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Chemical Investigation of Camu Camu (*Myrciaria dubia*) Leaves and Roots

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CHEMICAL INVESTIGAION OF CAMU CAMU (*Myrciaria dubia*) LEAVES AND ROOTS

A Thesis

by

JORGE L. FLORES

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Major Subject: Chemistry

CHEMICAL INVESTIGAION OF CAMU CAMU (*Myrciaria dubia*)

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ABSTRACT

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Being the largest on the planet, the Amazon rainforest also has the greatest biodiversity of plant life on earth. In the lowland regions of the Amazon grows the Peruvian camu camu (*Myrciaria dubia*) plant. A short and shaggy shrub, camu camu produces a tasty but sour fruit. Camu camu's berry-like fruit is widely consumed by the native people of the Amazon. Its distinguished richness in vitamin C, as well as the presence of antioxidants, minerals, and nutraceuticals, make it valuable to healthy eating. These qualities have also helped the fruit find their prominence in the international market.

Literature shows that the fruit of the camu camu plant has undergone chemical investigation and pharmacological examination, however, the investigation of neither the roots nor the leaves has been carried out in detail. Besides the fruit, various parts of the plant have been shown to be valuable to Amazonian folklore medicine, including the leaves and the roots. In order to identify new sources of medicinally important or industrially valuable compounds from existing but neglected botanicals, a chemical investigation of the non-polar to polar extracts from the camu camu plant have been conducted.

Ultra High Performance Liquid Chromatography (UHPLC-MS) studies have been performed for the polar extracts of both the roots and the leaves; the chemical composition of these plant parts and identification of several medicinally valuable compounds has been

accomplished. A Gas Chromatography-Mass Spectrometry (GC-MS) study has been performed for a non-polar fraction from the diethyl ether extract of the leaves; 100 compounds were found to be present, 49 known and 51 currently unknown. The isolation of 4 non-polar compounds has been carried out via column chromatography of the same diethyl ether leaf extract; Fourier-Transform Infrared Spectroscopy (FTIR) analysis has been conducted and the compounds have been determined to be pure. This study begins to uncover the privileged molecules from the camu camu plant's leaves and roots that may create useful applications for this untapped yet abundant natural resource.

DEDICATION

The completion of my thesis was made possible through the love and motivation of my family. My father, Dr. Jorge Flores, and my mother, Rossana Flores have always supported me and my effort to complete my degree. Thank you for always being there for me.

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I will always be grateful to Dr. Debasish Bandyopadhyay for his active guidance, unwavering support and constant encouragement. His mentoring has truly been invaluable to my academic accomplishments. I would like to thank my committee members for their advice, time, and guidance; their support has helped make my journey through graduate school possible. I would like to thank Dr. Allen, as department chair, she has always made sure that our laboratory receives the help that is needed to ensure the success of our research. I am very thankful for the help provided by Martha Flores Gallardo during my trip to Iquitos, Peru; it was with her help that I was able to identify and collect camu camu leaf and root samples. Finally, I am grateful to all of my peers who have worked alongside me in the lab and have always been willing to extend a helping hand when needed.

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CHAPTER I

INTRODUCTION

Mother Nature is the oldest reliable source of medicinal and nutraceutical compounds. Today, 25% of commercially available drugs are obtained directly from nature. Furthermore, 61% of drugs produced will either come directly from nature or have been synthesized from a natural product. The use of naturally occurring compounds and their unique cores as the base from which to develop and discover new drugs that can be brought to the market is a highly promising area of drug discovery research.

Declining Plant Biodiversity

In 2017, the number of known plant species across the globe was calculated to be 391,000, with an estimated average of 2000 new species being discovered every year [1]. Surprisingly, it was also found that only about 31,000 of these plants have documentation of human utility, be it as food, medication, poison, animal feed, for recreational use, or as a building material; medicinal use leads this group with 28,187 plants [2]. It is estimated that the Amazon basin alone contains up to 40,000 different species of plants [3]. Unfortunately, with the Amazon experiencing the highest rate of deforestation out of any rainforest in the world, these same models predict that 0.7% to 1% of the Amazon is disappearing every year. Furthermore, it has been estimated that by 2050 climate change and deforestation threaten up to 58% of species richness in the Amazon [4]. The threats facing plant life in the Amazon are astonishingly high; it

is of great urgency that actions take place to slow down these losses. Additionally, initiatives must take place to analyze these plants' potential as medicines and tools before they become scarce or no longer available.

Chemical Investigation Initiative

The goal of conducting a chemical investigation into an unstudied plant part is to be able to describe its chemical composition and to potentially identifying a possible new source of medicinally or industrially valuable compounds. From the early 1940s until 2010, the Federal Drug Administration (FDA) and comparable organizations in other countries approved 175 small molecules worldwide for anticancer use; of these 175 compounds, 131 (74.85%) were non-synthetic and 85 (49%) were either chemically modified natural products, or directly derived from nature [5]. The use of naturally occurring compounds as the basis from which to develop and discover new drugs that can be brought to the market is a highly promising area of drug discovery research. With the ongoing decline of the richest sources of biodiversity in the world, such as the Amazon, it is of great importance that researchers turn towards these overlooked goldmines before they disappear. By searching for these ignored plants and studying their chemistry, we may stumble upon a new source. The discovery of medicinal, industrial and commercial value in a plant part that was previously ignored creates the incentive for further studies into the plant as well as the conservation of these plant and its environment.

CHAPTER II

REVIEW OF LITERATURE

Medicinal Plants

Mother nature is the most experienced chemist capable of producing diverse molecules in a highly selective manner. Plants biosynthesize natural products as a part of their defense mechanism; these various biosynthetic routes have been developed and refined through millions of years of evolution. Eventually, humans use these plant-derived compounds as remedies for various ailments. For instance, the use of precursors such as TMCA (Figure 1) found in ancient Chinese herbs known as *Polygala tenuifolia* and *Gastrodia elata* whose ester derivatives may have anticonvulsant activity [6]. The ester derivatives of this precursor are synthesized and evaluated for their anticonvulsant and sedative properties. Natural products allow a plant to effectively compete and survive in its environment. Compounds provide a plant its antifungal, antimicrobial, poisonous, or toxic properties to defend against disease or consumption. Knowledge of these properties forms the basis of ethnomedicines worldwide, such as the cultivation done by Tibetan people for thousands of years resulting in the establishment of their regional medical system, *Sowa Rigpa* [7]. Additionally, plants may develop compounds that are beneficial to other organisms, promoting the consumption of the plants' fruits and seeds, allowing it to increase the distance over which its propagules may spread [8].

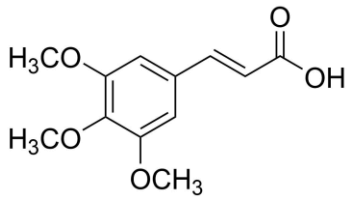


Figure 1. 3,4,5-trimethoxycinnamic acid (TMCA, 1) Traditionally Found in Chinese Herbs

Humans & Plants

Natural products share an intimate relationship with human civilization. Identifying the exact moment when humans first began to collect plant materials for medicinal benefit would be impossible to date; nature has been the provider of medicine for far longer than civilization has existed. *Pan troglodytes*, our closest living relatives, are known to consume plant material with known medicinal properties when experiencing parasitic infections [9]. This type of activity is done by younger chimps that learn through observation of their healthy adult members [10]. Self-medication is not limited to mammals; ants that have been infected by fungal pathogens will selectively consume harmful reactive oxygen species that are avoided by healthy ants in an effort to increase survivability [11]. When it comes to human beings, evidence of our ties with naturally derived medicine extends as far back as 60,000 years and currently 25% of commercial drugs are extracted from nature [12]. Evidence of the development and use of medicines from nature to treat ailments can be observed throughout all human cultures [13]. It was not uncommon for early humans to have experienced various difficulties in determining which plants were treasured with medicines. Consuming plants unknown to them would result in a medley of side effects, from vomiting and diarrhea to death. But it was only through this trial and error that early knowledge about which plants could be used to treat particular illnesses, that the first natural medicines were discovered [14].

Camu Camu

Describing Camu Camu

Peruvian camu camu (*Myrciaria dubia*) is a short bushy tree (Figure 2) that produces small round fruit which are sweet and sour in taste. The shrub is indigenous to the Amazon rainforest and can typically be found growing in the lowland regions of the Amazon alongside the Amazon river, from Peru to Brazil. The people native to the Amazon with knowledge of its sustenance utilize the plant's berry-like fruits by either eating them or more typically infusing them into cocktails, juices, and purees. The fruit has a remarkably high vitamin C content, as well as an array of antioxidants, flavonoids and other beneficial nutraceuticals that make it beneficial to ones health [15]. Camu camu fruit has seen a rise in popularity that has facilitated its prominence in international trade; you can find camu camu fruit as a powder or tablet sold in supplement and grocery stores worldwide. The nutritional benefits of the camu camu fruit have been well established [16]. Nevertheless, several in depth investigations into the fruits potential medicinal value are being pursued [17][18]. These advancements in our understanding of the fruit's properties are promising and warrant further investigation into other parts of the plant.

Figure 2. Camu Camu Tree

Unstudied Parts of Camu Camu

Ethnopharmacological studies have been conducted in which native people of the Amazon region were surveyed regarding the use of botanicals for shamanistic or ritualistic purposes, or in folklore medicine; surveys revealed that several different parts of the camu camu plant (stems, leaves, roots, seeds, and bark) besides the popular fruit are traditionally used in folklore practice for the treatment of various ailments [19]. These findings give promise to the success of chemical investigation of the camu camu. While folklore medicine is not directly indicative of the presence of medicinal compounds in a plant, the discovery of medicinally privileged compounds would explain the enduring belief in a plants healing benefits passed down through generations of people native to the region in which the plant grows. For these reasons, along with the lack of publications available regarding the chemical composition of either plant part, the leaves and roots of the camu camu plant were chosen as suitable candidates for a chemical investigation.

Reliable Technique for Camu Camu Investigation

A bioactivity-guided cold extraction using solvents ranging from non-polar to polar is a reliable method for creating the extracts from which this study can be conducted [20]. This is done separately for the leaves (Figure 3) and the roots (Figure 4)in order to fractionize their compounds into 8 polarity-based extracts, 4 for each plant part. If a non-polar solvent is used in an extraction, then it is expected that the compounds that are extracted will be non-polar; similarly, a polar solvent will extract polar compounds. Fractions can then be individually subjected to different forms of analysis that are most appropriate based on their polarity. These chemical constituents are characterized, identified and described in order to determine those that

provide therapeutic effects and those that have commercial or industrial value. Therefore, by looking deeper into the leaves and the roots, a more holistic understanding of the camu camu plant's overall chemical makeup. It is expected that the completion of a thorough study may reveal the presence of compounds that have industrial, commercial, and or medicinal value. The identification of medicinally and biologically relevant molecules could begin to explain the healing benefits described in Amazonian folklore.

Figure 3. Camu Camu Leaves

Figure 4. Camu Camu Roots

CHAPTER III

METHODOLOGY AND FINDINGS

This chapter provides the outline for the methods used to identify compounds present in camu camu leaves and roots – beginning with the methods used to process fresh leaves and roots into various dry extracts, the characterization methods used to identify compounds present in the extracts, and data analysis techniques used. Descriptions and organization of all notable compounds that were found as well as limitations of the research are provided.

Creating Extracts

Fresh Plant Parts to Powder

Camu camu trees were identified in Iquitos, Peru with the help of locals that are familiar with the plant's whereabouts and appearance. Once camu camu plants were found, 4.0kg of fresh leaves and 3.0kg of fresh roots were harvested from multiple trees in order to ensure diversity within the sample. The collected plant parts were air-dried in the absence of sunlight over the course of one week; shuffling and flipping once a day allowed for even drying (Figure 5). After being grinded to a powder (Figure 6), 1.64 kg of dry leaves and 1.05 kg of dry roots were recorded as the final weights of each plant part.

Figure 5. Dry Leaves

Figure 6. Pulverized Leaves

From Powder to Extract

From the 1.64 kg of powdered leaves, ??? g were stored, the remaining ??? kg is used to create extracts. From the 1.05 kg of powdered roots, ??? g were stored, the remaining ??? g is used to create extracts. The following process was repeated a total of eight times; four times for the leaves, four times for the roots, beginning with the least polar solvent to the most polar solvent, creating a total of eight extracts. Powdered plant material is added to an empty 4L glass bottle and solvent is added until the powder is fully submerged. Bioactivity-guided cold extraction method is conducted in conjecture with sonication for a total of 21 hours (Figure 7). The mixture of solvent and plant materials is vacuum filtered (Figure 8) to collect the extract-containing solute. Additional solvent is passed through the funnel to rinse the plant materials; this is done until the color of the solute coming out of the funnel is clear, signifying that all extract for the corresponding solvent has been extracted. The solvent is removed using a rotary evaporator. The remaining extract is placed in a desiccator for 72 hours in order to ensure that trace amounts of solvent are removed. This process is repeated using hexanes, dichloromethane, methanol, and finally water for the roots. Diethyl ether, dichloromethane, methanol, and finally water is used for the leaves. The use of low to high polarity solvents will separate the lowest polarity

constituents into hexanes, diethyl ether, and dichloromethane; the most polar constituents will be found in methanol and water. After the mass of each extract is recorded, the 8 extracts are securely sealed and placed in a freezer at -20^{o}C until they are analyzed. This is done to prevent the decomposing of compounds. Table 1 shows the mass of each extract that was obtained as well as percent by mass relative to the original weight of the dry plant materials.

Figure 7. Sonication Setup


Figure 8. Vacuum Filtration

Table 1. Weight and Percent by Mass of Dry Camu Camu Extracts

Extraction Solvent	Leaves	Roots
Hexanes	-	3.06
Diethyl Ether	101.0 g	-
Dichloromethane	5.44 g	7.23 g
Methanol	150.4 g	103.1 g
Water	50.37 g	25.47 g

Leaf Water Extract UHPLC-MS Methods

Preparation of Sample

To prepare powdered leaves for ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS) the following preparations took place. 50 mg of lyophilized water leaf extract was mixed with 500 µL HPLC grade methanol (containing 5 µg/mL 2-Chloro-L-phenylalanine as an internal standard). Solution is then mixing with vortexing for 1 min, the mixture is centrifuged at 12, 000 rpm, 4°C for 10 min. The supernatant is then filtered through a 0.45 µm filter and transferred to sampler vial for detection.

UHPLC-MS/MS Analysis of Sample

The Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6545 UHD and Accurate-Mass Q-TOF/MS was used for UHPLC-MS analysis. For the chromatographic column, Waters® XSelect HSS T3 (2.5 µm 100*2.1 mm) was used. The following conditionss were used to run the samples.

- Mobile phase:

  A: aqueous solution with 0.1% formic acid.

  B: HPLC grade acetonitrile solution with 0.1% formic acid.
- Flow rate: 0.35 mL/min.
- Column temperature: 40°C.
- Injection volume: 1 µL in positive mode & 2 µL in negative mode.
- Gradient elution condition optimized: 0-2 min, 5% B; 2-10 min, 5-95% B; 10-15 min, 95% B.
- Post time was set as 5 min for system balance.

Mass spectrometry was operated in the negative ion mode with the following parameters:

- Capillary voltage: 3.5 kV.
- Drying gas flow: 10 L/min.
- Gas temperature: 325°C.
- Nebulizer pressure: 20 psig.
- Fragmentor voltage: 120 V.
- Skimmer voltage: 45 V.
- Mass range: m/z 50–3000.

Data Analysis

Raw data was converted to the common (mz.data) format by Agilent Masshunter Qualitative Analysis B.08.00 software. In the R software platform, the XCMS program was used in peak identification, retention time correction, automatic integration pretreatment. Visualization matrices containing sample name, m/z-RT pair and peak area were obtained, from which tables were created.

Leaf Water Extract UHPLC-MS Results

Analysis of UHPLC-MS data revealed a series of medicinally important drugs and drug precursors, as well as various important nutraceuticals, and other common plant metabolites. UHPLC-MS was conducted in both the positive ion and negative ion mode; therefore, the results have reported separately. 614 features were obtained in the positive ion mode and 101 features were obtained in the negative ion mode. The total Ion Flow Chromatography (TIC) for negative ion mode is seen in Figure 12, the TIC for positive ion mode is seen in Figure 13. In order to better understand the leaves' chemical composition, both sets of compounds that were identified

were classified based on their class and percentages for each class was calculated. This was done for the set of compounds found in the positive ion mode (Figure 14) and compounds found in the negative ion mode (Figure 15).

Negative Ion Mode

Of the 101 components identified in negative ion mode, the 23 most abundant and notable compounds are listed in Table 2. The table lists the compounds' m/z Ratio, molecular weight, and peak area; the peak area provides an indication of the relative abundance of each compound within the water extract from the camu camu leaf. Any medicinal, commercial and nutraceuticals properties that these compounds possess can be described as followed: A first-generation cephalosporin antibiotic drug (Cephaloglycin), a phytodynamic therapy supporting photosensitizer (methoxsalen), an insomnia treating drug (Etizolam), an immunomodulator and antiviral drug (inosine), antioxidants (ellagic acid, plastoquinone-3), a basal cell carcinoma and genital warts treating drug (imiquimod), an antihypertensive (metyrosine), a compound with antibacterial activity (azelaic acid), an antimicrobial terpenoid (trishitin), and a sclerosant drug (sodium tetradecyl sulfate). Several known nutraceuticals and food ingredients were also found, these include the following: S-propyl 1-propanesulfinothioate, a plant non-protein amino acid (ascorbalamic acid), an anticancer compound (gingerol), humectant (pyroglutamic acid), 5-(Methylthio)-2-[(methylthio)methyl]-2-pentenal, 1-phenyl-1,3-nonadecanedione, pubescenol], a wine ingredient (ethyl 2-mercaptopropionate), a food flavoring agent (fragransol C), a bioactive alkaloid (aspidospermine), an antibacterial and bio-relevant compound (patulin), a phenolic derivative (pyrocatechol), a catabolite of vitamin B6 (4-pyridoxic acid), flavonoids, and bioactive pteridine derivatives. Notable structures from this group of camu camu leaf derived compounds can be seen in Figure 9.

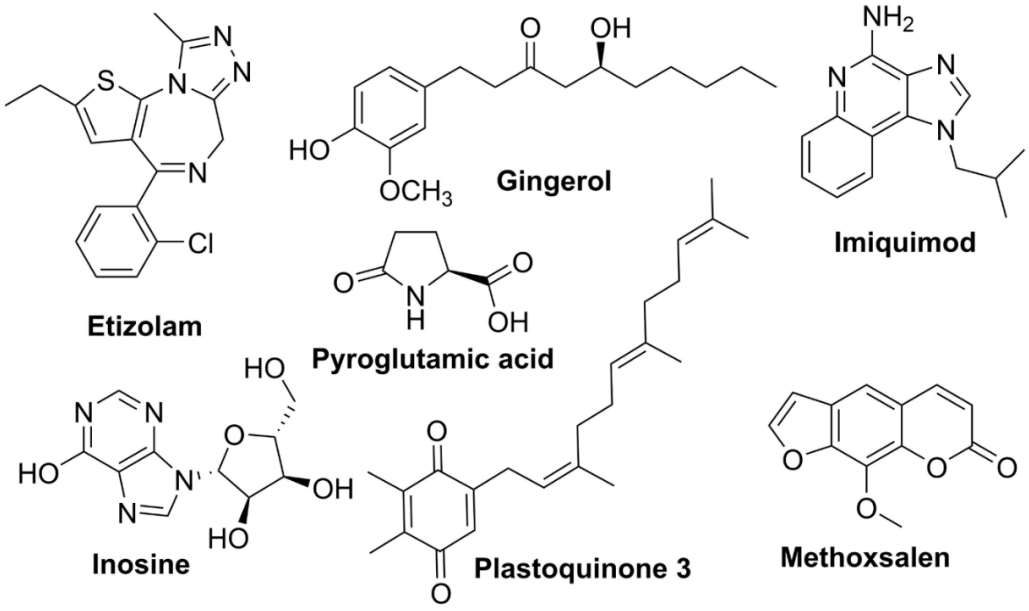


Figure 9. List of Structures for Medicinal Compounds Identified in UHPLC-MS Negative Ion Mode Analysis of Leaf Water Extract

Table 2. Top 23 Leaf Compounds Identified With UHPLC-MS Analysis in Negative Ion Mode

m/z	RT(min)	Peak Area	Molecular Weight	Name	Formula
339.2347	10.97	24560096.36	340.2402	Plastoquinone 3	C23H32O2
353.2138	10.21992	3820762.421	354.2307	(-)-Aspidospermine	C22H30N2O2
109.0297	4.2475	1733450.359	110.0368	Pyrocatechol	C6H6O2
301.0003	5.141001	1251963.668	302.0063	Ellagic acid	C14H6O8
293.1772	8.010885	1066081.873	294.1831	Gingerol	C17H26O4
189.0411	1.889464	853679.7895	190.0486	5-(Methylthio)-2-[(methylthio)methyl]-2-pentenal	C8H14OS2
165.041	0.746	843897.0843	166.0486	S-Propyl 1-propanesulfinothioate	C6H14OS2
153.0197	3.456943	594075.4759	154.0266	Patulin	C7H6O4
262.0581	0.771414	370099.9631	263.0641	Ascorbalamic acid	C9H13NO8
293.178	11.59932	277608.8223	294.1865	Sodium Tetradecyl Sulfate	C14H30O4S
194.083	8.477078	179280.0442	195.0895	Metyrosine	C10H13NO3
473.2842	13.33323	148860.2943	474.2981	Pubescenol	C28H42O6
239.1299	8.354	140431.2146	240.1375	Imiquimod	C14H16N4
187.0981	5.692993	77932.10306	188.1049	Azelaic acid	C9H16O4
128.0356	1.071622	76725.61701	129.0426	Pyroglutamic acid	C5H7NO3
221.1555	9.458	56625.26783	222.162	Rishitin	C14H22O2
341.0635	0.921977	46485.38846	342.0706	Etizolam	C17H15ClN4S
215.0327	0.735	36834.87598	216.0423	Methoxsalen	C12H8O4
133.0328	1.368326	36322.89091	134.0402	Ethyl 2-mercaptopropionate	C5H10O2S
182.0433	6.664712	33367.25789	183.0532	4-Pyridoxic acid	C8H9NO4
267.073	0.756991	21901.76484	268.0808	Inosine	C10H12N4O5
404.1036	0.921977	14538.41117	405.0995	Cephaloglycin	C18H19N3O6S
371.2934	10.385	10400.05506	372.3028	1-Phenyl-1,3-nonadecanedione	C25H40O2

Positive Ion Mode

The 20 most abundant compounds were organized in Table 3. Most notable from this list are the two medicinally relevant compounds betamethasone (Figure 10) and betaine (Figure 11). Betamethasone is a hydroxysteroid drug that is currently used to treat various skin conditions and diseases. This includes itching, inflammation, psoriasis and eczema. Betaine is an amino acid that is found in skin care products in order to retain moisture and keep skin hydrated. The other 18 most abundant compounds can be found in Table 3 with brief descriptions; if the compound does not not currently have a known use then their class or category of that compound was provided.

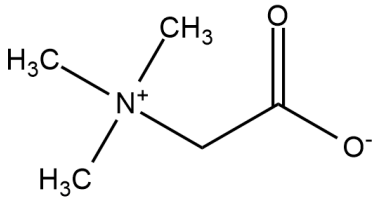


Figure 10. Betaine Structure

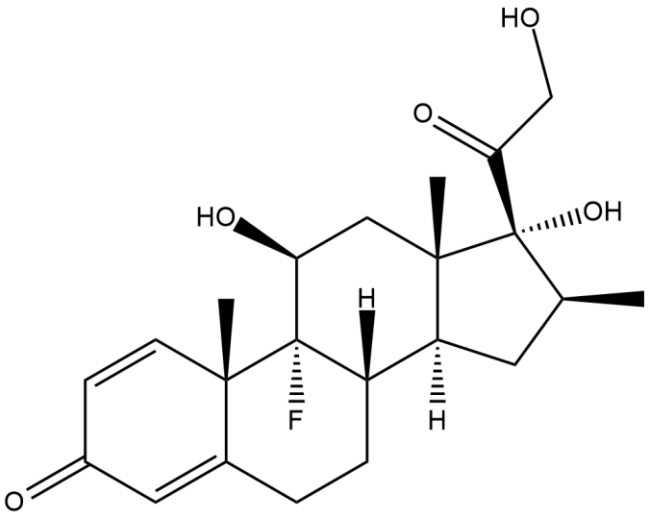


Figure 11. Bethamethasone Structure

Table 3. Top 20 Leaf Compounds Identified with UHPLC-MS Analysis in Positive Ion Mode

m/z	RT (min)	Peak area	Molecular Weight	Name	Formula	Description
679.5119	5.44963	27904581.1	656.5379754	DG(20:3n9/0:0/18:2n6)	C42H72O5	Fatty Acid, common food additive
393.2865	8.287042	8270295.113	392.2715304	3-Hydroxy-10'-apo-b,y-carotenal	C27H36O2	Terpene, Sesterterpenoid
701.4931	5.44963	6874465.034	678.5223254	DG(18:2n6/0:0/22:6n3)	C44H70O5	Diradylglycerol
318.3008	7.205731	5342304.458	317.2929941	Phytosphingosine	C18H39NO3	Organonitrogen compounds, structural component of plant cells
407.3022	8.287042	3690408.181	384.3239599	MG(0:0/20:1(11Z)/0:0)	C23H44O4	Endocannabinoids - agonist of cannabinoid receptors
702.5376	10.79332	2255684.362	701.5359403	PE(16:0/P-18:1(11Z))	C39H76NO7P	Glycerophospholipids
199.1704	11.45519	2124005.132	198.1619799	5Z-Dodecenoic acid	C12H22O2	fatty acid, plant metabolite
302.3056	7.822881	1772182.071	301.2980795	Sphinganine	C18H39NO2	signaling functions, inhibitor
415.1919	8.408099	1642707.855	392.1999022	Betamethasone	C22H29FO5	Hydroxysteroid drug, used to treat itching, inflammation and various skin conditions such as psoriasis and eczema
644.4955	10.36224	1588084.404	643.5023182	Glucosylceramide (d18:1/12:0)	C36H69NO8	Sphingolipids component of cell plasma membrane
322.2176	7.392811	1446409.623	299.2249146	2-Undecyl-4(1H)-quinolinone	C20H29NO	Quinolines, cell membrane component
672.5265	11.25727	1431988.851	671.5336183	Galactosylceramide (d18:1/14:0)	C38H73NO8	Sphingolipids, extracellular membrane component
118.0864	0.741921	1366440.435	117.0789786	Betaine	C5H11NO2	Amino acid, found in skin care products to keep skin hydrated
279.1604	9.976049	1338980.917	278.1518092	alpha-CEHC	C16H22O4	Metabolite of vitamine E
168.1375	6.487557	1300730.992	167.1310142	Myrtine	C10H17NO	Quinolizine
426.3002	7.580087	1252883.518	403.3086442	N-Palmitoyl phenylalanine	C25H41NO3	Amino acid
688.5216	10.32923	1201254.285	687.5202902	PC(14:1(9Z)/P-16:0)	C38H74NO7P	Glycerophospholipid
746.5638	10.76032	1135527.025	745.5621551	PC(15:0/18:1(11Z))	C41H80NO8P	Glycerophospholipid
790.5899	10.73832	1101802.884	789.5672404	PC(20:5(5Z,8Z,11Z,14Z,17Z)/P-18:1(11Z))	C46H80NO7P	Glycerophospholipid
421.3178	8.287042	1052739.719	398.329714	Nb-Palmitoyltryptamine	C26H42N2O	Indole

Figure 12. Total Ion Flow Chromatography (TIC) in Negative Ion Mode

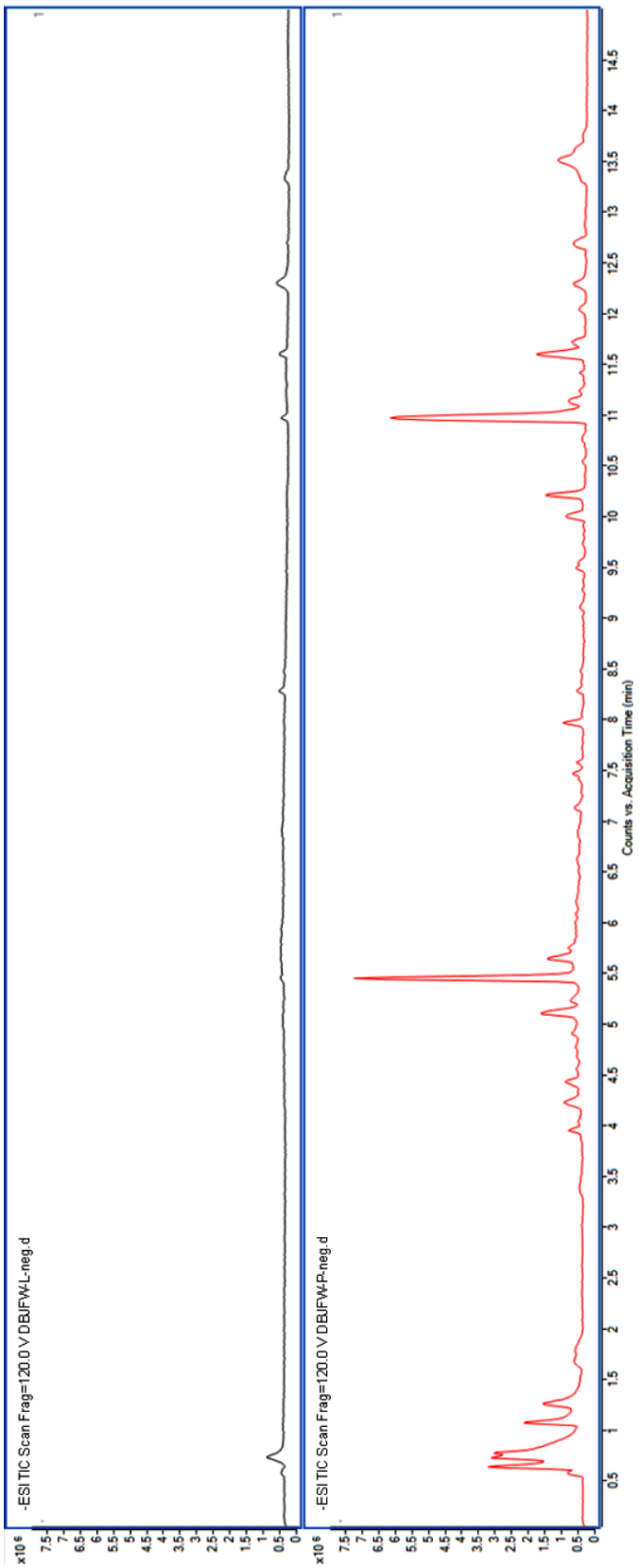
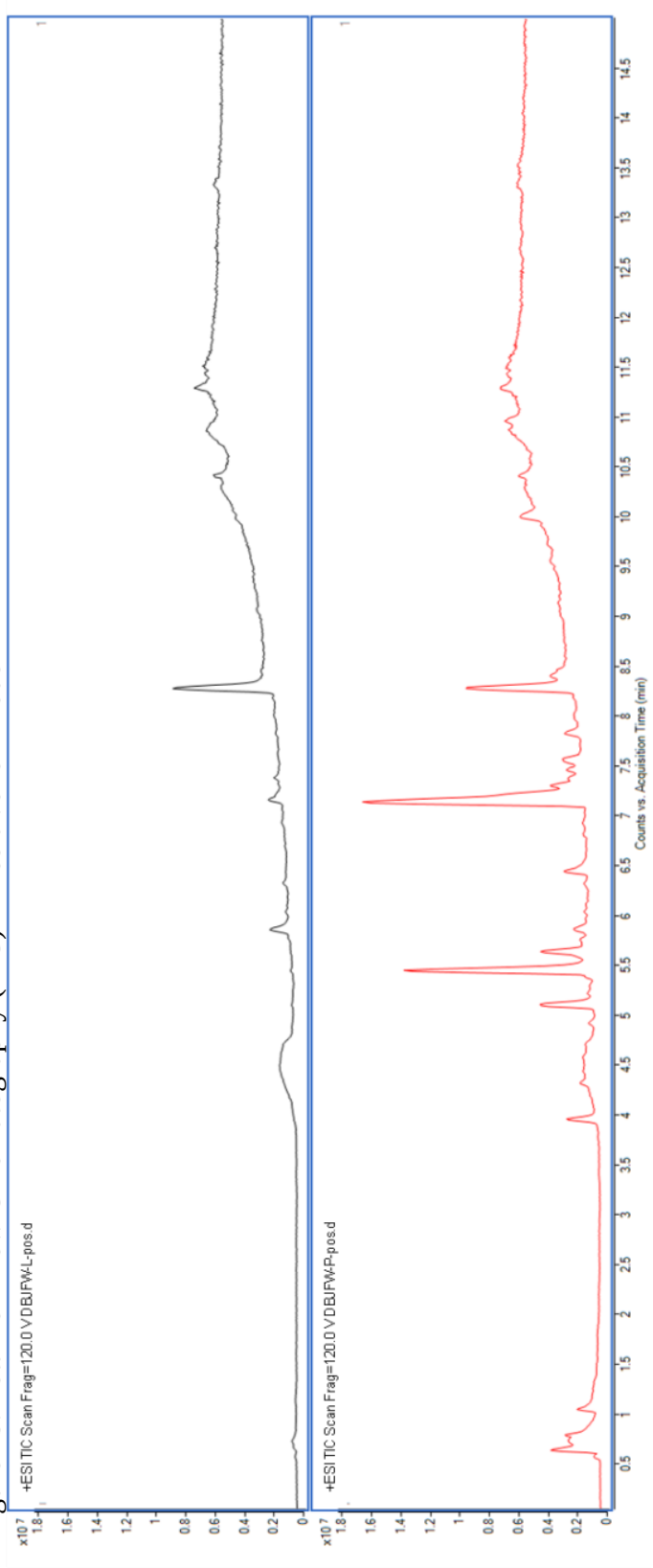


Figure 13. Total Ion Flow Chromatography (TIC) in Positive Ion Mode



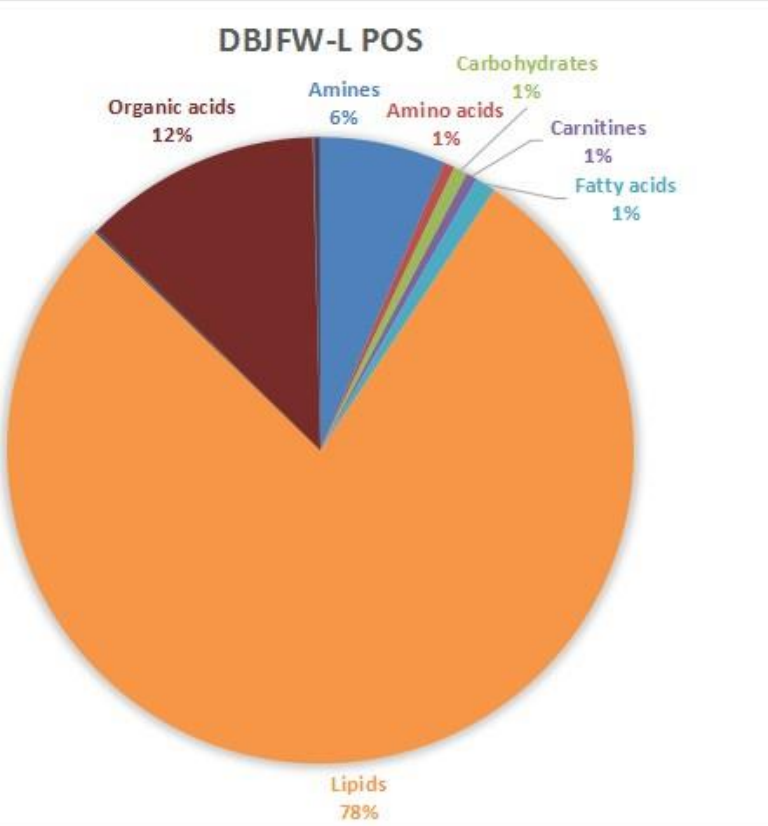


Figure 14. Leaf Classification Diagram of Positive Mode

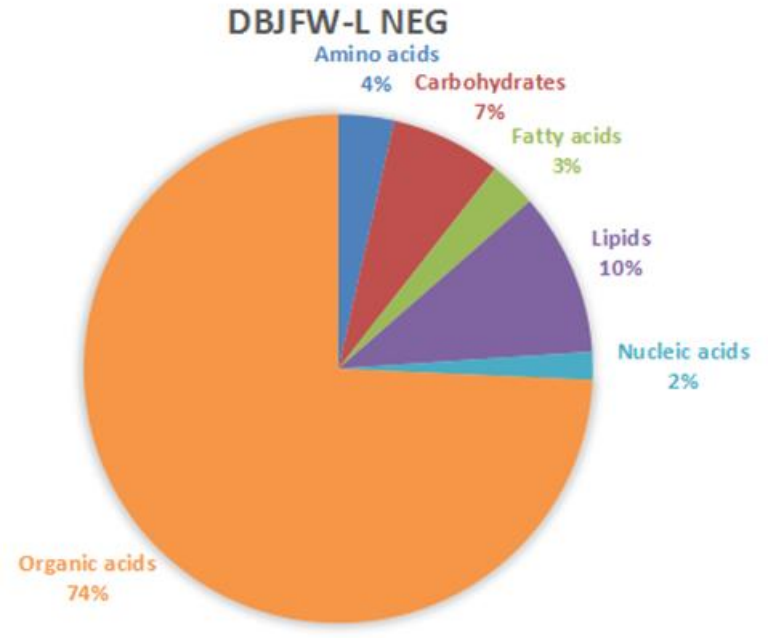


Figure 15. Leaf Classification Diagram of Negative Mode

Root Water Extract UHPLC-MS Methods

Preparation of Sample

25 mg sample of the water root extract was weighted out and dissolved in 300 μL deionized water. The solution was mixed well and a 100 μL sample was mixed with 300 μL HPLC grade methanol (containing 5 μg/mL 2-Chloro-L-phenylalanine as internal standard). The mixture was vortex for 1 min before being centrifuged at 13,000 rpm, 4°C for 10 min. The supernatant was collected and transferred to sampler vials for detected.

UHPLC-MS/MS Analysis of Sample

The Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6545 UHD and Accurate-Mass Q-TOF/MS was used for UHPLC-MS analysis. For the chromatographic column, Waters® XSelect HSS T3 (2.5 μm 100*2.1 mm) was used. The following conditions were used to run the sample.

- Mobile phase:

  A: aqueous solution with 0.1% formic acid.

  B: HPLC grade acetonitrile solution with 0.1% formic acid.
- Flow rate: 0.35 mL/min.
- Column temperature: 40°C.
- Injection volume: 1 μL in positive mode & 2 μL in negative mode.
- Gradient elution condition optimized: 0-2 min, 5% B; 2-10 min, 5-95% B; 10-15 min, 95% B.
- Post-run time was set as 5 min for system balance.

Mass spectrometry was operated in the negative ion mode with the following parameters:

- Capillary voltage: 3.5 kV.
- Drying gas flow: 10 L/min.
- Gas temperature: 325°C.
- Nebulizer pressure: 20 psig.
- Fragmentor voltage: 120 V.
- Skimmer voltage: 45 V.
- Mass range: m/z 50–3000.

Data Analysis

Raw data was converted from common (mz.data) format by Agilent Masshunter Qualitative Analysis B.08.00 software (Agilent Technologies, USA). In the R software platform, the XCMS program was used in peak identification, retention time correction, automatic integration pretreatment. The data was then subjected to internal standard normalization. Visualization matrices containing sample names, m/z-RT pair and peak area was obtained.

Root Water Extract UHPLC-MS Results

By conduction UHPLC-MS of water extract of roots 2798 features were obtained in the positive ion mode and 1719 features were obtained in the negative ion mode. Organization, classification and analysis of these features revealed a vast variety of plant metabolites with predominantly unknown use. In order to better understand the root's chemical composition, the vast array of compounds was organized and classified based on the class of each constituent. This was done separately for constituents found in the positive ion mode (Figure 16) and constituents found in the negative ion mode (Figure 17); The percentages of each class of

constituent was calculated. Notable compound classes were described to give a holistic understanding of what properties the camu camu roots may possess. Tables listing the 20 most abundant compounds in positive ion mode (Table 4) and the 20 most abundant compounds found in negative mode (Table 5) were made; retention time, m/z peak area, molecular weight, and chemical formula are also included. The total ion Flow Chromatography (TIC) for negative ion mode is seen in Figure 18, similarly the TIC for positive ion mode is seen in Figure 19.

Negative Ion Mode

Classes of compounds that are most notable in negative ion mode include the following: The abundance of tryptamines is highly notable; a subclass of alkaloids, tryptamines are known for their broad serotonergic activity in mammals and are often associated with psychoactive properties. Flavans are molecules known to provide a plant with resistance to fungi and insects. Triterpenoids are commonly known to have strong antioxidant activity as well as possible action against diabetic complications. Benzenediols are known to have skin lightening properties.

Positive Ion Mode

Classes of compounds that are most notable in positive ion mode include the following: Glycerophosphocholines are important choline compounds that are may be responsible for mental recovery, protection against aging related cognitive decline, and beneficial to overall brain health. Anilides are nitrogen-bound compounds that when found in plants will often serve as herbicides and fungicide. Eicosanoids function in various pathways as inhibitors of immune responses such as fever, inflammation, and allergies. Phosphate esters are typically intermediates in the conversion of food into usable energy. The temperature at which Phosphate esters auto-ignite is typically very high making these compounds highly fire resistant.

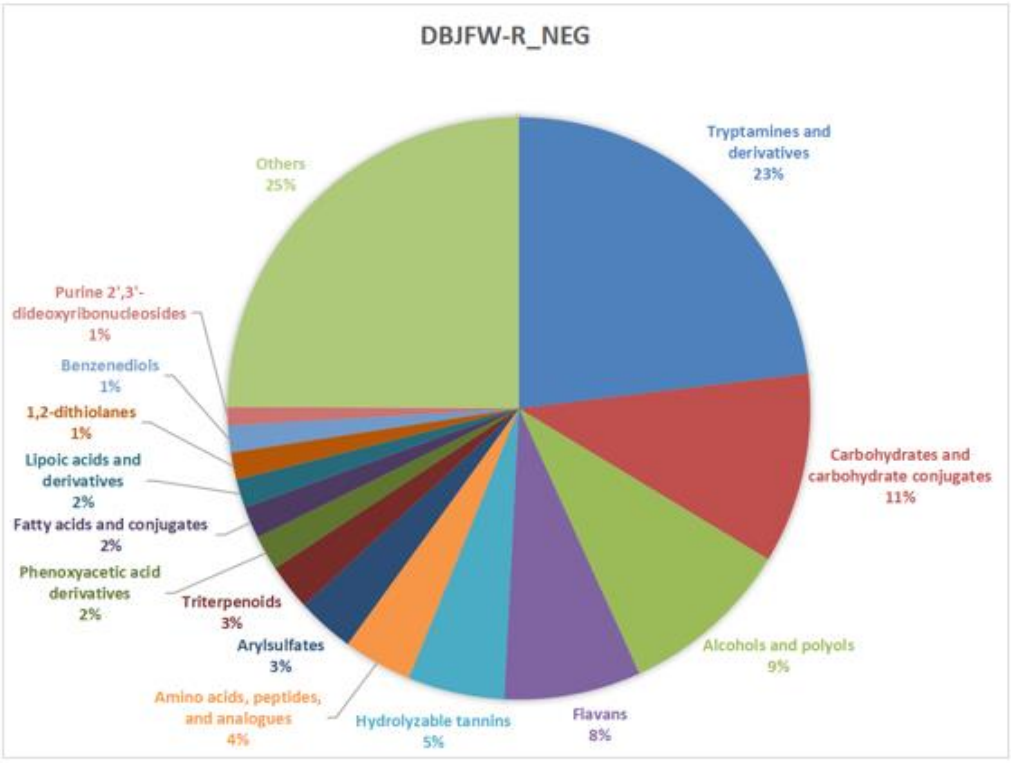


Figure 16. Root Classification Diagram of Positive Mode

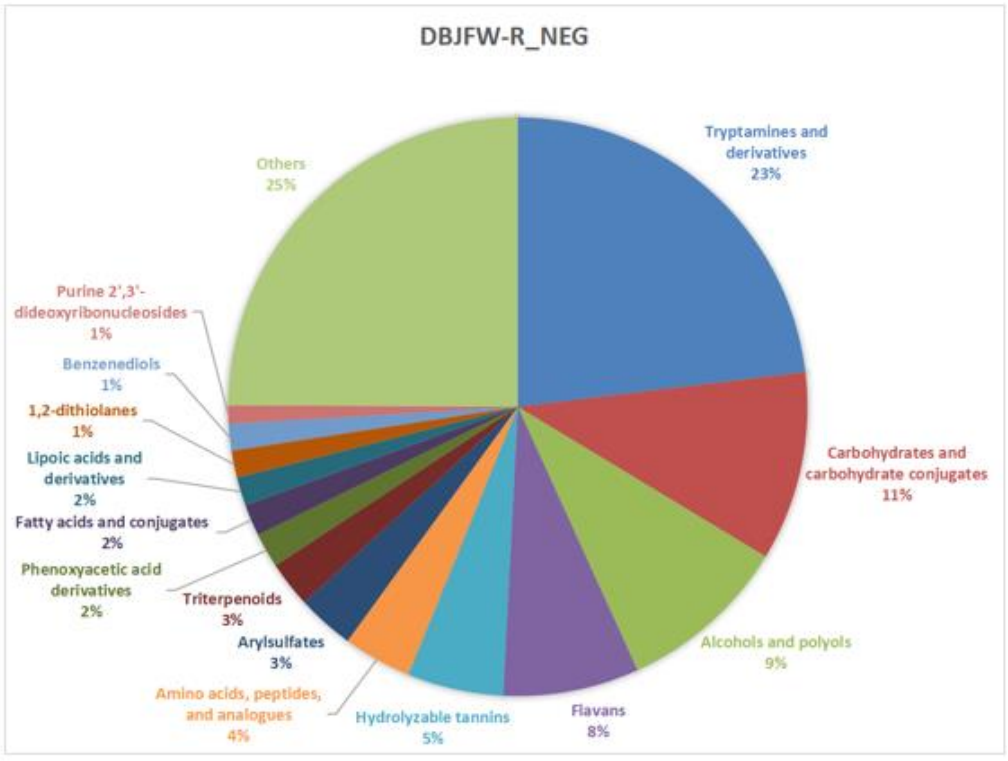


Figure 17. Root Classification Diagram of Negative Mode

Table 4. Top 20 Root Compounds Identified with UHPLC-MS Analysis in Positive Ion Mode

m/z	RT(min)	Peak Area	Molecular Weight	Name	Formula
144.1175	1.2592	8.406572961	143.111	(1E)-N-Cyclohexylethanimidoyl fluoride	C8H14FN
381.0858	0.739794	5.749219841	358.0966	1-(4-Bromo-3-hexylthiophen-2-yl)heptan-1-one	C17H27BrOS
496.3442	9.168525	5.320175427	495.3358	Ethanesulfonic acid, 2-[cyclohexyl(1-oxooctadecyl)amino]-, sodium salt	C26H50NNaO4S
935.0808	2.55171	5.049874129	934.0743	4,4',4'',4'''-(Porphine-2,3,5,21(23H)-tetrayl)tetra(benzene-1-sulfonic acid)	C44H30N4O12S4
502.233	5.781562	4.866318628	479.2434	N-desmethylimatinib	C28H29N7O
112.0906	1.25924	4.344756221	111.081	[(Cyclohex-2-en-1-ylidene)methyl]oxidanium	C7H11O
110.013	0.586553	4.158344853	87.02379	2-CYANOETHYLPHOSPHINE	C3H6NP
163.1367	5.687597	4.06137804	162.1283	ZINC02386537	C11H16N
153.1316	5.465653	3.826113789	152.1201	Perillyl alcohol	C10H16O
935.0804	3.69573	3.283212141	934.0743	4,4',4'',4'''-(Porphine-2,3,5,21(23H)-tetrayl)tetra(benzene-1-sulfonic acid)	C44H30N4O12S4
348.2805	6.844452	2.944746644	325.2913	Silane, azidotrihexyl-	C18H39N3Si
160.1131	1.359103	2.82403245	159.1059	1-(Azepan-1-yl)-2-fluoroethan-1-one	C8H14FNO
223.9962	0.586649	2.376351191	222.9888	PUBCHEM_71340648	C6H12AgO2
295.2328	9.712804	2.09479573	294.2243	Tetrabutylphosphonium chloride	C16H36ClP
1049.115	2.209016	2.035650722	1026.134	Camelliatannin F	C48H34O26
522.3638	9.422792	2.018726744	499.3742	N,N,N-Trihexylhexan-1-aminium hexafluorophosphate	C24H52F6NP
999.69	7.939949	2.018251939	976.698	PI(22:0/22:1(11Z))	C53H101O13P
952.1078	2.550783	1.992573544	929.1012	7233-01-4	C21H32F18O9Tb
268.1105	1.139334	1.966920589	245.1216	1-(4-Fluorophenyl)-N-[(4-methoxyphenyl)methyl]methanamine	C15H16FNO
151.0405	0.586553	1.958268118	150.0328	Dimethyl foromalonate	C5H7FO4

Table 5. Top 20 Root Compounds Identified with UHPLC-MS Analysis in Negative Ion

m/z	RT(min)	Peak Area	Molecular Weight	Name	Formula
233.0895	6.402	2121.034954	234.0968548	2-(3,4-Difluorophenyl)-5-propylpyrimidine	C13H12F2N2
394.9741	5.3865	1277.232744	395.9810806	N-[2-(3,5,6-Trichloropyridin-2-yl)ethyl]-2-(trifluoromethyl)benzamide	C15H10Cl3F3N2O
191.0554	0.983	878.0105996	192.0622809	s-Triazine, 2-methyl-4-methylamino-6-(trifluoromethyl)-	C6H7F3N4
305.0738	4.549	643.9292042	306.0811428	2-phenyl-2-(4-phenylphenyl)acetyl chloride	C20H15ClO
395.1257	5.967	593.6640853	396.1328934	Triethyl{3-[(triethylstannyl)sulfanyl]propyl}silane	C15H36SSiSn
169.0175	1.669	344.658916	170.0248944	Methanesulfonic acid--(oxiran-2-yl)methanol	C4H10O5S
411.1352	4.914	234.8883848	412.142425	Tributyltin benzoate	C19H32O2Sn
380.9572	4.964	234.2690788	381.9636827	7-(Dimethylamino)-4-iodo-3H-phenothiazin-3-one	C14H11IN2OS
291.0181	4.429	233.5888666	292.0252885	[2-hydroxy-5-(2-hydroxy-3-methoxy-3-oxopropyl)phenyl]oxidanesulfonic acid	C10H12O8S
234.0913	6.3915	214.7580449	235.0984123	N-Ethyl-N-(2,2,3,3-tetrafluoropropyl)aniline	C11H13F4N
395.9739	5.3815	199.1193054	396.9811059	4-(2-iodoethyl)phenyl 4-nitrobenzoate	C15H12INO4
933.0473	3.622	189.7896248	934.071231	7-[7,8,9,12,13,14,17,18,19,25-decahydroxy-24-(hydroxymethyl)-4,22,27-trioxo-3,23,26-trioxahexacyclo[13.10.3.1^{2,6}.0^{5,10}.0^{11,28}.0^{16,21}]nonacosa-5(10),6,8,11,13,15(28),16,18,20-nonaen-29-yl]-3,4,8,9,10-pentahydroxy-6-oxo-6H-benzo[c]chromene-1-carboxylic acid	C41H26O26
301.0022	5.1255	178.0531443	302.0094344	4'-Chloro-3'-cyano-3-nitro[1,1'-biphenyl]-4-carboxylic acid	C14H7ClN2O4
377.084	0.8745	171.1902794	378.0914947	3,5-Di-O-benzoyl-2-deoxy-2,2-difluoro-beta-L-erythro-pentofuranose	C19H16F2O6
409.0435	1.982	149.4242248	410.0507323	BAS 00393216	C19H14N4O3S2
205.0382	1.827	145.9088256	206.0453327	2-Methyl-3-(4-nitrophenyl)prop-2-enoate	C10H8NO4
133.0154	0.984	138.5478906	134.0228607	6H-Purin-6-one	C5H2N4O
469.0048	3.858	113.5251751	470.0121404	Sanguisorbic acid dilactone	C21H10O13
315.0155	5.62	105.93592	316.0227862	(((9-Oxo-9H-thioxanthen-2-yl)methyl)thio)acetic acid	C16H12O3S2
329.2366	6.879	102.1763785	330.2433043	1-Methyl-1-{2-[(1-phenylcyclohexane-1-carbonyl)oxy]ethyl}piperidin-1-ium	C21H32NO2

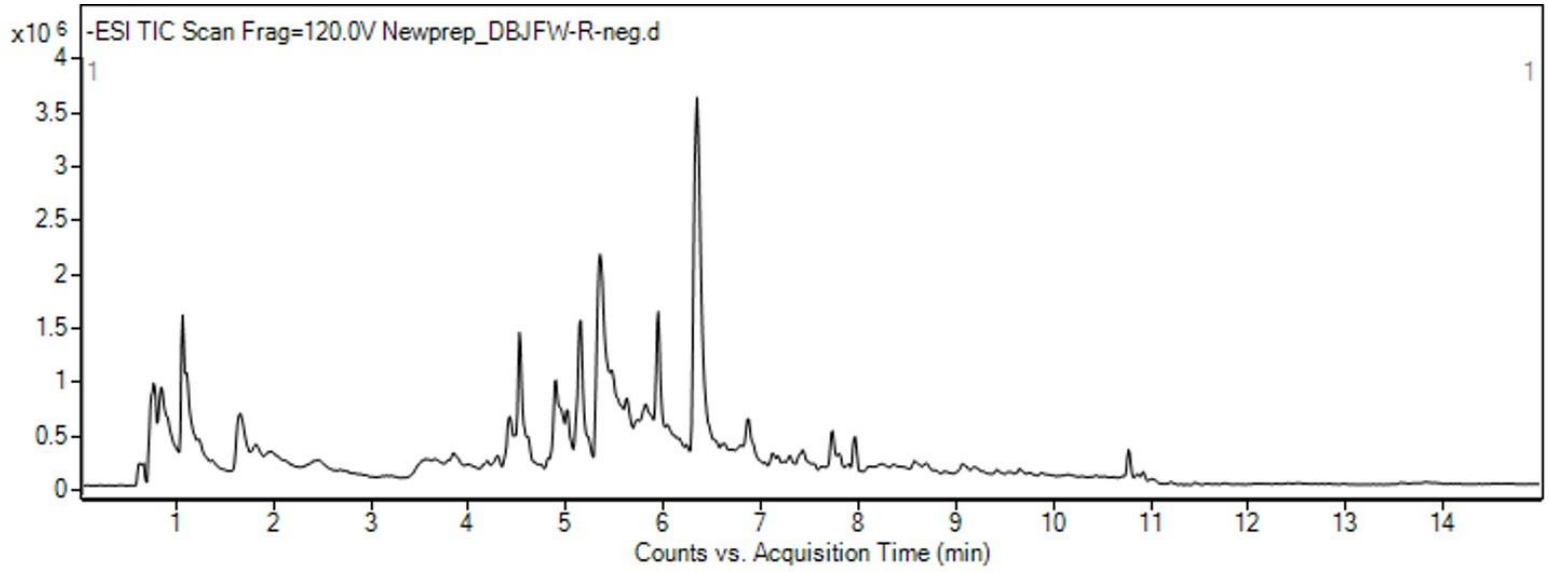


Figure 18. Total Ion Flow Chromatography (TIC) in Negative Ion Mode

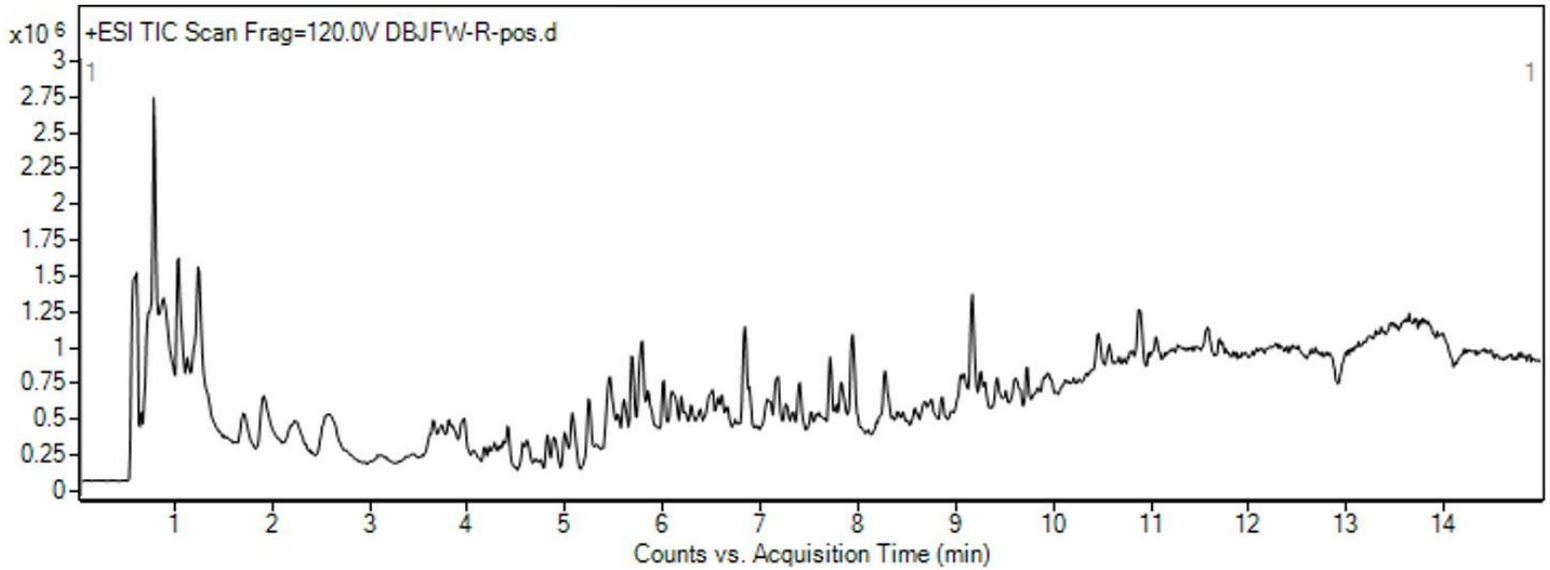


Figure 19. Total Ion Flow Chromatography (TIC) in Positive Ion Mode

Isolation of Compounds from DEE Leaf Extract

Column Chromatography for Isolation

Column chromatography was the method used in the isolation of compounds. This was completed by using 80.0 g of the diethyl ether leaf fraction in a gravity guided 2000 ml chromatography column. The mobile phase began with 100% Hexanes to elute non-polar compounds. Ethyl acetate was used to increase the polarity of the mobile phase and elute more polar compounds. Through this process 4 compounds were isolated in a pure form, labeled DBJF-1, DBJF-2, DBJF-3, and DBJF-4. Purity of each compound was verified with Thin-Layer Chromatography viewed under a 254 nm wavelength UV lamp and in an iodine chamber. The Melting point, weight, and the eluting solvent(s) of each compound was recorded on Table 6.

Table 6. Characteristics of Pure Compounds

Compound Label	Melting Point	Weight	% by weight	Eluting Solvent(s)
DBJF-1	63.1°C	24.4 mg	0.0305%	100% Hexanes
DBJF-3	56.0°C	51.1 mg	0.0639%	100% Hexanes
DBJF-4	174.5°C	54.1 mg	0.0676%	8% Ethyl Acetate 92% Hexanes
DBJF-5	133.8°C	138.0 mg	0.1725%	10% Ethyl Acetate 90% Hexanes

Interestingly, DBJF-4 has a crystalline structure; this makes it a potential candidate for X-ray crystallography analysis. DBJF-1, DBJF-3, and DBJF-5 appear to have amorphous structures. All 4 compounds were analyzed with Fourier-transform Infrared Spectroscopy (FTIR); the spectra of DBJF-1 is seen in Figure 20, the spectra of DBJF-3 is seen in Figure 21, the spectra of DBJF-4 is seen in Figure 22, and the spectra of DBJF-5 is seen in Figure 23.

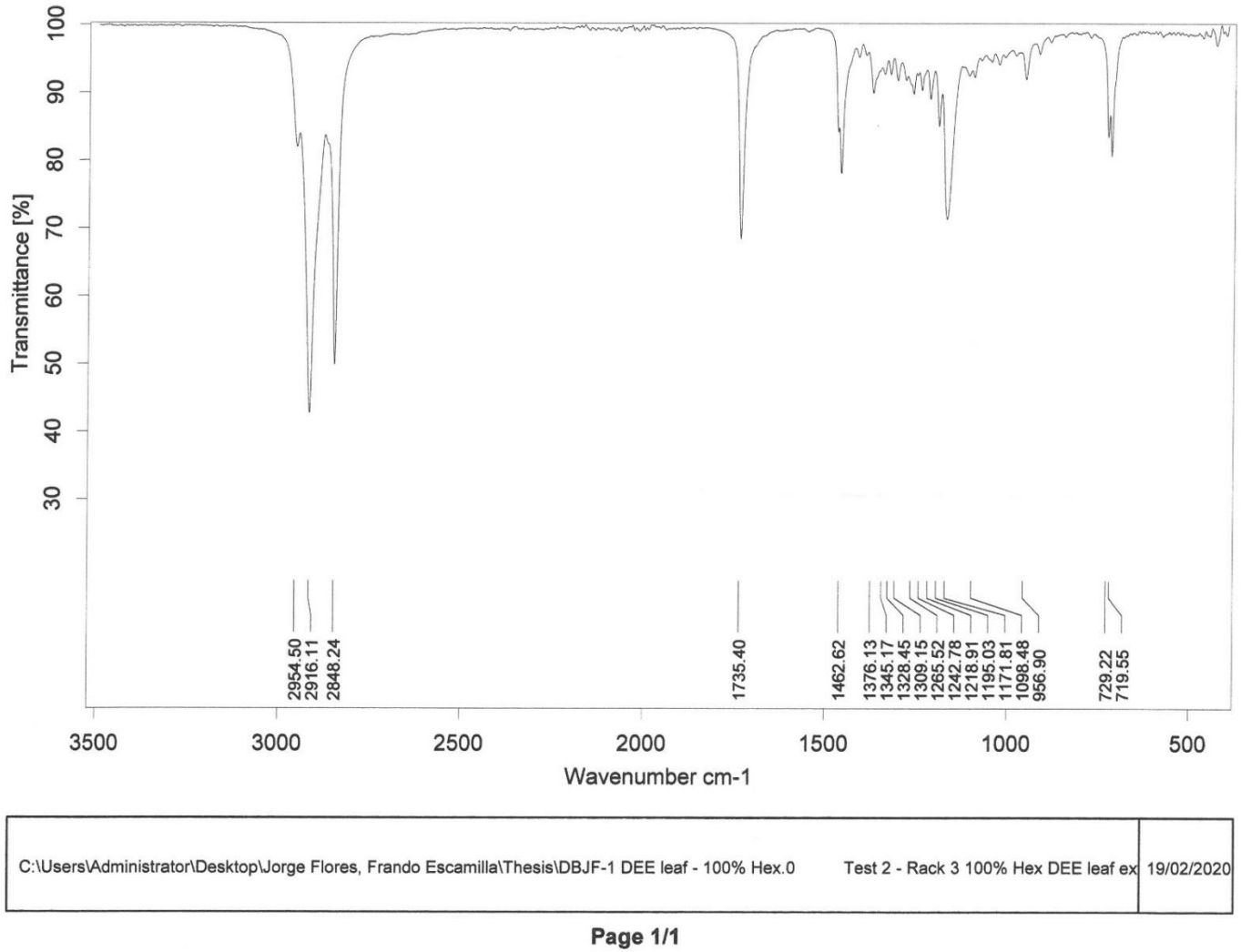


Figure 20. Fourier-Transform Infrared Spectroscopy (FTIR) Spectra of DBJF-1

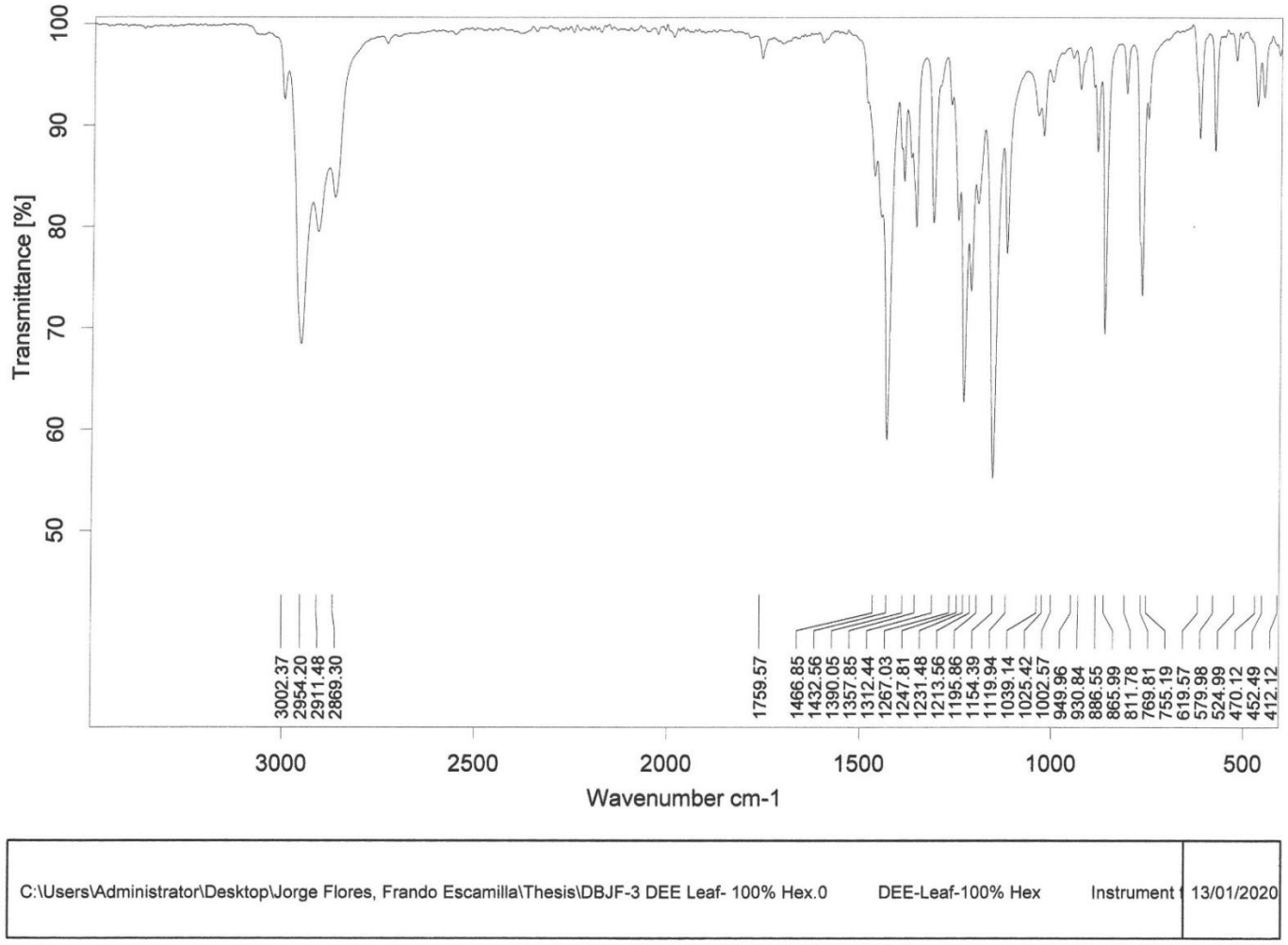


Figure 21. Fourier-Transform Infrared Spectroscopy (FTIR) Spectra of DBJF-3

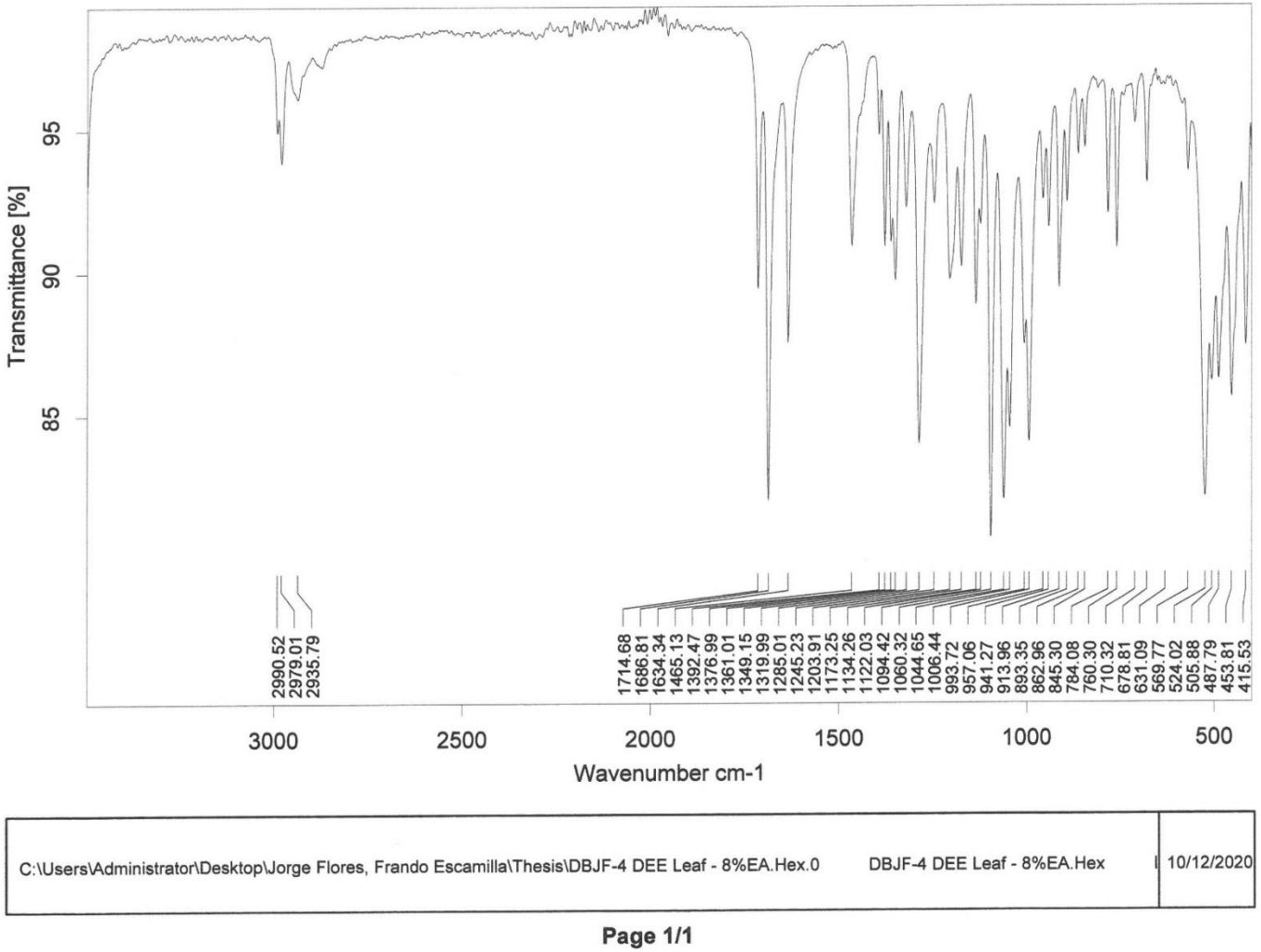


Figure 22. Fourier-Transform Infrared Spectroscopy (FTIR) Spectra of DBJF-4

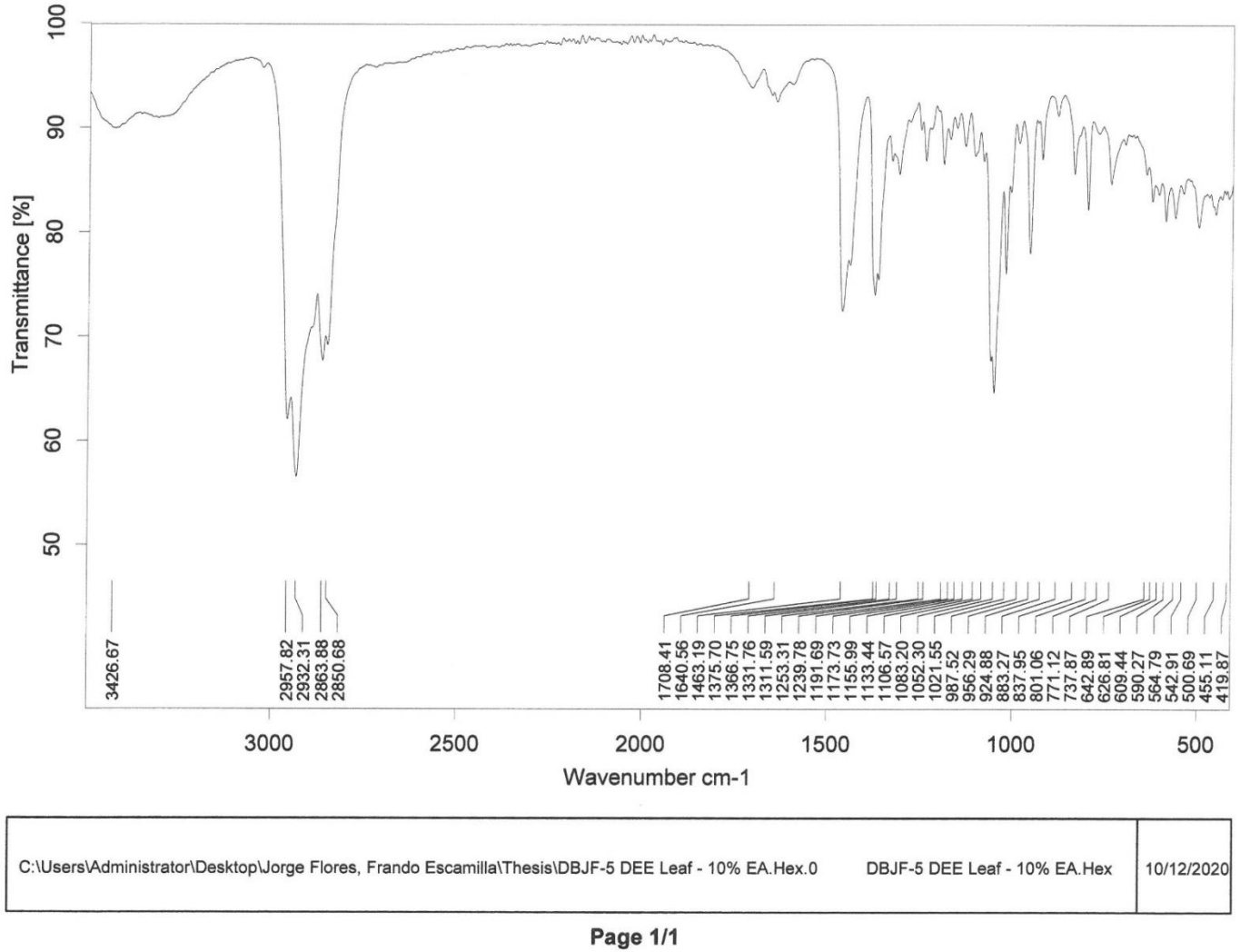


Figure 23. Fourier-Transform Infrared Spectroscopy (FTIR) Spectra of DBJF-5

Gas Chromatography–Mass Spectrometry (GC-MS) of Diethyl Ether Leaf Fraction

The first column chromatography fraction collected from the diethyl ether camu camu leaf extract showed to be abundant in non-polar compounds. This fraction was eluted with 100% hexanes. The 1D-NMR spectroscopy (Figure 28) of the fraction was conducted to detect the presence of aromatic rings. Gas Chromatography-Mass Spectrometry (GC-MS) analysis was conducted to identify the components of this fraction and to calculate the abundance of each.

Results

By performing GC-MS 100 compounds were found, of which 41 were found to be known and 59 were found to be unknown; the 19 compounds that were found to have an abundance greater than 1% were organized in table 7; of these, 8 compounds were found to be previously undiscovered. 9 of the known compounds are described in table 8. The mass spectrometry of a non-polar compound and its novel structure can be seen in Figure 26. Most notable is the compound 1-Dodecanol, 2-octyl- which is currently being used in topical applications because of its lubricating and emollient properties, seen in Figure 24. 1-Dodecanol, 2-octyl- is used in cosmetics and in pharmaceutical applications as an emulsifying and opacifying agent. The mass spectrometry of 1-Dodecanol, 2-octyl can be seen in Figure 27. The comprehensive mass spectrometry of the diethyl ether leaf fraction can be seen in Figure 28.

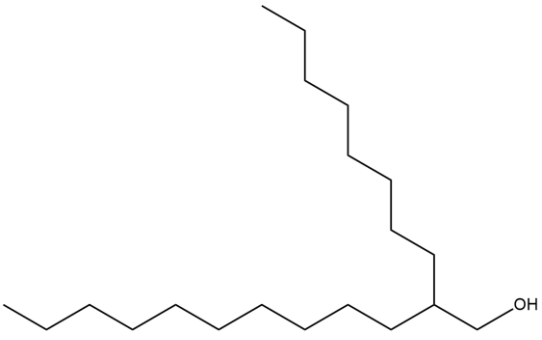


Figure 24. Structure of 1-Dodecanol, 2-octyl-

Table 7. Diethyl Ether Leaf Fraction Compounds with Abundance >1%

Compound Number	Area Sum % (mixture abundance)	Retention Time	Compound Name
1	17.46	44.556	Tetratriacontane
2	12.66	39.961	**Unknown**
3	5.37	41.780	**Unknown**
4	3.94	36.788	Nonacosane
5	3.14	27.404	Eicosane
6	2.79	26.184	**Unknown**
7	2.62	38.082	1-Dodecanol, 2-octyl-
8	2.31	31.134	**Unknown**
9	2.20	35.624	Octacosane
10	2.12	34.659	Octacosane
11	1.73	33.737	Hexacosane
12	1.50	31.797	Tetracosane
13	1.44	34.954	**Unknown**
14	1.43	34.048	**Unknown**
15	1.35	35.960	**Unknown**
16	1.32	32.791	Pentacosane
17	1.32	37.136	Hexacosane
18	1.20	30.767	Tricosane
19	1.07	36.303	**Unknown**

Table 8. Known Diethyl Ether Leaf Fraction Compounds with Abundance > 1%

Compound Label	Description
Tetratriacontane	It has a role as a plant metabolite.
Nonacosane	A volatile oil component that has as a role as a plant metabolite.
Eicosane	It has a role as a plant metabolite.
1-Dodecanol, 2-octyl-	Cosmetic & Pharmaceutical applications as emulsifier and opacifier.
Octacosane	It has a role as a plant metabolite.
Hexacosane	It has a role as a plant metabolite.
Tetracosane	It has a role as a plant metabolite.
Pentacosane	It has a role as a semiochemical and a plant metabolite.
Tricosane	It has a role as a plant metabolite and a volatile oil component.

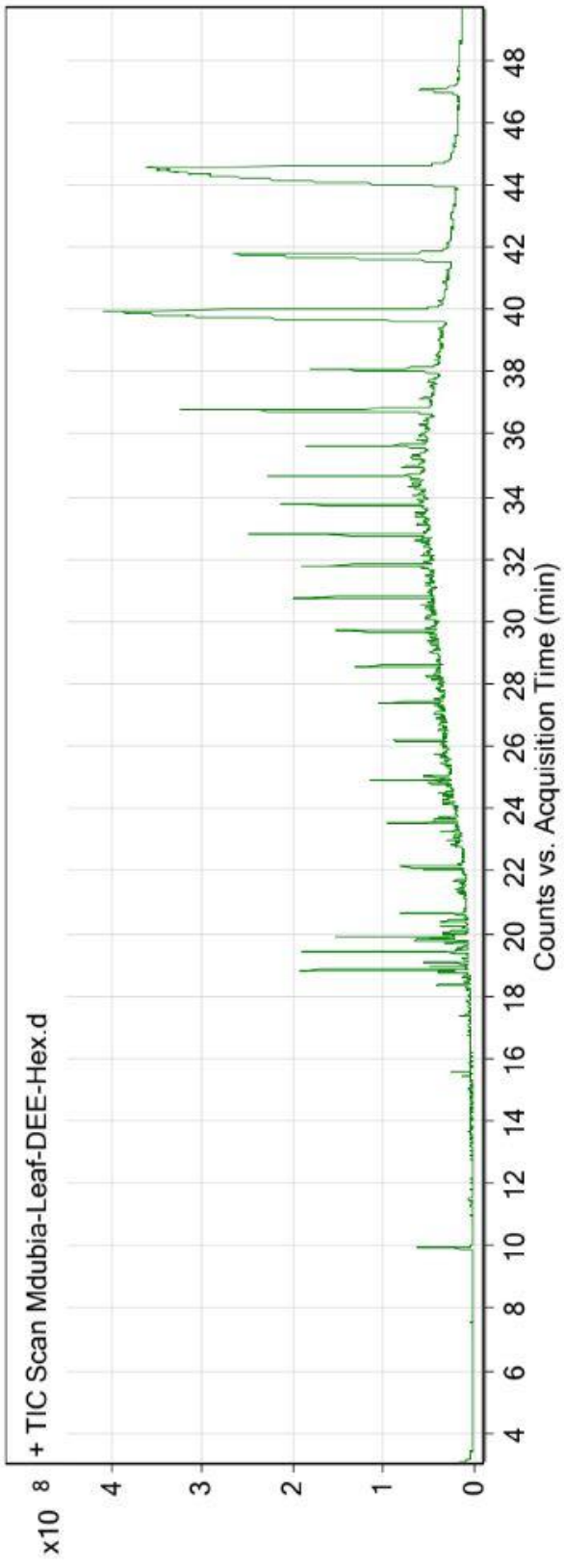


Figure 25. Comprehensive Mass Spectrometry of Diethyl Ether Leaf Fraction

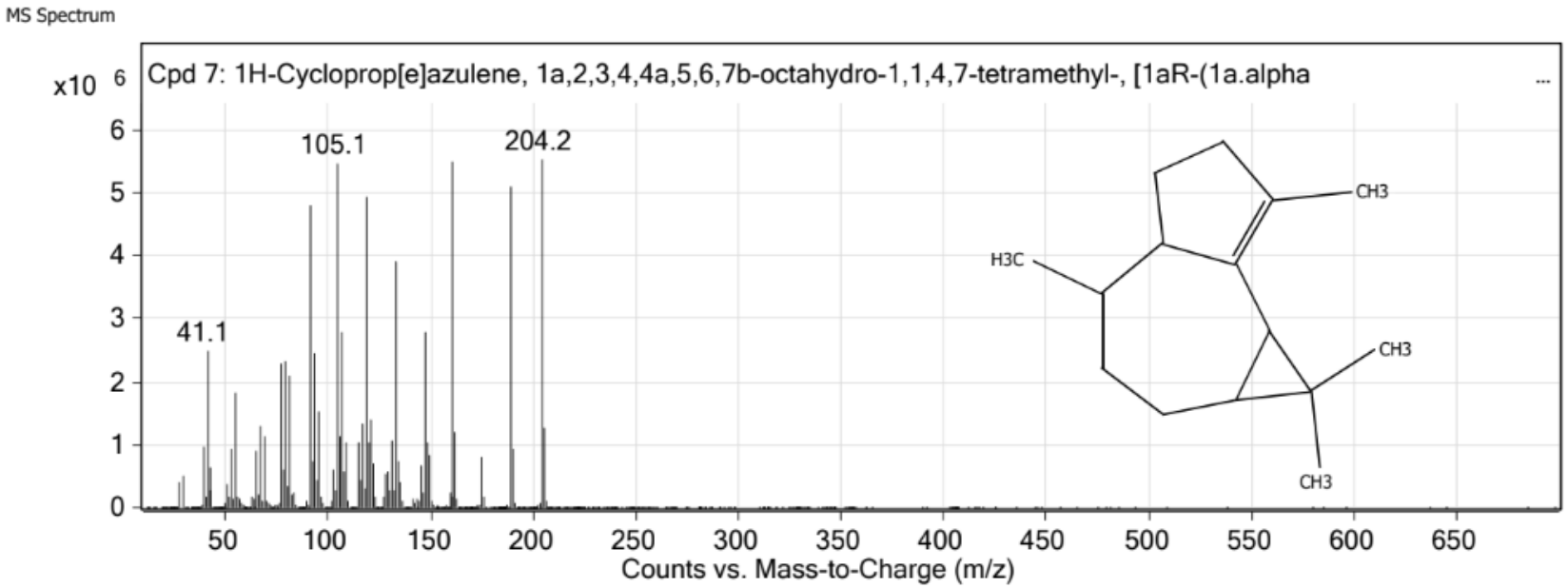


Figure 26. Mass Spectrometry of a Structurally Novel Hydrocarbon Identified

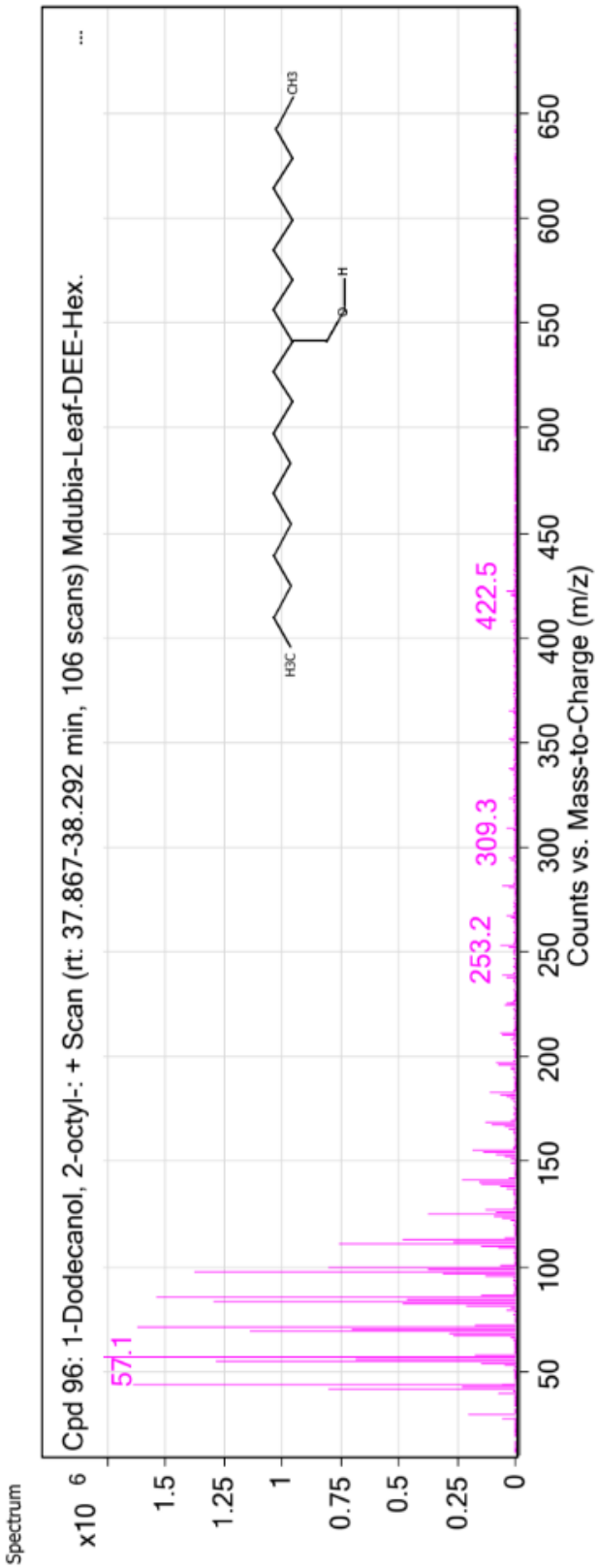


Figure 27. Mass Spectrometry of 1-Dodecanol, 2-octyl-

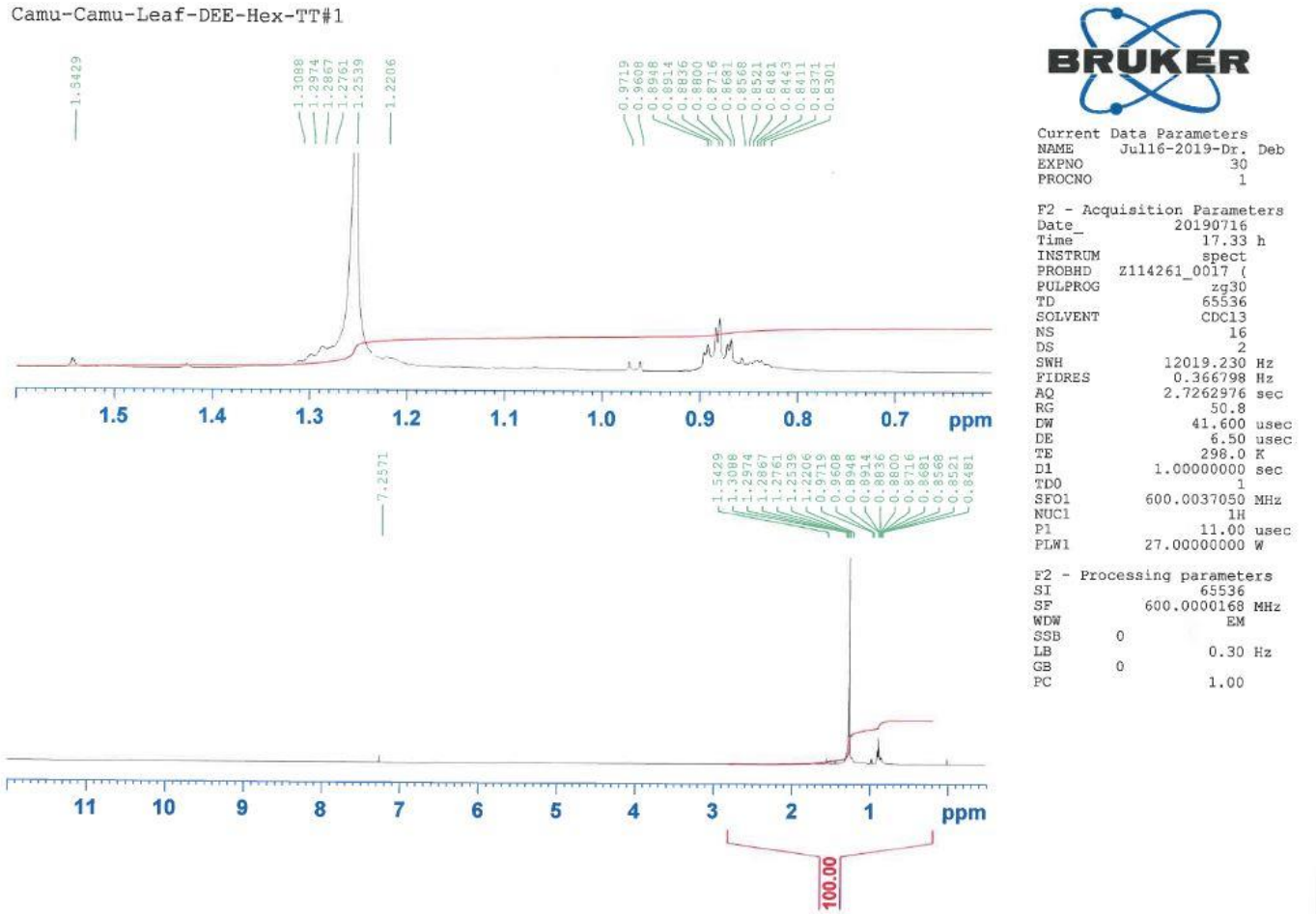


Figure 28. 1D NMR of Diethyl Ether Leaf Fraction

CHAPTER IV

SUMMARY & CONCLUSION

Summary

The term “phytochemicals” refers to a wide variety of compounds found in plants, but mainly describes a plant’s naturally produced compounds that show either a positive (as medicine) or negative (as narcotics/poison) effect on human/animal health [21]. The research of plants and natural products is crucial for the discovery of new drugs, for new sources of already established drugs, and has an important role in the areas of drug synthesis and development. Eight extracts were created from the leaves and the roots of the camu camu plant, four for each component of the plant; the extracts range from non-polar to polar. A chemical analysis of the water extract of camu camu’s leaves and roots via ultra-high performance liquid chromatography. Several phytochemicals have been found to be abundant in the categories of nutraceuticals, medicinal beneficial compounds, and industrially valuable compounds. These compounds range from a molecule with antioxidant properties to a highly valuable photosensitizer, methoxsalen. Photodynamic therapy is a highly promising area of treatment for certain skin ailments such as malignant skin cancer, vitiligo, and psoriasis [22]. This treatment is accomplished by combining light therapy with a specific drugs known as a photosensitizer. Gas chromatography-mass spectrometry was conducted for a fraction of the diethyl ether leaf extract. 100 compounds were found with GC-MS analysis, 41 of them were found to be known and 59 were found to be unknown compounds. Structurally diverse non-polar compounds were found

with GC-MS analysis. Column chromatography was conducted for the diethyl ether leaf extract. Four different pure compounds were isolated, purity was verified through different lab techniques including Fourier-Transform Infrared Spectroscopy (FTIR).

Conclusion

The novel discoveries that have been so far mark a newfound importance to the study of the camu camu plant. The hexane, dichloromethane, and methanol extracts of the camu camu roots along with the dichloromethane and methanol extracts of the leaves still need to undergo chemical studies. The extracts have been created and well preserved, paving the way for the continuation of camu camu leaf and root chemical investigations. Beyond this, we are hopeful that interest will be peaked into the study of other parts of the camu camu plant, as well as other overlooked Amazonian plants. Natural product research is essential to new drug discoveries and maintains an influential role in drug synthesis and development. The chemical properties of camu camu roots and leaves are not yet fully known. These findings are a promising start.

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BIOGRAPHICAL SKETCH

Jorge Flores was born in Lima, Peru on April 17th, 1994. He graduated from South Texas ISD Health Professions high school located in Mercedes, TX in May of 2012. His undergraduate studies were completed at The University of Texas Rio Grande Valley (formerly The University of Texas-Pan American) in December of 2017 with a bachelor's degree in Psychology (B.S.) and a Minor in Chemistry. Upon graduation he joined the research team of Dr. Debasish Bandyopadhyay in the chemistry department of UTRGV. His interest in chemical research focused on drug discovery and development led him to apply to the Master of Science in Chemistry program; he was accepted and granted the competitive Presidential Graduate Research Assistant award (PGRA) and began his studies during the fall semester of 2018. During this time, he submitted an abstract to the American Chemical Society (ACS) National Conference in March of 2020 which was accepted for a poster presentation. Again, in August of 2020 he submitted an abstract to the ACS National conference which was accepted for another poster presentation. He completed his Master of Science in Chemistry degree in August of 2021 at The University of Texas Rio Grande Valley. He may be contacted at l.flores.jorge01@gmail.com.