Lamiaceae: A Source of Nootropics with Utility in Neurodegenerative Diseases

Honors Thesis
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Abstract:

Nootropics are ingestible products that are presumed to improve mental functions such as cognition, memory, intelligence, motivation, attention, and concentration; and, are thought to work by altering the availability of the supply of neurochemicals (neurotransmitters, enzymes and hormones) to the brain by improving the brain's oxygen supply, or by stimulating nerve growth. The occurrence of plants with nootropic properties has been known since antiquity, and *Ginko biloba*, *Celastrus paniculatus*, *Salvia officinalis*, *Rosmarinus officinalis* and *Galanthus caucasicus*, among others, have been used for a memory enhancement benefit (Dastmalchi et al, 2007). However, the compounds of some nootropic plants, or their modes of action that results in improved brain function, has remained uninvestigated. The aim of this project is the investigation of suspected nootropic plants in anecdotal medicinal accounts to establish possible cholinergic activity and the chemistry associated with it, expecting to find novel compounds useful in the development of improved therapies for Alzheimer's disease (AD). Examination of these accounts led us to select a group of plants for study that include *Salvia verticillata*, *Melissa officinalis,* and *Angelica sinensis.*

We have established in the laboratory an in vitro bioautographic technique to measure Acetylcholinesterase inhibitory (AChEi) activity associated with the compounds of the plants chosen for this investigation. This exploratory study yielded activity in all three species with the highest associated with *M. officinalis*. We chose to further study the activity of *M. officinalis* and have proceeded with the chemical analysis of the active extract using TLC and diagnostic reagents, which suggest the phenolic nature of the most active chemical component. This procedure was followed by HPLC analysis, the chromatographic fractionation of the crude extracts, UV-VIS spectrophotometry, and ESIMS mass spectrometry. A major AChEi principle has been isolated from *M. officinalis* and its structure has been elucidated to be rosmarinic acid, an ester of caffeic acid, and a 3,4-dihydroxyphenyllactic acid (Avila et al, 2004).

Furthermore, it is argued that a contributing factor to the pathology of Alzheimer's disease is oxidative stress (Perry et al, 2002). Thus, neuronal protection from reactive oxygen species (ROS) is advantageous. We have therefore measured the antioxidant activity of members of the Lamiaceae plant family.

Chapter I

Acetylcholinesterase Inhibitory Activity in Alzheimer's Disease

Introduction:

The age-related neurodegenerative disorder, Alzheimer's disease (AD), is characterized by progressive deficits in memory and cognition resulting in dementia and death. Alzheimer's is the most common form of dementia that currently affects approximately 5.2 million Americans of all ages, making it the 6th leading cause of death in the United States. The Alzheimer's Association suggests that of those affected with Alzheimer's, 96% are age 65 and older. Those who are affected with Alzheimer's and are younger than 65 are diagnosed with younger-onset (also known as early-onset) Alzheimer's, while others experience late-onset Alzheimer's. Because there are an increasing number of individuals who reach the age of 65 and older, the annual number of new Alzheimer's cases is projected to double by the year of 2050 (7).

The hallmark pathologies associated with Alzheimer's are the accumulation of the protein fragments, β-amyloid (plaques), outside of the neurons, as well as the collection of twisted strands of tau proteins in neuronal axons (neurofibrillary tangles). These changes are associated with neuronal deficits, and therefore cognitive decline, resulting in the progression of Alzheimer's disease. These reported symptoms are in alignment with the Cholinergic Hypothesis of Alzheimer's disease, as cited by Francis et al (8). This hypothesis suggests that the loss of cholinergic neurotransmission in the basal forebrain, and in particular, its cholinergic projections to the cerebral cortex, the hippocampus, and other areas contribute to the deterioration of cognitive function, and therefore progression towards a demented state.

Neurofibrillary tangles and beta amyloid plaques are biological hallmarks of AD that have been proposed to stem from the reduction of Acetylcholine, as a consequence of the action of Acetylcholinesterase (AChE) within the synaptic cleft. A decreased concentration of Acetylcholine consequentially leads to the reduction of the activity of the postsynaptic muscarinic receptors (mAChR). The Cholinergic Hypothesis of AD suggests that a reduction in the activity of muscarinic receptors mediates the transformation of tau proteins to a hyperphosphorylated state. As cited by Avila et al., tau is a hydrophilic protein that normally stabilizes neuronal microtubules, allowing for the proper functioning of the neuronal messaging system (11). Although tau's function is regulated via kinase and phosphatase

activity, tau proteins aggregate in an hyperphosphorylated state, forming structures known as neurofibrillary tangles. Normally, muscarinic cholinergic receptor excitation leads to the inactivation of a Protein Kinase (GSK-3), inhibiting the phosphorylation of the tau protein (12) These neurofibrillary tangles are toxic and consequentially lead to neuronal death. Furthermore, the Cholinergic Hypothesis of AD suggests that a reduction in the activity of nicotinic receptors, as a consequence of a decrease in acetylcholine, leads to a mismetabolism of the neuro-protective amyloid precursor protein (sAPP). This mismetabolism leads to the production of β-amyloid. Overproduction, or the reduced clearance of β-amyloid, leads to amyloid aggregations, which consequentially lead to neuronal degeneration. It has also been cited that amyloid aggregation potentiates the accumulation and formation of neurofibrillary tangles, further progressing neuronal dysfunction (13).

There is currently no cure for this debilitating disease; however, there has been substantial evidence that cites the significant correlation between the inhibition of the enzyme Acetylcholinesterase (AChE) in the brain and an improvement of cognitive deficits experienced by Alzheimer's patients (2). Therefore, cholinergic modulation of the brain is used as a first-line treatment for symptoms associated with Alzheimer's disease (3). However, the drugs currently prescribed to manage this disorder exhibit poor clinical performance as a consequence of inadequate pharmacokinetics and pharmacodynamics leading to low bioavailability and rapid breakdown. Therefore, the search for new lead compounds to develop innovative drugs in the treatment of Alzheimer's is necessary.

Nootropic ingestible products are claimed to improve mental functions such as cognition, memory, intelligence, motivation, attention, and concentration. It has been speculated that their modes of action alter the availability of the brain's supply of neurochemicals by improving oxygen supply, or by stimulating nerve growth. The occurrence of plants with nootropic properties has been known since antiquity, and *Ginko biloba*, *Celastrus paniculatus*, *Salvia officinalis*, *Rosmarinus officinalis* and *Galanthus caucasicus* among others have been used for a memory enhancement benefit (1). However, in many cases the compounds of these plants and their modes of action that result in improved brain function has remained uninvestigated.

Aim 1 of our investigation was to explore the acetylcholine modulatory pharmacology of suspected nootropic plants cited in anecdotal medicinal accounts. Potential plants to be used in this investigation were identified through a thorough literature search for plants whose traditional use reflected nootropic activity, i.e., plants used for improving cognitive function, and as intelligence enhancers, mental strengtheners, etc. Ultimately, The plants *Salvia verticillata*, *Melissa officinalis,* and *Angelica sinensis,* all of the Lamiaceae plant family, were chosen due to chemical and pharmacological studies which evidenced their apparent efficacy in relieving symptoms associated with cognitive deficits such as memory loss, mental weakness, and an impaired capacity for learning (15).

Materials and Methods:

Collection and Processing of Plant Material

Salvia, *Melissa,* and *Angelica* were collected from the Cornell Plantations (Minns, Mundy and York gardens) in August 2013. Roots of *Angelica sinensis* were donated by ARCOMIG Inc. The collected plant material was air dried at room temperature and was ground using a Waring Blender. The ground material from *S. verticillata* and *M. Officinalis* were extracted using Ethanol (80%), and *A. sinensis* was extracted using Methanol. The solvents chosen for extraction were selected based on the literature, which cited effective solvents of extraction for each plant (4). The extracts were filtered, evaporated and redissolved at a concentration of 1M (based on an approximate estimate of the average molecular weight of all secondary metabolites in the extract to be 250 g/mol). The extracts were profiled for composition by analytical thin layer chromatography (TLC), and their respective chemical components on TLC were visualized through the use of short and long wave UV light, as well as by the use of various diagnostic reagents (vanillin and ferric chloride).

Vanillin Spray Reagent- Analytical Approach to TLC Analysis

1g of vanillin was dissolved in 18 mL of ethanol (95%). This solution was then placed in an ice bath. 1 mL of sulphuric acid and 6 drops of acetic acid were added drop-wise to the ethanolic solution. After the development of the TLC plate with the loaded plant extracts, 4-5mL of the prepared vanillin solution was sprayed onto the plate using a compressed air line. The TLC plate was then set aside to dry, and was subsequently exposed to an approximately 100°C hot plate for 2-3 minutes.

Ferric Chloride Spray Reagent- Analytical Approach to TLC Analysis

A 50 mL solution of MeOH and H_2O was prepared at a 1:1 ratio. 1% (wt./v) of $FeCl_3$ was added to the mixture. After the completion of the development of the TLC plate, 4-5 mL of the reagent was sprayed onto the plate using a compressed air line. The TLC plate was then set aside to dry.

Acetylcholinesterase Bioautographic Assay

The following procedure is an adaptation of an assay performed by Adhami et. al (2). A TLC bioautographic assay is used to screen plant extracts for their potential to inhibit AChE. 1000 U of AChE was dissolved in 149 mL of buffer A solution, which consist of a 50 mM Tris-Hydrochloric acid buffer at pH 7.8, resulting in a solution of 6.7 U/mL enzyme. Aricept, an AChEi drug, was used as a positive control during this assay. The extracts to be assayed were loaded onto a silica TLC plate, which was developed with a solvent mixture of ethyl acetate/methanol/water (10:2.7:2.0). After running the TLC, the plate was dried before 2 mL of the enzyme solution was sprayed onto the plate. After spraying the enzyme solution (which was maintained at room temperature) onto the TLC plate, the plate was dried completely. Subsequently, the plate was placed inside a sealed container, which included a moist wick, and had been previously incubating at a temperature of 37°C. The sealed container was then incubated at 37°C for a duration of 20 minutes. Afterwards, enzymatic activity was detected by spraying the TLC plate with a mixture containing a 2 mL solution of 13.4 mM napthyl acetate and a 8 mL solution of 7.4 mM Fast Blue Salt (FBS), a diazonium salt. The TLC plate was then set aside in the dark for 12 hours to allow for color development. Zones on the TLC plate presenting discoloration against a purple background were interpreted as zones containing compounds that effectively inhibited the enzyme, AChE.

High Performance Liquid Chromatography (HPLC)

A High Performance Liquid Chromatographic (HPLC) method for the analysis of plant crude extracts and fractions was adapted from Wang et al (6). Crude extracts were analyzed using a Waters 600 Pump System fitted with a Waters 2996 Photodiode Array Detector. UV spectra were monitored at 330 nm (6). Extracts were ran for 30 minutes through a reverse phase C-18 column with a 3um particle size, at a flow rate of 0.5mL/min. A two part solvent system was used: .1% aqueous orthophosphoric acid (Solvent A) and .1% orthophosphoric acid in methanol (Solvent B). Initial conditions were 60:40 (A:B), progressing linearly to 50:50 at 10 min, and then to 40:60 at 15 minutes with these conditions sustained for an additional 10 minutes, and then to 60:40 for the last five minutes. All crude extract samples were filtered using a syringe filter to remove particulates in suspension. The sample injection volume was 2 µL. The hypothesized

chromatographic peak associated with rosmarinic acid [(R,E)-3-(3,4-dihydroxyphenyl)-2-((3-(3,4-dihydroxyphenyl)acryloyl)oxy)propanoic acid] in *M. officinalis* was obtained and compared to the chromatogram of a commercial sample of rosmarinic acid (96%, Sigma-Aldrich chemical company). Data acquisition and processing was completed using Waters Millennium software.

Electrospray Ionization Tandem Mass Spectrometry (ESIMS/MS Analysis)

Plant samples were prepared for ESIMS/MS analysis at Cornell's Proteomics and Mass Spectrometry Facility, Institute of Biotechnology. Samples prior to analysis were cleaned from particulates with the aid of a syringe filter (PTFE or Nylon respectively; 0.2 mm, Alltech). The samples were analyzed in the negative and positive mode using Electrospray Ionization Tandem Mass Spectrometry (ESIMS/MS) in a 4000 Q Ion Trap LC/MS (Applied Biosystems) spectrometer, at an infusion rate of 10ml/hr and a capillary voltage of ±3500V. Mass spectra were acquired and selected parent ions were subjected to MS/MS analysis.

Crude Extract Fractionation Using Column Chromatography

A cotton swab was placed at the bottom of a chromatographic column (2.5 Aminex 90 cm) to create a plug in the system. Next, a sand layer of 1 cm was added to the column. A homogenized slurry mixture was prepared by adding hexane to 75 grams of silica gel. Hexane was drained from the column while this slurry mixture was simultaneously added. After the slurry was added to the column, another 1 cm layer of sand was added. The crude extract to be fractionated was loaded onto silica gel, and was deposited on top of the sand layer. A separatory funnel was used to facilitate changes in the solvent system. The solvent system, which was initially 100% hexane, underwent 10% incremental increases of ethyl acetate until a 1:9 ethyl acetate: methanol solvent system was achieved. Collected fractions were reanalyzed by TLC and were combined according to their chemical profiles.

Preparative Thin Layer Chromatography (TLC)

A Thin Layer Chromatography Tank was filled with 50 mL of following solvent system: ethyl acetate-methanol-water (10-2.7-2.0). For the purpose of sample application, the TLC plate was scored 1" from the bottom of the HPTLC Silica gel 60 F_{254} glass plate (20cm x 10cm). 2mL of the solution to be separated was placed in this scored indentation. Rosmarinic acid was added as a standard on the silica gel plate. The plate was then developed in the solvent tank and was allowed to run until the solvent line reached an inch from the top of the Silica Gel Plate. After development of the Silica Gel Plate, the plate was dried to completion and distinct bands were identified using long and short wave UV light. The bands were scraped from the HPTLC Silica plate and were processed separately. Each of the collected bands was pulverized with a mortar and pestle, extracted with 75 mL of methanol for 20 minutes, and was subsequently filtered using Whatman Filter Paper Disks. After filtration, the three bands were evaporated to 1.5 mL, filtered with a syringe filter, and were stored for further characterization.

Results and Discussion:

Based on anecdotal information retrieved from the literature, a group of purported nootropic plant species were targeted for study as potential sources of lead chemistry with therapeutic use in the treatment of Alzheimer's disease. Current drugs used to potentiate cognitive function are in the class of acetylcholinesterase inhibitors (AChEi) (26). An acetylcholinesterase inhibitory (AChEi) assay has therefore been utilized to determine the ability of the selected plants to inhibit the enzyme, AChE.

1.1 Preliminary Thin Layer Chromatographic Analysis Optimization

Prior to the assessment of AChEi activity of the crude extracts from the plant samples to be investigated, it is necessary to develop and optimize a thin layer chromatographic solvent system to allow for optimal crude component separation. The TLC plates were therefore developed using a 10:1.3:1 (ethyl acetate/methanol/water solvent system) solvent system. Long wave analysis of the plant compounds showed similar red and blue fluorescent spots, which are suggestive of the presence of structurally

similar compounds in the lanes corresponding to the alcoholic extracts of *S. verticillata* and *M. officinalis,* but not for the lanes correspond to the alcoholic extract of *Angelica sp.* and *A. sinensis*. Thus, utilizing UV light for TLC analysis allowed for preliminary chemical similarities to be established (figure 1).

1. *Salvia sp.* (Minns Garden)
2. *S. verticillata* (Minns Garden)
3. *M. officinalis* (Mundy Garden)
4. *M. officinalis* (York Garden)
5. *Angelica sp.* (Minns Garden)
6. *A. sinensis* (Commercial sample)

Figure 1. Comparison of alcoholic extracts using long wave UV Light.

As an attempt to further investigate the plant compounds of the Lamiaceae plant family, a vanillin spray reagent was utilized for the purpose of detecting terpenes and phenolics. This reagent detected further similarities between the alcoholic extracts of the plant species loaded onto the TLC plate (figure 2). Chromatographic behavior was most similar between the *S. verticillata* and *M. officinalis* species. The vanillin spray reagent therefore confirmed initial observations visualized using long wave UV light. A TLC solvent system change was made to enhance the separation of compounds associated with the mobile and stationary phases on TLC, consequentially allowing for better visualization of plant compounds (figure 2). The new solvent system had a 10:2.7:2.0 (ethyl

acetate/methanol/water) composition, and was used for the analysis of all future analytical TLC plates.

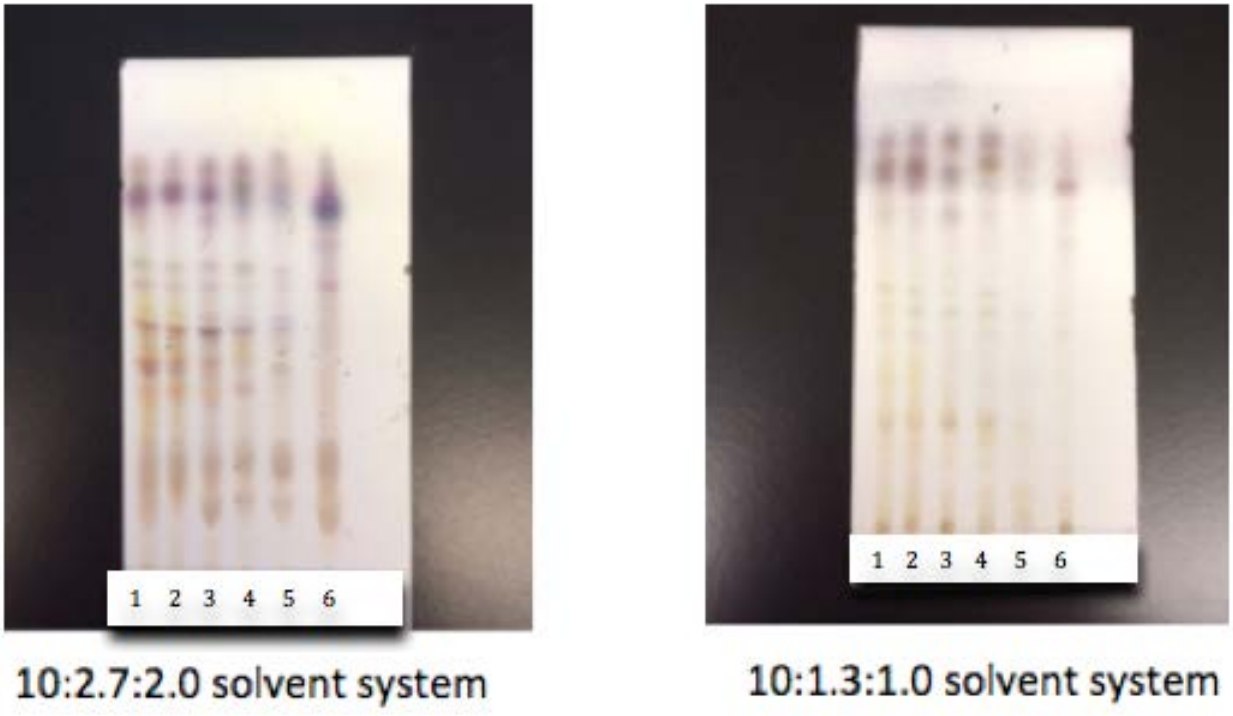


Figure 2. Comparison of the use of different solvent systems using the Vanillin Spray Reagent. From left to right; 1) Salvia sp. (Minns Garden) 2) S. *verticillata (Minns Garden)* 3) M. *officinalis* (Mundy Garden) 4) M. *officinalis* (York Garden) 5) Angelica sp. (Minns Garden) 6) Angelica *sinensis* (Commercial sample)

1.2 Assessment of Acetylcholinesterase Inhibition activity of the plant Crude Extracts

To investigate the cholinergic potential of the selected plant species, an autobiographic laboratory AChEi assay was utilized. This assay is based on the ability of AChE to cleave 1-naphthyl acetate into 1-naphtol, which subsequently reacts with Fast Blue Salt, forming a purple colored diazonium dye (5). That being said, regions of the TLC where acetylcholinesterase inhibition has occurred will be clearly distinguished as a white spot against a purple background, as shown in figure 3.

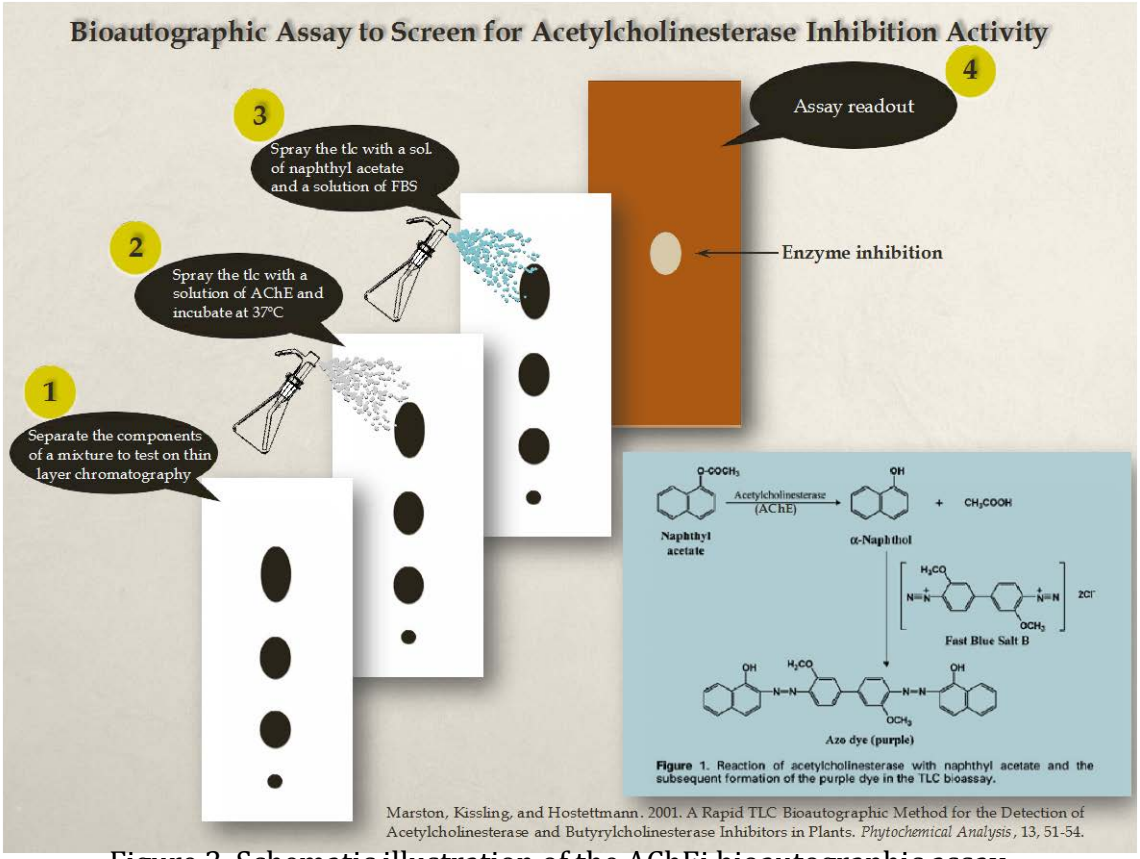
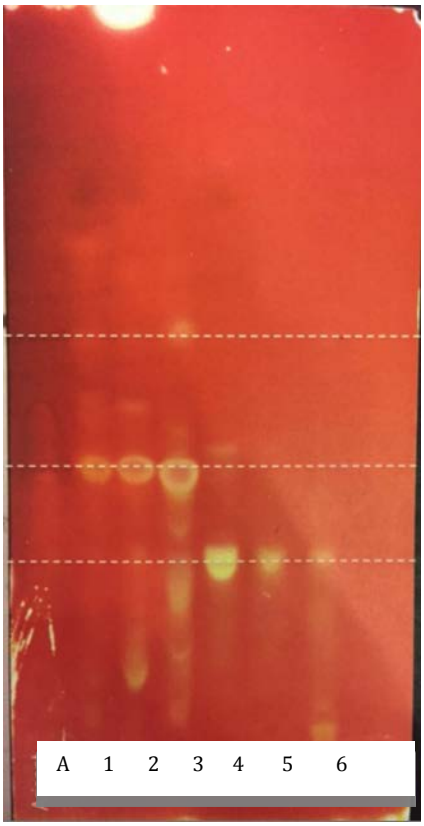


Figure 3. Schematic illustration of the AChEi bioautographic assay

After all plant samples, and the positive control, Aricept, were loaded onto the TLC plate, the plate was developed and the AChEi assay was performed. Results of the AChEi assay revealed the presence of various AChEi compounds within all plant extracts. However, the aqueous alcoholic extracts of most interest were those of *S. verticillata* and *M. officinalis* of the Mundy Garden, as these crude extracts contained prominent chemical constituents exhibiting high enzyme inhibitory activity, as observed by their comparatively large zones of inhibition (figure 4). Additionally, due to the similarities in the R_f values for the AChEi chemical constituents of the alcoholic extracts of *S. verticillata* and *M. officinalis* (Mundy), and the similarities in the chromatographic behavior of these two species during the preliminary chromatographic analysis, we proposed that analogous AChEi components existed in the alcoholic extracts of these two plants.



A. Aricept
1. *Salvia sp.* (Minns Garden)
2. *S. verticillata* (Minns Garden)
3. *M. officinalis* (Mundy Garden)
4. *M. officinalis* (York Garden)
5. *Angelica sp.* (Minns Garden)
6. *A. sinensis* (Commercial sample)

Figure 4. AChEi bioautographic assay

Ferric chloride was used to further diagnose on TLC the phenolic character of the major active chemical constituent. As seen in figure 5, the use of ferric chloride as a visualization reagent resulted in the phenolic nature of the major AChEi chemical constituent common to *S. verticillata* and *M. officinalis* to be elucidated (figure 5).

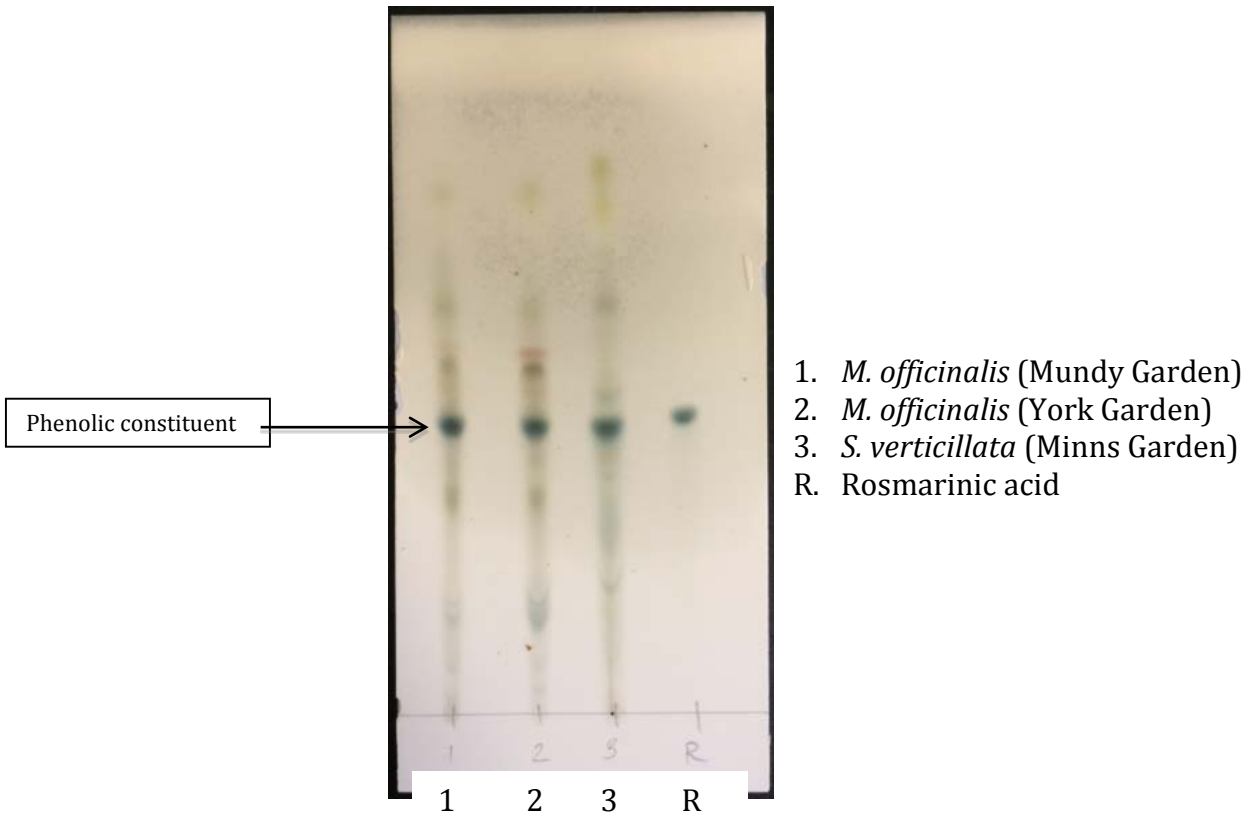
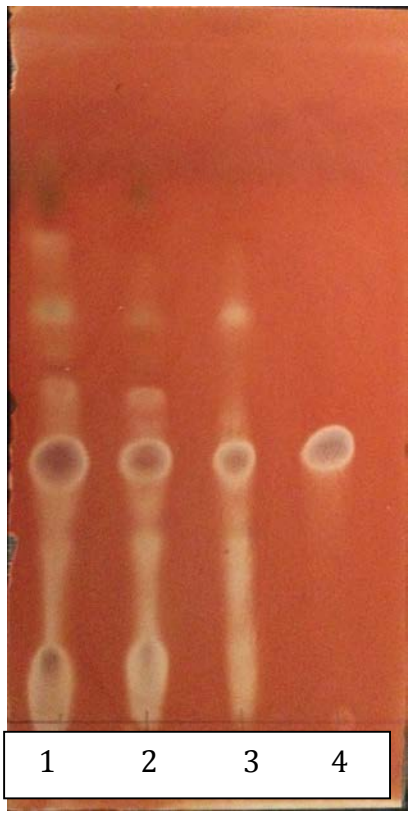


Figure 5. Comparison of Alcoholic Extracts using a Ferric Chloride Reagent.

As previously alluded to, the Lamiaceae plant family includes a large number of medicinal genera, such as, *Calamintha*, *Lavandula*, *Mentha*, *Melissa*, *Origanum*, *Rosmarinus*, *Salvia*, *Teucrium* and *Thymus*, which have been traditionally used to treat respiratory diseases, gastrointestinal problems and various nervous system disorders (27) . Some of the pharmacological activities have been attributed to the presence of polyphenols, along with other more common hydroxycinnamates, such as caffeic, ferulic, *p*-coumaric, chlorogenic acid, and rosmarinic acid, which are widely distributed in the plant kingdom, and occurring particularly in species of the Lamiaceae and Boraginaceae families (28). This reported chemical profile of the Lamiaceae prompted us to question if rosmarinic acid was present amongst the phenolic AChE inhibitors detected in *M. officinalis* and S. *verticillata*. To this end, an authentic sample of rosmarinic acid was included in the AChEi bioautographic assay. Results of this assay suggest rosmarinic acid's AChEi properties, as

well as its presence in the extracts of *M. officinalis* and *S. verticillata* (figure 6). This observation resulted in the focus of our continued investigation to shift towards the inspection of the similarities, or potential differences, between the active constituent of *M. officinalis* and rosmarinic acid.



1. *Salvia sp.* (Minns Garden)
2. *S. verticillata* (Minns Garden)
3. *M. officinalis* (Mundy Garden)
4. Rosmarinic acid (Commercial sample)

Figure 6. AChEi bioautographic assay.

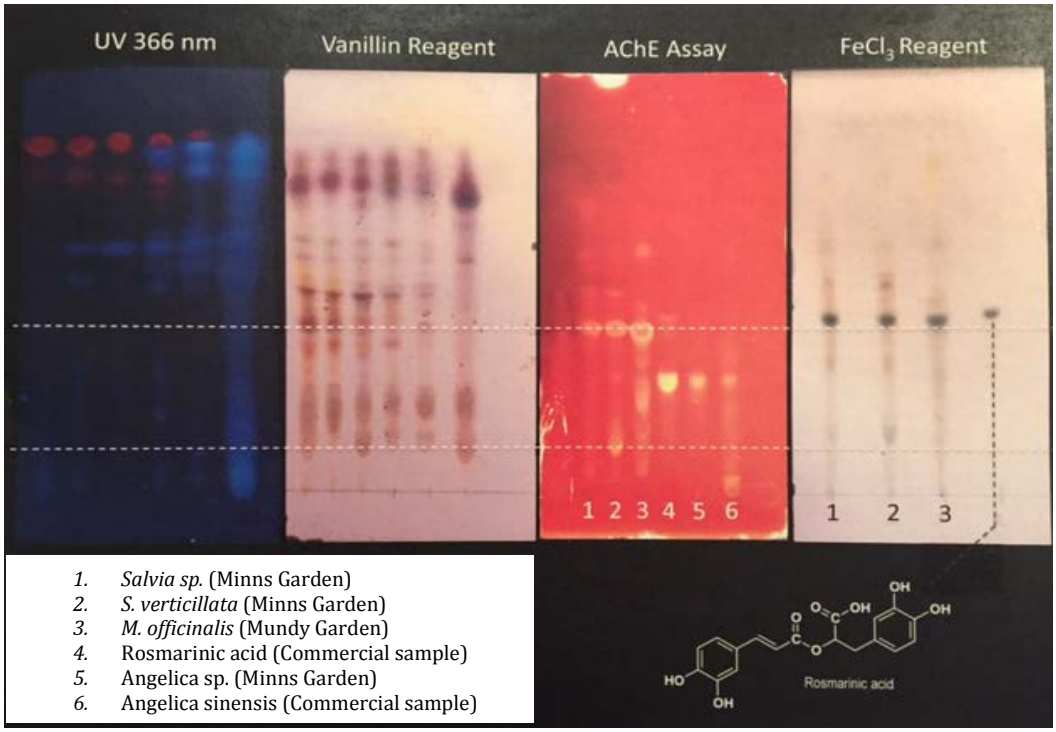


Figure 7. Summary of thin layer chromatography preliminary work.

1.3 High Pressure Liquid Chromatography (HPLC) Analysis

High Pressure Liquid Chromatography (HPLC) was utilized as a method to test the presence of rosmarinic acid in the aqueous alcoholic extract of M. *officinalis*. In order to do so, an analytical method has been adapted from the procedure described by Wang et. al (6). This procedure allowed for the chromatographic profile of M. *officinalis* to be compared to the chromatogram of a pure sample of rosmarinic acid. The results of this analysis showed the presence of a major chemical component in the alcoholic extract of *M. officinalis*, which exhibited the same retention time as a commercial sample of rosmarinic acid (figure 8, 9). This result further supported the hypothesis concerning the presence of rosmarinic acid within the aqueous alcoholic extract of *M. officinalis*.

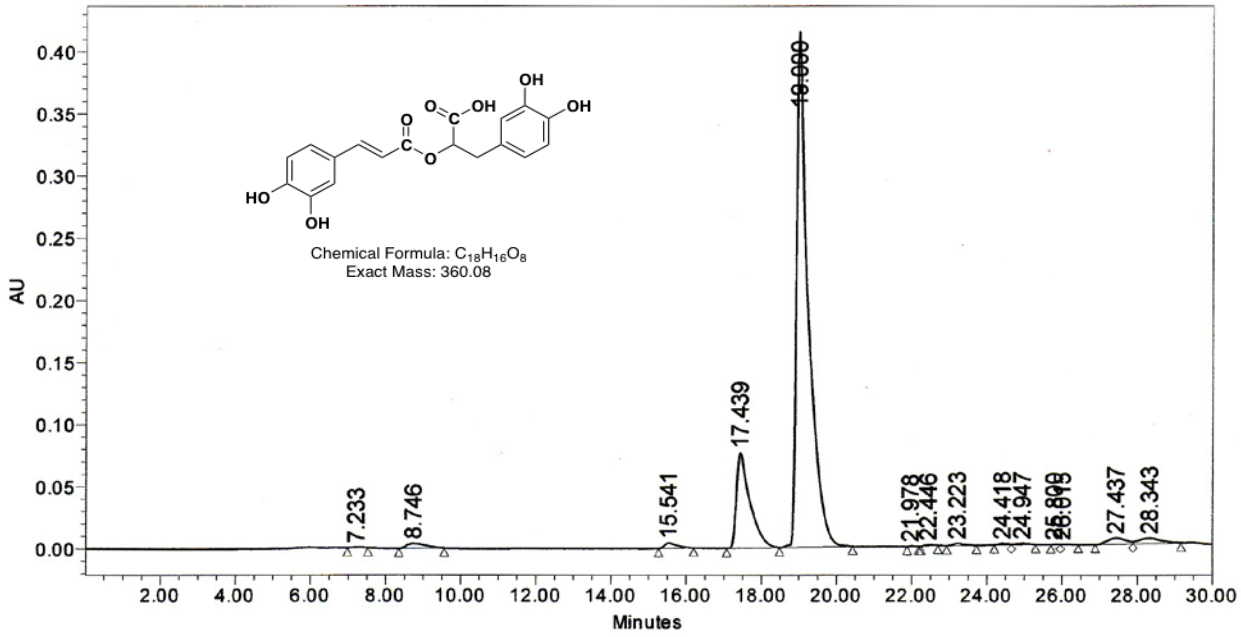


Figure 8. Retention time of a 96% pure sample of rosmarinic acid.

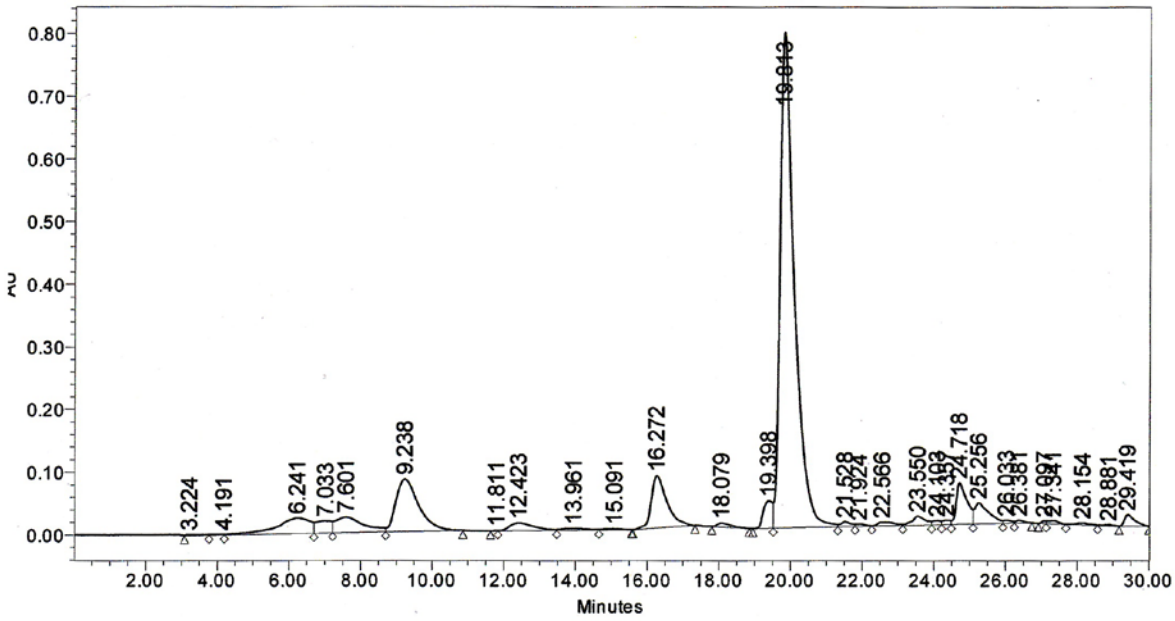


Figure 9. HPLC analysis of the alcoholic extract of *M. Officinalis.*

1.4 Electrospray Ionization Mass Spectrometry

Submission of the aqueous alcoholic extract of *M. officinalis* to electrospray ionization mass spectrometry (ESIMS/MS) analysis of the negative mode showed the presence of a corresponding molecular ion peak of rosmarinic acid, *m/z* 359.1 (figure 12). The ion, *m/z* 359.1, was subject to fragmentation (figure 13), and the fragmentation pattern is consistent with the expected fragmentation pattern of the rosmarinic acid standard (figures 10, 11).

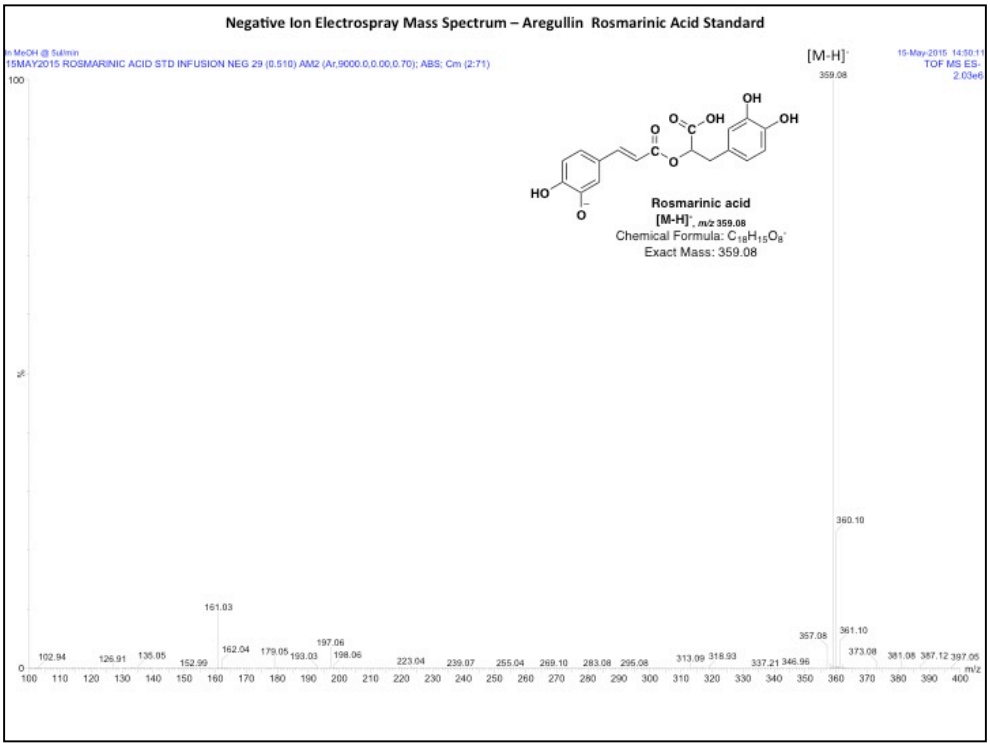


Figure 10. Negative Ion Electrospray Mass Spectrum of Rosmarinic Acid, Standard

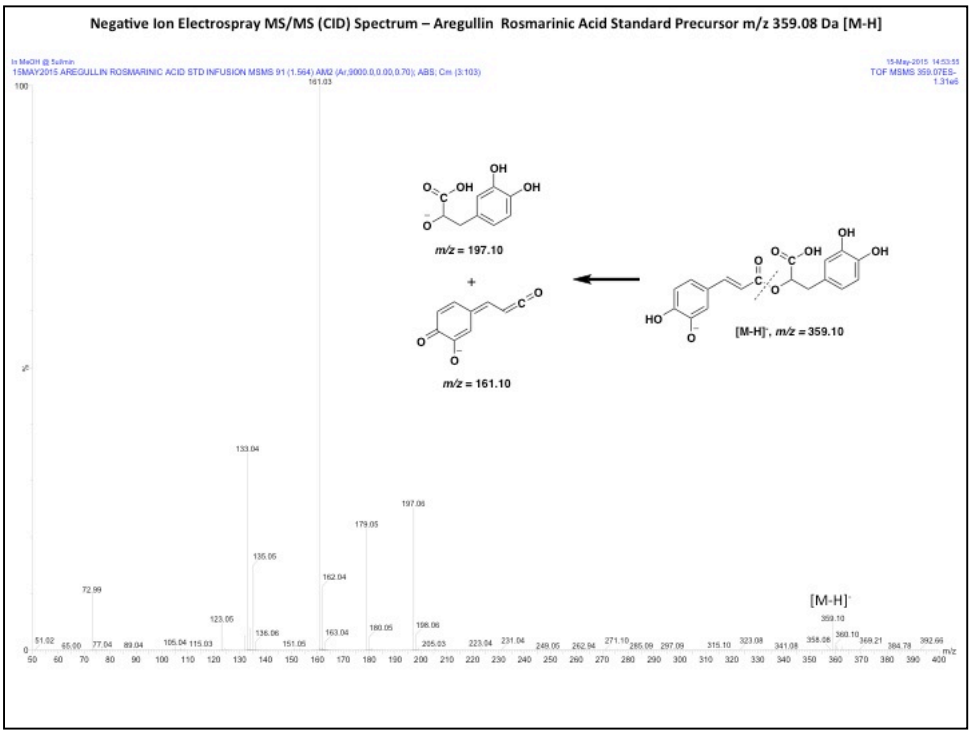


Figure 11. Negative Ion Electrospray MS/MS (CID) Spectrum of Rosmarinic Acid, Standard, Precursor m/z 359.08 Da [M-H]

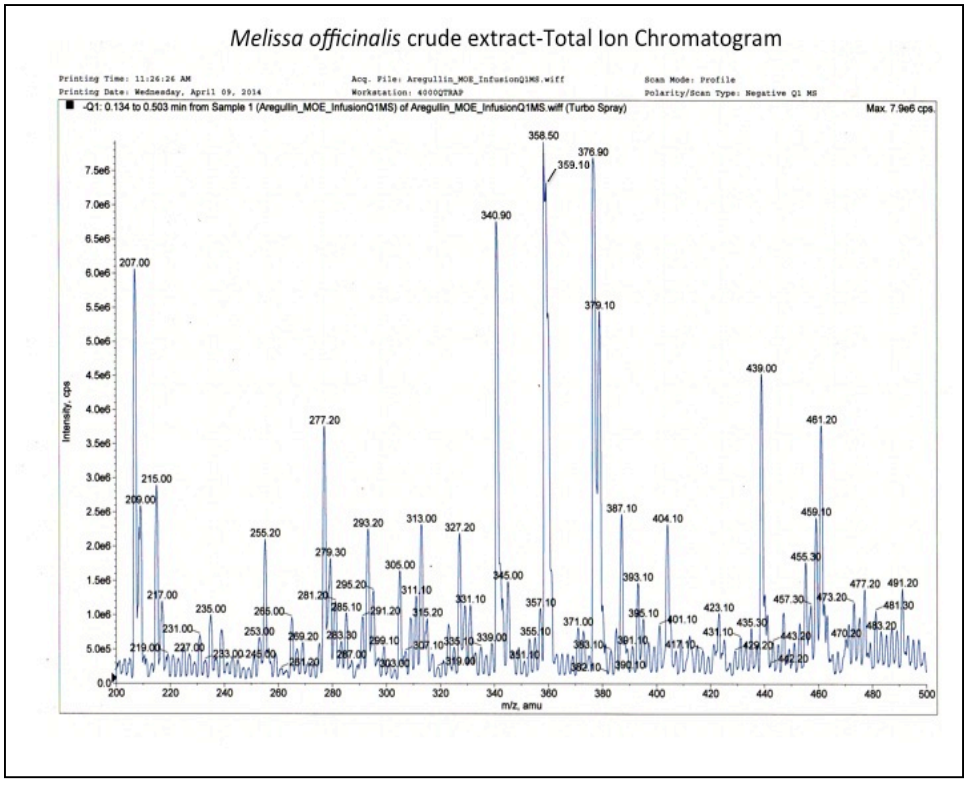


Figure 12. Total Ion Chromatogram of *M. officinalis*, Crude Extract

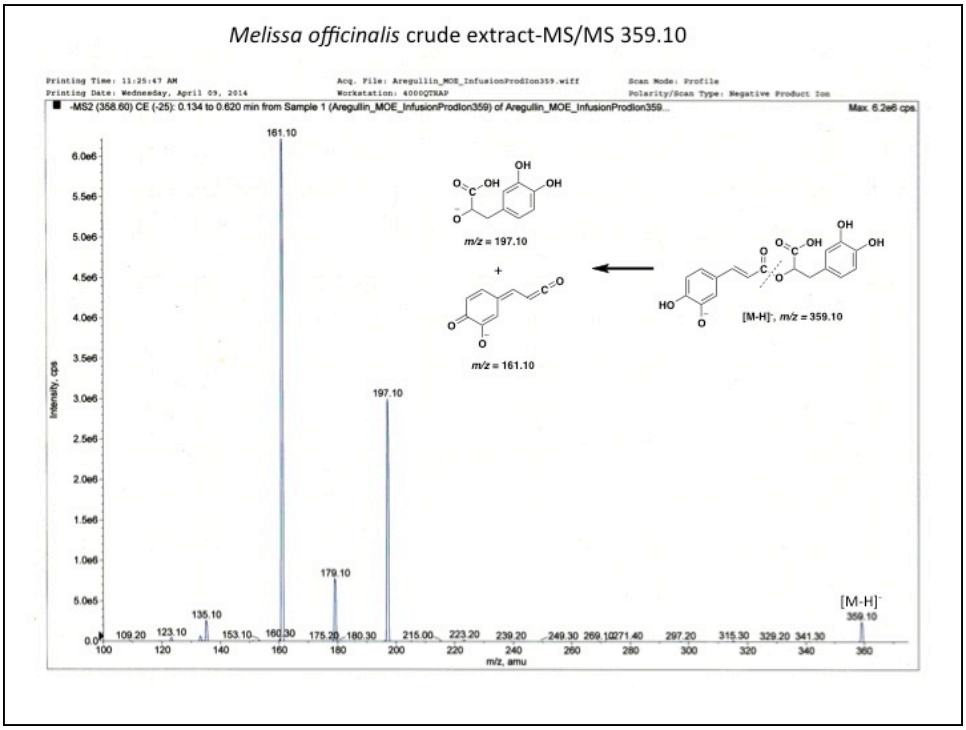


Figure 13. Total Ion Chromatogram of *M. officinalis* crude extract Precursor m/z 359.1 Da [M-H]

1.5 Column Chromatography

Having confirmed the presence of rosmarinic acid in the alcoholic extract of *M. officinalis*, silica gel column chromatography was utilized for the purpose of the isolation and purification of the target compound, rosmarinic acid. It was determined that establishing a solvent gradient would maximize the separation of phytochemicals in the alcoholic extract, and would furthermore allow for the efficient separation of rosmarinic acid. Given rosmarinic acid's behavior on TLC, it was resolved that this solvent system would consist of initially non-polar solvents, and would successively become more polar, which we predicted would eventually result in the elution of rosmarinic acid off the column. Having said this, the chromatographic column was developed by increases in solvent polarity, which resulted from successive 10% compositional changes of mixtures consisting of hexane/ethyl acetate /methanol. Each 10% compositional change consisted of an additional 250mL of solvent added to the chromatographic column. Rosmarinic acid's elution was monitored with the use of a hand-held UV lamp, as rosmarinic acid fluoresces blue under long wave UV light.

Approximately 300 15mL fractions were collected and analyzed comparatively by TLC against rosmarinic acid as a standard for the purpose of composition analysis and to determine which fractions contained compounds associated with the standard (figure 14, 15). The use of long wave and short wave UV light suggested that fractions 277-283 were enriched with rosmarinic acid (figure 14). In light of this, and due to their similar chromatographic behavior on TLC, fractions 277-283 were consolidated and were subjected to further analysis.

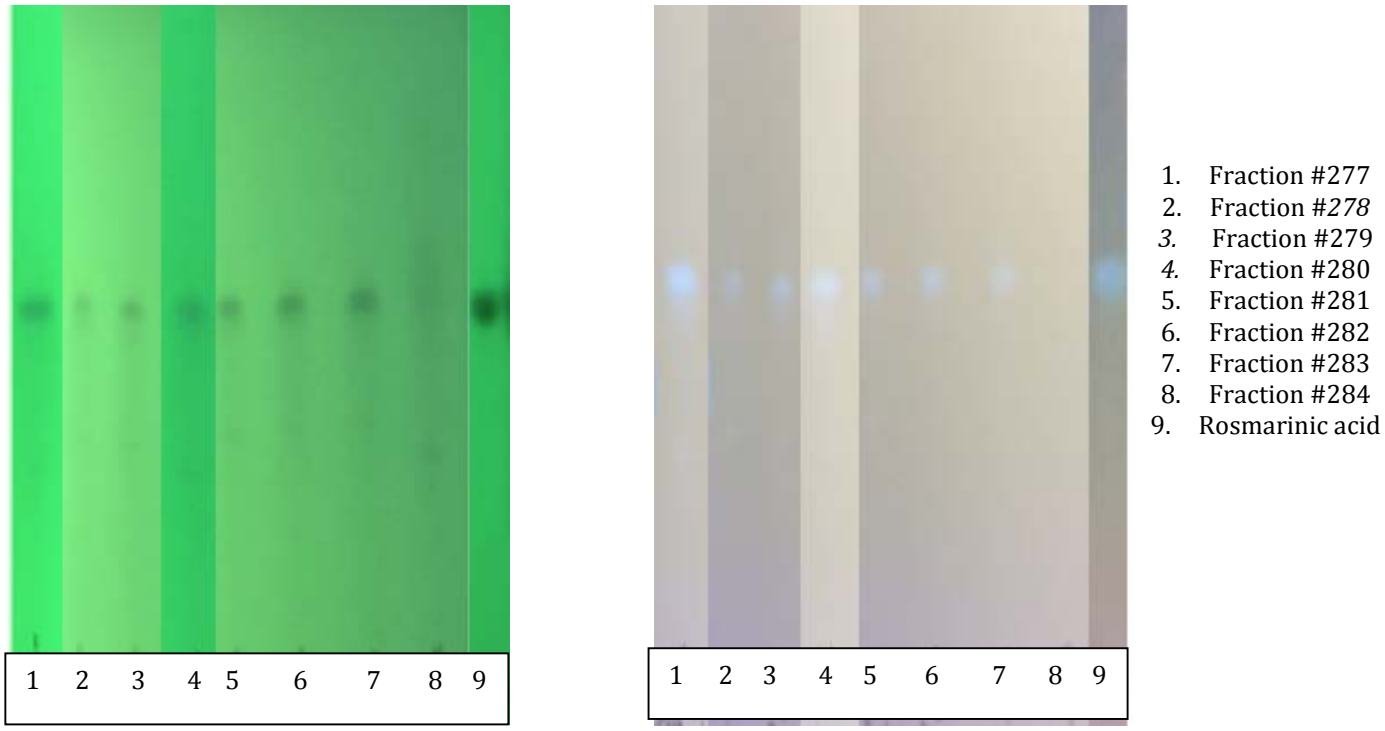


Figure 14. Comparative TLC analysis of fractions 277-283 using short wave (left) and long wave (right) UV light

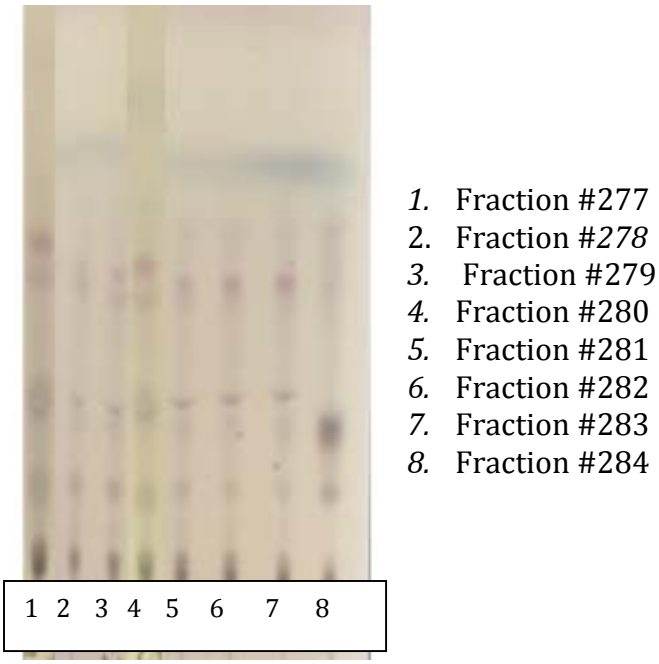
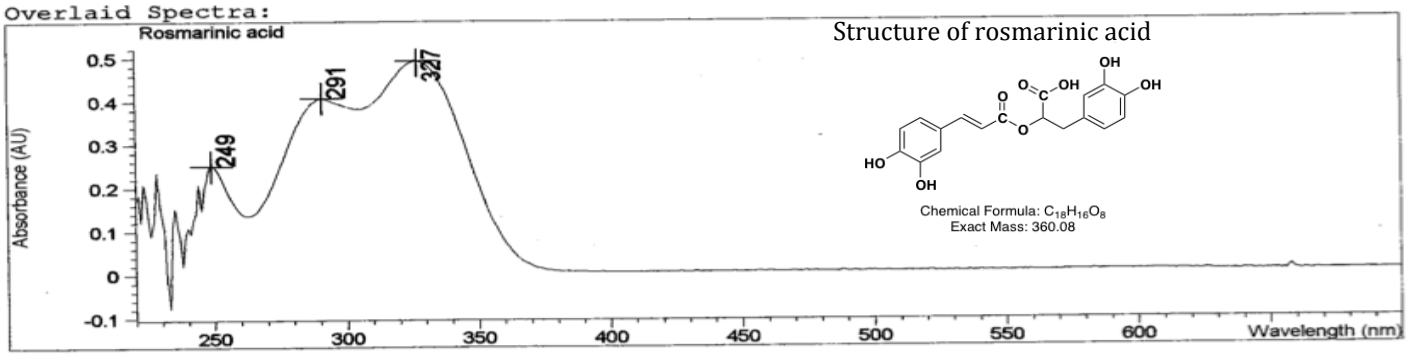


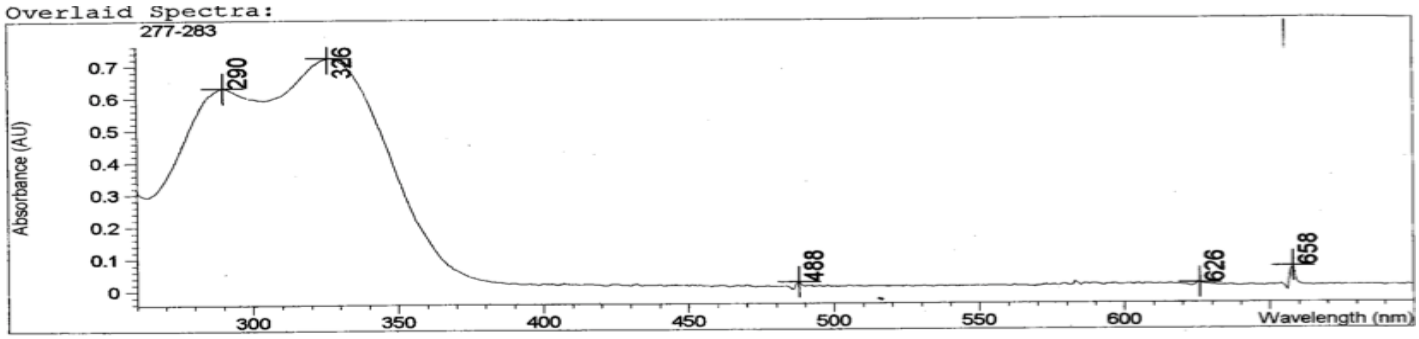
Figure 15. Comparative TLC analysis of fractions 277-283 using vanillin

The previously consolidated fractions were subjected to UV spectrophotometry as a means of further evidencing rosmarinic acid's presence, as rosmarinic acid has a λ_{max} at 327nm, which is cited to be due to its phenolic characteristic and its two aromatic rings. (30). The results of this procedure substantiates the similarities in the chromatographic behavior between fractions 277-283 and rosmarinic acid standard, as the consolidated fractions present a λ_{max} at 326nm, which is in close proximity to rosmarinic acid's characteristic λ_{max} at 327nm (figure 16, 17).



#	Name	Peaks(nm)	Abs(AU)
1	Rosmarinic acid	327.0	0.49181
1		291.0	0.40594
1		249.0	0.25037

Figure 16. UV spectra of rosmarinic acid, standard.



#	Name	Peaks(nm)	Abs(AU)
1	277-283	326.0	0.72457
1		290.0	0.63102
1		658.0	6.8541E-2
1		488.0	2.3973E-2
1		626.0	1.8100E-2

Figure 17. UV Spectra of Fractions 277-283.

Furthermore, the total ion chromatogram of fractions 277-283 in the negative mode also suggest a sample enriched with rosmarinic acid, as the molecular ion peak of *m/z* 359.1 is the most prominent (figure 18). The fragmentation pattern of this precursor ion of *m/z* 359.1 is consistent with the expected fragmentation pattern of rosmarinic acid (figure 10, 11). Therefore, this spectrometric data confirms the presence of rosmarinic acid, which furthermore attests to the efficacy of silica gel column chromatography as a means of eluting rosmarinic acid from the aqueous alcoholic extract of *M. officinalis*.

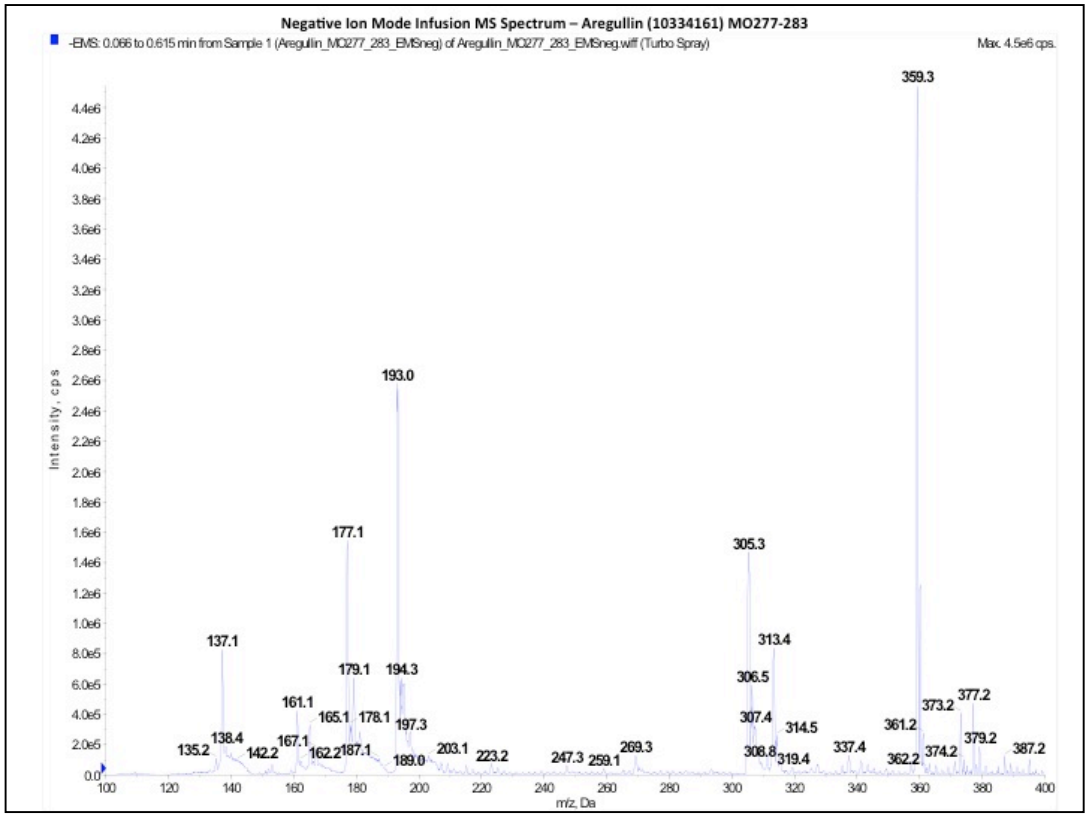


Figure 18. Negative Ion Mode Infusion MS Spectrum- Consolidated sample containing fractions 277-283.

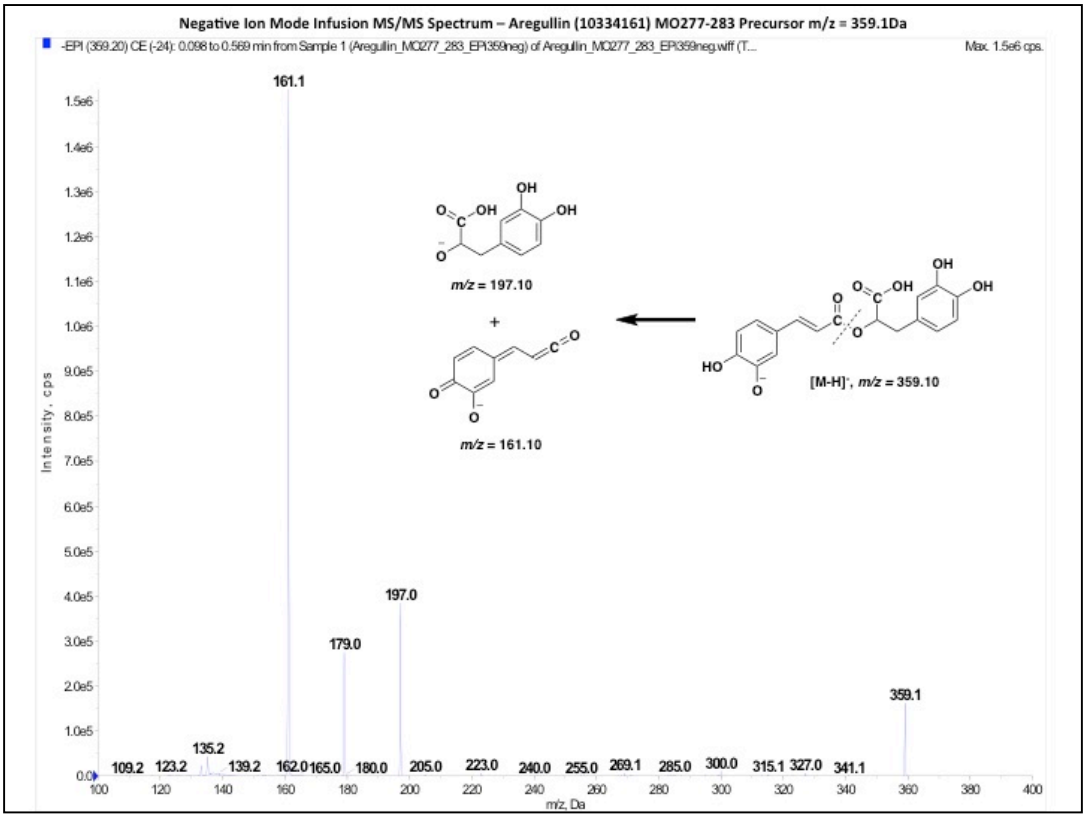


Figure 19. Fractionation pattern of precursor m/z=359.1.

Although the elution of rosmarinic acid from the chromatographic column was confirmed by mass spectrometry, analytical TLC with vanillin as a spray reagent (figure 15) and the total ion chromatogram of the consolidated fractions (figure 18) suggested the need for its further purification.

1.6 Preparative Thin Layer Chromatography (TLC)

Preparative TLC was the analytical procedure of choice to further purify the target compound of the consolidated sample. 2mL of the consolidated sample was loaded onto the HPTLC Silica gel glass plate, with rosmarinic acid used as a standard. After the preparative TLC plate had been fully developed in the 10:2.7:2 ethyl acetate/ methanol/ H_2O solvent system, it was observed that the fractions had separated into three distinct bands. A flat spatula was used to outline these bands and to scrape the three distinct bands off of the TLC plate. Each band was pulverized with a mortar and pestle, and was subsequently extracted with 75mL of methanol. The concentration of these filtered methanolic extracts were increased by evaporating them to a volume of 1.5mL. Afterwards, each 1.5mL solution was subjected to syringe filtration. We presumed that analytical TLC methods utilizing short and long wave UV light would allow for the determination of which 1.5mL syringe filtered solution of the three contained rosmarinic acid. However, results of the assay revealed rosmarinic acid's absence from all three syringe filtered methanolic extracts (labeled band 1, band 2, and band 3), thereby implying that the eluted rosmarinic acid contained in the original consolidated solution containing fractions 277-283 had undergone structural alterations to such a degree that resulted in its inability to be detected during the employment of analytical TLC methods (figure 20).

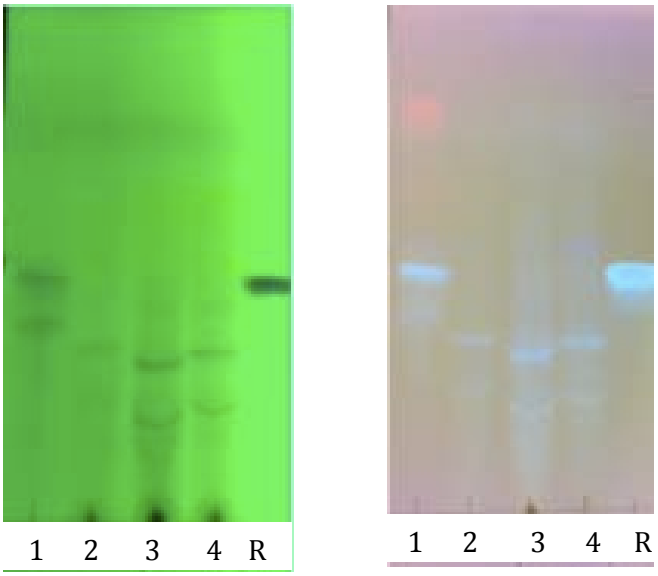


Figure 20. Analytical TLC using short wave (left) and long wave (right) UV light.

The degradation of rosmarinic acid, which understandably led to its loss of bioactivity, is further substantiated in the AChEi bioautographic assay, which was utilized in an attempt to detect AChEi constituents in the syringe filtered solutions derived from the bands collected from preparative TLC (figure 21).

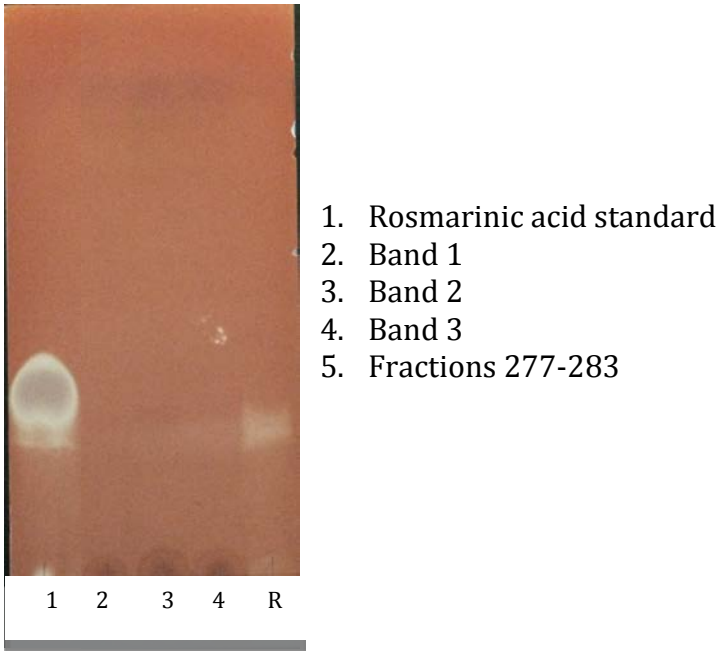


Figure 21. AChEi bioautographic assay

The degradation of rosmarinic acid was confirmed after each syringe filtered solution was subjected to mass spectrometry, as the total ion chromatogram of each solution lacked chemical constituents with an *m/z* value of 359.1, the characteristic ion peak of rosmarinic acid (figures 22, 23, and 24).

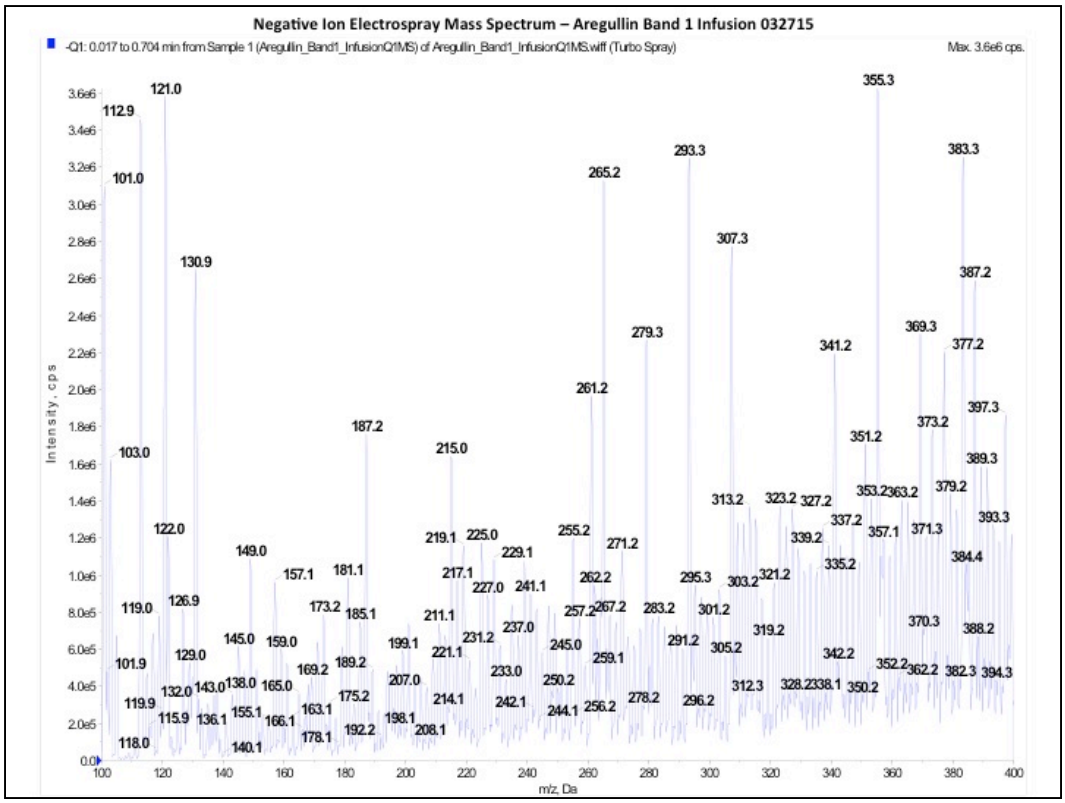


Figure 22. Negative Ion Electrospray Mass Spectrum of band 1.

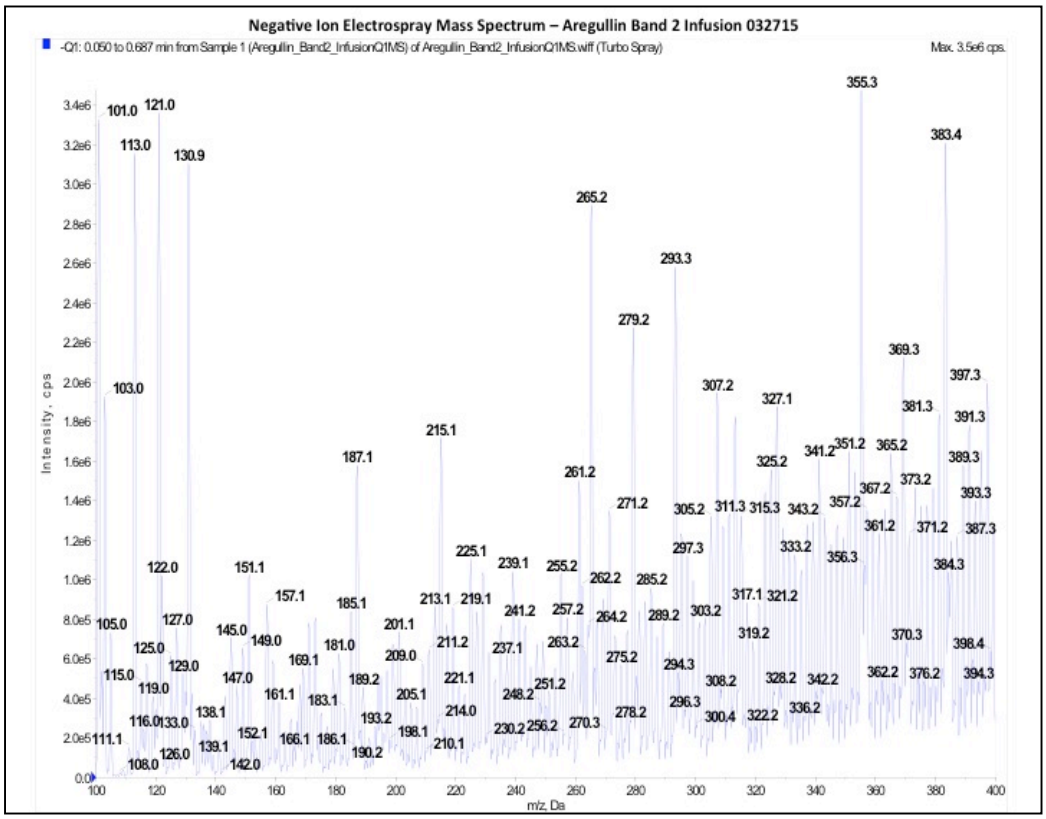


Figure 23. Negative Ion Electrospray Mass Spectrum of band 2.

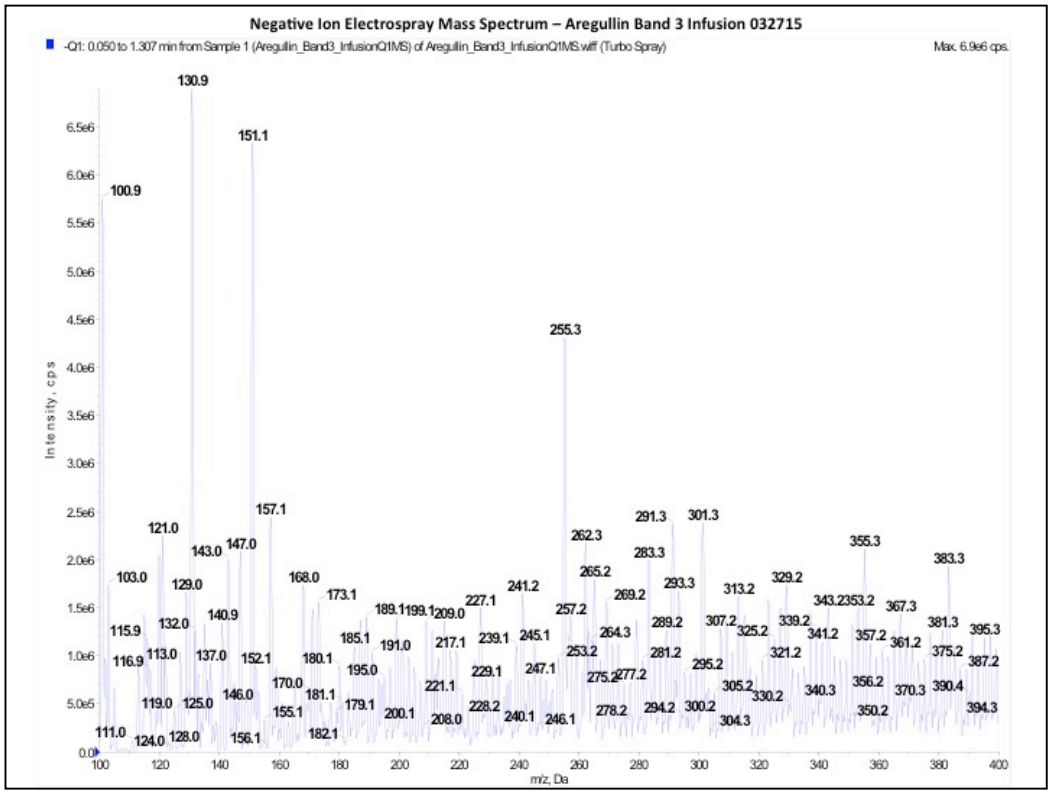


Figure 24. Negative Ion Electrospray Mass Spectrum of band 3.

It was hypothesized that the chemical arrangements that rosmarinic acid experienced was catalyzed either by the fractions' interaction with the silica gel during the extraction of each band in methanolic solution, or as a consequence to the application of heat during the evaporative process. To test these hypotheses, a 50 µL aliquot of RA was transferred to a vial containing 500 µL of methanol and silica gel. To reproduce the hypothesized chemical reaction catalyzed by the silica gel, rosmarinic acid was left in the presence of silica gel for a duration of 20 minutes, as this was the length of time that the bands collected from preparative TLC were extracted for. Another 50 µL sample of rosmarinic acid containing 500 µL of methanol was prepared and was left in the presence of silica gel for 3 weeks. This would allow for the consequences of rosmarinic acid's prolonged exposure to silica gel to be determined. The 3 bands collected from preparative TLC, the samples of rosmarinic acid exposed to silica gel for 20 minutes and for 3 weeks, consolidated fractions 277-283 (from which the 3 bands were originally derived), and a pure sample of RA were all compared and analyzed on TLC (Figure 25). With concerns to the results of this assay, it was no surprise that the consolidated sample containing fractions 277-283 contained compounds associated with rosmarinic acid (lane 3), as this was previously proven through the use of mass spectrometry (figure 18, 19). What was most striking however was not only the apparent loss of chemistry associated with rosmarinic acid (lane 2), but also the apparent product that had formed within the bands collected from preparative TLC (lanes 4-6) and in the sample of rosmarinic acid exposed to silica gel for 3 weeks (lane 7). These qualitative observations confirmed our initial hypothesis with concerns to the silica-gel catalyzed structural arrangements of rosmarinic acid. Silica gel's ability to catalyze structural arrangements is further affirmed by the investigations of Wu et al (29).

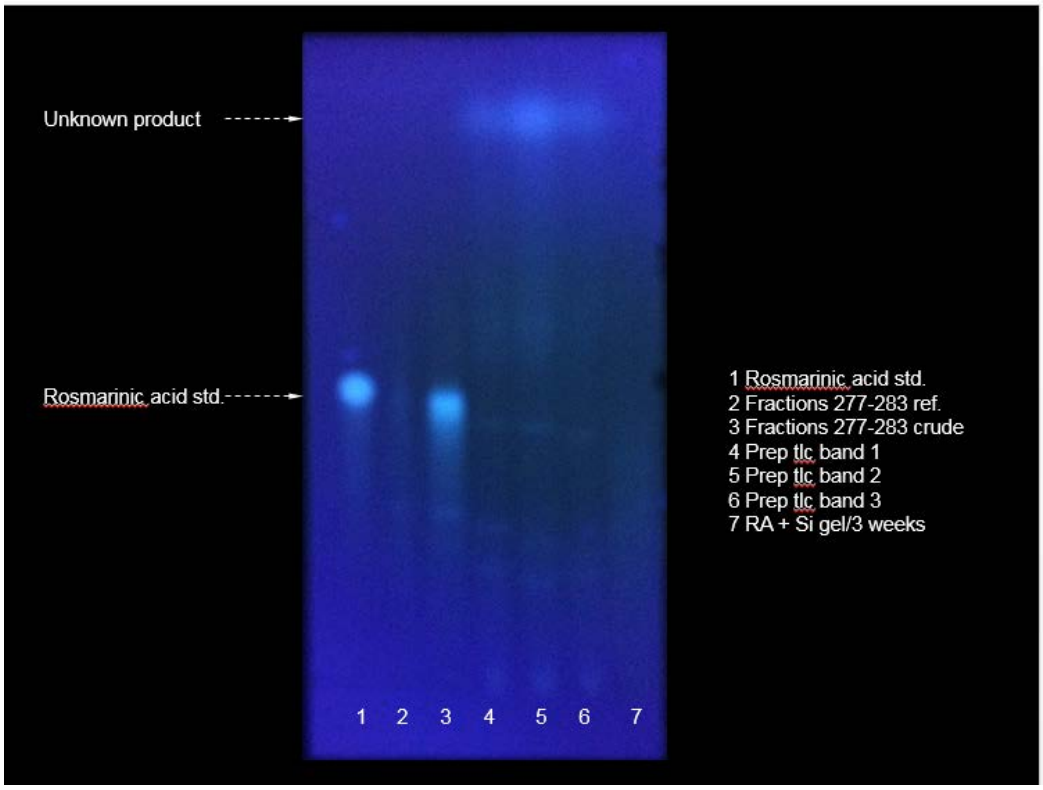


Figure 25. Comparative TLC

As an attempt to determine the structure of the product formed in the presence of silica gel, the sample of rosmarinic acid exposed to silica gel for a duration of three weeks was subjected to mass spectrometry. This method of analysis confirmed the formation of a product, which had an *m/z* value of 385.3, and had a concentration similar to that of the precursor ion with an *m/z* value 359.4, which was confirmed to be rosmarinic acid (figure 27). Having said this, it is deduced that this newly formed product must therefore be a derivative of rosmarinic acid. Additionally, the fragmentation pattern of the precursor ion of *m/z* 385.3 allowed for the structure of the compound formed in the presence of silica gel to be elucidated (figure 28).

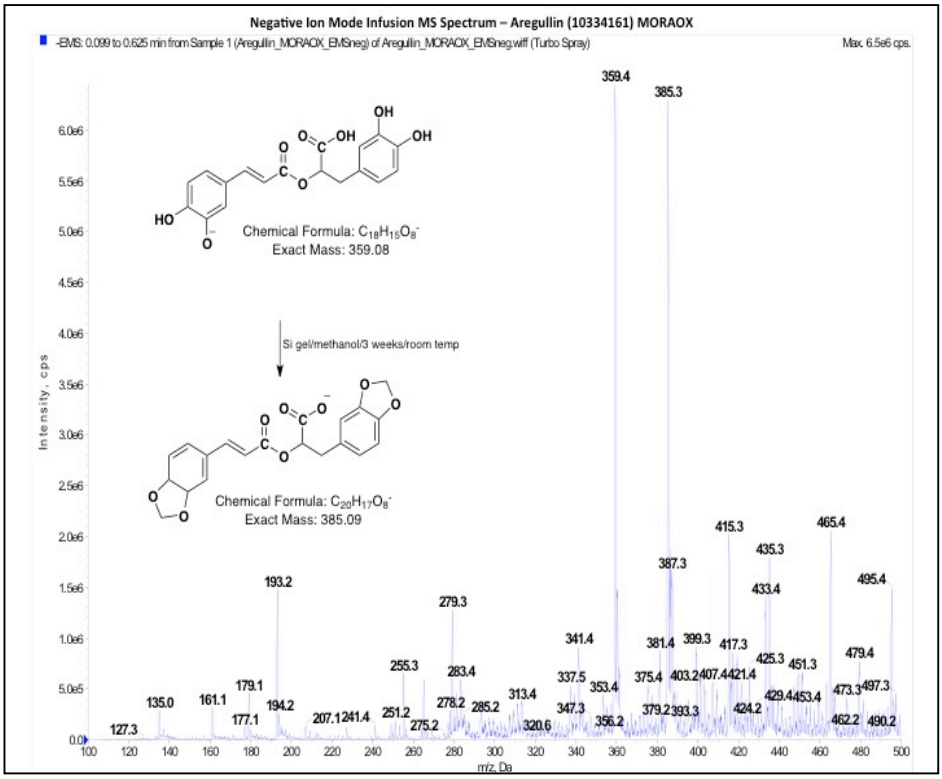


Figure 26. Total Ion Chromatogram of Rosmarinic acid + silica gel solution.

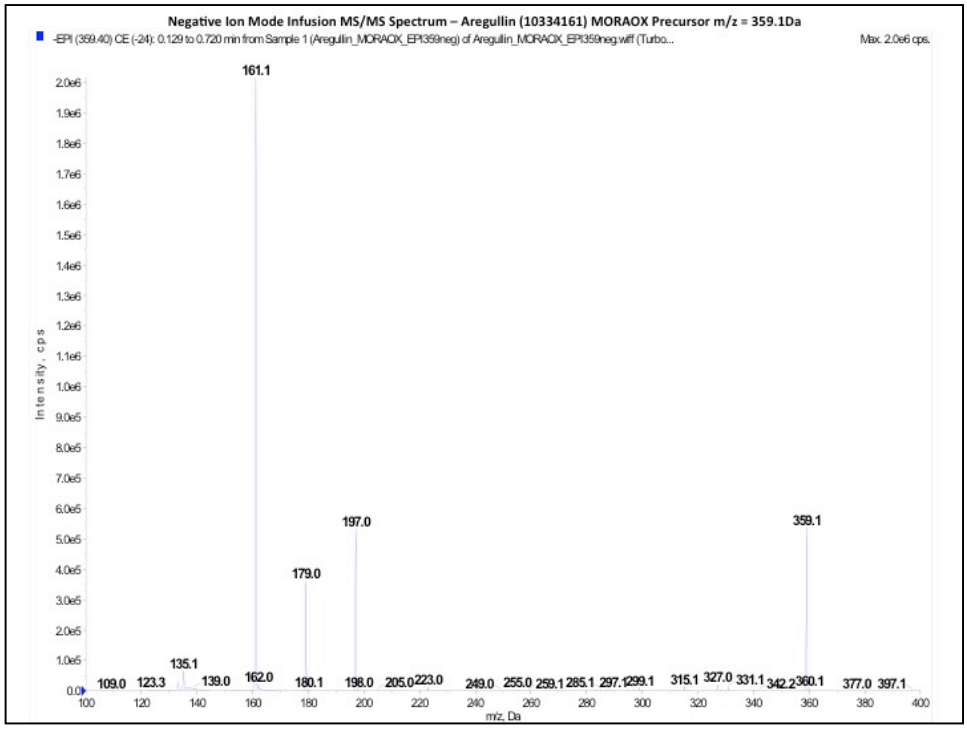


Figure 27. Fraction pattern of precursor *m/z*= 359.1

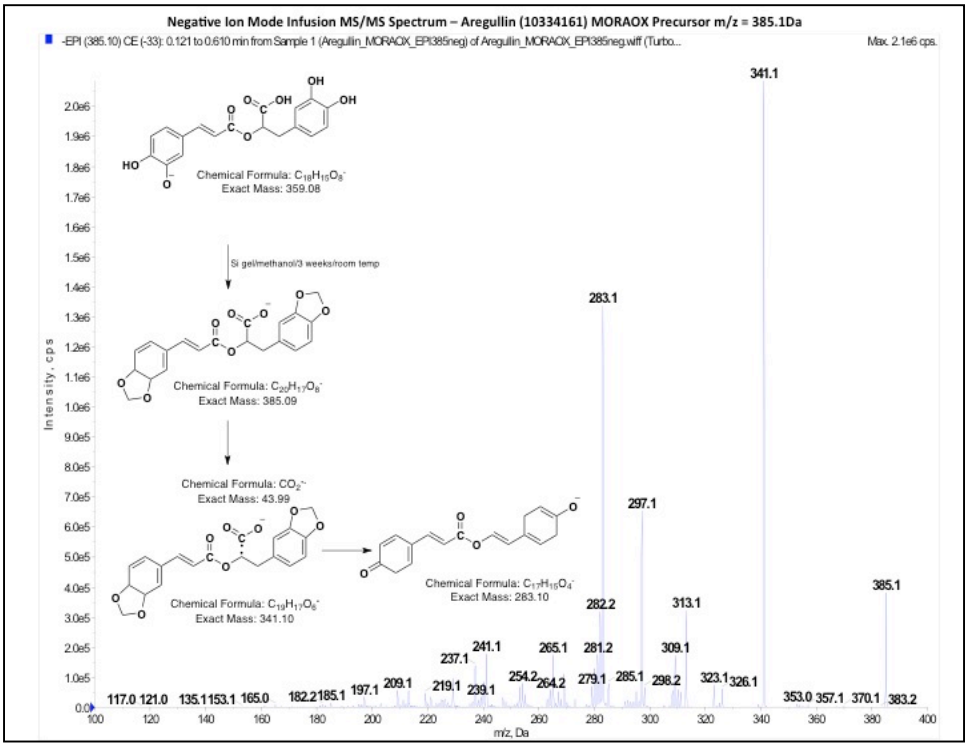
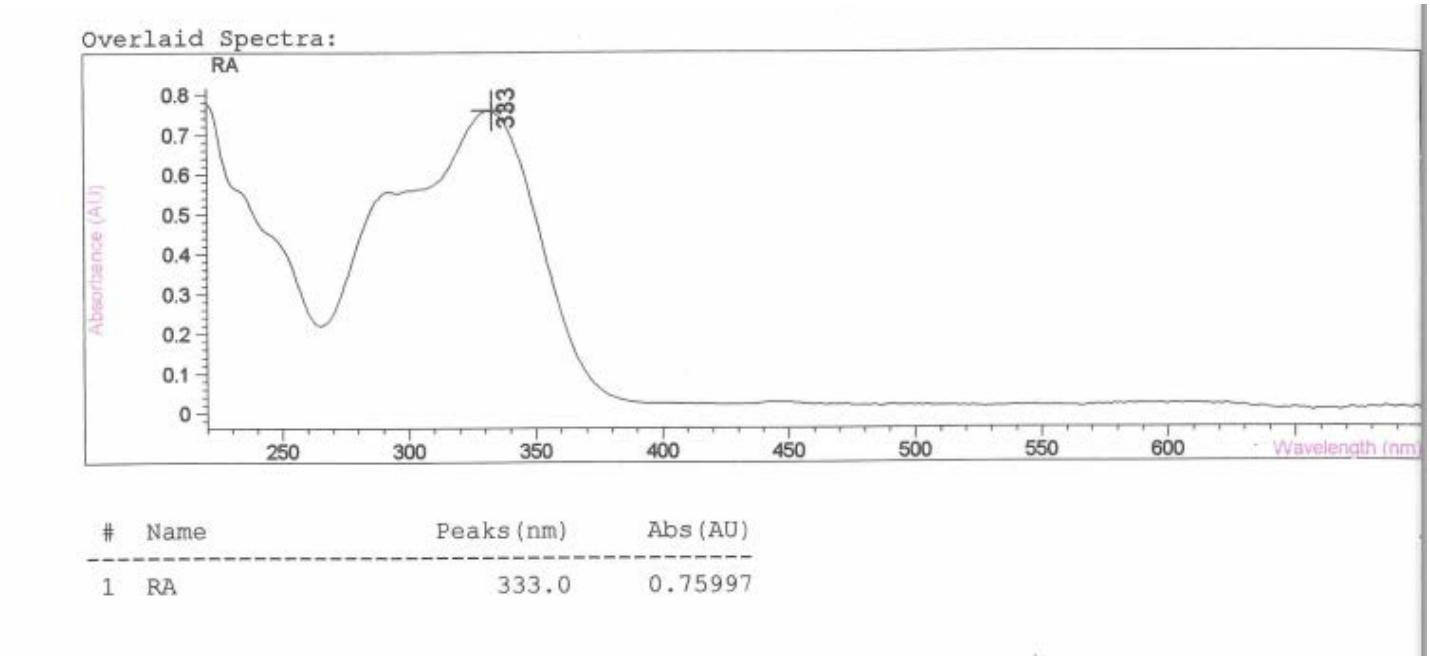


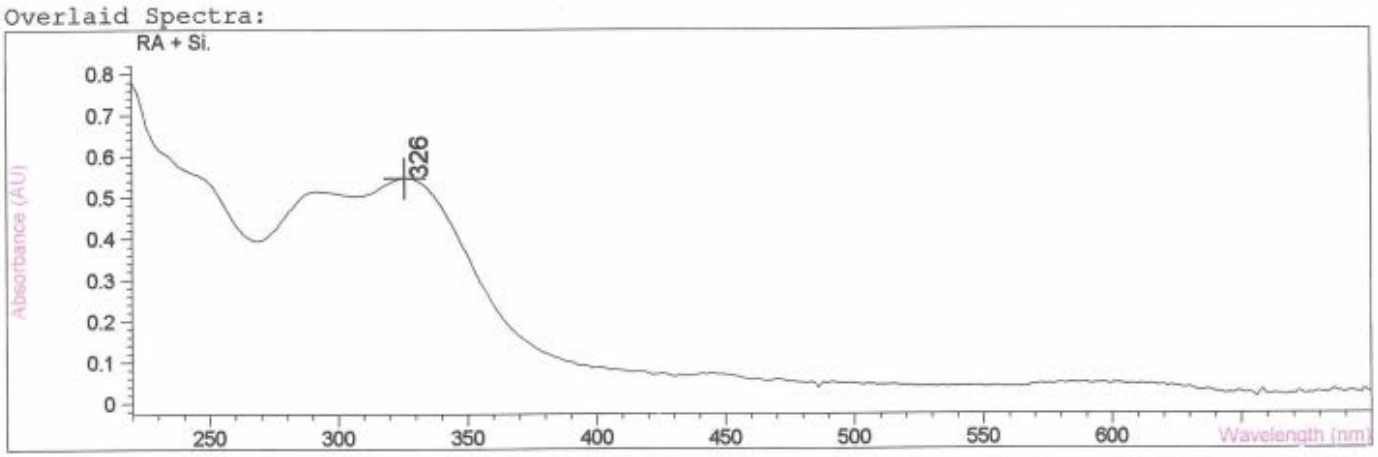
Figure 28. Fractionation pattern of precursor *m/z*=385.1

UV spectrophotometry was utilized as an alternative method to observe the chemical arrangements that occurred as a consequence of prolonged exposure to silica gel. These chemical arrangements would be determined through the degree of change in the λ_{max} of rosmarinic acid + silica gel as compared to the λ_{max} of the rosmarinic acid standard. Chemical arrangements were again substantiated through the decrease in the λ_{max} of the rosmarinic acid solution exposed to silica gel (figure 29, 30). As concentrations were held constant during this method of analysis, the decrease in λ_{max} of the rosmarinic acid + silica gel solution is likely to be a consequence of the change in the molar absorptivity variable of the Beer-Lambert Law, which is proportional to electron delocalization. The reduction of the λ_{max} of the rosmarinic acid + silica gel solution, which alludes to compounds with a reduced capacity for electron delocalization, thereby supports the structure elucidated from the fragmentation pattern of the newly formed chemical constituent with a precursor ion of *m/z* 385.3 (figure 28) .



#	Name	Peaks(nm)	Abs(AU)
1	RA	333.0	0.75997

Figure 29. UV Spectra of rosmarinic acid exposed to Silica gel for 3 weeks.



#	Name	Peaks(nm)	Abs(AU)
1	RA + Si.	326.0	0.54669

Figure 30. UV Spectra of a pure sample of rosmarinic acid.

Chapter II

Antioxidant Potential of the Lamiaceae

Introduction:

Oxidative Stress is defined as the imbalance between the production of oxygen-containing free radicals and the biological system's ability to detoxify, or to quench, reactive oxygen species (ROS) (16). Such ROS include superoxide, peroxide, and hydroxyl radicals. Free radicals are molecular species that contain an unpaired electron in an atomic orbital and are able to exist independently. The unpaired electron allows the species to behave as an oxidant or a reductant, consequentially resulting in the species as a highly unstable and reactive one. Free radicals have the ability to damage cellular components, such as DNA, proteins, carbohydrates, and lipids, ultimately leading to the onset and progression of many diseases (17). Endogenous free radicals have many origins, and can be a consequence of biochemical pathways that include prostaglandin biosynthesis, are implicated in the immune response, and certainly participate in the electron transport chain of the mitochondrion. However, Perry et al. suggests that mitochondrial abnormalities are a major source of oxidative free radicals and oxidative precursors, as abnormal mitochondria may produce excess hydrogen peroxide (H_2O_2) through the conversion of superoxide (18). Alternatively, exogenous factors that lead to the increased concentration of free radicals include X-rays, ozone, cigarette smoking, air pollutants, as well as industrial chemicals (17).

There is a considerable amount of research that has been performed on Alzheimer's disease, linking the effects of oxidative stress to the etiology and pathogenesis of AD. The central nervous system is particularly vulnerable to free radical damage, since the brain has a large oxygen consumption rate, its enrichment with lipids, and the relatively low concentrations of antioxidants, as compared with other systemic tissues. In support of this, Markesbery has collected data that exemplify the fact that AD brain proteins undergo free radical oxidation more so than age-matched control subjects (19). Furthermore, oxidative stress in AD patients is further evidenced by increased peroxidation in ventricular fluid, a microenvironment of transition medals, such as increased Fe, Al, and Hg in the brain, alterations in COX activity in the brain, increases in protein and DNA oxidation in the brain, and aggregations of β-amyloid plaques, all of which contribute to the increased concentrations of ROS (19). Cellular damage by ROS lead to cellular death, by the means of

necrosis or apoptosis mechanisms, leading to deficits in the existence of cholinergic neurons, and the continued progression of AD.

Due to the possible role of ROS in the pathogenesis of AD, antioxidants are considered to be a potential therapeutic component in the treatment of AD. Antioxidants have various modes of actions; they can remove superoxide free radicals, scavenge ROS as their precursor, inhibit ROS formation, and can bind metal transition metals, thereby inhibiting the generation of ROS. Clinical studies have shown that consumption of Vitamin E (2000 IU/d) slows the progression of AD. Additionally, clinical studies have suggested that consumption of vitamin E or C supplements are effective at improving cognitive performance. Due to clinical findings that suggest the efficacy of antioxidants in improving cognitive function, treatment with antioxidants are of benefit for those in early stages of AD, or those who are genetically at risk for developing the disease. Therefore aim 2 of this project is to explore the antioxidant capacity of members of the Lamiaceae plant family.

Materials and Methods:

Antioxidant Assay

Measurements of relative antioxidant capacity of the crude extracts to be analyzed were carried out according Williams et al (20). This technique makes use of the stable free radical, DPPH (2,2-diphenyl-1-picrylhydrazyl), and the capacity of components of crude extracts to quench it. DPPH, a stable and synthetic free radical, is often used as a standard when determining the antioxidant potential of certain compounds. It has a characteristic λ_{max} at 517nm, but can receive an electron or hydrogen from an antioxidant source, resulting in the reduction of its λ_{max} and its conversion to DPPH-H (Figure 29). The antioxidant capacities of compounds are therefore determined by the extent to which the λ_{max} of DPPH is reduced. Experimental data was collected using a spectrophotometer and disposable cuvettes. 0.197 g of DPPH was dissolved in 10ml of a suitable solvent (i.e., ethanol) to prepare a Stock Solution of concentration 5 x 10^{-2} M (molarity was calculated based on an approximate estimate of the average molecular weight of all secondary metabolites in the extract to be 250 g/mol). An aliquot of the DPPH ethanol stock solution was taken and diluted in ethanol to make a Working Solution of concentration 2.5 x 10^{-4} M. Similarly, working solutions of 2.5 x 10^{-4} M were made for all crude extracts to be analyzed in this assay. Before use, the spectrophotometer was blanked using 95% EtOH. Next, 5 mL of the DPPH working solution was added to .5 mL of 95% EtOH. This spectra was used as a zero to which all other readings were compared. Subsequently, .5 mL of DPPH working solution was added to .5 mL of the working plant extract to be analyzed. The decrease in absorbance was determined at 517nm every minute for a duration of 4 minutes.

Results and Discussion:

1.1 Antioxidant Potential of Alcoholic Extracts

The antioxidant capacity of a set of standards and alcoholic extracts of plants of the Lamiaceae plant family were investigated using the conventional DPPH method reported in the literature (20, 23). Antioxidants are of phenolic form and are able to donate hydrogen

atoms from their hydroxyl groups, and are furthermore able to form a stable phenoxyl radical (figure 30). As seen in figure 5 of chapter 1, *M. officinalis* and *S. verticillata* are enriched with phenolic compounds, which are likely to have a profound impact on the antioxidant potential of these species.

The antioxidant capacity of the alcoholic extracts of *M. officinalis and S. verticillata* are assessed using quercetin and rosmarinic acid as positive controls in the DPPH assay. The functionality of the DPPH assay was confirmed after observing successive decreases in absorbance at 517nm when DPPH was exposed to quercetin and rosmarinic acid. *S. verticillata* and *M. officinalis'*s relative antioxidant potential are evidenced, as the DPPH solution was measured to have a reduction in its absorbance at 517nm over time. *S. verticillata*, however, showed a higher free radical scavenging rate when compared to the alcoholic extract for *M. officinalis*, which suggests its more potent antioxidant properties.

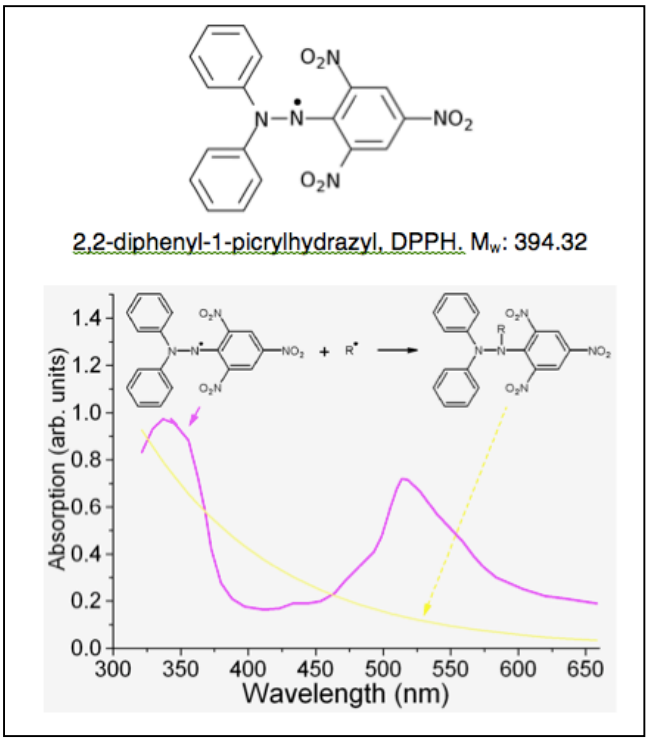


Figure 29. Schematic of the DPPH antioxidant assay.

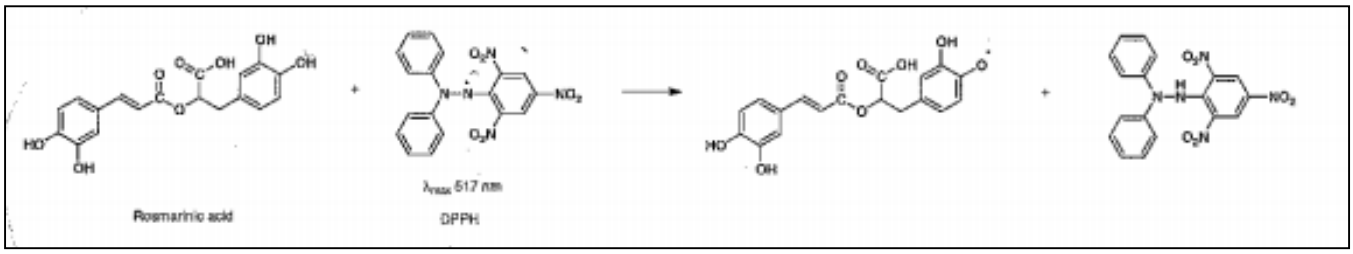


Figure 30. Mechanism of free radical quenching for rosmarinic acid.

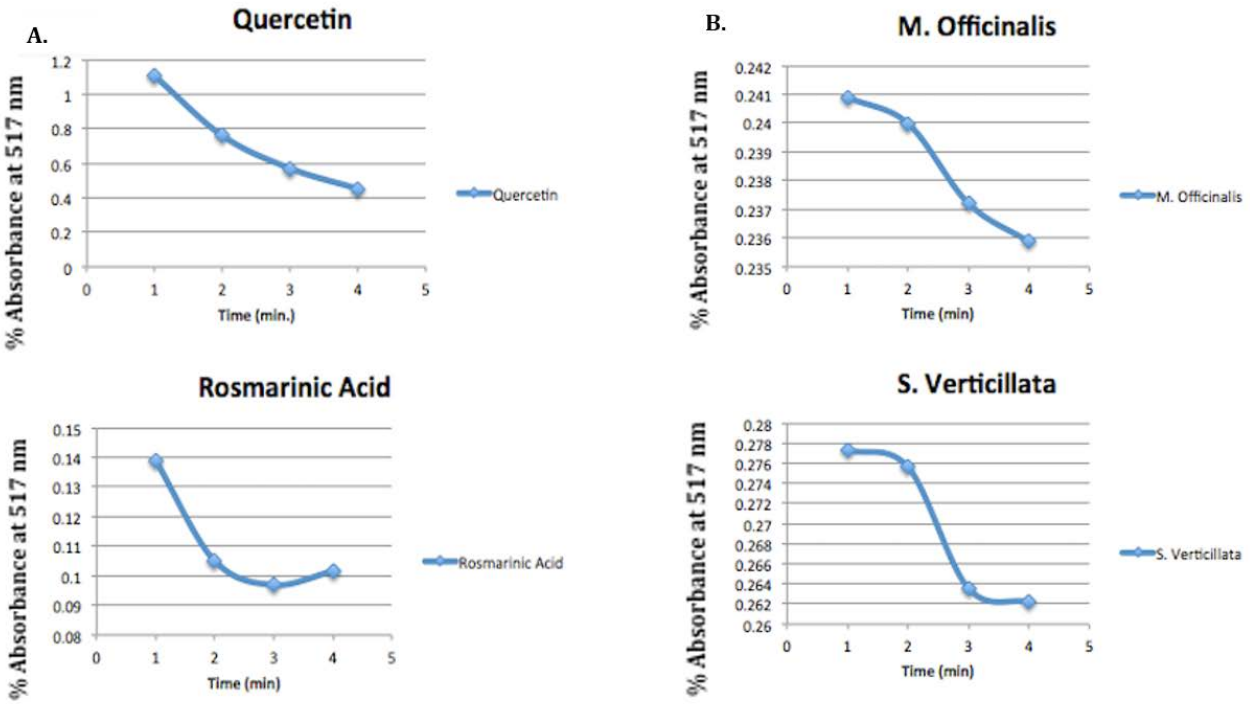


Figure 31. A) Free radical scavenging rates of quercetin, rosmarinic acid B) Free radical scavenging rates of *M. officinalis*, and *S. verticillata*.

Conclusion:

Utilization of the bioautographic assay for acetylcholinesterase inhibitory activity as a guide in the search for novel compounds useful in therapeutics for Alzheimer's disease ultimately led to the identification and targeting of the pharmacologically active compound, rosmarinic acid. This investigation was ultimately a success, as the active compound exhibiting both antioxidant and AChEi activity within the novel nootropic plant, M. *officinalis*, has been successfully isolated and characterized using chromatographic, spectroscopic and spectrometric techniques. Further purification of the isolated target compound was ineffective, as rosmarinic acid experienced silica-gel catalyzed structural changes, resulting in the inactivation of its pharmacological properties, and ultimately led to its degradation. Furthermore, the relative antioxidant capacities of *S. verticillata* and *M. officinalis* has been exemplified in Aim 2 of this investigation, thereby evidencing the antioxidant properties of the Lamiaceae plant family, and furthermore, its usefulness in alleviating the oxidative stress experienced by those who are burdened with Alzheimer's disease.

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