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Medicinal Properties of the Araliaceae, with Emphasis on Chemicals Affecting Nerve Cells

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Printed Name

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Medicinal properties of the Araliaceae, with emphasis on chemicals affecting nerve cells

by

Rana Alharbi

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I HEREBY RECOMMEND THAT THIS THESIS BE ACCEPTED AS FULFILLING THIS PART OF THE GRADUATE DEGREE CITED ABOVE

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Abstract

In recent times, medicinal plants have become the focus of scientists and research. However, many have used traditional medicine to take advantage of plant extracts to treat many diseases, especially neurological diseases. Various central nervous system receptors have been shown to associate with plant extracts influencing the pharmacology and in this manner conceivably assuming a role in human sickness and treatment. For instance, extracts from *Cussonia paniculata* Eckl. & Zeyh targeted several early nervous and mental disease, and *Kalopanax pictus* has been traditionally used for the treatment of rheumatoidal arthritis, nerve pain and diabetes mellitus.

In the present study, we investigated the chemical constituents of 12 species of Araliaceae: *Aralia elata, Hydrocotyle umbellata, Aralia racemosa, Aralia spinosa, Kalopanax septemlobus, Eleutherococcus sieboldianus, Fatsia japonica, Polyscias fruticosa, Oplopanax horridum, Hydrocotyle sibthorpoiaes, Hedera hibernica, Oplopanax horridus, Centella asiatica,* and *Schefflera arboricola*. Several species contained madecassic acid and asiatic acid.

In addition, we investigated the effects of these last two compounds on the growth of culture nerve cells. Madecassic acid showed significant activity in the neurite bearing, neurite extension, and combined length.

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I. Introduction

The **Araliaceae** are a family of flowering plants, also known as the **Ginseng family** or **ivy family**. The family includes trees, shrubs, lianas, and perennial herbs. The family from tropical area origin is present in cooler climates, too. They are found in the Americas, Eurasia, Africa, Australia, New Zealand, New Caledonia, and Pacific islands. Endemic Araliaceae are found in the pluvial montane forests, very humid montane, and humid lowland river forest regions and forested areas generally in temperate regions. They are present, too, in laurel forest, cloud forest, and warm, humid habitats. The Araliaceae (also known as the ginseng family) and the Apiaceae are the sole families in the order Apiales, belonging to the Subclass Asteridae II. The Apicaceae (also called Umbelliferae) or Parsley Family, are much larger than the Araliaceae, with about 4500 species and about 450 genera (Constance & Affolter 2004). The Araliaceae family comprises 55 genera and about 1,500 plant species, widely distributed in tropical, subtropical and temperate regions (Frodin 2004). Many species are used as medicines, such as species in the genus *Panax*, *Eleutherococcus* and *Aralia* (Brussell 2004). According to taxonomical studies, Araliaceae encompasses two large monophyletic groups: the *Aralia-Panax* group and the Asian Palmate group (Kim et al. 2017). The *Aralia-Panax* group consists of the two closely-related genera, *Aralia* and *Panax*. The Asian Palmate group is represented by the genera *Eleutherococcus*, *Dendropanax*, and *Schefflera* characterized as distinctive woody plants.

In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potentials of medicinal plants used in various traditional systems. The World Health Organization (WHO) estimates that 70 to 80% of the people in developing countries use traditional medicine as a major source of health care. However, many people underestimate the toxicity of natural products and do not realize that these agents could be as toxic or more than synthetic drugs. So far, many plants have been reported to be toxic to both human and animals. It should therefore, be emphasized that the traditional use of any plant for medicinal purposes, by no means, warrants the safety of such plant. Plants in folk medicine should therefore, be evaluated for safety or toxicity and necessary recommendations made on their use.

Background

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, *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 (Kumar et al. 2009). 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 (Gray et al. 2016). In vivo, CA extracts enhanced dendritic arborization in the hippocampus and amygdala, and accelerated nerve regeneration and functional recovery following sciatic nerve crush injury (Gray et al. 2016). In addition, CA treatment during postnatal period improved learning and memory in rats. Also, long-term treatment with CA extract ameliorated colchicine-induced memory impairment in rats. Aqueous extracts of CA ameliorated 3-nitroprorionic acid-induced oxidative stress and mitochondrial dysfunctions in mice brains. A water extract of CA increased the expression of antioxidant and mitochondrial genes in mice, and also improved their cognitive function (Gohil et al 2010). 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.

Limited human studies have examined the effects of CA. For example, a single 12-g oral administration of CA significantly reduced acoustic startle response in healthy subjects as compared with placebo group (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. CA has also been used to treat a variety of non-neurological diseases and conditions including ulcers, cancer, hypertension, atherosclerosis, eczema, wounds, and leprosy. In recent years, popularity of *Centella asiatica* has soared, and it is now used worldwide as an herbal dietary supplement called Gotu kola.

Chemical analysis of CA extracts found a variety of polyphenols and triterpenes (Rao et al. 2009). The most common triterpenoids in CA extracts include asiatic acid, madecassic acid (MA), asiaticoside, and madecassoside (Kai et al. 2008) Three of the 28 asiaticoside derivatives, asiatic acid, asiaticoside 6, and SM2, tested in cell cultures studies showed neuroprotective effects against b-amyloid induced neurotoxicity. All three asiaticoside derivatives reduced H_2O_2-induced cell death and lowered intracellular free radical concentration. The exact component of CA extract and the molecular mechanism whereby it confers neuroprotection is still unclear.

In this project, we examined the effects MA on cell proliferation, neurite outgrowth, and mitochondrial function in Neuro-2a (N2a) neuroblastoma cell line. We found that treatment of N2a cultures with MA increased the percentage of neurite bearing cells, and also increased neurite extension and combined length of neurites per cell.

II. Objectives

1. Extract and identify phytochemicals from the leaves of twelve species of Araliaceae.
2. Explore the effect of Madecassic acid on nerve cells.

III. Materials and Methods

A. Plant Specimens

The species of Araliaceae used in this study (*Aralia elata, A. racemosa, A. spinosa, Kalopanax septemlobus, Eleutherococcus sieboldianus, Fatsia japonica,*

Polyscias fruticosa, Oplopanax harridum, Hydrocotyle sibthorpoides, H. umbellata, Oplopanax horridus, Centella asiatica, Schefflera arboricola, Hedera helix and, *H. hibernica*) were obtained locally (Charleston) or outside of the state (Seattle or South Carolina). Voucher specimens have been deposited in the Stover-Ebinger Herbarium (EIU) in Charleston, Illinois.

B. Phytochemical Extraction and HPLC Analysis

HPLC analysis was used to characterize leaf and examine the variation in the amount or type of chemicals present. Metabolites were extracted using 1 mL of HPLC-grade methanol per 100 mg of fresh leaf, and dried leaf samples that were ground to a fine powder with a mortar and pestle using liquid nitrogen. Peak areas were quantified using R program and recorded in Powerpoint.

C. 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). Madecassic acid (MA) was 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% CO2.

D. Measurement of neurite outgrowth

To examine the effects of MA 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 48 hours in DMEM containing 10 mM of glucose, 1X PSA, and with either 1 mM 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 condition (MA versus ethanol alone).

D. 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.

IV. Results and Discussion

A. Objective 1: Identify and extract phytochemicals from the leaves of twelve species of Araliaceae.

ii. Bioactive Phytochemicals

a. Asiatic Acid

Centella asiatica is the main source for Asiatic Acid, but on the other hand is found in another plant's extraction that have been broadly utilized in medicines. Asiatic Acid incites apoptosis in human hepatoma cells due to Ca2 discharge and p53 up-regulation.8. Also, asiatic acid from C. *asiatica* can repress development of HT-29 colon malignant growth cells by influencing Bcl-2 and Bcl-xL correlative apoptosis. Asiatic Acid enlistment of apoptosis in SK-MEL-2 human melanoma cells can happen after expanding intracellular responsive oxygen species (ROS) level, which upgrades the declaration of Bax, however not Bcl-2 protein, in the melanoma cells. Asiatic acid is one regular items recognized as biofilm inhibitors in a biofilm.

Our analysis show that, the retention time of the asiatic acid standard was determined to be 28.5 min (using the parameters outlined in the Materials and Methods). Based on the common retention time a peak representing asiatic acid was found in the leaf samples of *Kalopanax septemlobus, Polyscias fruticosa, Oplopanax horridus, Aralia spinosa,* and *Hydrocotyle sibthorpoides*.

b. Coumarin

Coumarin is a constituent from natural of numerous plants and fundamental oils including woodruff, tonka beans, oil of cassia, sweet clover and lavender. Coumarin regularly exists as an unscented complex conjugated to sugars and acids, is discharged by the activity of acids, compounds, or bright (UV) radiation (Egan et al. 1990). Coumarin mixes have been utilized to treat such various diseases as bums, brucellosis, and rheumatic sickness, cancer, and they have been utilized as antispasmodics. Coumarin was first confined in 1822 and combined in 1868 based on animal data. It was restricted by the Nourishment and Medication Organization during the 1950s, being named a class 1 cancer-causing agent and hepatotoxin. Numerous derivatives of coumarin have been appeared to have anticoagulant, tumoristatic, and immunostimulatory properties, and a few derivatives have been utilized for fluorescent naming and as laser colors. In this study, Coumarin had a retention time of 17.31 min with our analysis system. The compound was shown to be present only in *Hedera hibernica*.

c. Curcumin

Curcumin, a polyphenolic compound got from dietary zest turmeric, has different pharmacologic impacts including mitigating, cell reinforcement, antiproliferative and antiangiogenic exercises. Stage I clinical preliminaries have demonstrated that curcumin is protected even at high portions (12 g/day) in people however show poor bioavailability**.**

HPLC analysis of Curcumin (25.83 min retention time) revealed that it may be present in *Fatsia japonica*.

d. . Quercetin

Quercetin is the most widespread of the flavonoid molecules and distributed broadly in the plant kingdom. It is the aglycone (lacks the sugar group) of various different flavonoids including, hyperoside, rutin, and isoquercetin. Quercetin seems to provide numerous benefits to human health, along with anticancer movement, waterfall anticipation, hostile to ulcer impacts, antiviral action, cardiovascular security, against hypersensitivity action, mitigating impacts, and antiviral action.

Quercetin had a retention time of 17.60 min in our HPLC-based analytical system. Just one species (*Aralia spinosa*) had detectable levels of quercetin.

g. Flavanones

Flavanones is a biochemically related mixes of confined occurrence. It has a filled C-ring. The flavanones are less solvent than the chalcones, will in general separate first in fragmentary crystallization and are effectively hastened at low pH, particularly if arrangements are chilled or solidified. These hastens stay

insoluble in water, methanol, ethanol or CH3)2CO (and blends thereof). Warming, or ideally the utilization of solid solvents, for example, dimethyl sulphoxide, dimethyl sulphoxide/methanol blends (1:1) or dimethylformamide, is basic to guarantee effective recuperation. The major dietary source of flavanones are Citrus fruits and associated products.

In our study, the presence of Flavanones (28.03 min retention time) was not demonstrated in any species.

h. Madecassic Acid

Madecassic acid (MA) is a triterpenoids, shows in Centella asiatica and other tropical plants. This compound due to its anticancer activity has lately been the subject of examination. current researches showed that MA had important impacts in treatment of inflammation, anti-oxidant, skin wound, tumor and nerve damage. Some studies revealed that MA could be found in some edible plants including basil (*Ocimum basilicum*), daylily (*Hemerocallis fulva* L.), and Okinawan spinach (*Gynura bicolor* DC.). An examination of Madecassic Acid levels (22.10 min retention time) in this study; it was not present in any of the 12 species we examined.

e. Uridine

The uridine diphosphate (UDP)- glucuronosyltransferases (UGT)are a group of enzymes that catalyze the covalent expansion of glucuronic corrosive to a wide scope of lipophilic synthetic substances. They assume a noteworthy role in the detoxification of numerous exogenous and endogenous mixes by creating items that are more polar and, in this manner, all the more promptly discharged in bile or urine. Acquired insufficiencies in

UGT frames are injurious, as exemplified by the incapacitating impacts of hyperbilirubinaemia. In addition, neurotoxicity in subjects with changes in the compound that changes over bilirubin to its increasingly polar glucuronide.

In the present study, uridine had a retention time of 4.13 min with our analysis system. The compound was not shown to be present in any species.

Table 1. Overview of chemicals found in Araliaceae species examined using HPLC.

Note: Uridine was not found in any species that we examined.

Species Name	*Asiatic Acid*	*Coumarin*	*Curcumin*	*Quercetin*	*Flavanones*	*Madecassic Acid*
Aralia elata						
Hydrocotyle						
Aralia racemosa						
Kalopanax	√					
Eleutherococcus sieboldianus						
Fatsia japonica				√		
Polyscias fruticosa	√					
Aralia spinosa	√			√		
Hydrocotyle sibthorpoides	√					
Hedera hibernica		√				
Oplopanax horridus	√					
Centella asiatica						
Schefflera arboricola						
Hedera helix						
Apium graveolens						

Results and Discussion

B. Objective 2

Madecassic acid increases neurite outgrowth in N2a cells

Previous studies have shown that extracts from CA induces neurite outgrowth. We examined whether MA, the main component of the CA, promotes neurite outgrowth in N2a cells. The cells were incubated for 2 days in medium containing 5 mM MA in ethanol or ethanol alone (vehicle). Following incubation, the cells were photographed and various parameters of neurite outgrowth were measured using NeuronJ software. The measurements revealed that the percentage of neurite bearing cells were significantly ($p<0.001$) higher in cells incubated with MA as compared to vehicle (ethanol) (Figure 31). MA incubation also significantly ($p<0.05$) increased neurite extension as compared to cells incubated with vehicle alone (Figure 34). 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 35).

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). Additional experiments showed that MA and asiatic acid are the main neurite outgrowth promoting factors in the CA extract. However, this previous study used a high concentration (14.4 mM) of purified MA to induce neurite outgrowth as compared to 5 mM MA 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 MA 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 [19]. 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 [17]. Presently we are examining signal transduction pathways known to regulate neurite outgrowth in N2a cells treated with MA.

Conclusions

The results from this study showed that MA treatment increased the percentage of neurite bearing cells in N2a cells, and also increased neurite extension and the combined length of neurites in N2a cells. MA decreased the doubling time in cell proliferation assay and increased cell viability in N2a cultures. MA treatment also increased mitochondrial electron transport activity in N2a cells, which was correlated with decrease in lactate level in the culture medium.

These findings demonstrate that MA has positive effects on neuronal structure and function in N2a cultures. Future studies to examine the beneficial effects of MA in animal models of neurological diseases would help elucidate its therapeutic potential in neurodegenerative disease involving mitochondrial dysfunction and compromised nerve regeneration.

Figure 1. Representative samples of *Hydrocotyle umbellata* (a), *A. Aralia elata*(b), *A. Hedera Hibernica* (c), and *A. schefflera* (d), that were grown in the Thut Greenhouse at Eastern Illinois University or at the Whiteside Gardens.

Figure 2. Representative sample of *Aralia elata* (e), *Polyscias sp.* (f), that were grown in the Thut Greenhouse at Eastern Illinois University

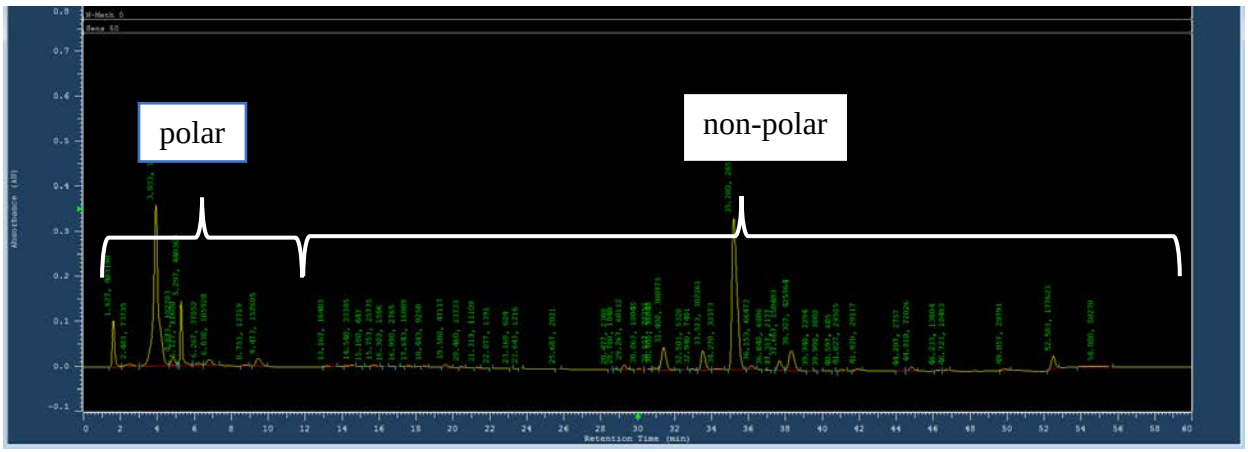

Figure 3. HPLC chromatogram showing polar and non- polar constituents detected for a smple of *AsiaticAcid, Coumarin, Curcumin, Curcumin, Flavanones, Madecassic Acid, Uridine.*

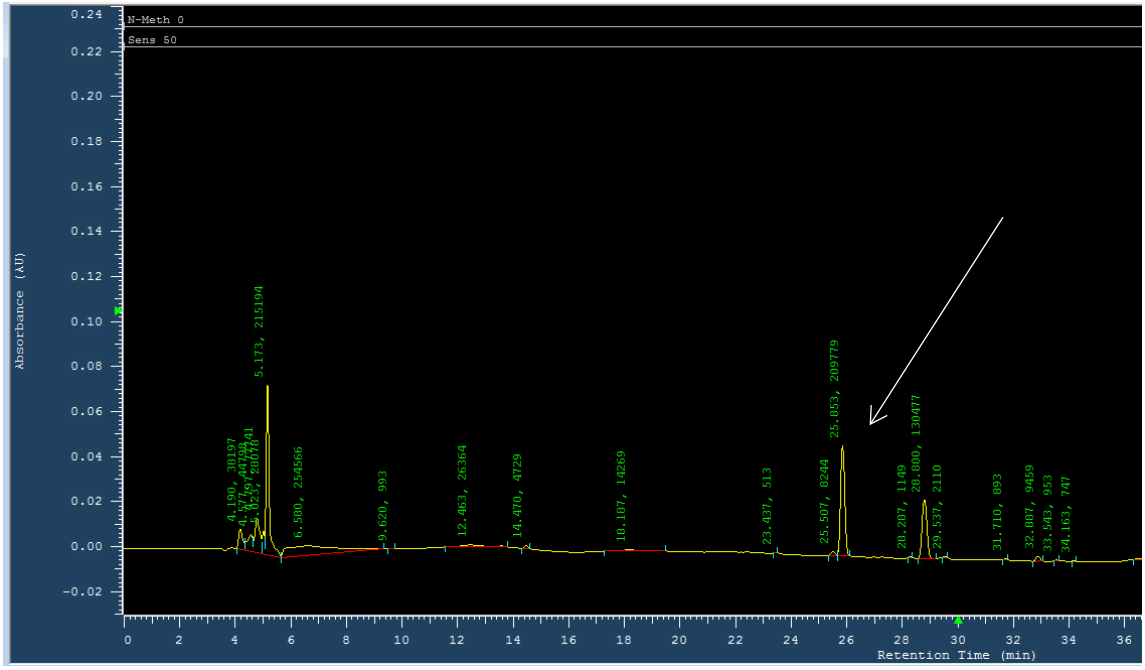

Figure 4. HPLC chromatogram from a pure asiatic acid sample diluted in methanol Arrow indicates peak for Asiatic acid.

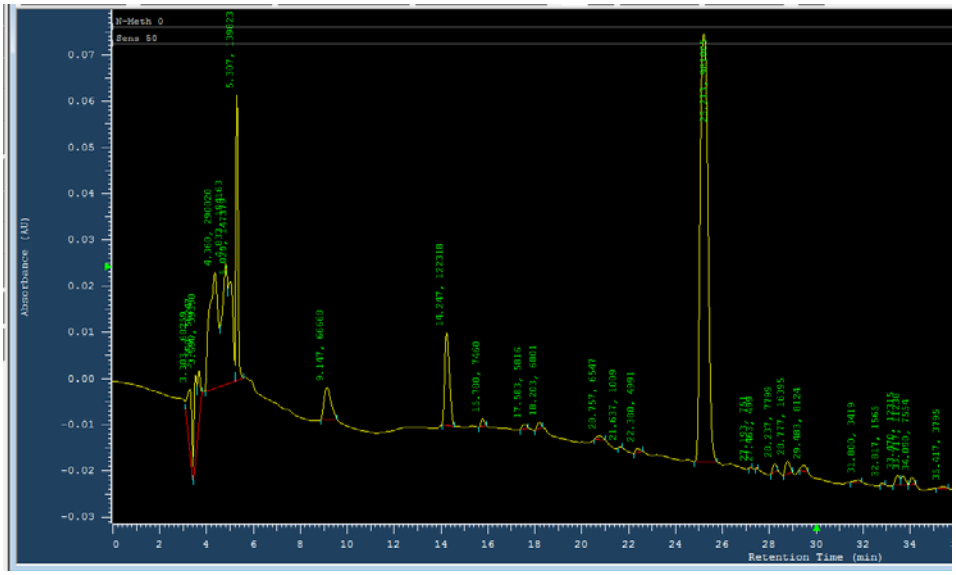

Figure 5. HPLC chromatogram from a pure asiatic acid sample diluted in methanol (Ideal wavelength: 200 nm)

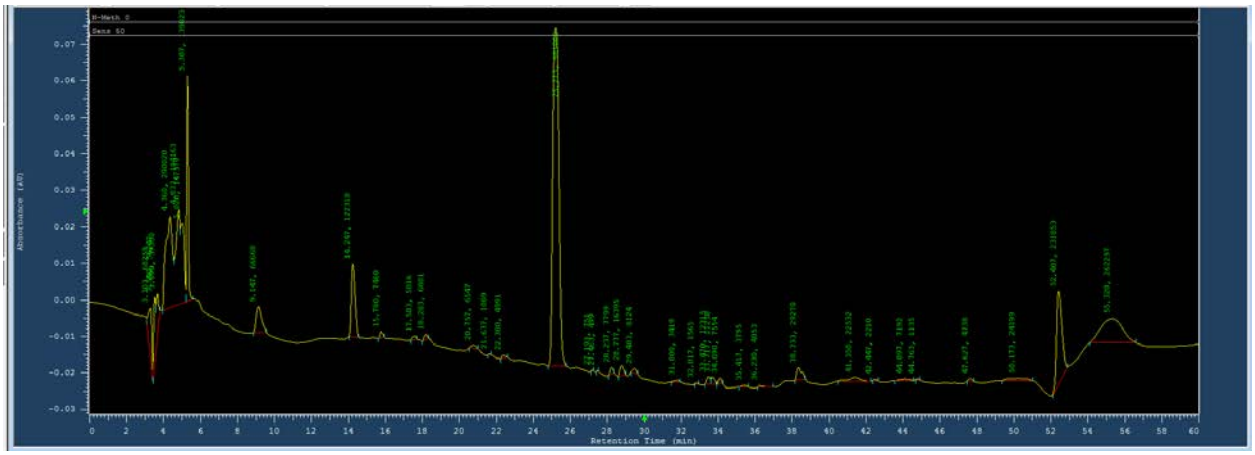

Figure 6. HPLC chromatogram from a pure Coumarin sample diluted in methanol.

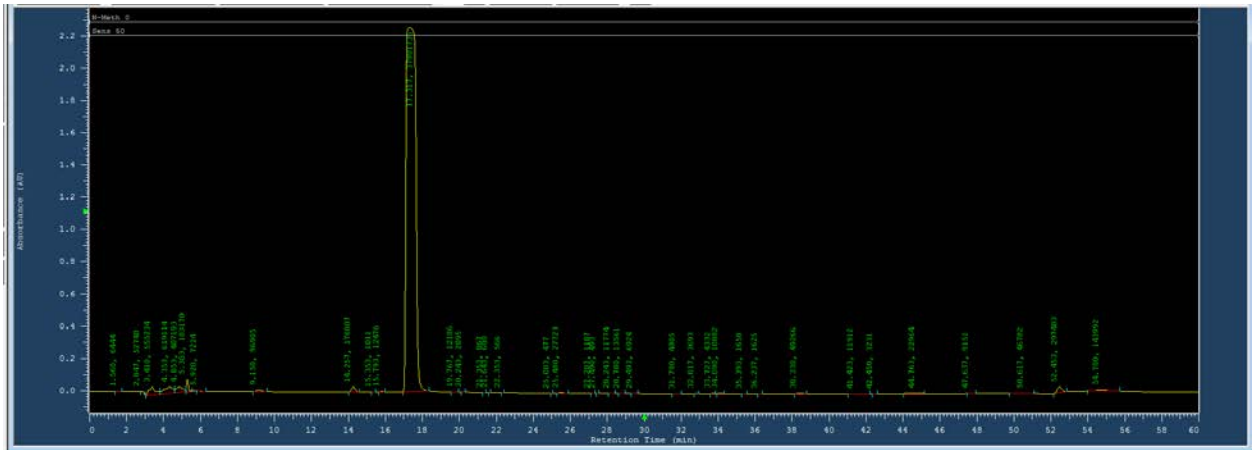

Figure 7. HPLC chromatogram from a pure Coumarin sample diluted in methanol(Ideal wavelength: 200 nm)

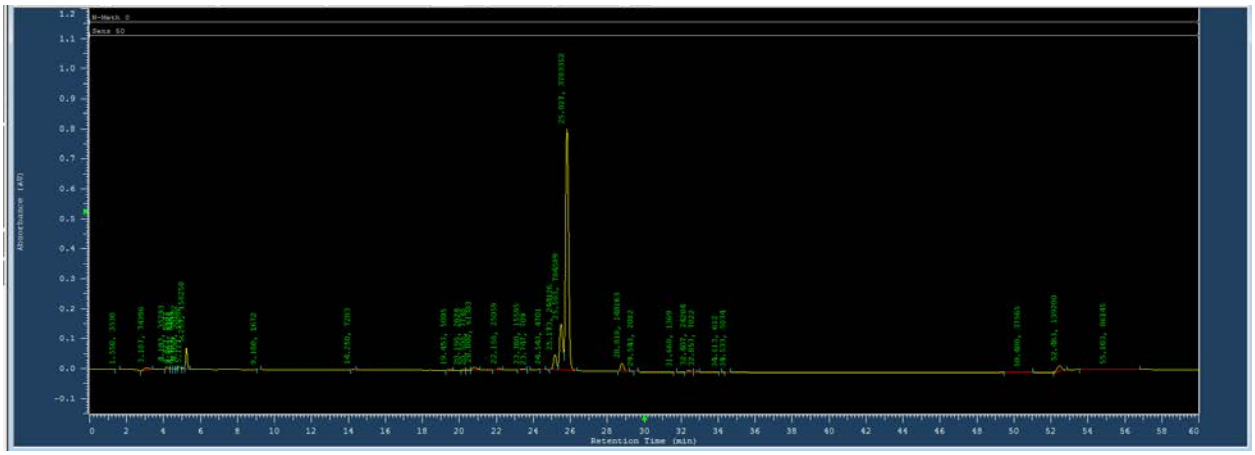

Figure 8. HPLC chromatogram from a pure Curcurmin sample diluted in methanol.

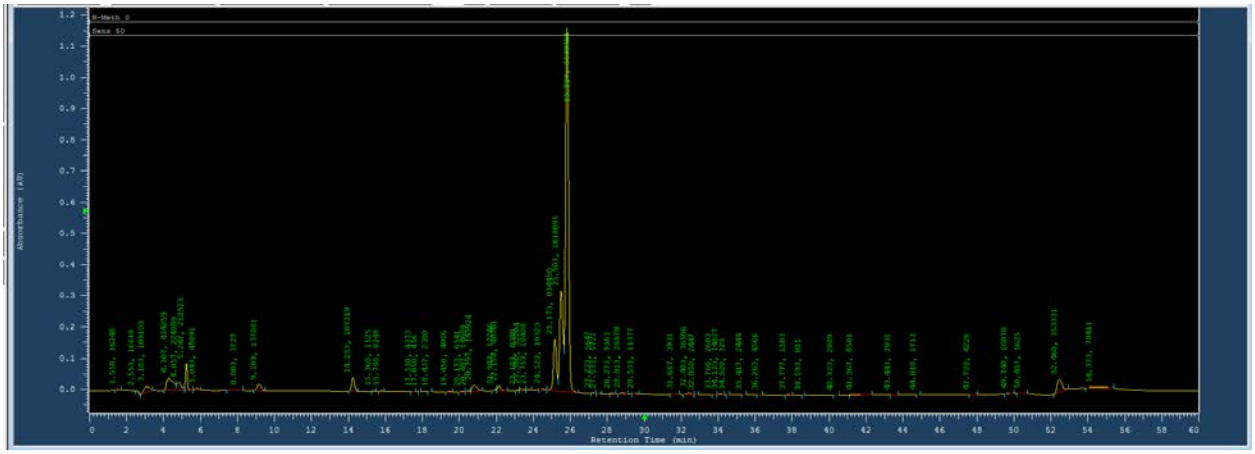

Figure 9. HPLC chromatogram from a pure Curcurmin sample diluted in methanol (Ideal wavelength: 200 nm)

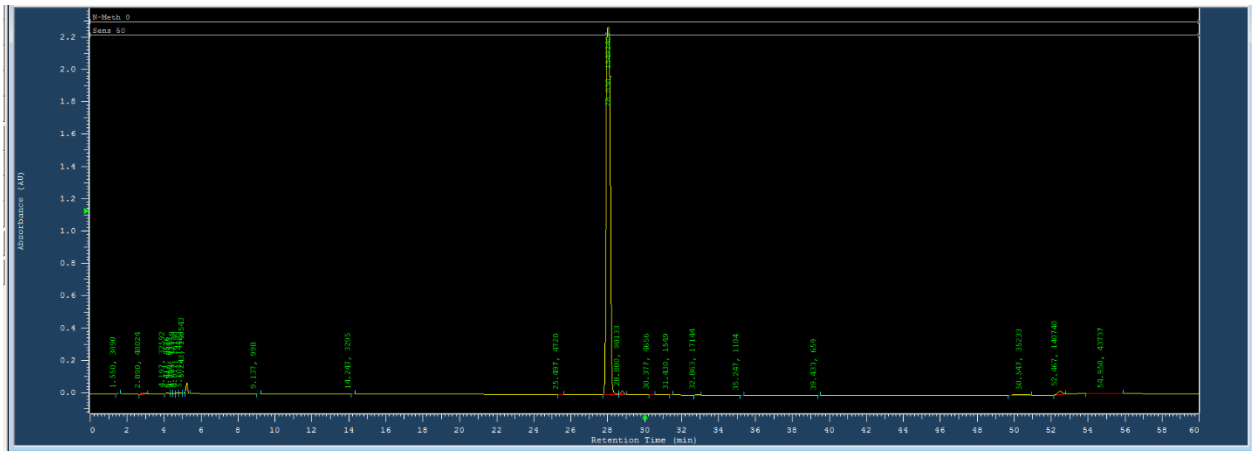

Figure 10. HPLC chromatogram from a pure Flavonone sample diluted in methanol.

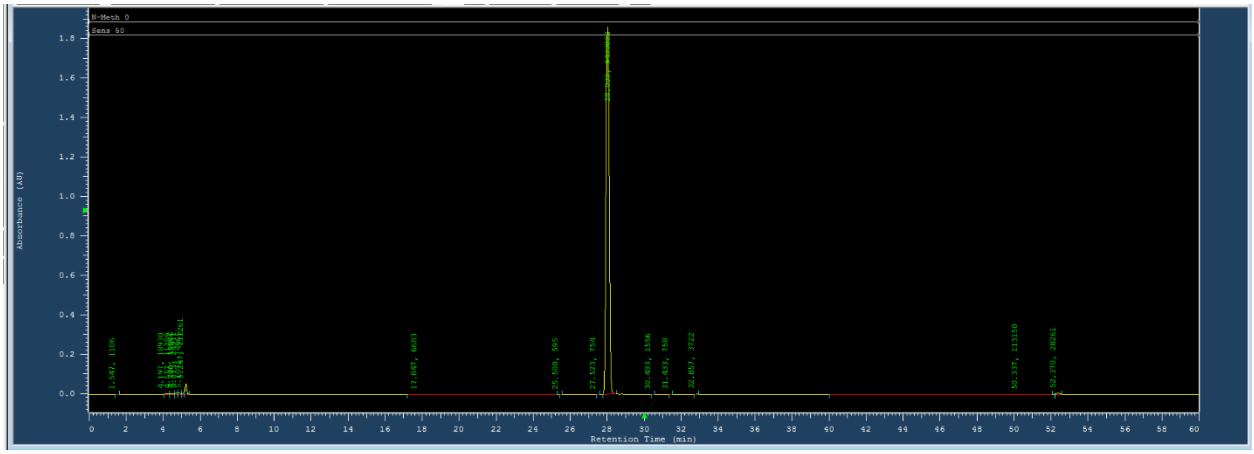

Figure 11. HPLC chromatogram from a pure Flavonone sample diluted in methanol (Ideal wavelength: 250 nm)

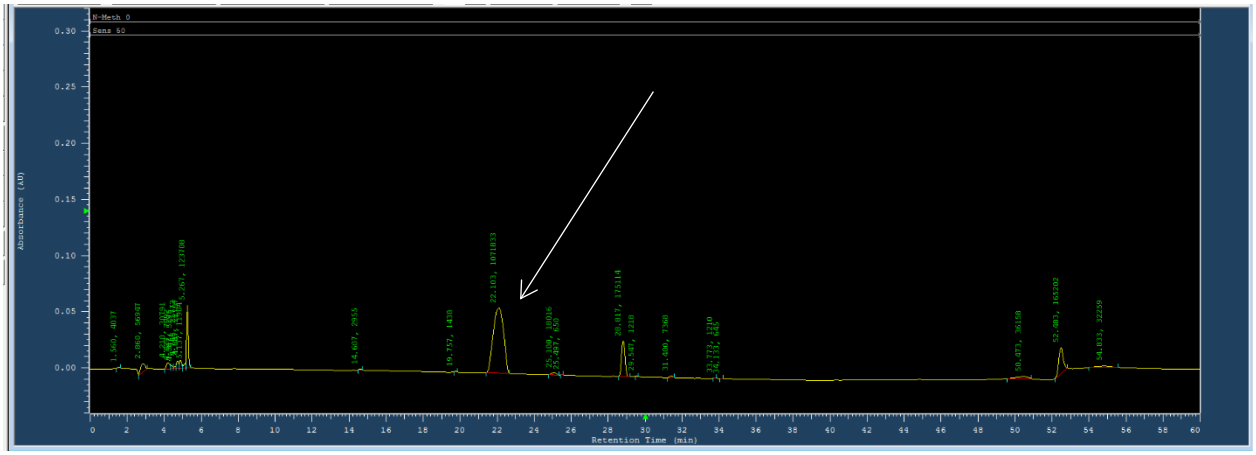

Figure 12. HPLC chromatogram from a pure Madecassic Acid sample diluted in methanol.

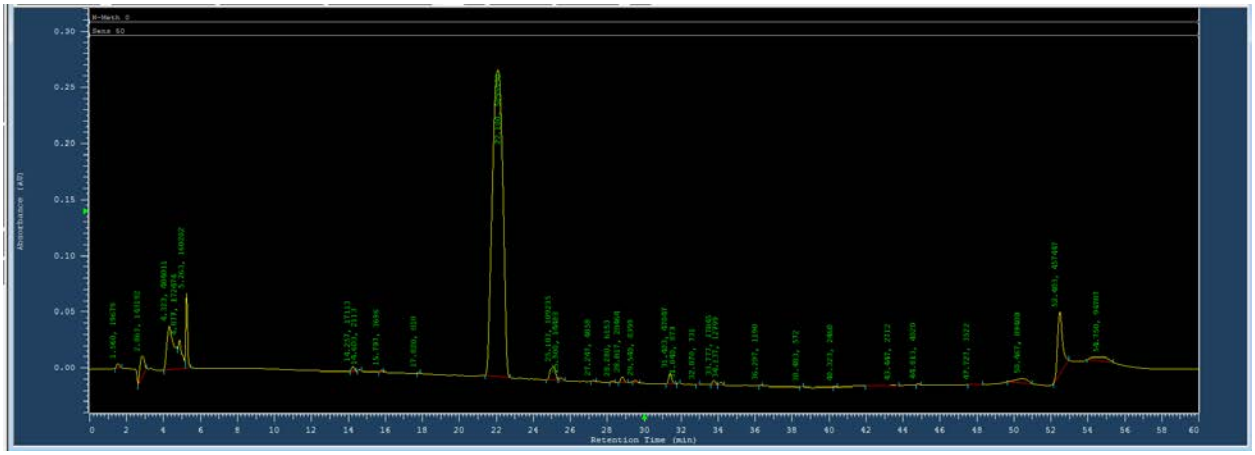

Figure 13. HPLC chromatogram from a pure Madecassic Acid sample diluted in methanol (Ideal wavelength: 200 nm)

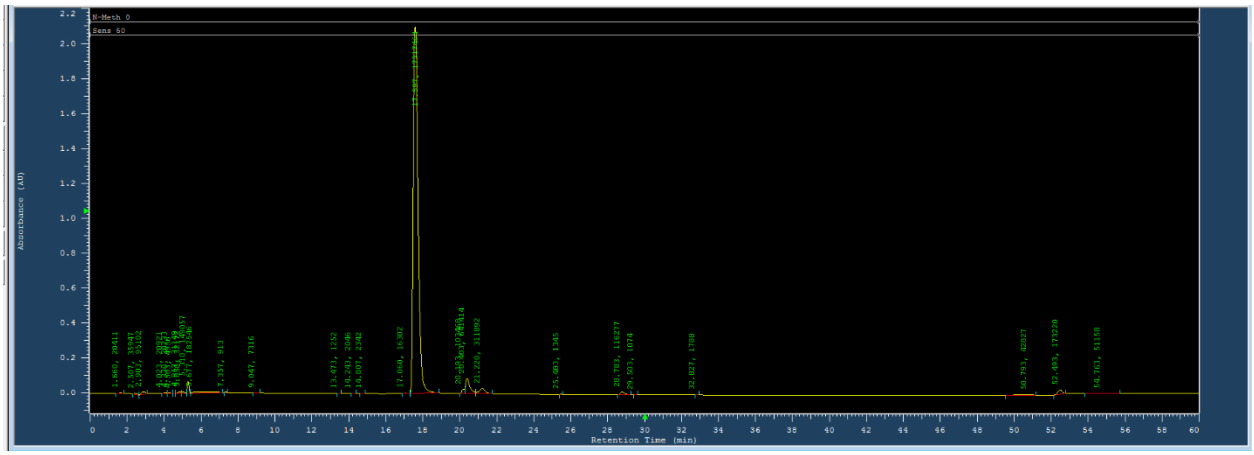

Figure 14. HPLC chromatogram from a pure Quercitin sample diluted in methanol.

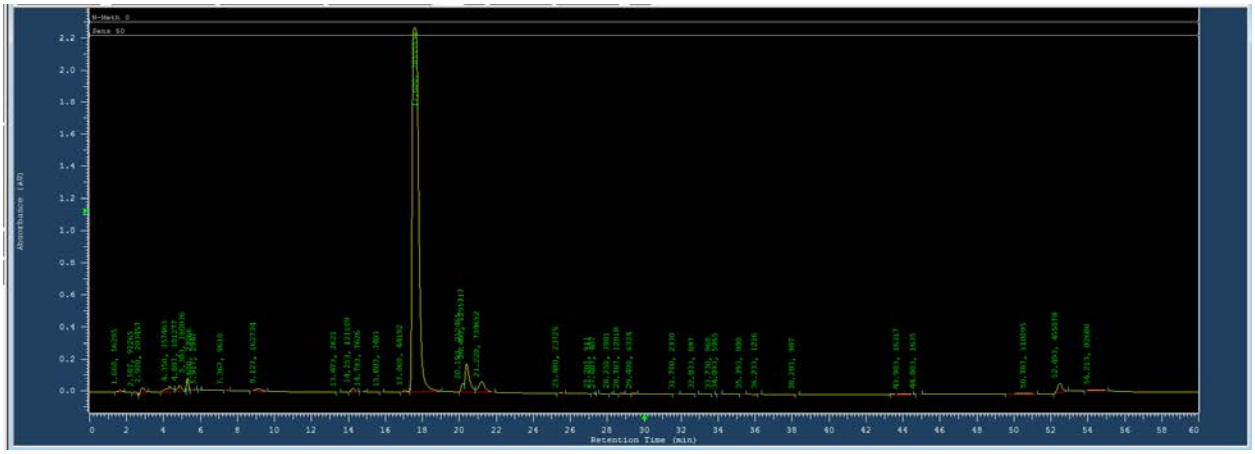

Figure 15. HPLC chromatogram from a pure Quercitin sample diluted in methanol (Ideal wavelength: 200 nm).

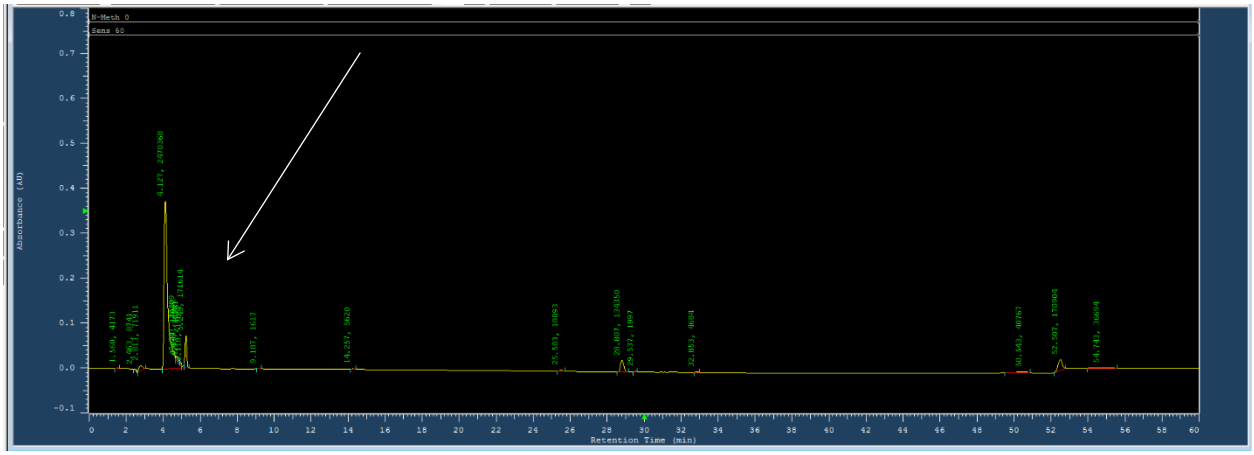

Figure 16. HPLC chromatogram from a pure Uridine sample diluted in methanol.

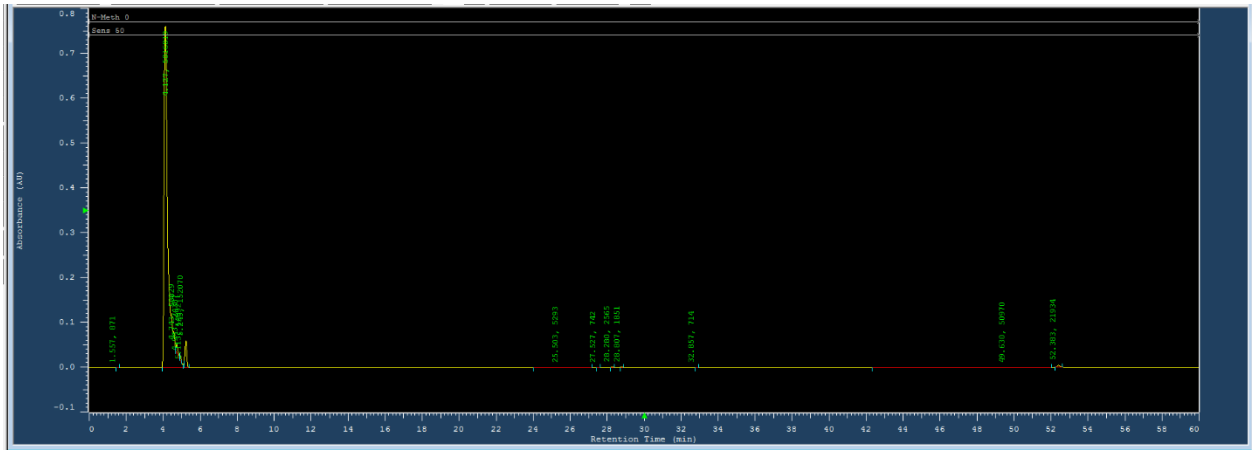

Figure 17 HPLC chromatogram from a pure Uridine sample diluted in methanol (Ideal wavelength: 260 nm)

Compound	Retention time (min)
Asiatic	28.85
Coumarin	17.31
Curcurmin	25.83
Flavonone	28.03
Madecassic	22.10
Quercetin	17.60
Uridine	4.13

Table 2. *he retention times of the Asiatic acid, Coumarin, Curcurmin, Flavonone, Madecassic, Quercetin, Uridine standards*

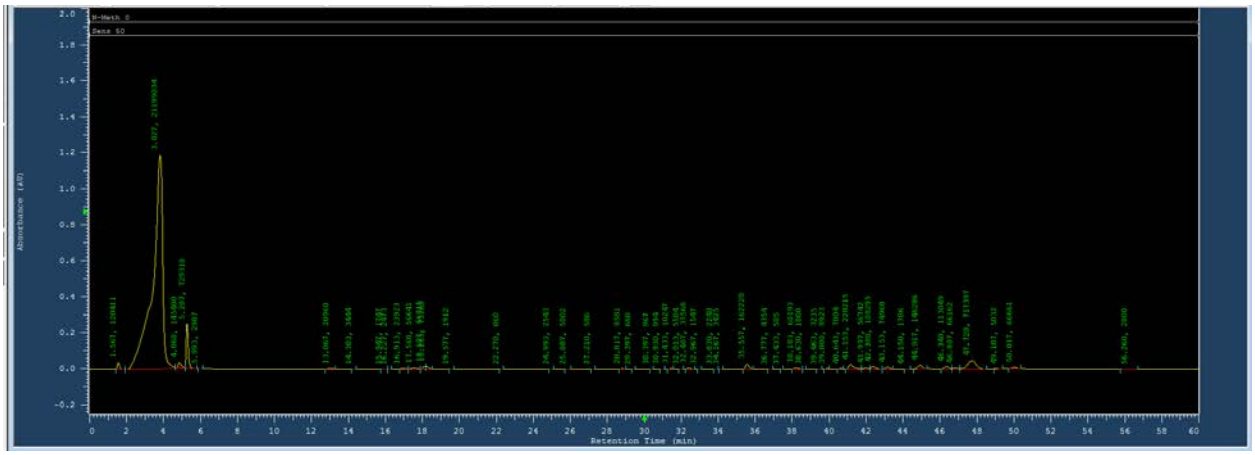

Figure 18. HPLC chromatogram of fresh *Aralia elata* leaf sample(220 nm).

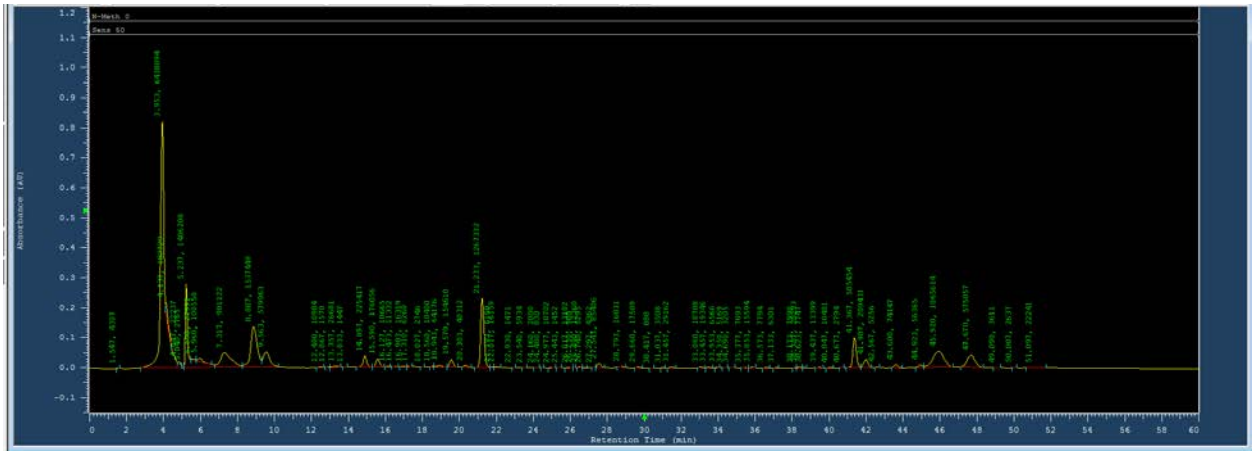

Figure 19. HPLC chromatogram of fresh *Hydrocotyle* leaf sample (220 nm).

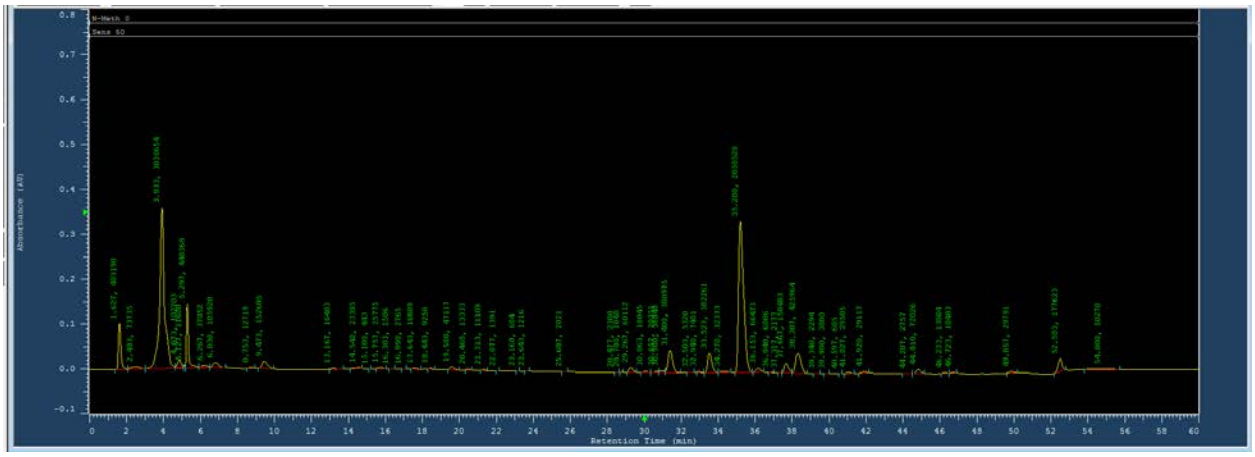

Figure 20. HPLC chromatogram of fresh *A. Aralia racemosa* leaf sample (220 nm).

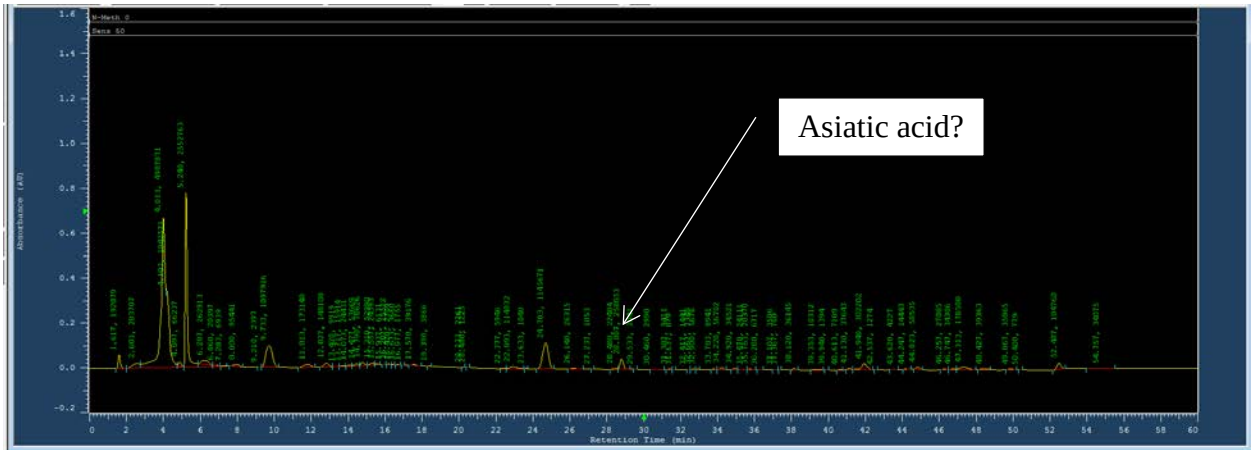

Figure 21. HPLC chromatogram of fresh *Kalopanax* leaf sample (220 nm).

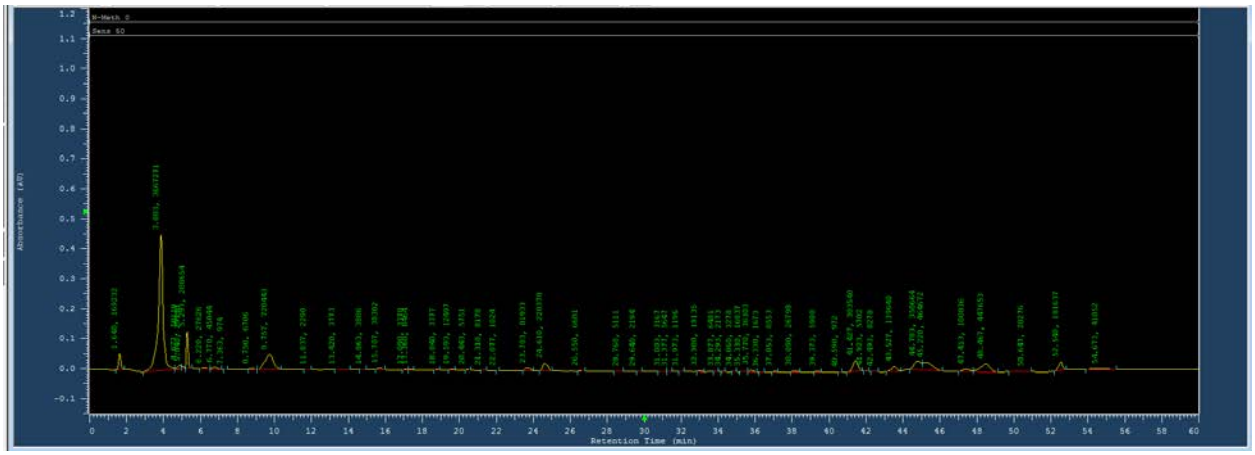

Figure 22. HPLC chromatogram of fresh *Eleutherococcus sieboldianus* leaf sample (220 nm).

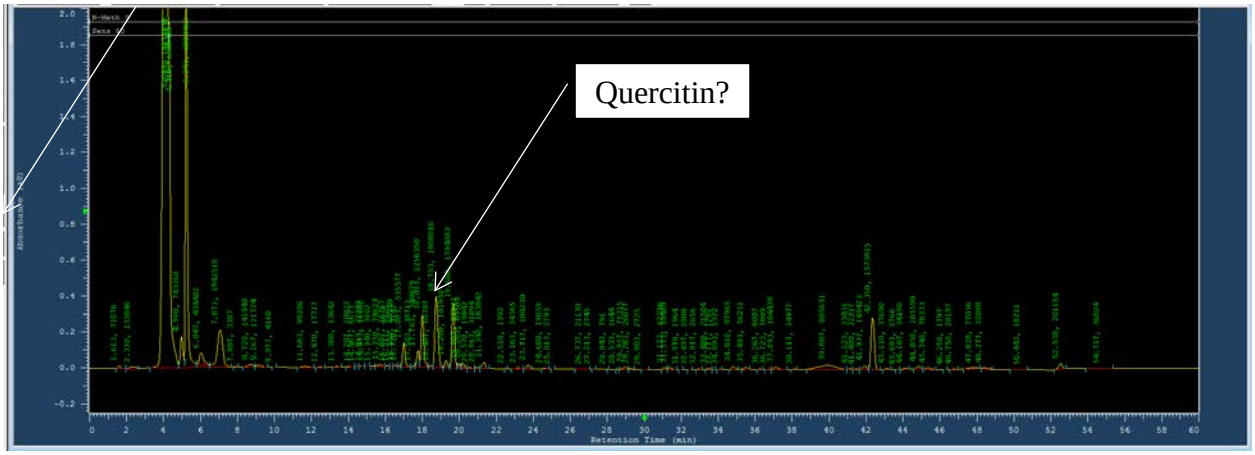

Figure 23. HPLC chromatogram of fresh *Fatsia japonica* leaf sample (220 nm).

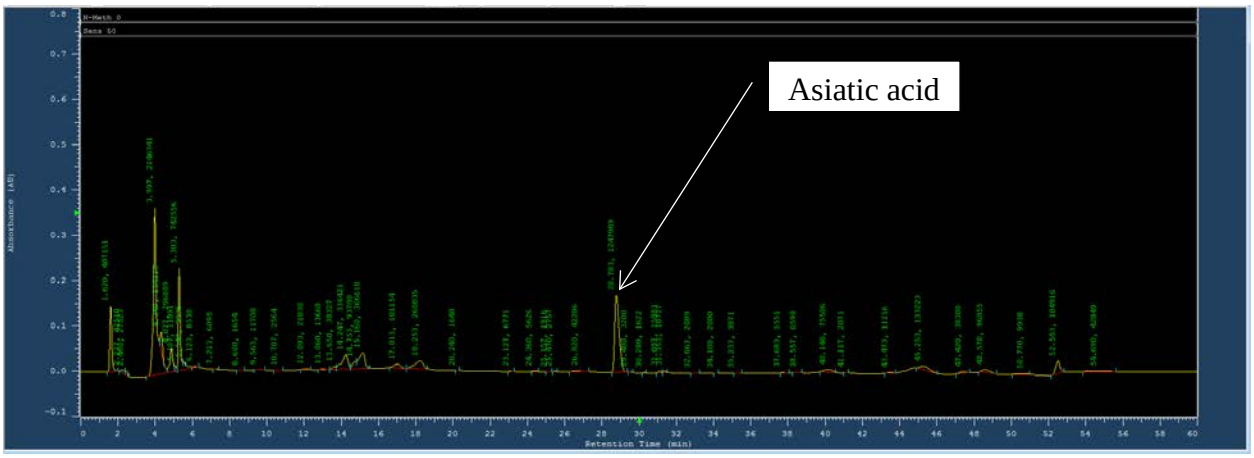

Figure 24. HPLC chromatogram of fresh *Polyscias fruticosa* leaf sample (220 nm).

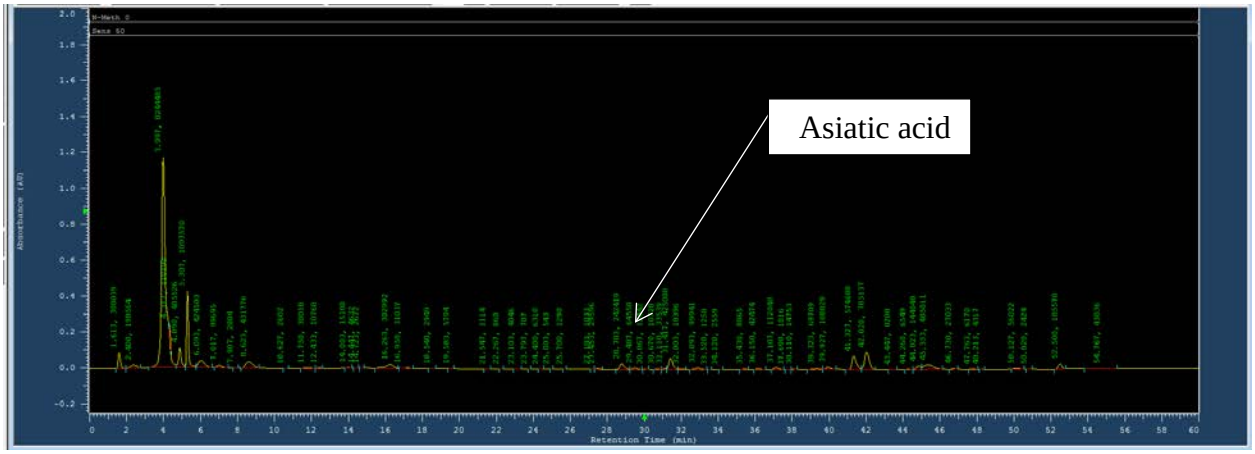

Figure 25. HPLC chromatogram of fresh *Oplopanax horridum* leaf sample (220 nm).

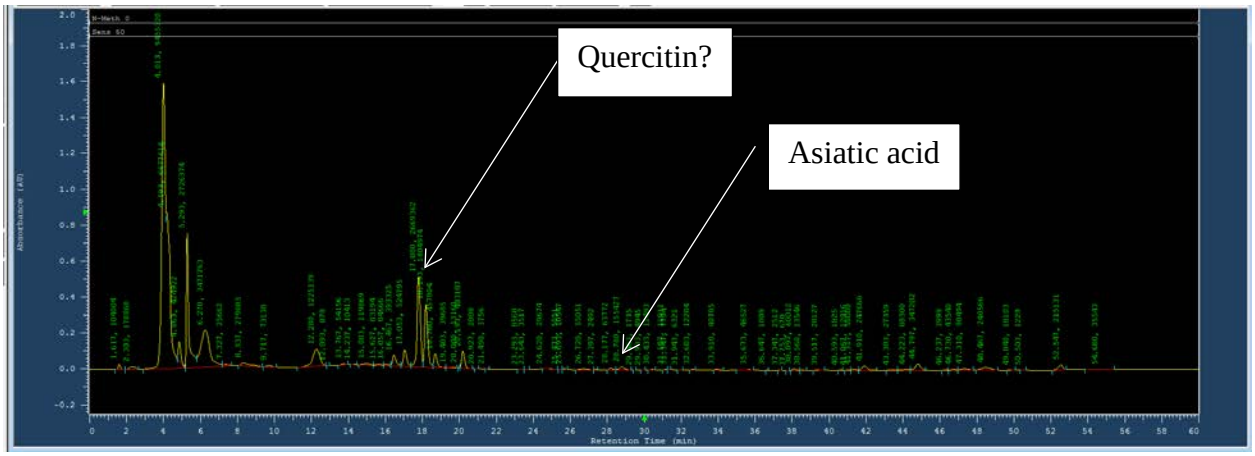

Figure 26. HPLC chromatogram of fresh *Aralia spinosa* leaf sample (220 nm).

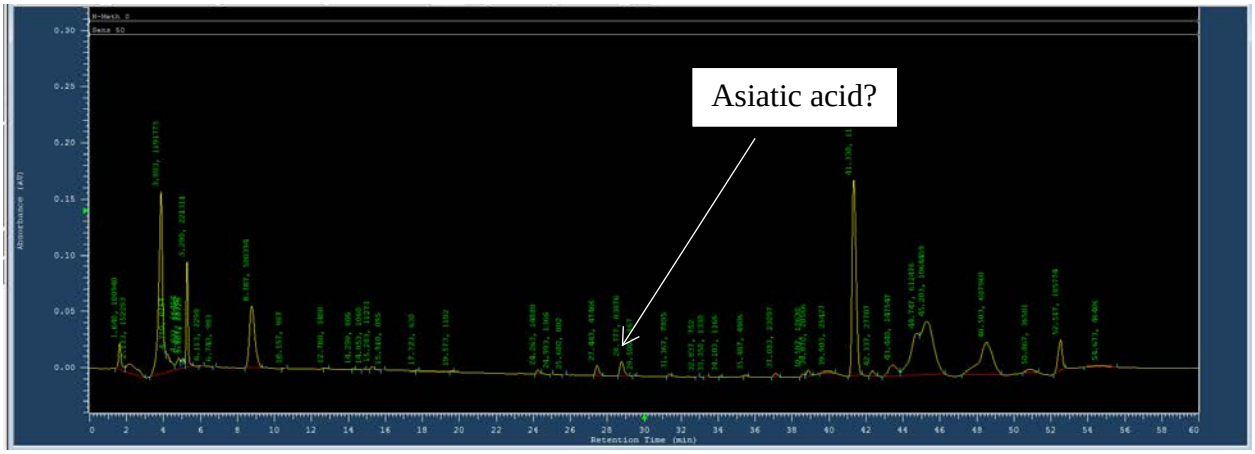

Figure 27. HPLC chromatogram of fresh *Hydrocotyle sibthorpoides* leaf sample (220 nm).

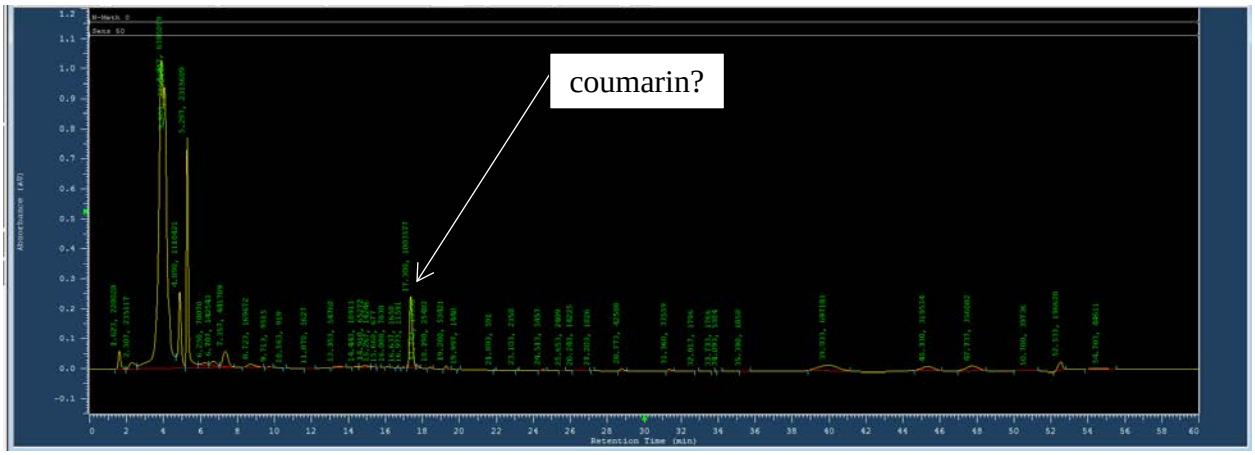

Figure 28. HPLC chromatogram of fresh *Hedera hibernica* leaf sample (220 nm).

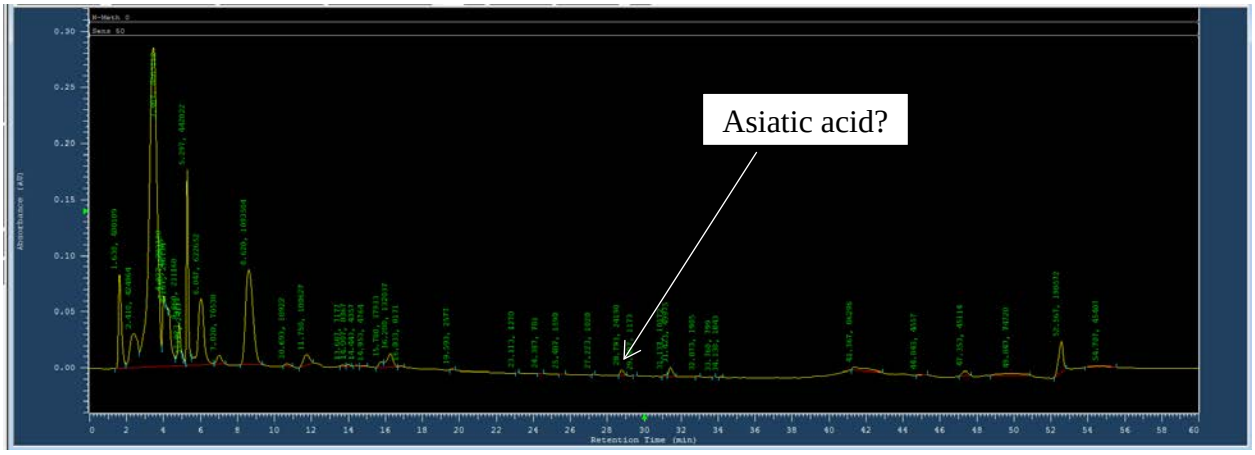

Figure 29. HPLC chromatogram of fresh *Oplopanax horridus* leaf sample (220 nm).

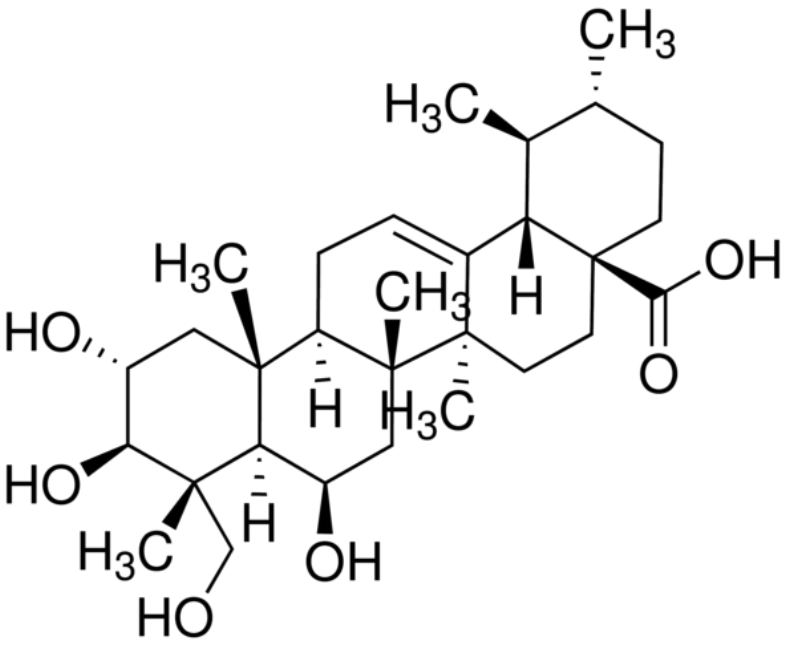

Figure 30. The structure of madecassic acid (from www.sigmaaldrich.com).

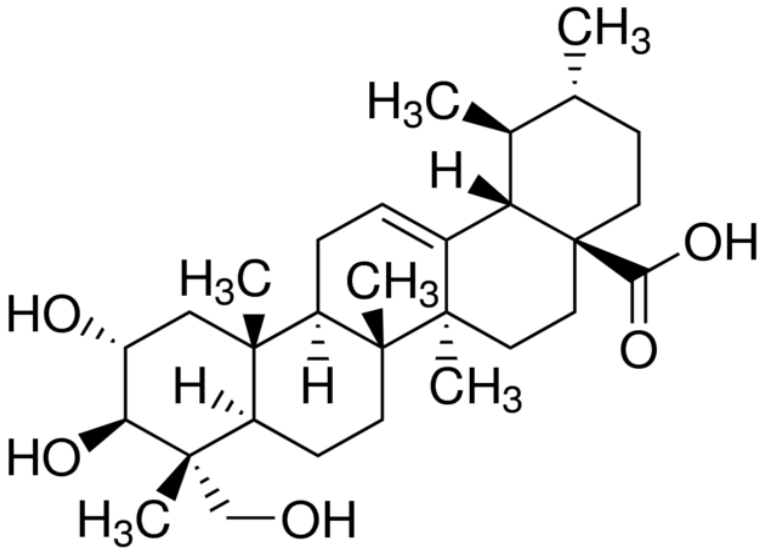

Figure 31. The structure of asiatic acid (from www.sigmaaldrich.com).

Medium ***Ethanol.*** ***Madecassic Acid***

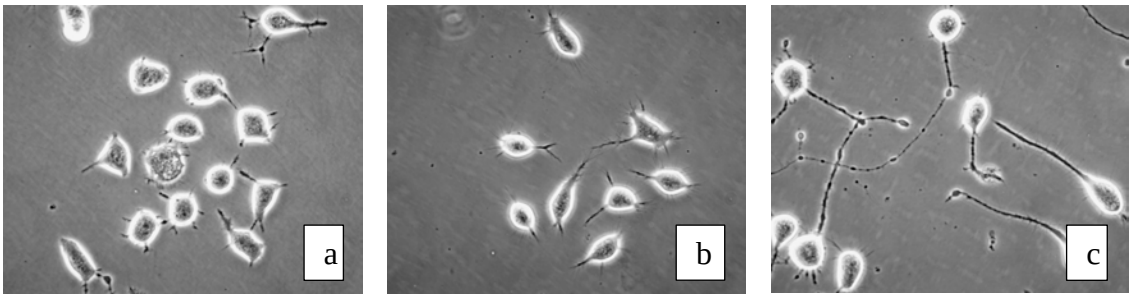

Figure 32. Examples of neurite cells samples in *Medium control* (a), *Ethanol* (b), *Madecassic acid* (c).

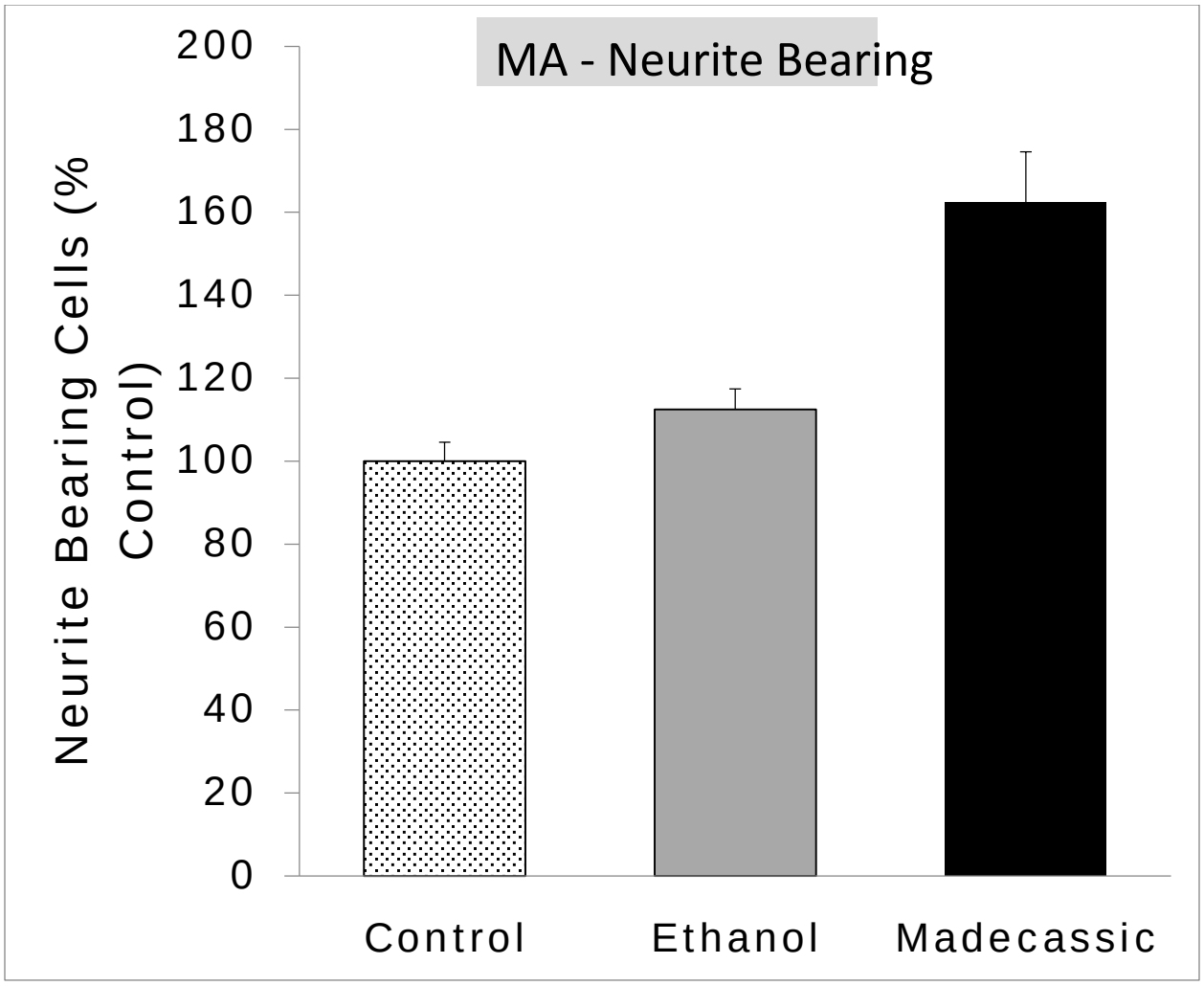

Figure 33. Neurite bearing cells control.

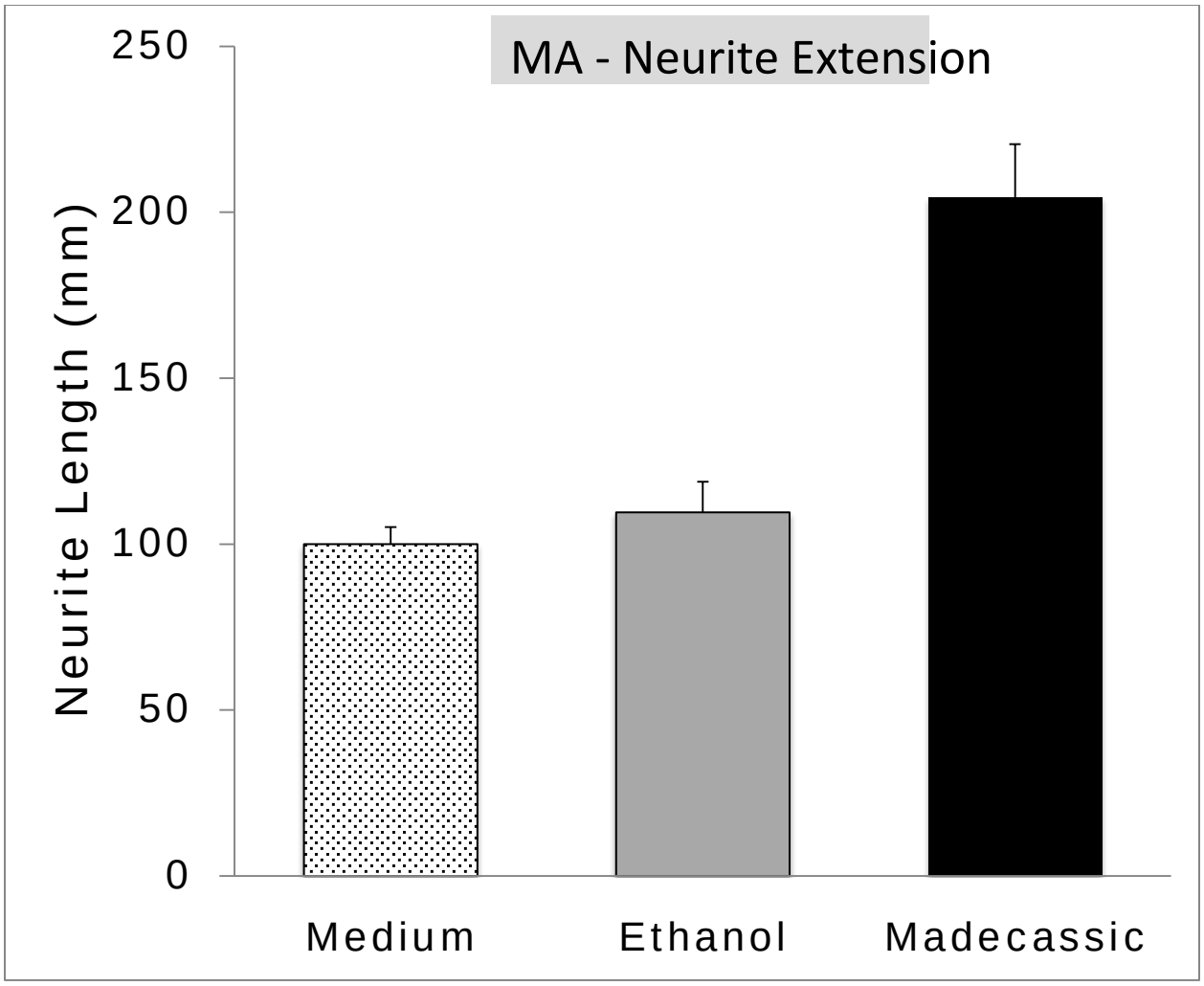

Figure 34. Neurite Extension.

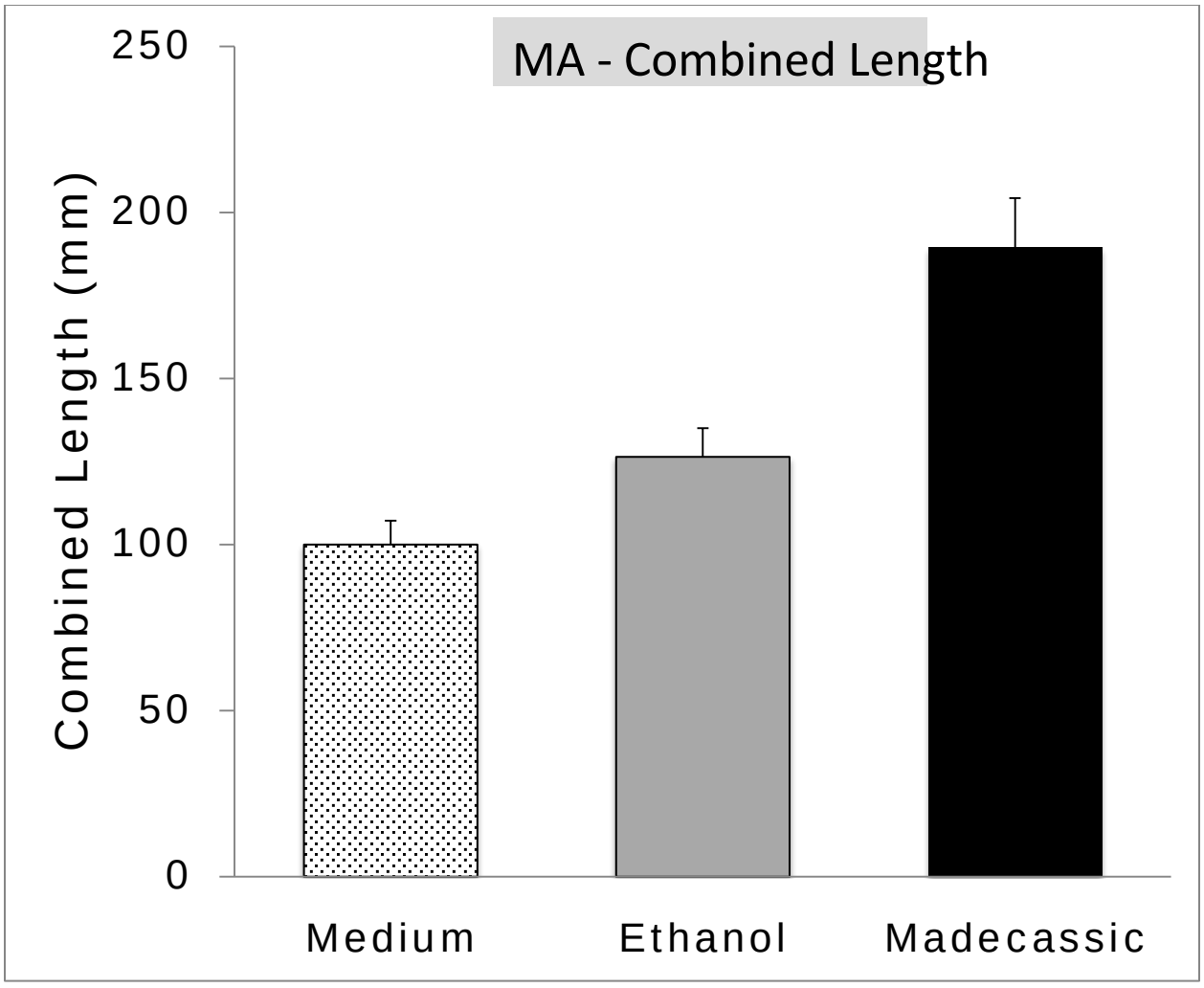

Figure 35. *Combined Length.*

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