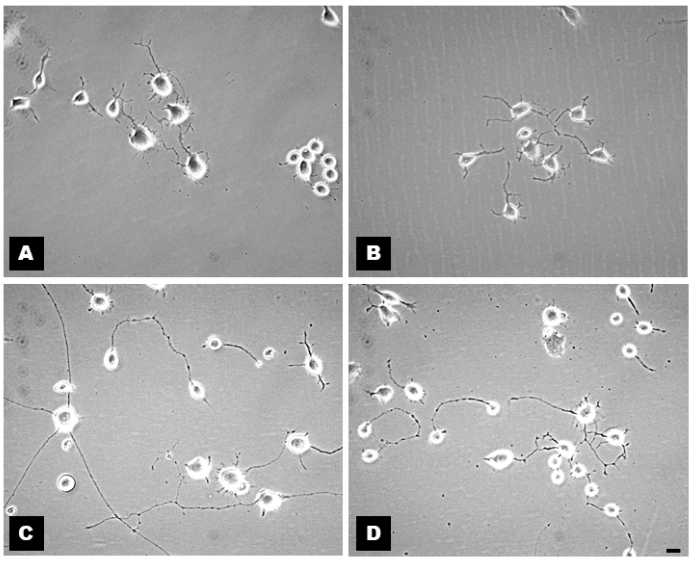

Figure 3. Phase contrast photographs of representative neurons in N2a cells incubated in medium alone (A), in medium containing ethanol (vehicle; B), madecassic acid (C), or asiatic acid (D). Scale bar = 10 μm.

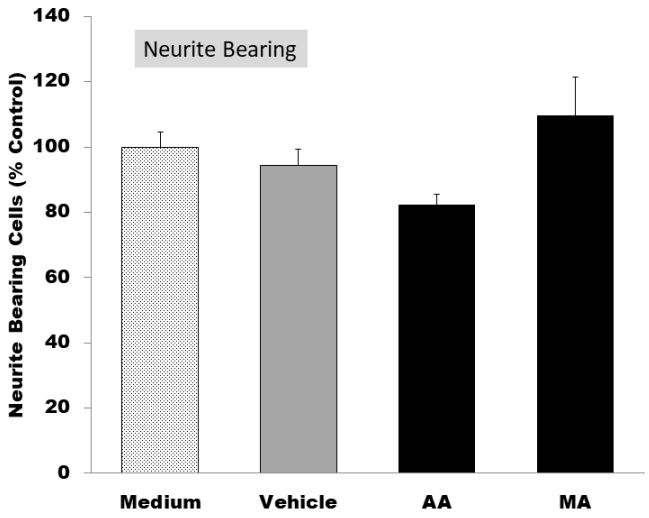

Figure 4. Percentage of neurite bearing cells in N2a cultures incubated with medium, ethanol, asiatic acid (AA), or madecassic acid (MA). Data are mean +/- SE from four different experiments.

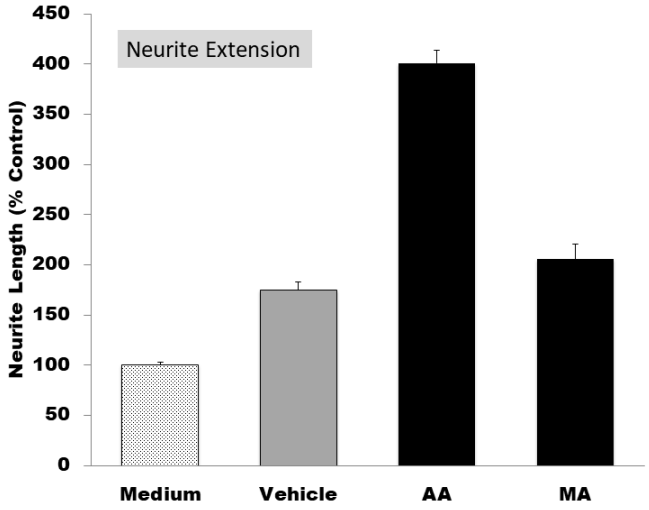

Figure 5. Neurite extension in N2a cultures incubated with medium, ethanol, asiatic acid (AA), or madecassic acid (MA). Data are mean+/- SE from four different experiments.

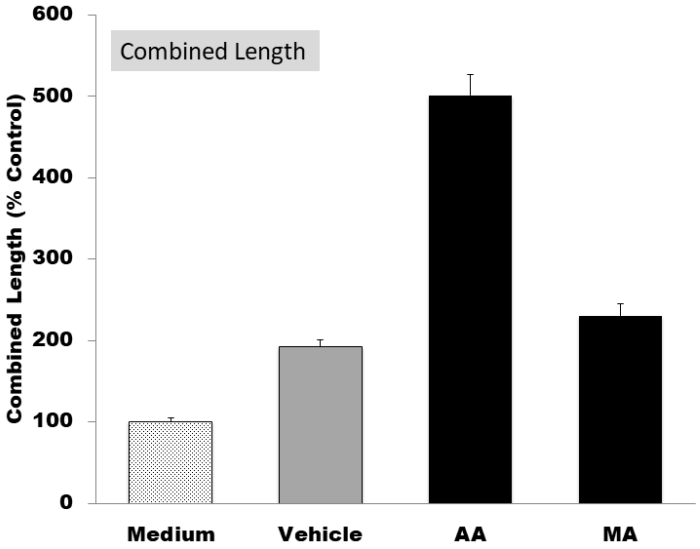

Figure 6. Combined length of neurites in N2a cultures incubated with medium, ethanol, asiatic acid (AA), or madecassic acid (MA). Data are mean+/- SE from four different experiments.

Incubation of N2a cells with vehicle or MA had no significant ($p>0.05$) effect on the percentage of neurite bearing cells as compared to cells grown in medium alone (Figure 4). Similar to AA, MA significantly ($p<0.05$) increased neurite extension as compared to cells incubated with vehicle alone (Figure 5). 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 6).

The results from our studies are consistent with previous studies showing increased neuronal differentiation with ethanol extract of CA (Jiang et al., 2016). However, that study used a relatively high concentration of AA (14.4 μM) compared to 5 μM AA, and also used a different cell type (PC12). Nevertheless, the underlying molecular and biochemical mechanisms whereby AA and MA increases neurite outgrowth remains uncertain. As a first step toward understanding this mechanism, we performed transcriptome analysis on N2a cells after treatment with AA, MA, or ethanol alone.

3.2. Transcriptome response to asiatic acid and madecassic acid

Treating N2a cells with ethanolic solution of AA and MA (5 μM) significantly induced neurite outgrowth *in vitro.* These physiological changes in N2a cells led us to investigate potential changes in gene transcription associated with the observed neurite outgrowth. Transcriptome-based studies involving the effect of AA and MA on neurons appear to be limited. Three RNA samples each of neurons without treatment (medium alone), treated with ethanol only (vehicle), or ethanolic stock solutions of AA or MA (Figure 7) were subjected to transcriptome analysis. The twelve samples generated an average of 29.5 million

reads each using the RNA-seq platform from Illumina (Table 1). For subsequent analysis, the transcript values were normalized as RPKM and only transcripts showing significant differences between replicate sets ($P<0.05$) were interpreted. Furthermore, the transcriptomes of the medium samples were excluded from further analysis to simplify interpretation of the results.

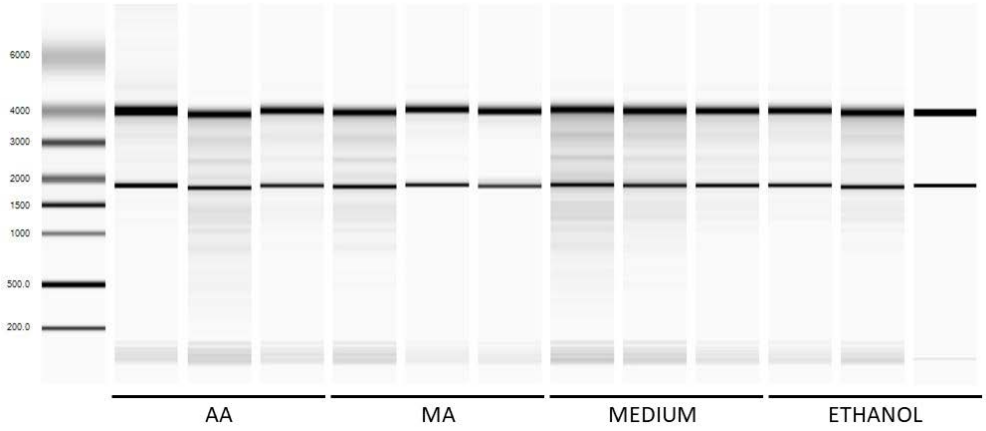

Figure 7. Experion digital gel electrophoresis image of RNA from N2a cell cultures grown in standard medium, ethanol vehicle, ethanolic asiatic acid (AA), or ethanolic madecassic acid (MA). The two darkest bands represent 28S rRNA (at approximately 4000 nucleotides) and 18S rRNA (at approximately 1800 nucleotides).

Table 1. The number of RNA-Seq reads from each sample as measured by a HiSeq 4000 instrument at the Roy J. Carver Biotechnology Center (University of Illinois at Urbana-Champaign). The AA and MA samples were treated with asiatic acid and madecassic acid, respectively. ETH represents samples that received only the ethanol vehicle, and MED refers to those grown in standard medium.

Sample	Number of Reads
AA-1	29,534,051
AA-2	28,029,685
AA-3	28,457,627
MA-1	28,462,147
MA-2	29,717,364
MA-3	29,122,888
ETH-1	30,332,758
ETH-2	29,255,847
ETH-3	28,446,532
MED-1	32,395,676
MED-2	29,269,087
MED-3	30,976,852
Total Reads	**354,000,514**

A heatmap was generated based on the 2500 most abundant transcripts among the three replicate samples for each treatment: asiatic acid (AA) madecassic acid (MA), and ethanol (ETH). This heatmap clearly demonstrated that asiatic acid and madecassic acid samples had unique transcriptomes compared to the ethanol vehicle (Figure 8). Moreover, the expression patterns of the replicates within a sample type show excellent reproducibility (Figure 8). This holistic visualization suggests that AA and MA influence gene expression in N2a cells in a manner unique from ethanol alone.

Transcripts were sorted based on their relative expression levels (e.g. MA compared to ethanol, AA compared to ethanol) to identify the core set of transcripts common to both AA and MA samples. In addition, this analysis allowed us to identify the transcripts that were highly expressed in only AA or MA. For this analysis, transcripts that were 10X higher and above in AA and/or MA compared to ethanol vehicle were considered. Using this threshold, a total of 23 high-expression transcripts were identified as being shared between AA and MA samples, representing the core transcripts (Figures 9 and 10). This core set had more transcripts than were unique to AA (5 transcripts) and MA (9 transcripts) at the 10X threshold (Figure 9).

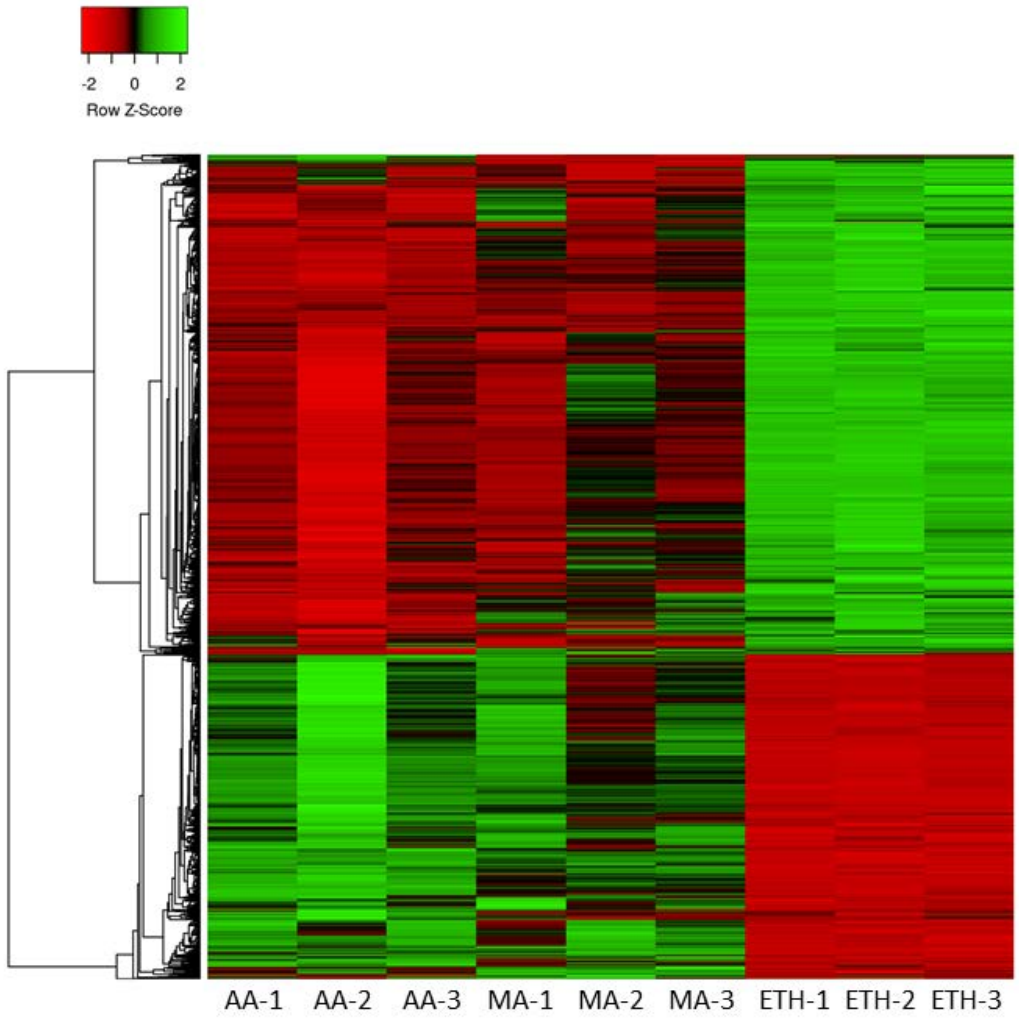

Figure 8. Heatmap of the most abundant 2500 transcripts from N2a cells showing excellent reproducibility between the three treatment replicates: asiatic acid (AA), madecassic acid (MA), and ethanol vehicle (ETH).

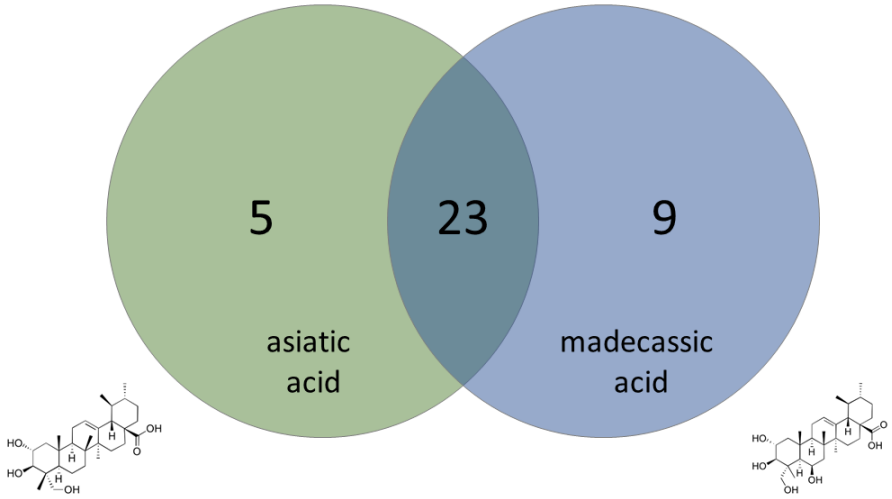

Figure 9. Venn diagram showing the number of highly expressed transcripts (10X and higher compared to ethanol vehicle) common and unique to asiatic acid (AA) and madecassic acid (MA) treatments of N2a cells.

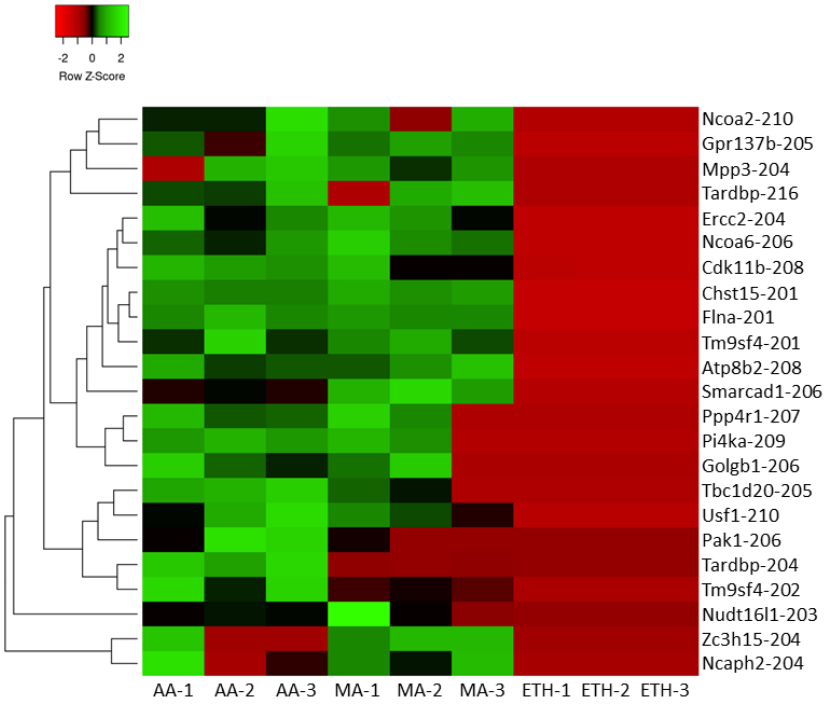

Figure 10. Heatmap showing the relative expression levels and identities of the 23 transcripts in the high-expression (10X or greater than ethanol vehicle) core set common to asiatic acid (AA) and madecassic acid (MA) treatments.

Similarly, the levels of low-expression transcripts in the AA and MA samples compared to ethanol vehicle were also explored. The cutoff for the low-expression transcripts was 0.1X or lower than ethanol alone. Unlike the high-expression analysis, AA and MA shared fewer low-expression transcripts (8) than were uniquely low to AA (35 transcripts) and MA (9 transcripts) at the 0.1X threshold (Figures 11 and 12). This indicates that the neurons receiving the AA treatment had more genes experiencing a drastic reduction in transcription (0.1X or lower) than those exposed to MA, despite the structural similarities of these two phytochemicals.

3.2.1. Genes related to the cytoskeleton and neuronal migration

Among the core set of highly expressed transcripts of AA- and MA-treated neuron cells was *Flna-201* (Figure 10). Flna (filamin A) is an actin-binding protein that plays a vital role in regulating the cytoskeleton by interacting with multiple proteins, thus inducing actin changes on the cell's periphery to sustain cell structure (Carreno et al., 2004; Smythe and Ayscough, 2006). These dynamic changes in the actin cytoskeleton are essential for migration and cell adhesion (Nakamura et al., 2011). A prior study showed that the Flna mutation in humans leads to defects in neuronal migration in the cerebral cortex in X-linked periventricular heterotopia (PH) (Fox et al., 1998).

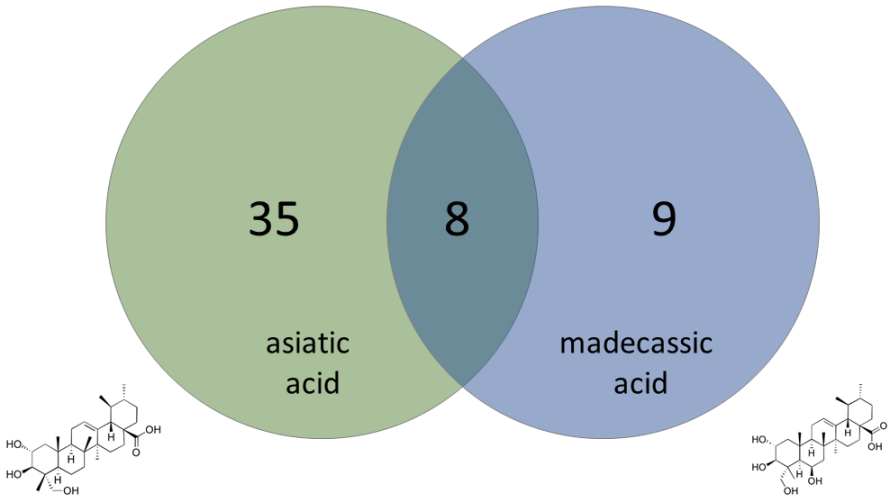

Figure 11. Venn diagram showing the number of low-expressed transcripts (0.1X and lower compared to ethanol vehicle) common and unique to asiatic acid (AA) and madecassic acid (MA) treatments of N2a cells.

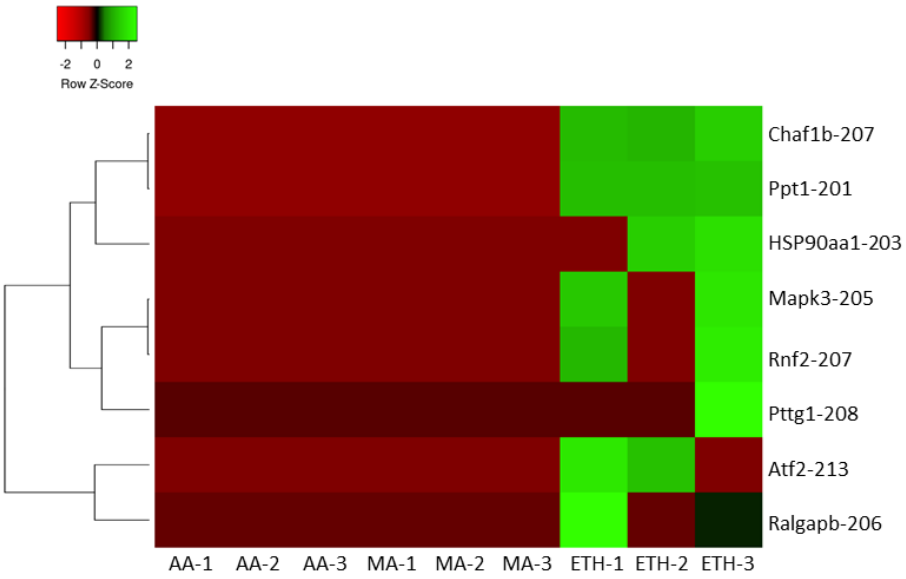

Figure 12. Heatmap showing the relative expression levels and identities of the 8 transcripts in the low-expression (0.1X or lower than ethanol vehicle) core set common to asiatic acid (AA) and madecassic acid (MA) treatments.

Flna also indirectly affects the activation of Big2 (brefeldin-A-inhibited guanine exchange factor), a vesicle trafficking protein, by localizing Big2 to the cell membrane and activating Arf1, which regulates neuronal migration by permitting the turnover of receptors and cell adhesion molecules (Zhang et al., 2013). Moreover, Flna overexpression can also lead to migration deficiency (Sarkisian et al., 2006). Likewise, the loss of Mpp3, MAGUK-family protein, causes delay in progenitor cell migration resulting in ectopically localized pyramidal neurons and interneurons in layer II-IV (Dudok et al., 2013). *Mpp3-204* was found in the core set of highly expressed transcripts (Figure 8).

3.2.2. Genes related to signal transduction

Another notable member of the core set of highly expressed transcripts in the present study was *Pak1-206* (Figure 10). Pak1 is a member of the serine/ threonine protein kinases family (Bokoch, 2003). It is known to be involved in cortical development. Results suggest that Pak1 is crucial for neuronal proliferation, survival, differentiation, and/or migration in the developing cortex. At the p7 (postnatal day 7), Pak1 knock-out mice experienced a significant decrease in pyramidal neurons in many cerebral cortex layers linked to neuronal migration impairment and smaller neuronal progenitor pool (Pan et al., 2015). Dynamic changes in the actin cytoskeleton are essential for migration (Nakamura et al., 2011). In postmitotic neurons, cytoskeletal remodeling during neurite outgrowth is regulated by p35/Cdk5 phosphorylation of Pak1 on Thr-212 (Rashid et al., 2001). Also, Thr-212-phosphorylated Pak1 is co-localized with F-actin, and it wraps distally, extending the microtubule bundle. Thus, phosphorylation of

Pak1 by p35/Cdk5 may impact the cross-talk between microfilaments and microtubules during neuronal growth cone movement (Lei et al., 2000).

Phosphatidylinositol (PtdIns) 4-kinase IIIα (PI4KA) is a cellular lipid kinase (Balla, 2013), which was present as *Pi4ka-209* in the core set of highly expressed transcripts as a result of AA and MA treatment (Figure 10). It has been identified as an enzyme that contributes to PtdIns(4,5)P2 synthesis in the plasma membrane via PtdIns(4)P production. PtdIns(4,5)P2 is one of the most critical regulatory lipids in the plasma membrane. It is a regulator of transporters and ion channels; it also regulates endocytosis and exocytosis by interacting with proteins (Balla, 2013).

PI4KA is ubiquitously expressed in the brain, but there is little research on its role in the CNS. In the brain, PI4KA inactivation in the embryonic germline of mice is fatal (Nakatsu et al., 2012), and PI4KA has been linked to brain abnormalities in the human embryo (Pagnamenta et al., 2015). Conversely, in the peripheral nervous system, PI4KA inactivation has been shown to reduce impulse conduction and motor function in Schwann cells of the sciatic nerves due to PI4KA involvement in nerve myelination (Alvarez-Prats et al., 2018). In addition, PI4KA knock-out mice showed actin cytoskeleton redisposition (Alvarez-Prats et al., 2018).

CDKs (cyclin-dependent kinases) are vital protein kinases for many cellular functions, including transcription and the cell cycle. CDK activation requires the presence of specific cyclin subunits (Malumbres et al., 2009). In this study, *Cdk11b-208* appears in the core set of highly expressed transcripts

(Figure 10). It is a member of the serine/threonine-protein kinase family, paralogous gene of Cdk11, which plays a critical role in splicing, transcription, apoptosis, and mitosis (Ahmed et al., 2019). However, unlike Cdk5, Cdk11b has not been linked to brain function. Cdk5 is a serine/threonine protein kinase that has an essential role in nervous system development, such as neurite outgrowth and neuronal migration (Humbert et al., 2000). Actin changes influence the p39/Cdk5 complex, and also p39 is found to be colocalized with Cdk5 in neuron growth cone in COS7 cells. This implies that p39/Cdk5 may be involved in dynamic cytoskeleton regulation in neurons (Humbert et al., 2000).

3.2.3. Genes related to transcriptional regulation

In neurons, upstream stimulator factors, or USFs (USF1/2), regulate the transcription of brain-derived neurotrophic factor (BDNF) by binding to Ca^{2+}-responsive E-box element (CaRE2) on BDNF promoter III. This makes USFs essential for the activation of CaRE2-dependent transcription factors. The Ca^{2+}-activated signaling pathway in neurons regulates the transcriptional activity of USFs. *In vivo*, USFs bind to the promoter of neuronal activated-regulated genes, making USFs important for activity-dependent transcription regulation in neurons (Chen et al., 2003). In the present study, the transcript *Usf1-210* was expressed in both AA- and MA-treated neurons above the 10X threshold compared to neurons receiving ethanol alone (Figure 10).

NCoA2, which is also known as SRC-2, GRIP1, or TIF2, is a member of the p160 steroid receptor co-activator (SRC) family (Sun & Xu, 2020). It can mediate the transcription activation of a number of members of the steroid

receptor superfamily (Xu et al., 1998). It is highly expressed in the anterior pituitary, but is expressed only at low levels throughout the brain. In contrast, NCoA1 (SRC-1) expression is abundant throughout the brain, including in the hypothalamus, amygdala, basal ganglia, hippocampus, and isocortex (Meijer et al., 2000). In the present study, NCoA1 did not appear in the high-expression core gene set, but NCoA2 did as *Ncoa2-210* (Figure 10).

Similar to NCoA2, NCoA6 also binds to a nuclear receptor in a ligand-dependent manner as a co-activator protein. NCoA6 acts also as a co-integrator for nuclear receptors (NRs) and various transcription factors associated with proliferation, growth, metabolism, the immune response, apoptosis, and cytokine signaling (Mahajan & Samuels, 2008). It is notable that *Ncoa6-206* was among the most highly expressed transcripts in neurons receiving either phytochemical treatment in our study (Figure 10). Deletion of NCoA6 in knock-out mice is lethal due to NCoA6's involvement in brain, liver, and heart development (Mahajan & Samuels, 2008).

4. Conclusions

The results from this study showed that AA treatment of N2a cultures decreased the percentage of neurite bearing cells as compared to vehicle-treated cells. Furthermore, AA treatment increased neurite extension and combined length of neurites. In contrast, MA treatment had no significant effect on the percentage of neurite bearing cells. However, MA treatment in N2a cells significantly increased neurite extension and combined length of neurites. These findings demonstrate that AA and MA facilitate neuronal extension in N2a cultures. For further support, the transcriptome responses to AA and MA were analysed. It was found that AA and MA samples had unique transcriptomes compared to ethanol (vehicle), suggesting that AA and MA significantly influence gene expression in N2a cells. Several of the protein products of those transcripts can be attributed to neurite outgrowth and related cellular functions. Future studies should examine the effects of AA and MA in *in vivo* animal models and in humans to further explore their therapeutic potential to prevent or cure neurological diseases.

5. References

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