ISOLATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM PLANT BASED TRADITIONAL CHINESE MEDICINE

by

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I dedicate this research to my family.

ACKNOWLEDGMENTS

I P L I Q O H V E Z O V P E X L
Y T F J H O I A S U W N O R T K
T B O Y R A B U I M C O H V E S
C I N C S O M U L O D M N E Z T
H O A A V N E D E O N L Y E G C
F K H I B T E W I Y T H O N P D
W O D P U T N Y W O P I U R Y N
A B N T J D H L B E S V P O E U
J E T S X P E Z N H C D E J G R
U P N D A G O R E L B X B S M A
S Y A H K L X A N O G H K A Q E

ABSTRACT

Plants used in Traditional Chinese Medicine (TCM), which have been cultivated and expanded for thousands of years, provide a rich source for screening bioactive compounds with modern assays. These plants have traditionally been proven to provide some activity in the treatment of human disease and with modern isolation techniques and manipulation of the plant metabolome, the bioactive compounds can be isolated and provide new leads in drug discovery. The project described in this work details the bioassay guiding fractionation of two plants, *Scutellaria baicalensis* and *Cyclocarya paliurus.* In addition to the bioassay guided fractionation, optimization into the isolation of the respective bioactive compounds for each plant was made in order to improve efficiency. Finally, in order to look at the expression profile of the polar fractions compared to the undesirable lipophilic fractions that occur in excess with normal plant based extracts, preliminary work was completed with cell culture studies in the plant *Cichorium intybus*.

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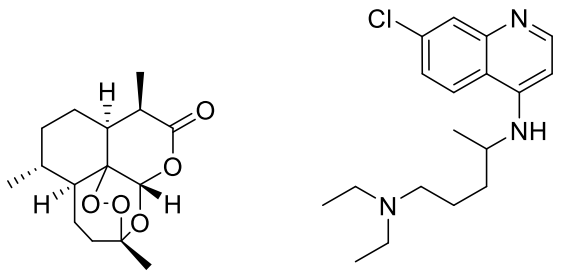
CHAPTER I: BACKGROUND AND REVIEW OF PLANTS USED IN TRADITIONAL CHINESE MEDICINE

Plants used in Traditional Chinese medicine (TCM), which have been cultivated and used as the main source of treatment in China for thousands of years, provide a rich source for screening bioactive compounds using modern assays. These plants have traditionally been proven to provide some activity in the treatment of human disease and with modern techniques the bioactive compounds can be isolated and provide new leads in drug discovery.[1] One issue that has arisen during the introduction of modern approaches to identify bioactive compounds in Chinese herbal remedies is that the TCM prescription is based on a whole body perspective. If there are multiple therapeutic targets for a single TCM formula, then interpreting which compounds are interacting with which targets can be very difficult. In 2008, the Chinese government established the Innovative New Drug Development and Manufacturing project with more than 10 billion yuan (U.S. $1.6 billion) in public funding. The aim of the project was to find new drugs from TCM using modern drug discovery techniques.[2] Two major discoveries from TCM laid the groundwork for the project and the potential success.

The first major discovery that developed into a Western approved drug was artemisinin shown in Figure 1 for the treatment of malaria. This parasitic disease is caused by *Plasmodium* parasites carried by female mosquitos as a vector and transmitted with a bite. According to the World Health Organization, in 2015, 214 million new cases and 438,000 deaths due to malarial infection were reported.[3] Two species of parasites are responsible for the disease in humans, *Plasmodium falciparum* occurs mostly on the

African continent and is responsible for the most malaria related deaths worldwide and *Plasmodium vivax*, which occurs throughout the rest of the world. Both are life threatening and require treatment. Artemisinin, discovered by Youyou Tu in 1971 from the plant *Artemisia annua L.*, was the first effective antimalarial drug to replace existing therapies such as chloroquine, shown in Figure 1. Resistance to chloroquine had developed by that time and was no longer an effective treatment. Tu had trouble replicating the activity from the initial mouse study showing activity from *A. annua.* Finally, she reviewed traditional texts and found the first mention by Ge Hong (284-363 AD), to "soak a handful of qinghao (Chinese name for *A. annua*) in two liters of water, wring out the juice and drink it all".[4] This led Tu to believe that modern techniques of heating for extraction might be destroying the active compound and once she tried the traditional technique the activity returned. Artemisinin or derivatives used as a combination therapy are now the first line of defense in antimalarial treatment. The discovery of artemisinin is a good example of how TCM and western drug discovery and development techniques can work together and develop a new drug based on traditional practices.



artemisinin chloroquine

Figure 1. Structures of artemisinin and chloroquine

A second major drug developed from TCM was camptothecin, shown in Figure 2. Camptothecin was discovered in 1966 by Monroe Wall while screening natural products for antileukemic activity.[5] Camptothecin was isolated from *Camptotheca acuminata*, also known as the Happy Tree, and used in TCM. Although the original natural product showed promising activity in animal models, the compound had solubility issues and cytotoxicity problems. The mechanism of action was later discovered to be acting as a topoisomerase I poison. This led to structure-activity relationship (SAR) studies to improve upon the solubility and toxicity issues of the original camptothecin. Currently two analogs of camptothecin, topotecan and irinotecan, shown in Figure 2, are approved and are used for cancer therapy.

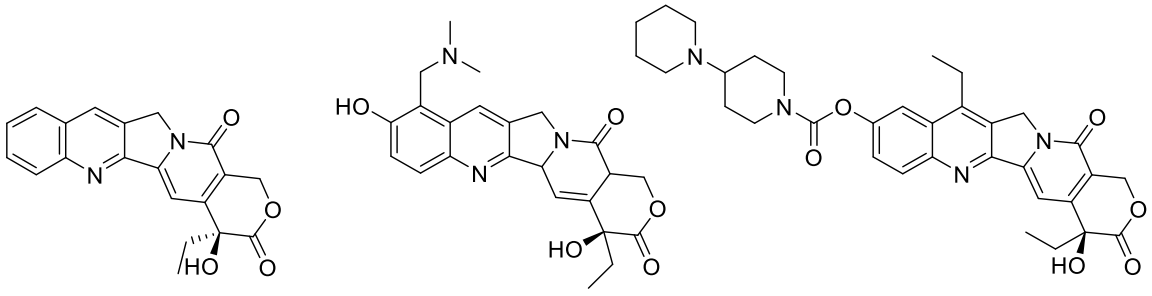
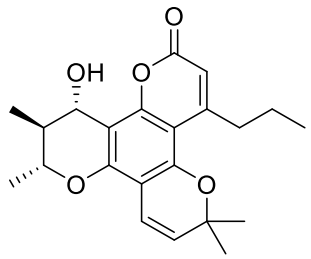


Figure 2. Structures of camptothecin, topotecan, and irinotecan

During the 1980s, with the emergence of Acquired Immune Deficiency Syndrome (AIDS), the National Cancer Institute (NCI) screened their cancer library of over 60,000 plant and marine organism extracts to search for a treatment for this new pandemic. Although this library was originally collected for the screening of cancer lines, several

hits were identified as having activity against the Human Immunodeficiency Virus (HIV), the causative agent of AIDS. These included the class of compounds known as calanolides shown in Figure 3 below, isolated from the tropical rainforest tree *Calophyllum lanigerum*.[3]



calanolide A

Figure 3. Structure of calanolide A

Despite natural products being a major inspiration for many drugs on the market today, the past few decades have seen the pharmaceutical industry turn towards a more synthetic approach for discovering new drug compounds. Prior to 1990, about 80% of drugs were natural products or natural product analogs. The focus on synthetics, beginning in the 1940s, caused that number to drop to 50% by the 1990s.[6]

Due to the methods used in isolating natural products at the time of library collection, the extracts or compounds were inefficient compared to preparation of synthetic compounds. Additionally, improvements in High Throughput Screening (HTS) and different methods for producing large synthetic libraries caused a shift from natural products that do not fit the HTS method well to the large, less diverse synthetic libraries that were designed for HTS. The loss of diversity and low hit rate with synthetics,

however, has recently caused resurgence in screening natural products. Improvements in structural elucidation and isolation methods of natural products now allow a more automated approach that works with modern HTS. Enzyme assays or non-cell based assays have also streamlined the screening process and expanded the number of diseases and targets beyond the traditional cancer screens to include parasitic, viral, and immune assays that were not feasible for most laboratories in the past. The traditional uses of the TCM plants also provide a key to where screening activity might be focused. Whether the traditional use was for fever, diarrhea, vomiting, or inflammation, these symptoms of infectious disease can be seen in modern assays testing anti-parasitic, anti-bacterial, immuno-modulation, and anti-viral activity.

Compounds derived from TCM plants show correlation between the traditional use and the activity of the isolated bioactive compounds. This correlation can be seen in Table 1 below:

Table 1. TCM-derived compounds used in Western medicine[7]

Drug	Clinical use	Traditional use	Plant name
Aescin	anti-inflammatory	anti-inflammatory	*Aesculus hippocastanum*
Berberine	anti-dysentery	treatment of gastric ailments	*Berberis sargentiana*
Caffeine	stimulant	stimulant	*Camellia sinensis (L)*
(+)- Catechin	haemostatic	haemostatic	*Potentilla fragarioides L.*
Curcumin	choleretic	choleretic	*Curcuma longa L.*
Deserpidine	anti-hypertensive	anti-hypertensive	*Rauwolfia serpentina*
Digitoxin	cardiotonic	cardiotonic	*Digitalis lanata*
Quinidine	anti-arrhythmic	anti-malarial	*Cinchona officinalis*
Quinine	anti-malarial	anti-malarial	*Cinchona officinalis*
Salicin	analgesic	analgesic	*Salix alba L.*
Strychnine	stimulant	stimulant	*Strychnos nux-vomica L.*
Theobromine	diuretic, vasodilator	diuretic	*Camellia sinensis (L)*

Isolation of Bioactive Compounds

Isolation strategies for natural products are constantly evolving. Originally, all compounds that could be purified were isolated from a plant that was used traditionally to treat diseases without concern if the specific compound was responsible for activity. This resulted in isolation of many inactive compounds, and gave way to bioassay-guided isolations that let the disease lead the way to the responsible compound or compounds.[8] Bioassay-guided isolations are currently the most common technique to purify the responsible compound for a certain bioactivity.

Bioassay-guided fractionation starts with a crude extract from the dried plant material using either aqueous ethanol or aqueous methanol. The crude extract is then taken through a liquid-liquid extraction using solvents of increasing polarity, from hexanes to water, to produce five fractions (1-5) as shown in Figure 4. These initial five fractions are then used for prescreening using a bioassay to see if the extract warrants further fractionation. If one of the fractions is bioactive, that fraction is further purified with either gravity column chromatography or flash column chromatography, depending on the complexity of the crude sample. The column fractions are then rescreened with the original bioassay to confirm activity. This process is continued until a final pure compound or compounds responsible for the bioactivity is isolated and characterized. Final purification often requires high performance liquid chromatography (HPLC) in order to obtain clean nuclear magnetic resonance (NMR) spectra and high resolution mass spectrometric (HRMS) data for compound characterization. Even a small amount of an impurity can lead to mis-assignment of peaks in the spectrum.

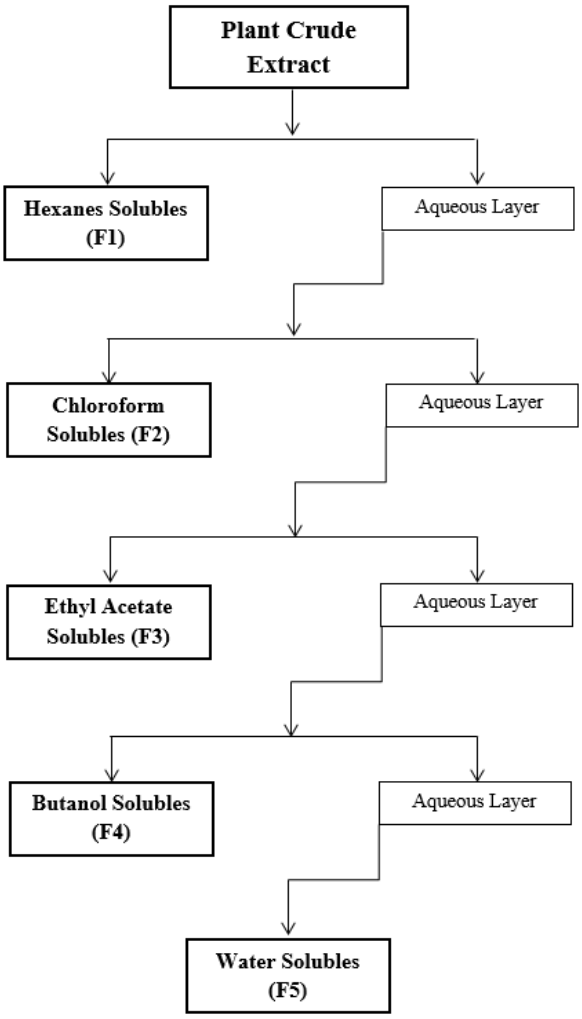


Figure 4. Flow diagram of initial liquid-liquid extraction based on polarity

Advances in isolation and structural elucidation technologies now provide a more complete picture of the entire plant extract. For example, entire extracts can be separated into just one to four compounds per well on a 96-well plate using HPLC and liquid handlers. A typical process outlined in Figure 5 below, would start with one gram of a

crude extract from the plant obtained from maceration in a 50:50 ethanol-ethyl acetate mix. The extract is loaded onto an automated flash cartridge that creates six flash column chromatography fractions based on elution with solvents of different polarity. Often, the first hexanes fraction is discarded since it typically contains inactive lipids. Each of those fractions is injected into a semi-preparative HPLC with different solvent gradients to match the predetermined flash fraction properties. Using a liquid handler, the HPLC fractions on average contain one to four compounds that are loaded into a 96 well plate.

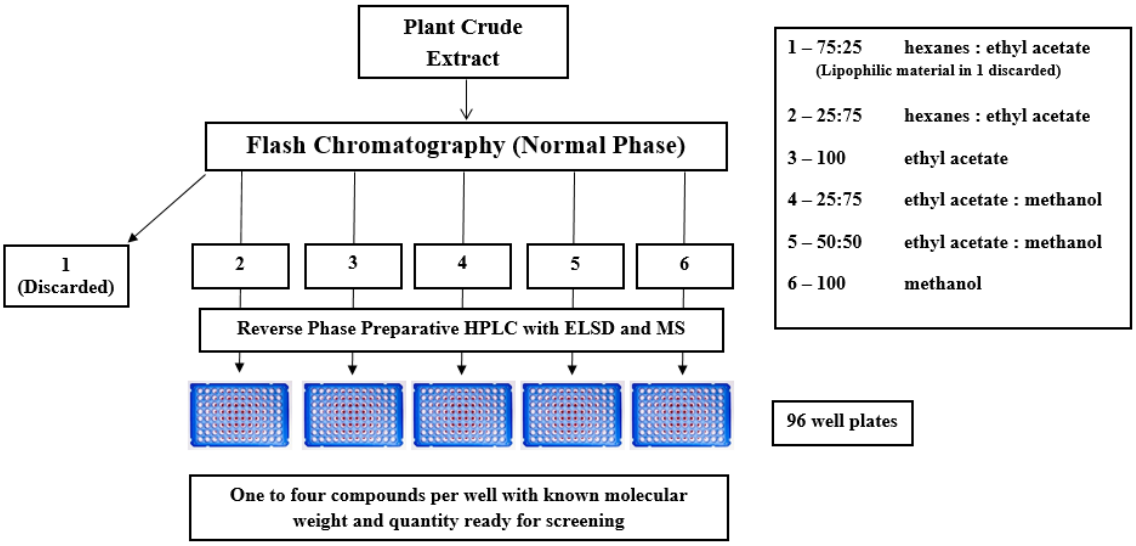


Figure 5. Flow diagram of advanced separation and purification of crude plant extract

The fractions are further quantified by mass spectrometry (MS) and an Evaporative Light Scattering Detector or ELSD.[9] An ELSD detector does not require a UV active chromophore in order to detect a compound and, once standards are set, enables quantification of the amount of material going into each well of the 96-well plate. This

insures that compounds occurring in minute quantities will be given a weighted average and screened accordingly. Otherwise, these compounds that occur in low amounts would be lost in the overall complexity of the initial crude extract.

Classes of Bioactive Compounds Found in Plants

In general, three broad classes of secondary metabolites based on structure and biosynthetic pathway make up the majority of bioactive compounds found in plant based natural products. The classes include terpenes, phenylpropanoids, and alkaloids.[10] These classes can be further broken down into subclasses within these major areas.

Terpenes are composed of multiple isoprene units. The category the terpene falls under is dependent on the number of isoprene units that make up that compound: monoterpenes, diterpenes, triterpenes, etc. The triterpenes are made up of 30 carbons and may undergo cyclizations to become the steroidal and oleanane class of compounds. Figure 6 below shows examples of some different subclasses of terpenes, including the basic isoprene unit, which is the building block for all of the compounds in this class.

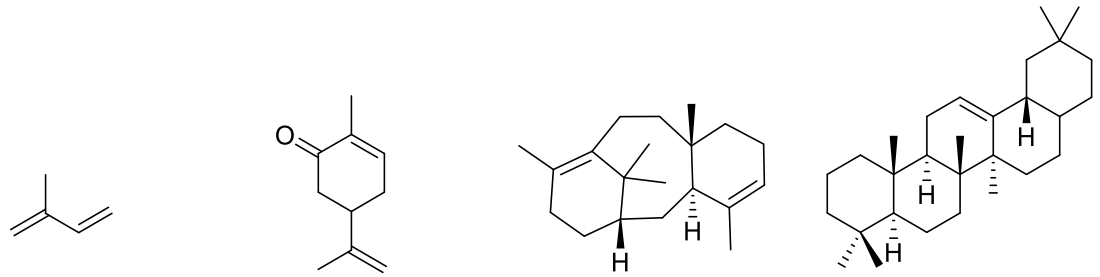
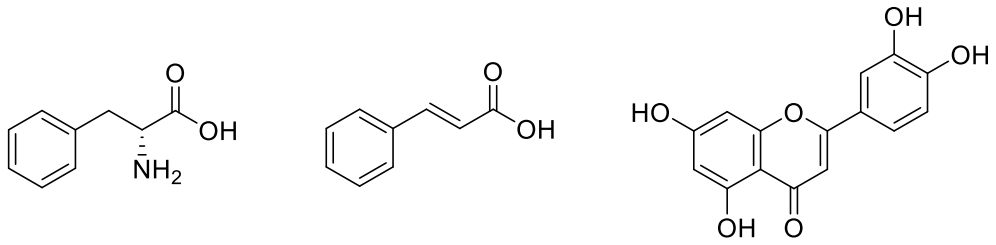


Figure 6. Terpene examples

Phenylpropanoids are a large class of compounds that share the amino acid phenylalanine as the basis of the compound structure. Cinnamates, flavonoids, natural phenols, and polyphenols all make up the phenylpropanoids class and a common characteristic shared among most of the phenylpropanoids is the tricyclic ring system seen in flavonoids.[10] Flavonoids make up the largest group of compounds in the phenylpropanoid class and are generally responsible for bioactivity in many different assays. Occasionally, this activity is too non-specific and many flavonoids turn into problem compounds with off-target bioactivity. Examples of the amino acid phenylalanine and other compounds in this class are shown below in Figure 7.



phenylalanine cinnamic acid luteolin

Figure 7. Structures of phenylalanine, cinnamic acid, and the flavonoid luteolin

The alkaloids, probably the most well-known of the natural product classes when thinking of plant compounds with human biological activity, are characterized by the presence of nitrogen. One well known example, isolated from *Papaver somniferum*, is morphine shown in Figure 8. Morphine is one of the first pure compounds isolated from plants, in 1804 by Friedrich Sertürner, and is still the basis for many analgesic drugs on

the market today, as well as continued research into analogs for additional therapeutic compounds.

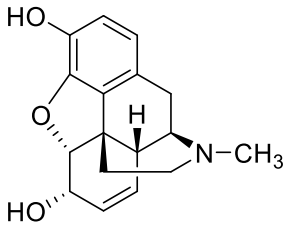
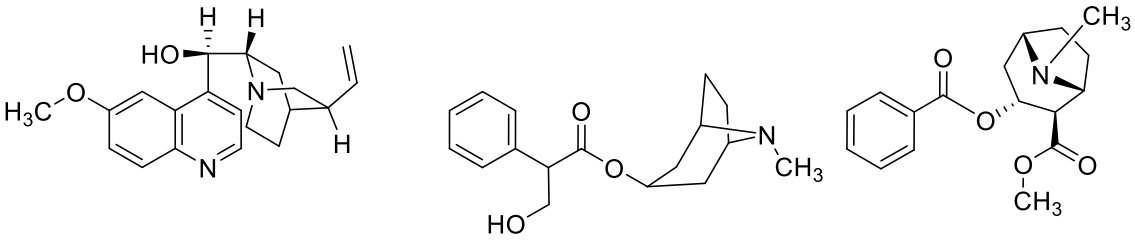


Figure 8. Structure of morphine

Traditional isolation techniques, including acid/base extractions, were very amenable for the isolation and purification of alkaloids. This resulted in alkaloids being some of the first pure compounds to be isolated from plants. Examples of other early alkaloid discoveries are shown below in Figure 9.



quinine
Discovered 1820
from *Cinchona calisaya*

atropine
Discovered 1819
from genus *Mandragora*

cocaine
Discovered 1860
from *Erythroxylon coca*

Figure 9. Structures of quinine, atropine, and cocaine

The two classes of compound that form the basis of the research for this dissertation include the oleananes and the flavonoids. Pure compounds in each class were identified by bioassay-guided fractionation of *Cyclocarya paliurus* and *Scutellaria baicalensis* respectively.

Oleananes are a class of natural triterpenoid compounds found in flowering plants. The core pentacyclic structure consists of six membered rings (rings A through E) along with seven distinct methyl groups shown in Figure 10 with oleanolic acid with the carbon skeleton numbered. The oleanane class of compounds has shown great promise in natural product drug screening with a wide spectrum of activity in various diseases.

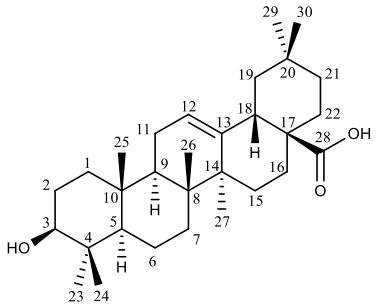
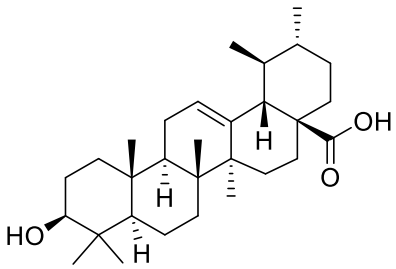


Figure 10. Oleanolic acid and numbering system

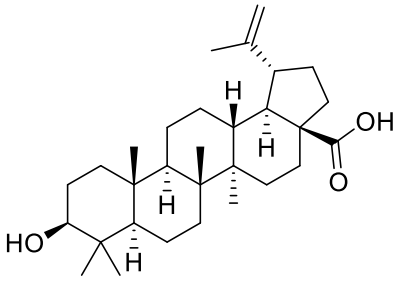
Different oleanane compounds have demonstrated activity in anti-tumor, anti-viral, anti-inflammatory, and antimicrobial screens [11] Ursolic acid, shown in Figure 11, has shown activity at various stages of tumor development: tumorigenesis, differentiation, and promotion.[12–14]



ursolic acid

Figure 11. Ursolic acid

Oleananes have also shown potent anti-viral activity, including activity against HIV. Structure activity based profiling has been completed with ursolic, oleanolic, betulinic acid (Figure 12) and derivatives showing anti-HIV activities.[15,16]



betulinic acid

Figure 12. Betulinic acid

Flavonoids, from the larger class of phenylpropanoids, make up one of the largest collection of compounds in this class of natural products. Currently over 4000 varieties of flavonoids have been identified and the core structure consists of two phenyl rings and

a single heterocyclic ring shown in Figure 13.[17] Flavonoids have many off target interactions or general cytotoxicity issues that have thus far interfered with development into an approved drug.

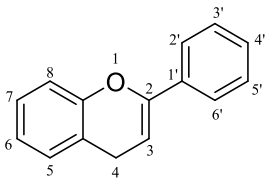
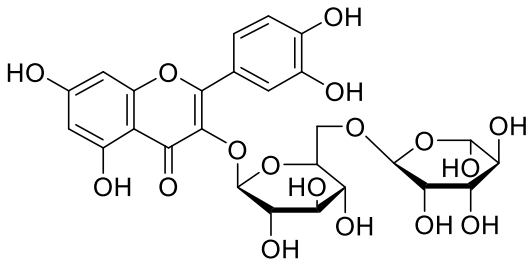


Figure 13. Flavonoid backbone and numbering system

Flavonoids have been well studied since first isolated as a possible vitamin in oranges in 1930 (rutin) shown in Figure 14. Since that time, flavonoids have been attributed with many mechanisms including: antioxidant effects, direct radical scavenging, nitric oxide, xanthine oxidase, leukocyte immobilization, and interaction with many other enzyme systems.[17] Even though flavonoids have wide ranging and off target activity, this class of compounds still shows signs of selectivity in certain diseases and are therefore merit continued research as possible drug leads.



rutin

Figure 14. Structure of the flavonoid rutin

Plant Cell Culture

While most compounds isolated from plants have followed a traditional extraction of the whole plant as it occurs in nature, a newer alternative is the use of plant cell culture. Plant cell culture is a sterile method for propagating plant cells or plant tissue *in vitro.* Once an undifferentiated cell culture is established, then treatment with chemicals or plant hormones allows for manipulation of the plant cells for research, conservation purposes or metabolic screening. The metabolic screening of plant cell cultures has many advantages over screening extracts from plant material collected from the wild. Traditional screening of plants collected in the wild does not represent the full metabolic profile of the plants. Plants have an innate immune system that will only produce certain secondary metabolites when the plant is stressed, either by infection or other physical damage. This stress can be simulated in culture to force the plant to produce metabolites that might not be produced in the wild at the time of cultivation. Additionally, nuisance compounds like chlorophyll and tannins existing in large quantities from plants collected in the wild, occur in small quantities in cultured plant cells. Culture conditions also allow for manipulation of certain pathways that are only activated in response to chemicals or hormones generated during a stress response from the plant, also known as the innate immune response. Advances in understanding the biosynthetic pathways responsible for the innate immune system allows for manipulation and activation of the defensive pathways that produce compounds that help the plant fight microbes and other pests. Defensive compounds in response to stress, are most often the compounds responsible for activity in bioassay screening. Growing the plants in cell culture or an *in vitro* environment gives greater control in manipulating the culture media to activate these

defensive pathways and test the changes in the metabolic profile. Activating the defensive pathways should be part of any wide scale screening effort to insure the full metabolic profile of the plant is being tested.

CHAPTER II: SYNTHESIS OF CYCLOCARIC ACID A AND COMPARISON TO MATERIAL FROM *CYCLOCARYA PALIURUS*

Cyclocarya is a flowering plant in the family Juglandaceae. The *Cyclocarya* genus is comprised of a single species, *Cyclocarya paliurus* (wheeled wingnut), an endemic tree growing in southern China, commonly known as the "sweet tea tree" shown below in Figure 15.[18] Interest in the tree comes from biological activities including hypoglycemic, hypolipidemic, and antihypertensive activity. The leaves of *C. paliurus* have long been a source for treatment of hypertension and diabetes.[19–21]

Figure 15. *Cyclocarya paliurus* or wheeled wingnut

Numerous components have been isolated from the leaves, including polysaccharides, flavonoids, phenols, steroids, and triterpenoids, with most attention focused on the polysaccharides. Two triterpenoids previously reported is the oleananes cyclocaric acid A and cyclocaric acid B, shown in Figure 16 below, with subsequent

reports of the isolation of cyclocaric acid A referring back to the data in the original 1996 article.[22–24] One exception is a reference to sapindic acid from *Sapindus laurifolius*, assigned the same structure as cyclocaric acid A in 1968.[25]

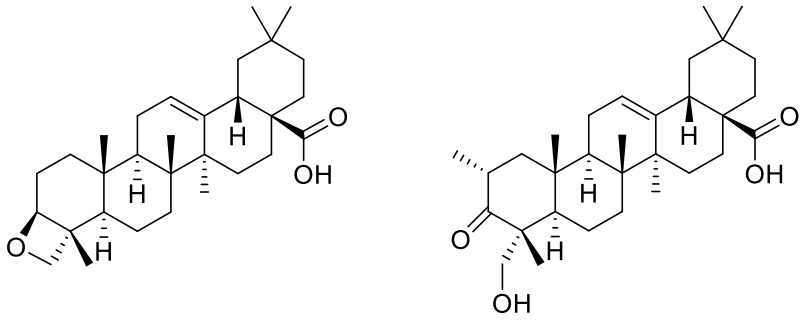


Figure 16. Structure of cyclocaric acid A and cyclocaric acid B

Initially, a literature review concerning any plant of interest is conducted prior to any isolation and structural work. At the time of this work, *C. paliurus* had been reported in various journal articles concerning compound isolations without relation to activity and a few journal articles discussing the bioactivity of the polysaccharides and phenolic compounds in the aqueous fraction.[26,27] Therefore, the literature review and initial bioassay screening made *C. paliurus* a prime target to start an initial investigation into anticancer compounds from this plant.

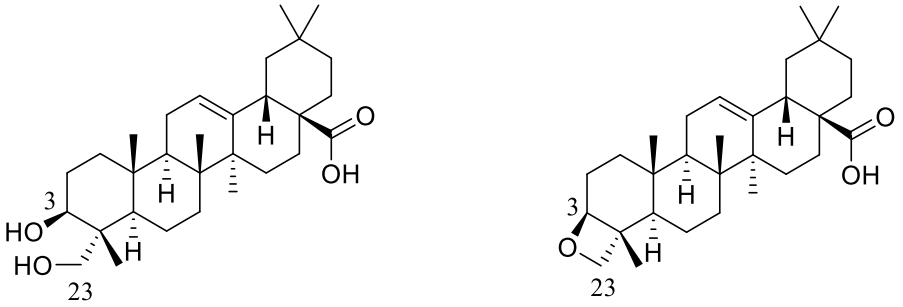
Interest in pursuing *C. paliurus* for bioactive compounds originated with cancer cytotoxicity screens against BT20 (breast cancer line) and A549 (lung cancer line). The original fractions provided by Dr. Chun-nian He and screened by Dr. Ying Gao included

those in Table 2. High levels of inhibition of cancer cell growth were observed in the ethanol, petroleum ether and chloroform fractions (Cp1, Cp2 and Cp3 respectively). This pattern of activity is not uncommon at the crude extract level. The ethanol fraction contains a majority of the compounds from the other extractions. The active compounds still showed solubility in the petroleum ether fraction and chloroform fraction. Many compounds that are not soluble once purified can be made readily soluble when still part of a crude mixture.

Table 2. Initial results of cancer cytotoxicity for fractions of *C. paliurus* (Cp)

Fraction	**Solvent**	**BT20 % Inhibition**	**A549 % Inhibition**
Cp1	Ethanol Fraction	94.1	90.8
Cp2	Petroleum Ether	97.8	98.5
Cp3	Chloroform	97.4	99.5
Cp4	Ethyl Acetate	70.9	59.8
Cp5	Butanol	67.8	NA
Cp6	Water	54.6	NA

The ethanol extract of dried plant material from *C. paliurus* was purified to afford two pentacyclic triterpenoids belonging to the oleanane series, which were initially thought to be cyclocaric acids A and B. Spectroscopic analysis, followed by independent synthesis, shows that the structure initially reported as cyclocaric acid A is actually hederagenin shown in Figure 17 below. The key carbons (C3 and C23) are labeled in Figure 17, and were crucial in determining the correct structure of the plant isolate.



hederagenin cyclocaric acid A

Figure 17. Structure of hederagenin and cyclocaric acid A

A crude ethanol extract of *C. paliurus* was first extracted with hexanes to remove unwanted lipophilic compounds, and then extracted sequentially with $CHCl_3$, EtOAc, and n-BuOH. Purification of the $CHCl_3$ fraction by gravity column chromatography on silica gel afforded seven fractions. Fractions 1−3 were combined and further purified by flash column chromatography to provide two pure compounds as shown in Figure 18. Two separate methods were developed for the isolation of the bioactive compounds for *C. paliurus*. Method A, shown below in Figure 18, was the traditional bioassay guided fractionation method.

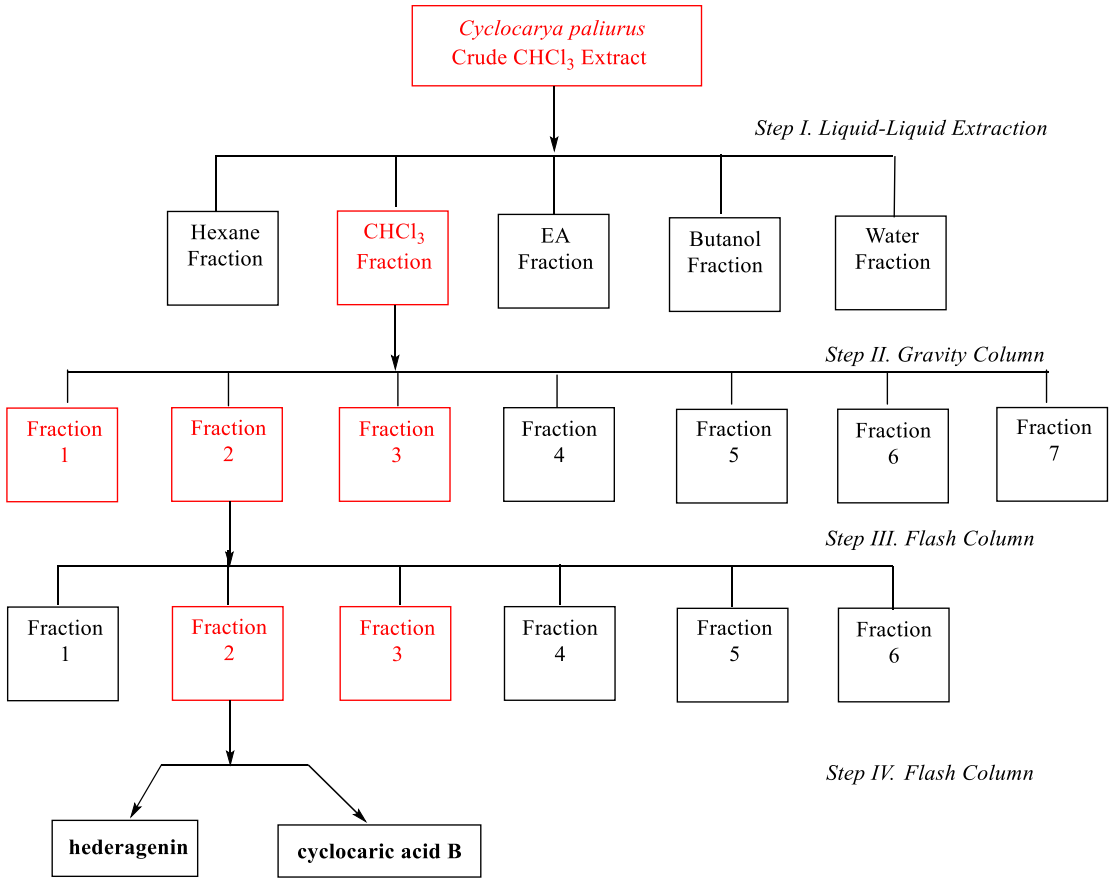


Figure 18. Flow chart for Method A bioassay guided fractionation of *C. paliurus*

Once the compounds were identified, certain properties of the compounds became apparent, and allowed for optimization of the isolation. Method B shown below in Figure 19, was developed as an expedited isolation to quickly access the pure compounds. Method A was four steps and still showed signs of impurities in the isolates. Method B, however, was three steps with highly purified compounds at the end of step 3. The key difference in the methods was dissolving the initial crude extract in a small amount of chloroform, then adding hexane until a precipitate formed. This precipitate was then

filtered out and only contained a handful of compounds, including the hederagenin and cyclocaric acid B.

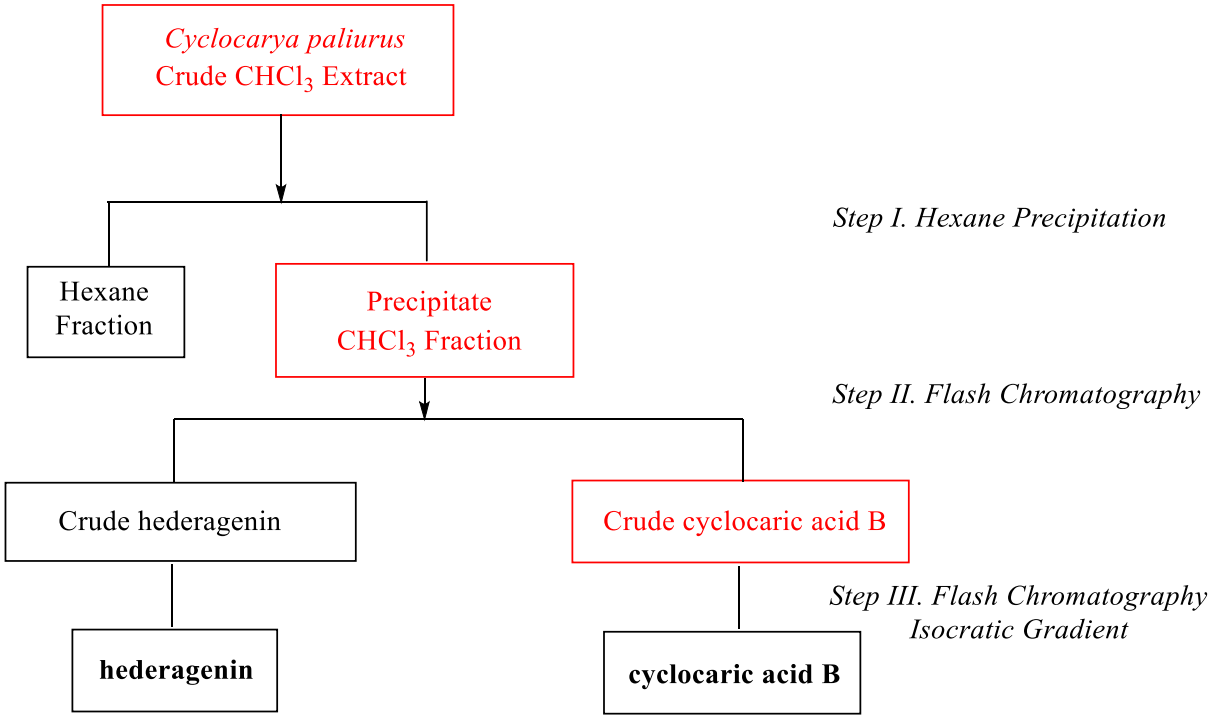
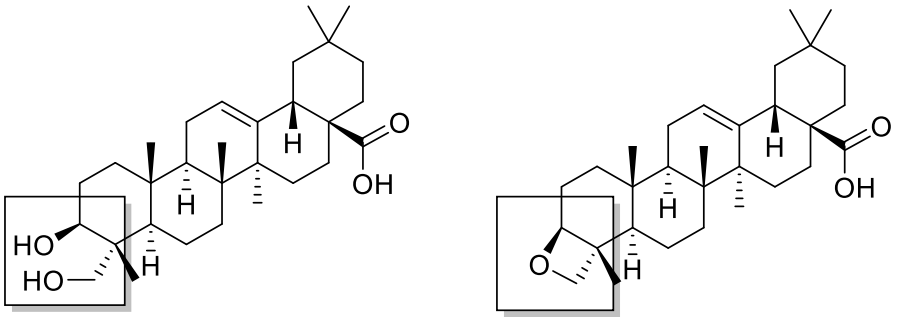


Figure 19. Flow chart for Method B fractionation of *C. paliurus* to improve efficiency of isolation

In 1996, Zhong et al. had reported the isolation of the oleananes cyclocaric acids A and B.[22,28] The structure of the higher Rf compound, which retained the cancer cytotoxicity, in our extract was originally assigned as the oxetane cyclocaric acid A, and the lower as cyclocaric acid B. The NMR data for the compound assigned as cyclocaric acid A was identical to the original report. However, on closer investigation of the ^{1}H and ^{13}C NMR data from that report, particularly the coupling constants for the C-23

methylene protons of cyclocaric acid A, it became evident that the compound was in fact not the oxetane, but was the alcohol, hederagenin. This is a common oleanane found in ivy (*Hedera helix*) and has been isolated from many different plants.[29,30] Hederagenin has previously been reported to have cancer cytotoxicity in the same cell line used for the bioassay guided fractionation.[31]

Although the NMR data of the high Rf plant material was identical to that reported in 1996 for cyclocaric acid A, High-Resolution Mass Spectrometry (HRMS) data using Electrospray Ionization (ESI) showed a molecular ion of m/z 473.3625 [M + H] that corresponds not to cyclocaric acid A, but to the molecular weight of hederagenin. There is also an [M − 18] ion at m/z 455.3491 corresponding to the loss of the alcohol of hederagenin. Figure 20 below, shows the structures and exact mass of hederagenin and cyclocaric acid A. An [M – 18] signal is common in alcohols such as hederagenin and with older MS techniques a molecular ion is often not seen for alcohols. In this case it could easily be confused for the oxetane structure. In the original report, using Electron Impact Mass Spectrometry (EIMS) data, it is presumed that the true molecular ion was not seen, and the [M − 18] ion was taken as the molecular ion, which would match that of the oxetane structure.



Exact Mass: 472.3553
hederagenin (alcohol)

Exact Mass: 454.3447
cyclocaric acid A (oxetane)

Figure 20. Structure and molecular weights of hederagenin (alcohol) and cyclocaric acid A (oxetane)

In order to confirm these speculations, a synthesis of the oxetane was carried out. This would allow unequivocal comparison to the isolated natural product. The synthetic route began with hederagenin. Protection of the acid as the allyl ester was followed by conversion of the primary alcohol into tosylate **2** as seen in Figure 21.[32] Oxidation of the 3β-alcohol with pyridinium chlorochromate (PCC) afforded ketone **3**. Reduction with sodium borohydride afforded primarily the 3β-alcohol **2** again, which is the wrong stereoisomer for synthesis of cyclocaric acid A. Use of a different reducing agent, L-selectride, which preferentially affords axial alcohols from cyclohexanones was attempted next. Reduction with L-selectride afforded the oxetane **5** as the major product and minor amounts of the intermediate 3α-alcohol. Reduction first affords the intermediate 3α-alkoxide **4**, which displaces the tosylate *in situ* to provide the oxetane **5**. Deprotection of the ester to the acid afforded cyclocaric acid A, as shown in Figure 21.

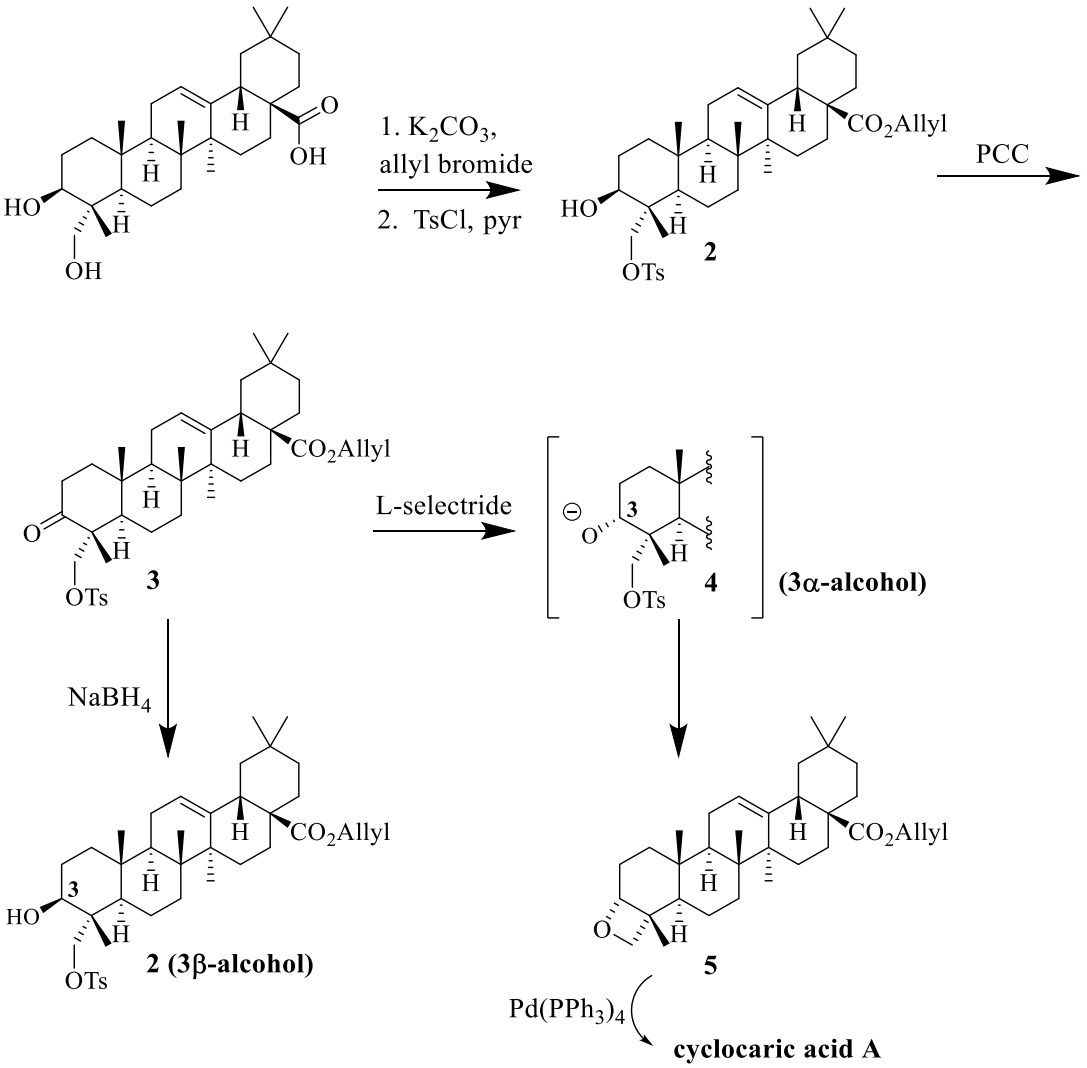


Figure 21. Synthesis of cyclocaric acid A from hederagenin

A more lengthy synthesis of the methyl ester of cyclocaric acid A had been reported in 1976 as part of an investigation of oleananes, although it had not been given the name cyclocaric acid A at that time.[33] Although the MS data indicate that the structure of cyclocaric acid A, originally reported as a component of *C. paliurus*, is indeed hederagenin, the NMR data comparing the synthetic material and the plate isolate

are also conclusive. Table 3 collates the ^{1}H and ^{13}C NMR data of hederagenin (from *C. paliurus*) and the synthetic cyclocaric acid A. Data from the original report are identical to hederagenin and are listed in the Experimental Section for comparison.

Table 3. NMR Data (500 MHz, pyridine-*d5*) of cyclocaric acid A and hederagenin

	cyclocaric acid A		**hederagenin**	
position	δ_C, type	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	35.3, CH_2	α 1.36, m	38.8	α 1.07, m
		β 1.51, m		β 1.59, m
2	24.8, CH_2	1.61, m	27.7	1.89, m
3	86.4, CH	4.66, d (4.0)	73.4	4.23, dd (11.2, 5.1)
4	42.5, C		42.9	
5	51.8, CH	1.86, m	48.6	1.55, m
6	20.6, CH_2	α 1.16, m	18.6	α 1.48, m
		β 1.31, m		β 1.69, m
7	32.6, CH_2	1.53, m	33.0	α 1.32, m
				β 1.67, m
8	40.0, C		39.8	
9	45.4, CH	1.24, m	48.2	1.80, m
10	35.5, C		37.3	
11	23.9, CH_2	1.95, m	23.9	1.95, m
12	122.8, CH	5.55, t (2.9)	122.6	5.51, t (2.9)
13	144.7, C		144.9	
14	41.3, C		42.4	
15	28.3, CH_2	α 1.21, m	28.4	α 1.18, m
		β 2.13, m		β 2.13, m
16	23.8, CH_2	2.15, m	23.7	α 1.99, m
				β 2.13, m
17	46.8, C		46.7	
18	42.2, CH	3.34, dd (13.8, 4.1)	42.0	3.32, dd (13.8, 4.0)
19	46.4, CH_2	α 1.33, m	46.5	α 1.33, m
		β 1.83, m		β 1.81, m
20	31.0, C		31.0	
21	34.3, CH_2	1.23, m	34.3	1.45, 1.22, m
22	33.4, CH_2	α 1.81, m	33.1	α 1.80, m
		β 2.04, m		β 2.03, m
23	82.7, CH_2	α 3.95, d (4.6)	68.0	α 3.74, d (10.3)
		β 4.30, d (4.6)		β 4.21, d (10.3)
24	13.5, CH_3	0.67, s	13.2	1.07, s
25	19.5, CH_3	0.93, s	16.0	0.99, s
26	17.4, CH_3	1.06, s	17.5	1.07, s
27	26.0, CH_3	1.27, s	26.2	1.26, s
28	180.2, C		180.2	
29	33.3, C_3	0.98, s	33.3	0.95, s
30	23.9, CH_3	1.03, s	23.8	1.02, s

One clear indication of whether the oxetane is present or not is the coupling constant of the C-23 methylene protons. These protons show a similar pattern (two doublets) in both hederagenin and the oxetane. However, in the seco (open) form of hederagenin, the coupling constant (J value) is 10.3 Hz, whereas in the cyclic form of cyclocaric acid A, it is 4.6 Hz. The smaller J value is typical for oxetanes and comparable to values found in the earlier oxetane synthesis.[33] Generally, in syntheses involving a ring closure to give an oxetane, the J value decreases and the signals are deshielded.[34,35] The differences are evident in Figure 22 shown below, which shows an overlay of the plant isolate, hederagenin (purchased), and the synthetic cyclocaric acid A. The plant isolate and hederagenin are clearly identical and do not have the oxetane structure of cyclocaric acid A. The MS data of the synthetic cyclocaric acid A showed a molecular ion at m/z 455.3511 [M + H]+ as well as a fragment from loss of CO_2H at m/z 406.2161.

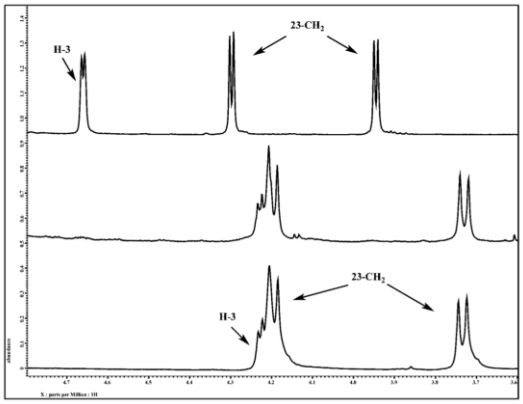


Figure 22. Overlay of ^{1}H-NMR (δ 3.6-4.8) of cyclocaric acid A (top), plant isolate (middle) and hederagenin (bottom), showing a comparison of the C-23 hydrogens and the C-3 hydrogen.

It is also important to address some confusion regarding the C-3 configuration. For the oxetane of previously reported cyclocaric acid A, the oxygen is reported as 3β, which would equate to a *trans*-fused oxetane moiety, however it is drawn as the 3α-orientation in most references. Although a 3β oxygen may make more sense as a hederagenin derivative, there are very few reports of *trans*-fused bicyclo[4.2.0]oxetanes.[36,37] In fact, Tsuda and co-workers were unable to synthesize *trans*-fused oxetanes of oleananes and recovered only retro-aldol, ring-opened products.[33] Similar results were obtained in the current investigation, with attempts to form a *trans*-oxetane from the tosylate **2** resulting in multiple decomposition products and no oxetane.

In conclusion, two components of *C. paliurus* have been isolated, and independent synthesis confirms that "cyclocaric acid A" was not previously isolated from this plant and the compound originally reported as an isolate is, in fact, hederagenin. In all likelihood, cyclocaric acid A is not actually a natural product. The similarity of the NMR data, combined with the MS techniques available at that time, shows how this mistake could easily have been made based on the available information. Although the same structure was reported in 1968 as sapindic acid, this was done without NMR data, and the assignment was based on chemical tests and elemental analysis. Thus, it is unclear whether or not that isolate was actually the same structure as that assigned to cyclocaric acid A.

This investigation of *C. paliurus* was based on bioassay guided fractionation with cancer cytotoxicity as the bioassay. Promising activity was observed, leading to isolation of a pure bioactive compound originally thought to be cyclocaric acid A, based on

literature review. With the proof that this is actually hederagenin the observed activity is not surprising and matches previously reported assays for hederagenin. Also, the synthetic cyclocaric acid A was found to be inactive.

Materials and Methods

General Experimental Procedures

The NMR data were obtained on a 500 MHz FT-NMR model ECA-500 JEOL (Peabody, MA, USA) purchased with funding provided by the National Science Foundation. Coupling constants (J values) are recorded in hertz (Hz). All signal assignments are based on COSY, HMQC, and DEPT data. Polarimetry was performed using an Autopol III polarimeter (Rudolph Research, Fairfield, NJ, USA). High-resolution ESIMS was performed at Notre Dame University, Notre Dame, IN, USA. Solvents and chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (Milwaukee, WI, USA). Hederagenin was purchased from Biopurify Phytochemicals (Chengdu, Sichuan, China).

Plant Material

The leaves of *Cyclocarya paliurus* (Batal.) Iljinskaja (CPs) were collected in Zhangpu County, Fujian Province, China, in May 2012, and identified by Dr. Chun-nian He (Institute of Medicinal Plant Development, Chinese Academy of Medical Science, Peking Union Medical College, Beijing, P. R. China). A voucher specimen (No. 2012 cP003) has been deposited at the Herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. Guangxi Botanical Garden of Medicinal Plants (Nanjing, China) provided the

crude ethanol extract from *C. paliurus*. The dried leaves of *C. paliurus* (1.0 kg) were pulverized and extracted successively four times with 95% EtOH (each 10 L, each 24 h) at room temperature. The combined EtOH extract was concentrated under reduced pressure at 60 °C to afford a dark brown residue (100 g).

Bioassay Guided Isolation

Method A

The crude EtOH extract (11.2 g) was partitioned by liquid/liquid extraction to provide five fractions: hexanes, EtOAc, $CHCl_3$, n-BuOH, and H_2O. The $CHCl_3$ fraction was then purified by gravity column chromatography on 70−230 mesh silica gel, eluting with a $CHCl_3$−MeOH gradient system (1:0 to 0.1:2), to afford seven fractions. Fractions 1−3 were combined and chromatographed by flash column chromatography eluting with an EtOAc−hexanes−MeOH gradient (50:50:0−100), affording six fractions. Fractions 2 and 3 were combined and purified further by flash column chromatography to afford 1.1 mg of hederagenin (originally assigned as cyclocaric acid A) and 12.4 mg of cyclocaric acid B as shown in Figure 23.

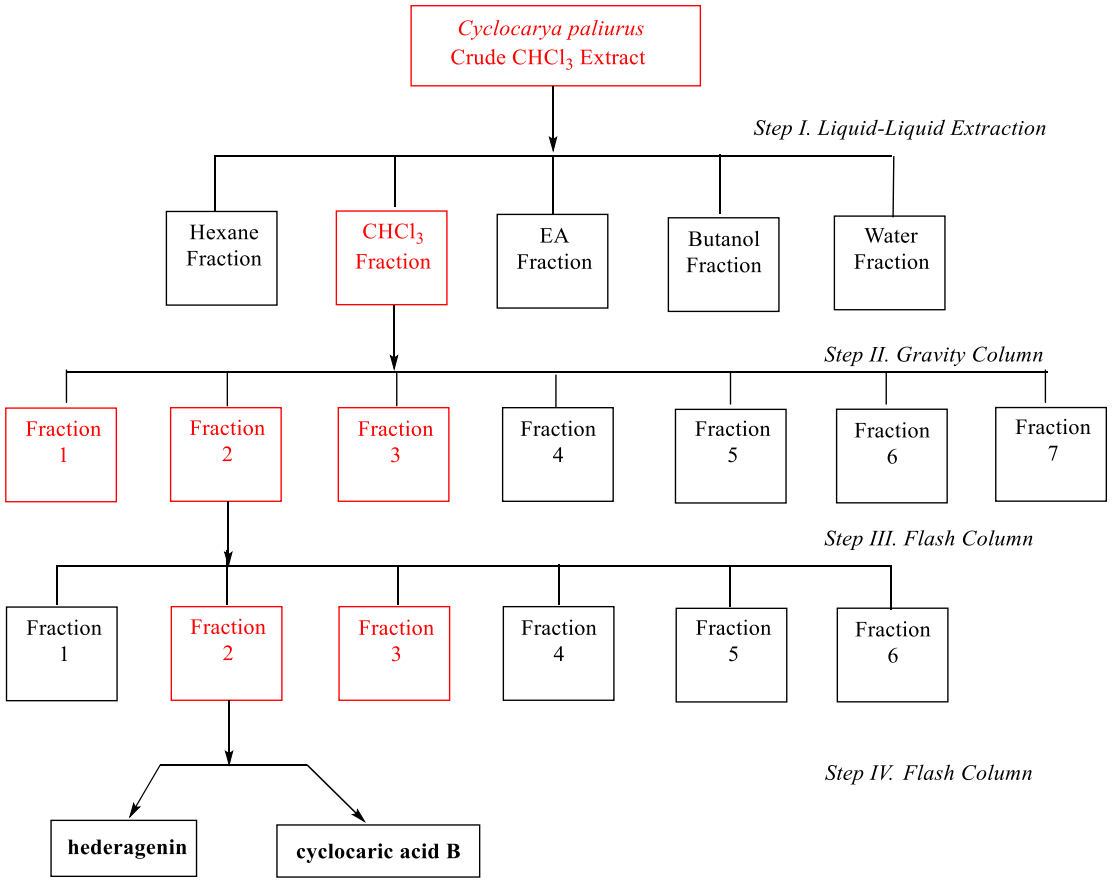


Figure 23. Flow chart for Method A bioassay guided fractionation of *C. paliurus*

Method B

The crude EtOH extract (21.2 g) was dissolved in a small amount of $CHCl_3$, and hexanes were added to form a precipitate. The precipitate was filtered and washed with hexanes. The precipitate (1.6 g) was dissolved in $CHCl_3$ and suspended on silica gel. Flash column chromatography, eluting with $CHCl_3$–acetone (0–40), afforded a mixture of hederagenin and cyclocaric acid B. Further purification by flash column chromatography (isocratic, 10% acetone– 90% DCM) afforded hederagenin (7.1 mg) and cyclocaric acid B (16.6 mg). These processes are shown in Figure 24.

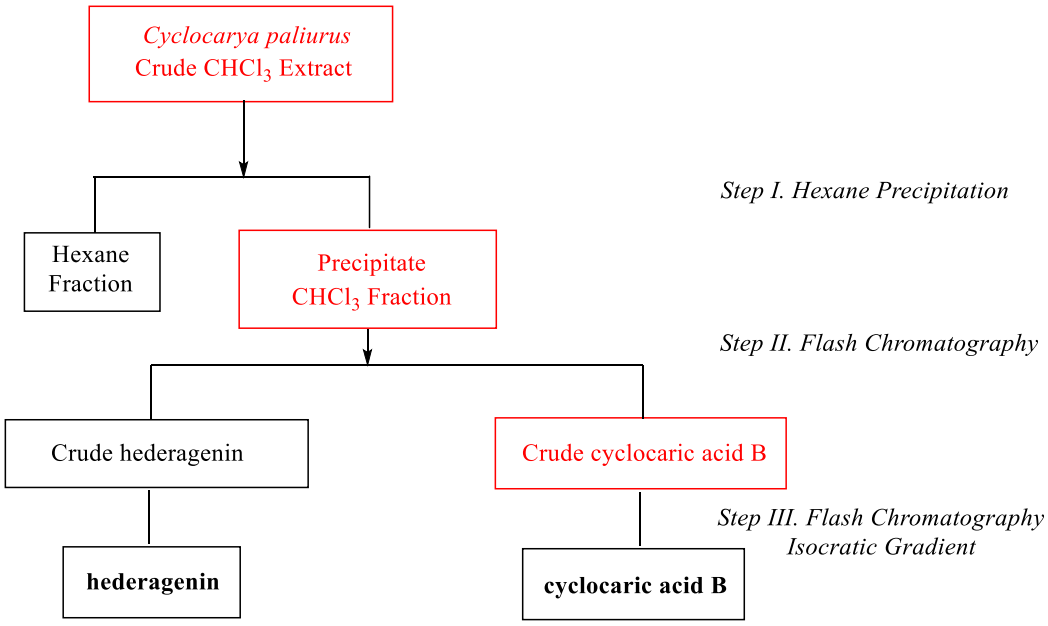


Figure 24. Flow chart for Method B fractionation of *C. paliurus* to improve efficiency of isolation

Synthesis

Allyl Hederagenate (1)[32]

To a solution of hederagenin (0.314 g, 0.66 mmol) in DMF (8 mL) were added K_2CO_3 (0.132 g, 0.96 mmol) and allyl bromide (83 µL, 0.96 mmol). The solution was stirred at room temperature for 24 h, extracted with EtOAc, and washed with H_2O. The organic layer was dried over $MgSO_4$ and filtered, and the solvent evaporated. The crude product was chromatographed on a 25 × 120 mm silica gel column eluting with a gradient of 1:9 to 1:1 EtOAc−hexanes to afford 313 mg of the known allyl ester (92%) as an amorphous solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.72 (s, 3H, H-26), 0.88 (s, 3H, H-24), 0.90 (s, 3H, H-29), 0.93 (s, 3H, H-30), 0.95 (s, 3H, H-25), 1.13 (s, 3H, H-27), 2.88

(dd, J = 14.0, 4.0 Hz, 1H, H-18), 3.42 (d, J = 10.9 Hz, 1H, H-23a), 3.64 (t, J = 8.0 Hz, 1H, H-3), 3.70 (d, J = 10.8 Hz, 1H, H-23b), 4.52 (m, 2H, CH_2O-allyl), 5.20 (dd, J = 10.3, 1.2 Hz, 1H, vinyl-CH2), 5.28 (t, J = 3.4 Hz, 1H, H-12), 5.32 (dd, J = 17.1, 1.7 Hz, 1H, vinyl-CH_2), 5.90 (m, 1H, CH-vinyl); ^{13}C NMR (125 MHz, $CDCl_3$) δ 11.4 (C-24), 15.7 (C-25), 17.0 (C-26), 18.5 (C- 6), 23.0 (C-16), 23.4 (C-11), 23.6 (C-30), 25.9(C-27), 26.6 (C-2), 27.6 (C-15), 30.7 (C-20), 32.4 (C-22), 32.5 (C-7), 33.1 (C-29), 33.9 (C-21), 36.9 (C-10), 38.1 (C-1), 39.3 (C-8), 41.3 (C-18), 41.7 (C- 14), 41.7 (C-4), 45.9 (C-19), 46.7 (C-17), 47.6 (C-9), 49.7 (C-5), 64.8 (CH-allyl), 71.8 (C-23), 77.0 (C-3), 117.7 (vinyl-CH_2), 122.4 (C- 12), 132.5 (CH-vinyl), 143.7 (C-13), 177.4 (C-28).

23-Tosyloxy Allyl Hederagenate (2)

To a solution of hederagenin allyl ester (0.313 g, 0.61 mmol) in pyridine (5 mL) was added p-toluenesulfonyl chloride (0.233 g, 1.22 mmol). The mixture was stirred at room temperature for 24 h and then poured into 1 M HCl, extracted with EtOAc, washed with $NaHCO_3$ and brine, dried with $MgSO_4$, and filtered, and the solvent was evaporated. The crude product was chromatographed on a 25 × 120 mm silica gel column eluting with a gradient of 1:9 to 1:5 EtOAc−hexanes to afford 240 mg of the monotosylate 1 (59%) as an amorphous solid: [α] 25_D +58 (c 0.0006, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.67 (s, 3H), 0.68 (s, 3H), 0.89 (s, 3H), 0.92 (s, 3H), 0.93 (s, 3H), 1.12 (s, 3H), 2.45 (s, 3H, tosylCH_3), 2.88 (dd, J = 14.0, 4.0 Hz, 1H, H-18), 3.61 (m, 1H, H-3), 3.71 (d, J = 9.7 Hz, 1H, H-23a), 3.98 (d, J = 9.7 Hz, 1H, H-23b), 4.52 (m, 2H, allyl-CH_2O), 5.20 (dd, J = 10.6, 1.2 Hz, 1H, vinyl-CH_2), 5.27 (t, J = 3.4 Hz, 1H, H-12), 5.31 (dd, J = 17.2, 1.7 Hz, 1H, vinyl-CH_2), 5.89 (m, 1H, vinyl-CH), 7.35 (d, J = 8.0 Hz, 2H, aryl-H), 7.81 (d, J = 8.6

Hz, 2H, aryl-H); ^{13}C NMR ($CDCl_3$) δ 11.7 (C-24), 15.8 (C-25), 16.9 (C-26), 17.9 (C-6), 21.7 (tosyl CH_3), 23.0 (C-16), 23.3 (C-11), 23.6 (C-30), 26.0 (C-27), 26.5 (C-2), 27.6 (C-15), 30.7 (C-20), 32.1 (C- 22), 32.4 (C-7), 33.1 (C-29), 33.9 (C-21), 36.9 (C-10), 37.8 (C-1), 39.3 (C-8), 41.3 (C-18), 41.7 (C-14), 42.3 (C-4), 45.9 (C-19), 46.7 (C-17), 46.9 (C-9), 47.5 (C-5), 64.8 (allyl-CH_2O), 71.1 (C-3), 71.7 (C-23), 117.7 (allyl-CH_2), 122.2 (C-12), 127.9 (aryl C), 129.9 (aryl C), 132.6 (allyl-CH), 133.0 (aryl C), 143.8 (C-13), 144.8 (aryl C), 177.4 (C-28); HRESIMS m/z ($C_{40}H_{59}O_6S$) calcd for $[M + 1]^+$ 667.4027; found 667.4049.

3-Didehydro-23-tosyloxy Allyl Hederagenate (3)

To a solution of hederagenin monotosylate **2** (0.235 g, 0.35 mmol) in DCM (7 mL) was added pyridinium chlorochromate (0.304 g, 1.41 mmol). The mixture was stirred for 2 h at room temperature, the reaction mixture was filtered through Celite, and the solvent was evaporated. The crude material was chromatographed on a 25 × 120 mm silica gel column with a gradient of 1:9 to 1:5 EtOAc−hexanes to afford 194 mg of the ketone 2 (83%) as an amorphous solid: [α] 25_D +33 (c 0.003, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.77 (s, 3H), 0.91 (s, 3H), 0.92 (s, 3H), 0.94 (s, 3H), 1.04 (s, 3H), 1.15 (s, 3H), 2.46 (s, 3H, tosyl-CH_3), 2.90 (dd, J = 13.0, 4.0 Hz, 1H, H-18), 3.84 (d, J = 9.2 Hz, 1H, H-23a), 4.05 (d, J = 9.2 Hz, 1H, H-23b), 4.53 (m, 2H, allylCH_2O), 5.21 (dd, J = 10.6, 1.2 Hz, 1H, allyl-CH_2), 5.31 (t, J = 3.4 Hz, 1H, H-12), 5.32 (dd, J = 17.2, 1.7 Hz, 1H, allyl-CH_2O), 5.90 (m, 1H, CH-vinyl), 7.35 (d, J = 8.0 Hz, 2H, aryl-H), 7.77 (d, J = 8.6 Hz, 2H, aryl H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.9 (C-24), 17.0 (C-25), 17.5 (C-26), 19.1 (C-6), 21.7 (tosyl-CH_3), 23.0 (C-16), 23.4 (C-11), 23.6 (C-30), 25.9 (C-27), 27.6 (C-15), 30.7 (C-2),

31.7 (C-20), 32.4 (C-22), 33.1 (C-7), 33.9 (C-29), 34.8 (C-21), 36.3 (C-10), 37.1 (C- 1), 39.3 (C-8), 41.4 (C-18), 41.8 (C-14), 45.8 (C-19), 46.4 (C-17), 46.7 (C-9), 47.1 (C-4), 50.4 (C-5), 64.8 (allylCH_2O), 72.2(C-23), 117.8 (allyl-CH_2), 122.0 (C-12), 128.0 (aryl-C), 129.8 (aryl-C), 132.5 (allyl-CH), 132.7 (aryl-C), 143.9 (C-13), 144.8 (aryl-C), 177.4 (C- 28), 212.8 (C-3); HRESIMS, m/z ($C_{40}H_{57}O_6S$) calcd for $[M + 1]^+$ 665.3970; found 665.3904.

3β – alcohol (2)

To a solution of ketone **3** (0.028 g, 0.042 mmol) in MeOH (2 mL) was added $NaBH_4$ (0.003 g, 0.088 mmol). The mixture was stirred at room temperature for one hour, and checked for reaction completion with TLC. The crude mixture was washed with brine, extracted with EtOAc, and then the organic layer was dried with $MgSO_4$. Finally the organic layer was filtered, and the solvent was evaporated. The organic layer was dried over $MgSO_4$ and filtered, and the solvent evaporated. The crude product was checked by NMR for the placement of the C3 hydrogen. Product was confirmed from previous work to be 3β – alcohol.

Cyclocaric Acid A Allyl Ester (5)

To a solution of ketone **3** (0.028 g, 0.042 mmol) at −78 °C in anhydrous THF (1 mL) was added L-selectride (150 μL, 1 M solution). After 10 min the dry ice − acetone bath was removed, and the reaction mixture was allowed to warm to room temperature. After 2 h, 2 M NaOH (1.1 mL) and 30% H_2O_2 (0.25 mL) were added to the reaction mixture. The reaction mixture was stirred for one additional hour, poured into H_2O, and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and filtered, and the

solvent evaporated. The crude product was chromatographed on a 25 × 120 mm silica gel column eluting with a gradient of 1:9 to 1:5 EtOAc−hexanes to afford 6 mg of oxetane 3 (29%) as an amorphous solid: [α] 25_D +40 (c 0.001, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.78 (s, 3H), 0.80 (s, 3H), 0.91 (s, 3H), 0.93 (s, 3H), 1.03 (s, 3H), 1.17 (s, 3H), 2.90 (dd, J = 13.8, 4.0 Hz, 1H, H-18), 3.87 (d, J = 5.2 Hz, 1H, H-23a), 4.29 (d, J = 5.2 Hz, 1H, H-23b), 4.53 (m, 2H, allyl-CH_2O), 4.67 (d, J = 2.9 Hz, 1H, H-3), 5.21 (dd, J = 10.6, 1.2 Hz, 1H, allyl-CH_2O), 5.32 (dd, J = 17.5, 1.7 Hz, 1H, allyl-CH_2), 5.34 (t, J = 3.4 Hz, 1H, H-12), 5.90 (m, 1H, allyl-CH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.5 (C-24), 17.0 (C-26), 19.4 (C-25), 20.3 (C-6), 23.1 (C- 16), 23.5 (C-11), 23.6 (C-30), 24.3 (C-2), 25.7 (C-27), 27.6 (C-15), 30.7 (C-20), 32.2 (C-22), 32.4 (C-7), 33.1 (C-29), 33.9 (C-21), 34.8 (C-10), 35.2 (C-1), 39.6 (C-8), 41.3 (C-18), 41.6 (C-14), 42.1 (C-4), 44.9 (C-19), 45.8 (C-9), 46.9 (C-17), 51.3 (C-5), 64.8 (allyl-CH_2O), 83.1 (C-23), 87.0 (C-3), 117.7 (allyl-CH_2), 122.7 (C-12), 132.6 (allylCH), 143.6 (C-13), 177.4 (C-28); HRESIMS m/z ($C_{33}H_{51}O_3$) calcd for $[M + 1]^+$ 495.3833; found 495.3839.

Cyclocaric Acid A

To a solution of oxetane allyl ester 3 (0.039 g, 0.079 mmol), triphenylphosphine (0.013 g, 0.048 mmol), and pyrrolidine (0.011 g, 0.16 mmol) in anhydrous THF (1 mL) was added tetrakis(triphenylphosphine) palladium(0) (0.028 g, 0.024 mmol). The solution was stirred at room temperature for 24 h, and then the solvent was evaporated. The crude product was chromatographed on a 25 × 120 mm silica gel column eluting with a gradient of 1:9 to 1:2 EtOAc−hexanes to afford 14.4 mg of cyclocaric acid A (40%) as an

amorphous solid: [α] 25_D +45 (c 0.007, $CHCl_3$); NMR data in Table 2; HRESIMS m/z ($C_{30}H_{46}O_3$) calcd for $[M + 1]^+$ 454.3447; found 454.3451.

Original Report

Data reported in the original paper,[22] which match those of hederagenin and not the actual cyclocaric acid A, are recorded here for ease of comparison. ^{1}H NMR (pyridine-d5) δ 0.93 (s, 3H), 0.98 (s, 3H), 1.01 (s, 3H), 1.05 (s, 3H), 1.06 (s, 3H), 1.24 (s, 3H), 3.30 (dd, 1H, H-18), 3.72 (d, J = 10 Hz, 1H, H-23a), 4.18 (d, J = 10 Hz, 1H, H-23b), 4.19 (d, J = 5.16 Hz, 1H, H-3), 5.51 (t, 1H, H-12); ^{13}C NMR (pyridine-d5) δ 13.1 (C- 24), 17.5 (C-26), 16.1 (C-25), 18.7 (C-6), 23.7 (C-16), 23.9 (C-11), 23.8 (C-30), 27.7 (C-2), 26.2 (C-27), 42.3 (C-15), 31.0 (C-20), 33.1 (C-22), 33.3 (C-7), 33.3 (C-29), 34.3 (C-21), 37.5 (C-10), 38.9 (C-1), 39.8 (C-8), 42.1 (C-18), 42.3 (C-14), 42.9 (C-4), 46.5 (C-19), 48.2 (C-9), 46.7 (C-17), 48.8 (C-5), 68.3 (C-23), 73.7 (C-3), 122.6 (C-12), 144.9 (C-13), 180.2 (C-28).

CHAPTER III: BIOASSAY GUIDED FRACTIONATION OF THE PLANT *SCUTELLARIA BAICALENSIS*

Scutellaria baicalensis, also known as Chinese skullcap, shown in Figure 25 below, is a member of the Lamiaceae or mint family. *S. baicalensis* is a common herb used in China and Japan for traditional medicine but is also used as a food additive. *S. baicalensis* has been attributed with a wide range of beneficial bioactivity and is therefore of great interest in natural product research.

Figure 25. *Scutellaria baicalensis* or Chinese skullcap

Over fifty different compounds have been identified from the roots of *S. baicalensis.* It has been reported that many of the polyphenols, including the flavonoids baicalein and wogonin shown in Figure 26, have potent anti-cancer activity.[38,39] Other

flavonoids from *S. baicalensis* have shown anti-inflammatory, anti-bacterial, anti-oxidant, and anti-viral activity. [40–42]

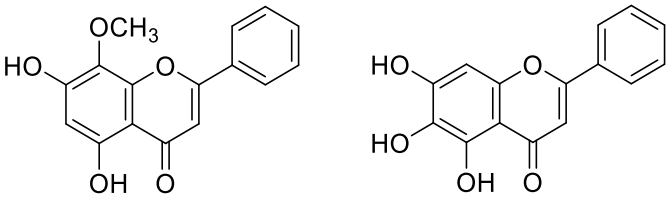


Figure 26. Wogonin and baicalein are two flavonoids isolated from *Scutellaria baicalensis*

At MTSU, screening of the initial crude ethanol extract identified anti-trypanosomal, immune-modulation, and anti-tumor activity. The most robust activity was the anti-trypanosomal, showing inhibition against *T. brucei.* The initial bioassay guided fractionation was used to pursue the active compounds in the assay against *T. brucei.* Anti-trypanosomal activity was first reported on the extract of *S. baicalensis* in 1998, showing a minimal effective concentration (MEC) of 30 µg/mL and an effective concentration (EC) of 20 µg/mL.[43] This report did not involve purification of the extract and only identified the extract itself as being of interest. Later, *S. baicalensis* and other species in the genus *Scutellaria* were characterized, and various compounds were identified with anti-trypanosomal, anti-cancer, and anti-microbial activity. Scutellarin, apigenin, and chrysin, shown in Figure 27, were the main compounds that showed the most promising activity against *T. brucei* with IC_{50} values in the low µM range.[44]

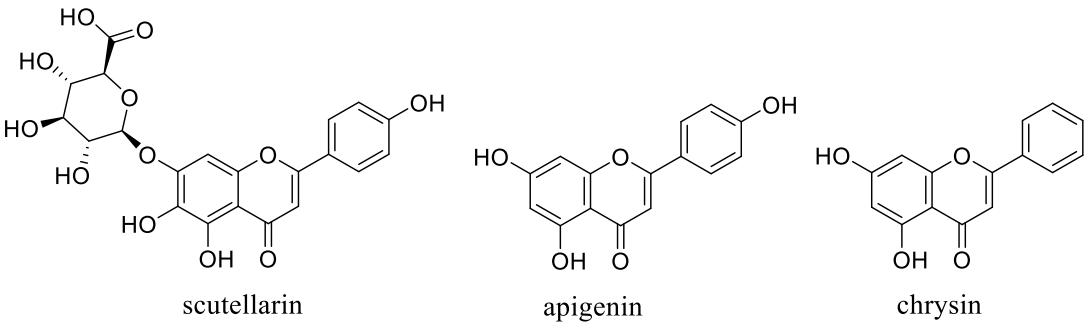


Figure 27. Structures of scutellarin, apigenin, and chrysin

The crude ethanol extract of *Scutellaria baicalensis* was dissolved in ethanol and stirred overnight. A precipitate was formed, which was filtered, washed with ethanol and then dried. The precipitate was then dissolved in water and purified by chromatography on reverse-phase HPLC. Chromatography, eluting with acetonitrile-water, afforded seven fractions labeled Sb1a1-Sb1a7, with the major fraction being Sb1a3. Fortuitously, the major fraction Sb1a3 was also identified as the active compound. An outline of the isolation is given below as a flow chart in Figure 28.

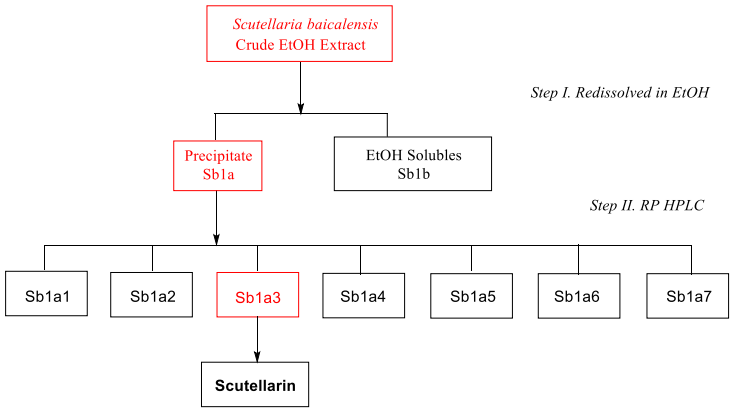


Figure 28. Flow chart for the isolation of scutellarin

The fraction Sb1a3 was isolated after reverse phase HPLC of the precipitate. HPLC analysis of the active fraction Sb1a (the precipitate) showed seven major components using a UV detector as shown in Figure 29. Each component was isolated using preparative HPLC and Sb1a3 was identified as the active component based on the bioassay.

HPLC chromatograph

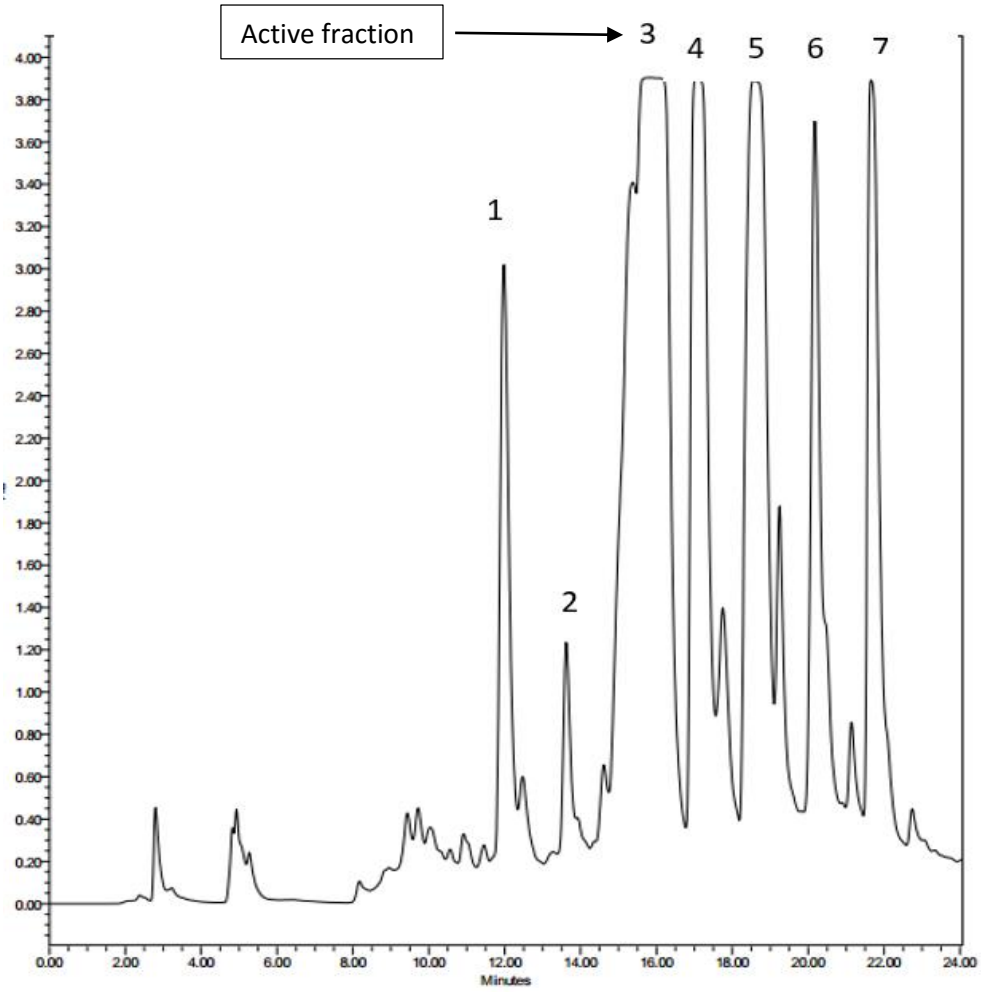
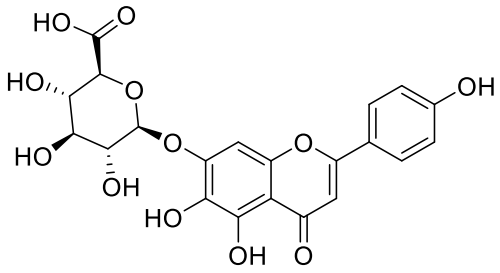


Figure 29. HPLC analysis of the precipitate from the EtOH extract of *Scutellaria baicalensis*

Once Sb1a3 was isolated in sufficient quantity, ^{1}H and ^{13}C NMR spectra were collected for structure characterization. Literature review linking this plant with anti-trypanosomal activity and NMR data published for the reported active compound, scutellarin, led to identification of the active compound from this bioassay guided fractionation to be scutellarin.[45,46] The structure of scutellarin is shown below in Figure 30. It is a glycoside of glucuronic acid at C7 of the parent flavone, scutellarein.



scutellarin (Sb1a3)

Figure 30. Structure of scutellarin

After the initial HPLC isolation of SB1a3 and confirmation of activity, a ^{1}H NMR spectrum of the resulting compound quickly classified the compound as belonging in the flavonoid class. This determination was made by the lack of signals in the range from 0.5 – 3.0 ppm and the presence of aromatics in the 6.5 – 8.0 ppm range, along with singlets for the hydroxyls present on the ring. The area around 4.0 ppm also showed signals consistent with the presence of a sugar indicating that the compound was glycosylated. The ^{1}H – NMR spectrum is shown in Figure 31.

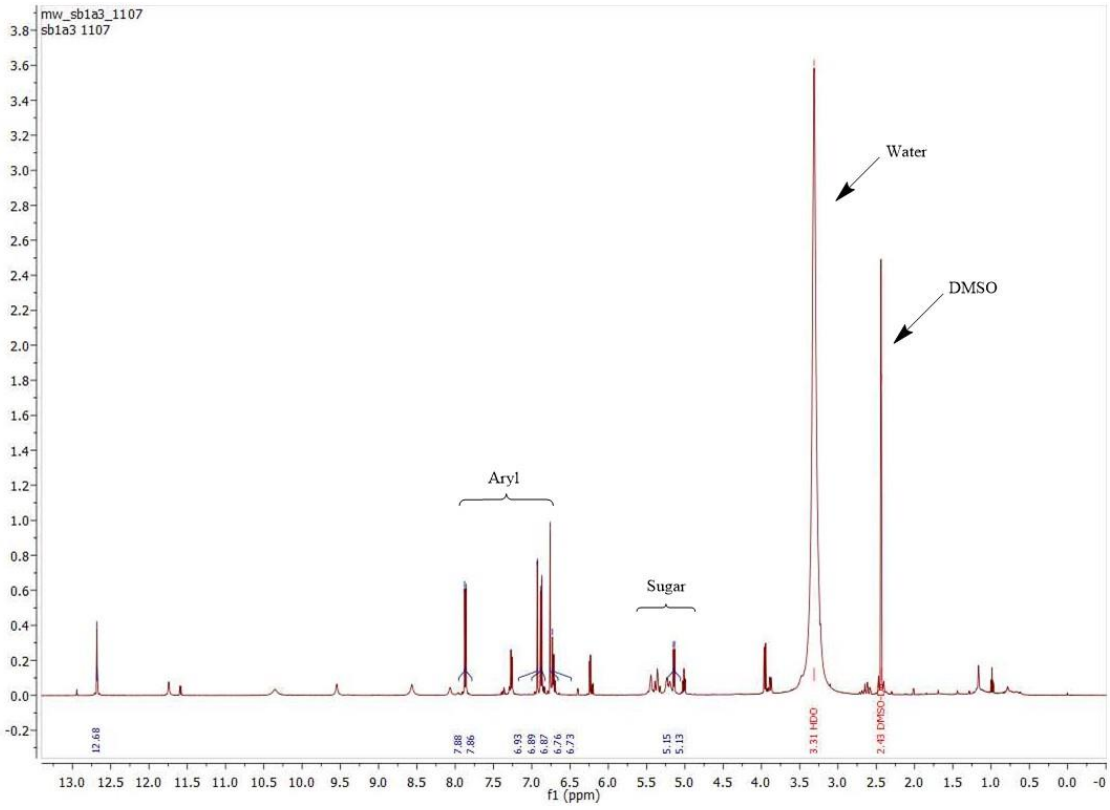


Figure 31. ^{1}H - NMR of Sb1a3 (scutellarin)

After determining the class by NMR, the mass spectral analysis showed the M+1 peak at 463.1, shown in Figure 32, and the loss of glucuronic acid (M-174) from the main compound at 289.1. Based on the information provided from the spectral analysis and a literature review from previously isolated flavonoids from *S. baicalensis*, the conclusion was that this compound is scutellarin.

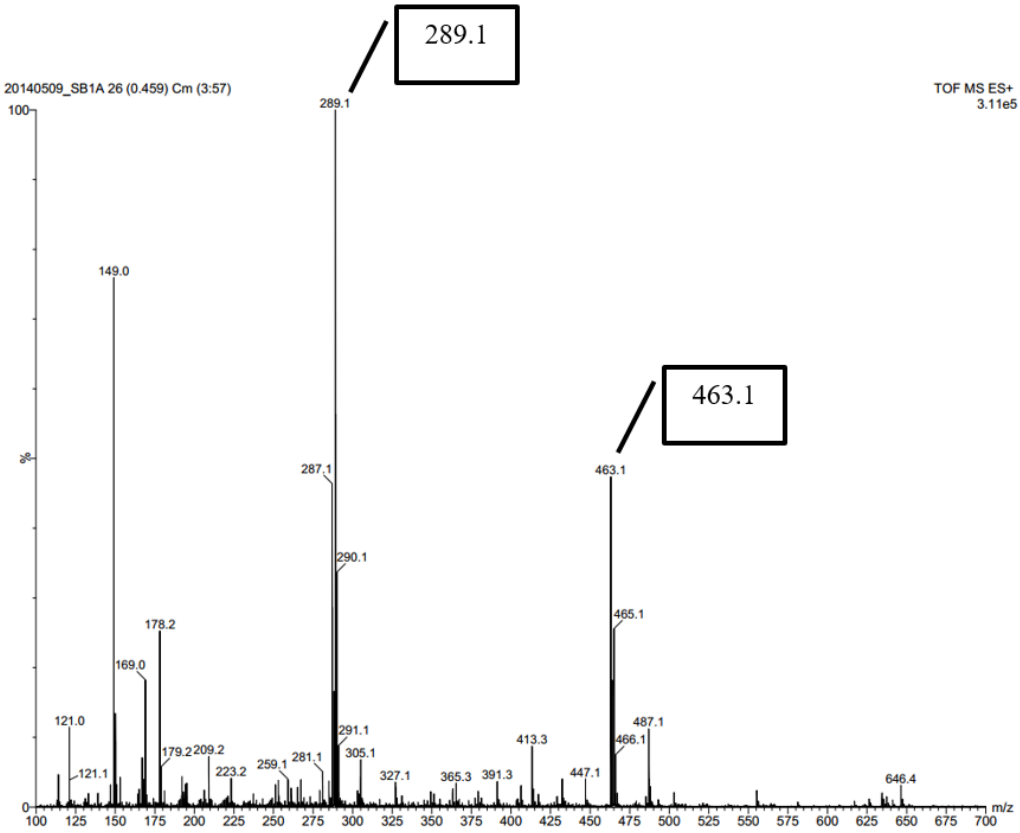


Figure 32. Mass spectroscopy analysis of SB1a3

Bioassay guided fractionation, using *T. brucei* inhibition as the guiding assay, resulted in the isolation of scutellarin. This confirmed the earlier working showing the anti-trypanosomal activity of scutellarin reported by Mamadalieva et al. An efficient isolation of scutellarin was developed from the precipitate of the crude extract of *S. baicalensis.* The efficiency was based on the compound's solubility characteristics and the ease of precipitation. Filtration afforded an almost pure compound from the initial crude extract.

Material and Methods

General Experimental Procedures

The NMR data were obtained on a 500 MHz FT-NMR model ECA-500 JEOL (Peabody, MA, USA) purchased with funding provided by the National Science Foundation. Coupling constants (J values) are recorded in hertz (Hz). All signal assignments are based on COSY, HMQC, and DEPT data. All mass spectra were acquired on either a Waters Synapt HDMS QToF with Ion Mobility. Solvents and chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (Milwaukee, WI, USA).

Plant Material

The leaves of *Scutellaria baicalensis* Georgi. (Sbs) were collected in Chengde County, Hebei Province, P. R. China, in May 2012, and identified by Dr. Chun-nian He (Institute of Medicinal Plant Development, Chinese Academy of Medical Science, Peking Union Medical College, Beijing, P. R. China). A voucher specimen (No. hq20120501) has been deposited at the Herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College.

Bioassay Guided Isolation

The crude ethanol extract of *Scutellaria baicalensis* (6.26g) was dissolved in ethanol and stirred overnight. The precipitate was filtered, washed with ethanol and then dried. The precipitate (1.9g), was then dissolved in water and purified by chromatography on reverse-phase HPLC. Chromatography, eluting with acetonitrile-water, afforded seven fractions Sb1a1-Sb1a7. Fraction Sb1a3 was a pure compound and

was identified as the active compound. An outline of the isolation procedure is given below as a flow chart in Figure 33.

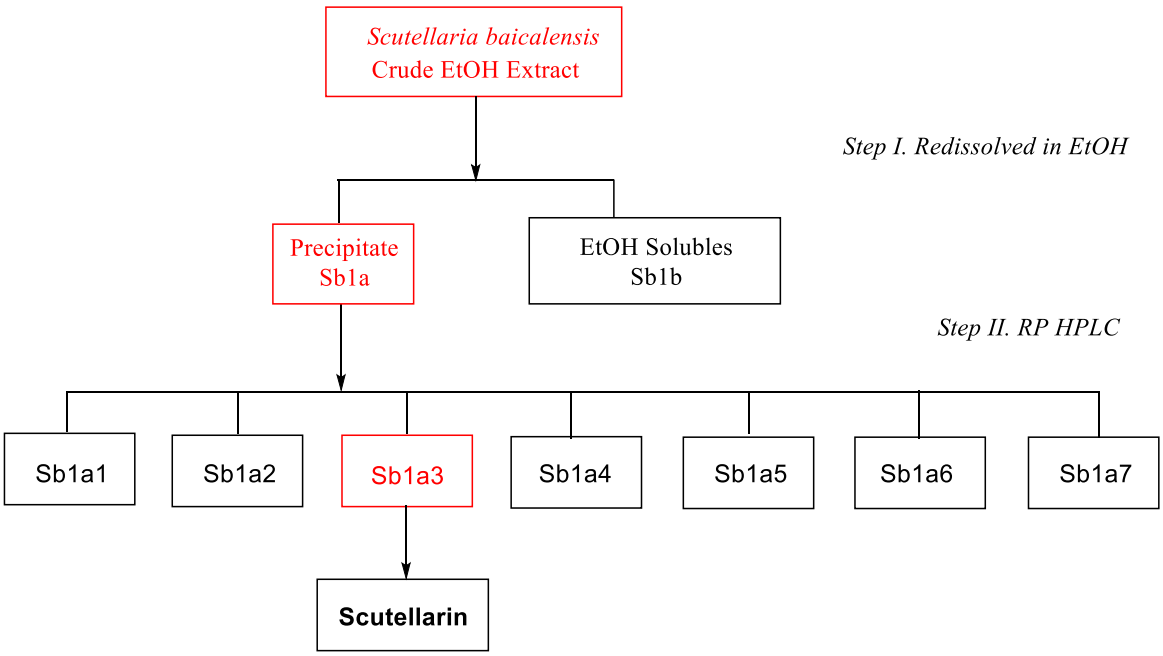


Figure 33. Flow chart for the isolation of scutellarin

The precipitate fraction Sb1a (1.902 g), was filtered, dried and the residue was dissolved in 10 mL of a 1:1 mixture of water and methanol for HPLC analysis. The sample (0.5 mL portions) was injected into a Waters 1525 HPLC equipped with a Waters UV/Visible Detector 2489. A Phenomenex Luna 5µ C18 (250 x 10 mm) column using a gradient elution from 5% acetonitrile to 100% over 25 minutes detecting at 254 nm was employed. Fractions were collected in one minute increments.

Scutellarin

Scutellarin was isolated as an amorphous solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.68 (s, 1H), 7.87 (d, J = 8.8 Hz, 1H), 6.93 (s, 1H), 6.88 (d, J = 8.9 Hz, 1H), 6.76 (s, 1H), 5.14 (d, J = 7.5 Hz, 1H); ^{13}C NMR (76 MHz, DMSO-D_6) δ 182.89 (C-4), 170.75 (C-6’’), 164.63 (C-2), 161.74 (C-4’), 151.55 (C-9), 149.53 (C-7), 147.35 (C-5), 130.98 (C-6), 129.41 (C-2’), 128.96 (C-6’), 121.81 (C-1’), 116.52 (C-2’), 115.66 (C-5’), 106.37 (C-10), 103.04 (C-3), 100.51 (C-1’’), 94.14 (C-8), 75.80 (C-5’’), 75.62 (C-3’’), 73.32 (C-2’’), 71.91 (C-4’’); ESIMS m/z ($C_{21}H_{18}O_{12}$) calcd for $[M + 1]^+$ 463.1; found 463.1.

CHAPTER IV: *CICHORIUM INTYBUS* – PLANT CELL CULTURE

Plant cell culturing provides a sterile method to grow and manipulate plants on a cellular basis versus dealing with the multicellular whole plant. Culture techniques include callus cultures, which are masses of undifferentiated cells grown on semisolid media, shown below in Figure 34.

Figure 34. Callus culture

Callus media is a mixture of salts, sugar, nutrients, and plant hormones that have to be optimized in the correct ratios for the callus to grow on a semi-solid media. Optimization requires a specific balance of the salts and hormones and can often be different among similar species in the same genus. Plant hormones are broken down into two main categories. First, cytokinins are for the promotion of cell division in plant roots and shoots. The second class of plant hormones, called auxins, is responsible for stem elongation. The ratio of these two classes of hormones is the most critical for successful plant cultures. Hairy root culture, initiated by infected plant tissue with the soil bacteria

Rhizobium rhizogenesis, causes the plant to produce fine roots in a minimal medium and also tends to up-regulate certain metabolites. *R. rhizogenesis* inserts a root inducing plasmid that causes this condition and the bacteria can then be eliminated with antibiotics while the culture is maintained. Suspension or single cell cultures in liquid media are accomplished once the callus media has been optimized. The calli are transferred to liquid media that is identical to the callus media, but without the agar. At this stage, many tasks can be accomplished with the plant cells, including cold storage, organogenesis to provide new plantlets for propagation, or metabolic manipulation to alter the regulation of secondary metabolites being produced by the plant. Metabolic screening of plant cell cultures allows for manipulation of the media and stressors that is either difficult or impossible to do with a plant in the wild. Plants rarely suffer from microbial infections and have adapted to fight infections and herbivorous attacks with an innate immune system. This innate system produces potent metabolites that often have biological activity in humans as well.

As an investigation into the use of plant cell culture, *Cichorium intybus* or common chicory shown below in Figure 35, was used. This plant has been well characterized in culture metabolically and provides a good reference species for this work. *C. intybus* is a member of the Asteraceae family and is an invasive plant commonly found along roadsides in the U.S. *C. intybus* is native to Europe but is now common in many countries, including the United States.

Figure 35. ***Cichorium intybus***

C. intybus contains many medicinally important secondary metabolites and has been studied extensively in the wild and in plant tissue culture systems. Secondary metabolites isolated from *C. intybus* include inulin, esculin, coumarins, and a large number of flavonoids.[47,48] The leaves and roots of *C. intybus* contain large quantities of sesquiterpene lactones, which gives chicory a bitter taste.[49,50] Ground chicory root was first used as a coffee substitute during the American Civil War and then in the Great Depression during coffee shortages. Chicory is a cheap substitute for coffee and lacks the caffeine, but due to the sesquiterpene lactones, has a similar bitter taste as the chlorogenic acid lactones in coffee. Figure 36 shown below, gives the structures for lactucin, lactupicrin, and 8-deoxylactucin. These compounds have shown antifungal, antiprotozoal and cytotoxic activity.[51,52]

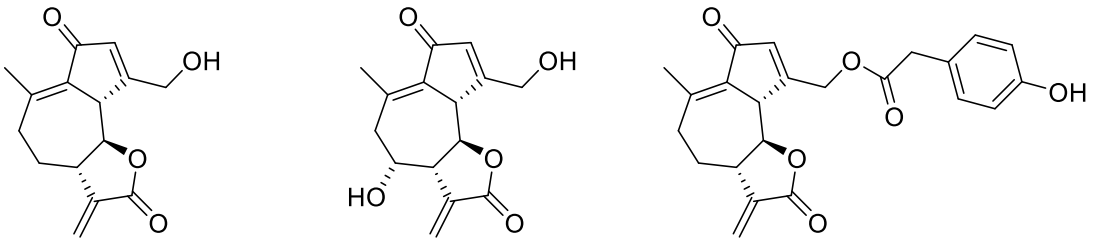


Figure 36. Structures of sesquiterpene lactones found in *C. intybus*

The abundance of medicinally important compounds found in *C. intybus* have resulted in many researchers focusing on improved extraction and methods for over-production of these compounds in tissue culture. One common problem in the use of crude plant extracts for bioassays is the presence of compounds that can result in false positives and mask results. These include chlorophyll, tannins and phenolic compounds that form aggregates, which bind to proteins in a non-specific fashion.[53] Tissue culture offers a method for manipulating the live plant in a sterile setting to alter the metabolic production of compounds. Some advantages are the reduction in amounts of undesirable lipophilic compounds and the increased production of more drug-like compounds in the polar fractions. However, essential oils as well as many medicinally important alkaloids including morphine and vinblastine, have resisted cell culture techniques in order to increase production. In some cases, these compounds are not produced at all in cell culture. The ultimate goal of this project is to study the production of 8-deoxylactucin and the different metabolic profiles of the wild plant, callus culture, stressed callus

culture and finally a hairy root culture. The initial goal was to study the weight distribution of the plant mass in regard to lipophilic and polar fractions.

Plant material and seed were initially collected and sterilized in order to optimize a method for callus culture. Due to contamination issues, the use of plant material from the wild was discarded and sterile seeds were germinated and used for induction of callus. Once callus media has been optimized on semi-solid media (agar medium), then initiation of a suspension culture begins by adding one gram of callus per 50 mL of liquid media (agar free medium). Cells will divide and begin to break away from the callus and form a single cell suspension culture. Callus material, once optimized, was also used for initiation of a hairy root culture by infecting the callus with *R. rhizogenesis.*

For comparison, an extraction of the wild plant was compared to extracts of callus culture and hairy root culture. All materials were extracted in the same manner. A 1:1 mixture of ethanol and ethyl acetate was added to the ground plant biomass in an Erlenmeyer flask and sonicated for 15 minutes. The flask was then moved to a stir plate and stirred for 24 hours. The extract was filtered and the process repeated a second time. Concentration under vacuum afforded the crude extract.

Results and Discussion

The most surprising difference between the suspension culture extraction and the extraction from the plant leaf material is the weight distribution of the fractions. In separate experiments, the plant material (*C. intybus* Wild) and suspension culture material (*C. intybus* CC-E) were purified on silica gel using an automated Combiflash system. As seen below in Figure 37, the fractions run in increasing polarity from F1 –

F11, eluting with hexanes, to ethyl acetate, and finally to methanol. Each fraction resulted from a gradient starting with 100% hexanes (F1), increasing the level of ethyl acetate in 20% increments until 100% ethyl acetate (F6). A second gradient was then performed with ethyl acetate (F7) increasing in 20% increments with methanol until 100% methanol (F11) was reached for the final fraction.

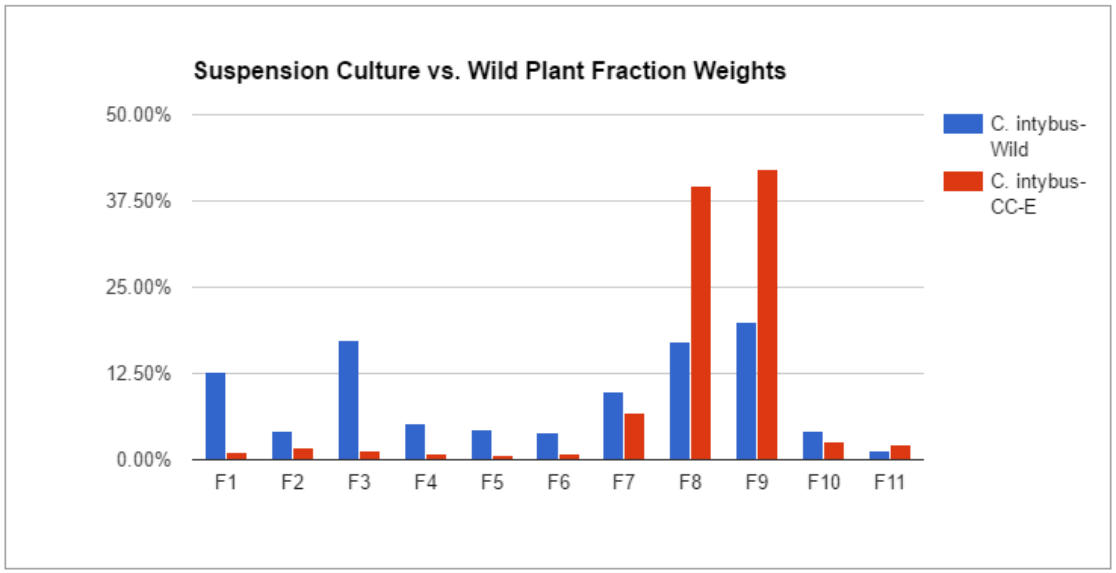


Figure 37. Chart of fraction weight distribution in *C. intybus* extracts showing Fractions 1 – 11 vs. percent of total weight

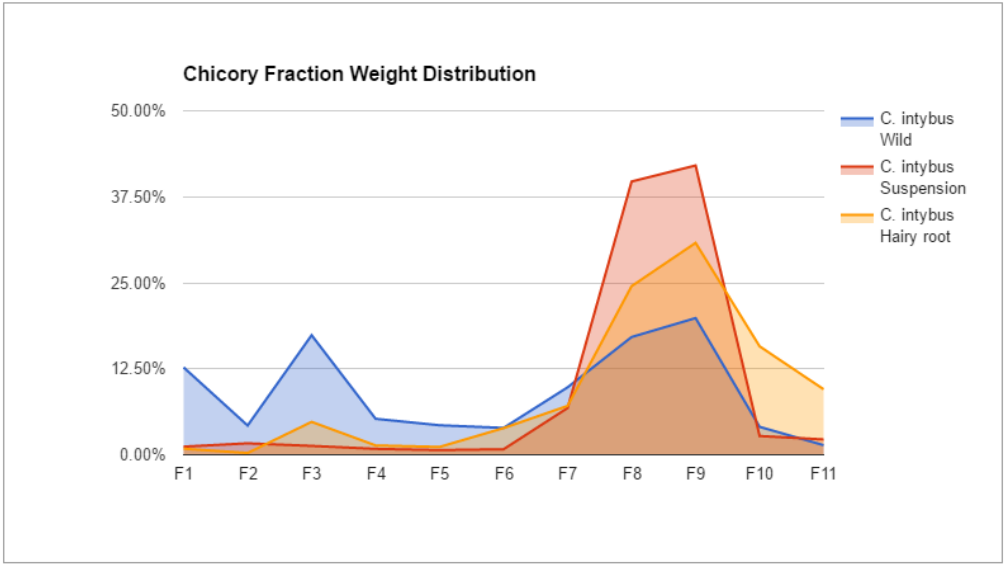
The leaf material had almost half of the total extract weight come out in the first five fractionations with the majority of that first half made up from F1 (100% hexanes) and F3, which contains the chlorophyll fraction. This was characterized with the UV detector showing a large peak measuring at 360 nm characteristic of chlorophyll. The callus culture extraction (CC-E) had 94% of the total extract weight come out from

fractions F6 – F11. This more polar region of the extract contains compounds that have the characteristics to be much more drug-like than the lipophilic compounds that come out in the early fractions. Even if a compound with activity comes out in an early fraction, the compound could possibly be diluted out by the excess amount of chlorophyll and other lipophilic compounds. This dilution might reduce the percentage of an active compound to undetectable levels.

Weight Distribution of Fractions

The polar gradient fractionation performed on the three types of *C. intybus* tissue, gave a clear distribution of the chemical profile of the extracts. The suspension and hairy root cultures lacked mass in the lipophilic regions that is abundant in the leaf extract. Table 4 below, shows the polar fractions F7 – F10 make up the majority of the weight in the two cultured tissue types especially in the suspension culture.

Table 4. Fraction weight distribution of *C. intybus* tissue types

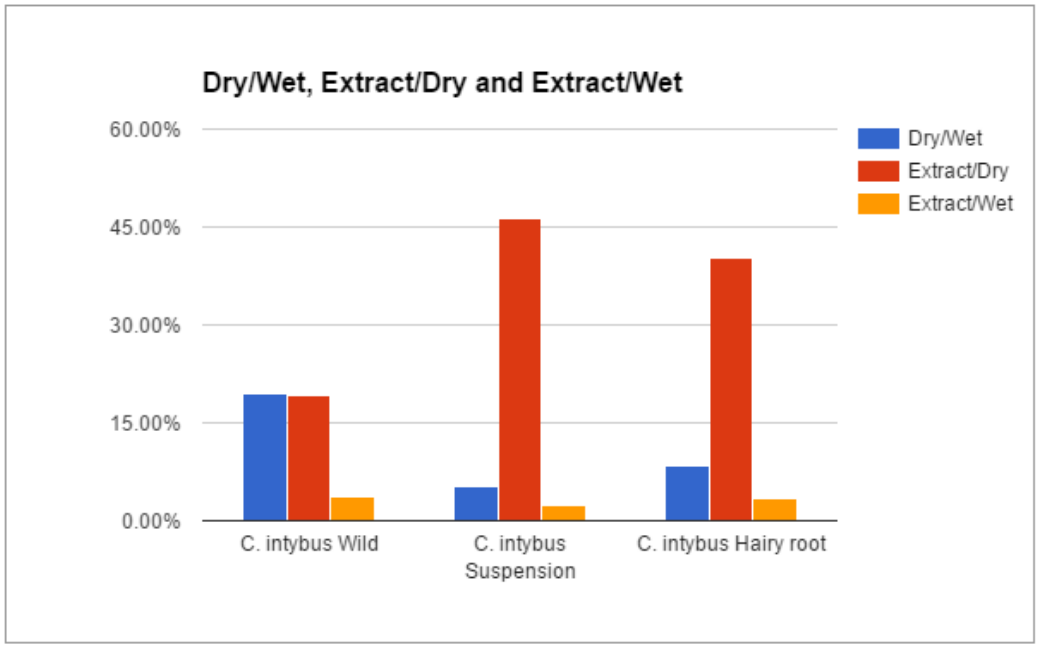


Another aspect that can be seen in Table 5 below, is the difference in the density of the biomass. The ratio of extract from the dried plant biomass is in the 40 percent range for each of the cultured tissue types versus 19% for the wild plant material. The final extract ratio to wet biomass weight balances out for all three tissue types, averaging about three percent.

Table 5. *C. intybus* biomass and extract ratios

Biomass and Extract Ratios

	Dry/Wet	Extract/Dry	Extract/Wet
***C. intybus* Wild**	19.53%	19.33%	3.78%
***C. intybus* Suspension**	5.37%	46.43%	2.50%
***C. intybus* Hairy root**	8.42%	40.27%	3.39%



This preliminary work did not go into identifying compounds in the extracts. The initial goal was to produce more of the desired areas of the extract and minimize the fractions or compounds that are less likely to contain bioactive compounds or are detrimental to bioassay work. Future work should cover production of compounds of interest and confirm that the ratio of these compounds to the total extract is increased. Certain alkaloids and essential oils are not produced in tissue culture and therefore, more work needs to be done in order to understand the processes involved and in order to overcome these obstacles.

Materials and Methods

C. intybus Leaf Extraction

Fresh leaf material was placed in a 1000 mL beaker and dried in an oven for 24 hours to produce dried plant material. Dried leaves were ground in a mortar and pestle to a fine powder, then the ground plant material was carried forward for extraction. The dried powder was extracted with a 1:1 mixture of EtOH and EtOAc. Once solvent was added, the mixture was sonicated for 15 minutes then placed on stir plate for 24 hours. This procedure was performed two times to produce the final crude extract.

Callus and Suspension Cultures

C. intybus seeds were initially washed in running tap water for 10 minutes and then surface sterilized as follows. The seeds were added to a 50 mL conical tube with one drop of detergent added with DI water and agitated for 5 minutes. Then the seeds were immersed in a 5% bleach solution for 10 minutes and agitated. Finally, the seeds were immersed in a 70% ethanol solution for 1 minute followed by rinsing with sterile DI

water. The seeds were then germinated on Murashige and Skoog's (MS) media containing 3% (w/v) sucrose and 0.8% (w/v) agar.[54] Petiole and leaf sections were excised from three week old seedlings and placed on a grid system with a salt and hormone gradient to optimize callus conditions shown in Figure 38.[55]

Full MS Zero Hormones	Full MS 10 µM NAA 5 µM BAP	Full MS 5 µM NAA 10 µM BAP	Full MS 10 µM NAA 10 µM BAP
1/2 MS Zero Hormones	1/2 MS 10 µM NAA 5 µM BAP	1/2 MS 5 µM NAA 10 µM BAP	1/2 MS 10 µM NAA 10 µM BAP
1/4MS Zero Hormones	1/4 MS 10 µM NAA 5 µM BAP	1/4 MS 5 µM NAA 10 µM BAP	1/4 MS 10 µM NAA 10 µM BAP

Figure 38. Callus optimization grid

The final optimized media was determined to be half strength MS salts with vitamins, 7.5 µM 1-Naphthaleneacetic acid (NAA), 10 µM 6-Benzylaminopurine (BAP), 10% coconut water, and a pH of 5.8. Once the semi-solid media is optimized, then the callus is divided out on 100 mm petri dishes to grow up biomass. After one month, there is enough callus material to inoculate 100 mL of media in a 250 mL Erlenmeyer flask to produce a suspension culture. After one week, individual cells start breaking off the original callus

to produce a single cell culture. Three to four weeks later the suspension cultures are ready for stressing and extraction.

Suspension cultures were filtered and rinsed with DI water and then frozen in liquid nitrogen before being placed on the lyophilizer. 80.5 g of wet callus was rinsed, freeze dried to a weight of 4.6 g, and then ground into a fine powder. Extraction was performed identical to that of the dried plant leaves to produce 1.1 g of extract.

Hairy Root Cultures

R. rhizogenesis (strain ATCC 15834) cultures were maintained in 100 x 25 mm culture tubes containing 25 mL Yeast/Mannitol Broth (YMB). Infection of the callus culture was made with a sterile scalpel dipped into the bacterial culture and subsequently sliced through the calli.[56] Once infected, the callus was placed on hormone free (basal) semi-solid media with 3% sucrose, and 100 mg myoinositol l^{-1} and incubated for three days. Callus was then transferred to fresh MS media containing 0.5 g l-1 Cefotaxime (PhytoTechnology Laboratories®). Bacteria were eliminated from culture by transferring to fresh media containing Cefotaxime every seven days for eight times and finally confirming by inoculating bacterial media plate with hairy root culture material. Final hairy root culture, shown in Figure 39, was maintained in half strength MS media on gyratory shaker at 100 RPM.[56]

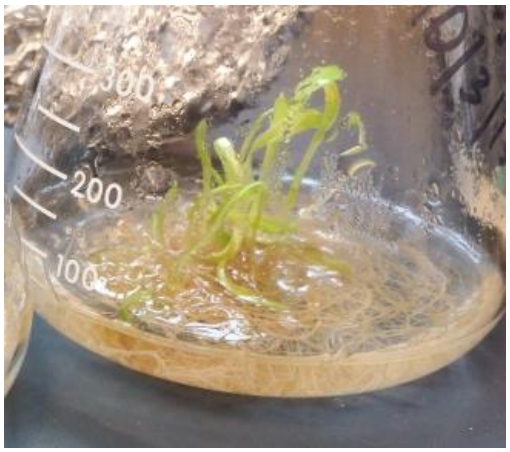


Figure 39. *C. intybus* hairy root culture

Extraction of hairy root culture material was completed once excess material had been subdivided and backup material was in place. *C. intybus* hairy roots were filtered and rinsed with DI water and then frozen in liquid nitrogen before being placed on the lyophilizer. 17.7 g of fresh hairy roots were rinsed, freeze dried to a weight of 1.5 g, and then ground into a fine powder. Extraction was performed identical to that of the dried plant leaves to produce 0.6 g of extract.

Plant Material

The stems, leaves, flowers, and seed of *Cichorium intybus* were collected in Washington County, Johnson City, Tennessee, in June 2014, and identified by Dr. Jeffrey Walck in the Biology Department of Middle Tennessee State University (MTSU). A voucher specimen has been deposited at the MTSU Herbarium by Dr. Ashley Morris in

the Biology Department. Seeds were stored in a -18° C freezer before being processed for sterile tissue culture.

Fresh leaf material was placed in a 1000 mL beaker and dried in an oven for 24 hours to produce dried plant material. Dried leaves were ground in a mortar and pestle to a fine powder, then plant material was carried forward for extraction. Other plant material, including suspension/callus culture and hairy root culture, were filtered, rinsed, and then freeze dried with a lyophilizer. The dried material was ground with a mortar and pestle in the same manner as the dried leaf material. The dried powder from each tissue type was extracted with a 1:1 mixture of EtOH and EtOAc in a 1000 mL Erlenmeyer flask. Once solvent was added, the mixture was sonicated for 15 minutes and then placed on a stir plate for 24 hours. The extract was then filtered and the residue suspended in fresh solvent. This procedure was performed two times to produce the final crude extract. Table 6 below, gives the percent of fraction weight for each tissue type.

Table 6. Material and extract weights of *C. intybus*

Chicory Project	**Wet Weight**	**Dry Weight**	**Percent Dry/Wet**	**Extract Weight**	**Percent Extract/Dry**	**Percent Extract/Wet**
C. intybus Wild	76.8	15.0	19.53%	2.9	19.33%	3.78%
C. intybus Suspension	52.1	2.8	5.37%	1.3	46.43%	2.50%
C. intybus Hairy root	17.7	1.49	8.42%	0.6	40.27%	3.39%
All weight in grams						

One gram of extract was carried forward from each tissue type for extraction on an automated Combiflash system using a stepped gradient. The fractions run in increasing polarity from F1 – F11, eluting with hexanes, then to ethyl acetate, and finally to methanol. Each fraction resulted from a gradient starting with 100% hexanes (F1), increasing the level of ethyl acetate in 20% increments until 100% ethyl acetate (F6). A second gradient was carried out with ethyl acetate (F7) increasing in 20% increments with methanol until 100% methanol (F11) was reached for the final fraction. This protocol was adapted from the standard protocol used by Sequoia Sciences in St. Louis, MO.[9]

CHAPTER V: CONCLUSION

Bioassay guided fractionation of extracts from *Cyclocarya paliurus* and *Scutellaria baicalensis* were used to investigate two plants used in TCM. The *C. paliurus* isolation was guided by an anticancer screen that led to the isolation of "cyclocaric acid A", previously isolated and characterized in 1996 from this plant.[22] Closer scrutiny of the NMR indicated the structure had originally been mis-assigned. This was confirmed through an independent synthesis of the proposed structure of cyclocaric acid A and then comparison to the plant isolate using NMR and HRMS. The plant isolate was determined to be hederagenin, which has known anticancer activity, and not cyclocaric acid A. Cyclocaric acid A is most likely not a natural product and the synthesized compound had no anticancer activity. The initial goal was isolation of either a novel compound or finding novel bioactivity for a known compound. Although these goals were not reached, the structure correction is also important. Based on the 1996 report of cyclocaric acid A (actually hederagenin), subsequent reports continued to be published characterizing the extract of *C. paliurus* and included references to cyclocaric acid A.[26,27,57] Along with several patents on isolation methods, these all referred back to data in the 1996 paper and were, in fact, incorrect and based on hederagenin.[23,24]

Scutellaria baicalensis initially showed anti-trypanosomal activity from the crude extract. Bioassay guided fractionation led to the isolation of the known flavonoid scutellarin. In this case, scutellarin has previously been reported to have anti-trypanosomal activity with known MICs that corresponded to the activity of the plant isolate.

An alternate to the use of whole plant bioassay guided fractionation is the use of plant cell culture. An investigation into the plant *Cichorium intybus* with plant cell culture showed the benefit of decreasing the lipophilic fractions and increasing the amount of compounds on the polar side of the extract. Typically the initial non-polar lipophilic fractions contain unwanted compounds, such as chlorophyll, that often have off-target activity and cause bioassay problems. Additionally, compounds of interest as a higher percentage of total extract weight and an increase in the polar fraction material as a whole, should provide much more successful screens and remove many false positives due to lipophilic compound interactions. Although the primary or most abundant bioactive compounds were isolated with the bioassay guided fractionation of cell culture, more work needs to be done to make sure compounds produced in minute quantities are given equal weight in the screening process.

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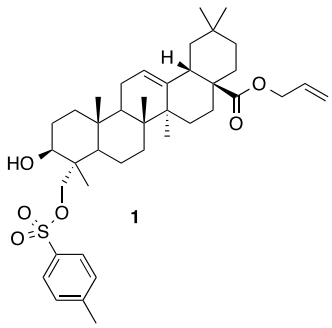
APPENDICES

APPENDIX A: SPECTROSCOPY FOR *CYCLOCARYA PALIURUS*



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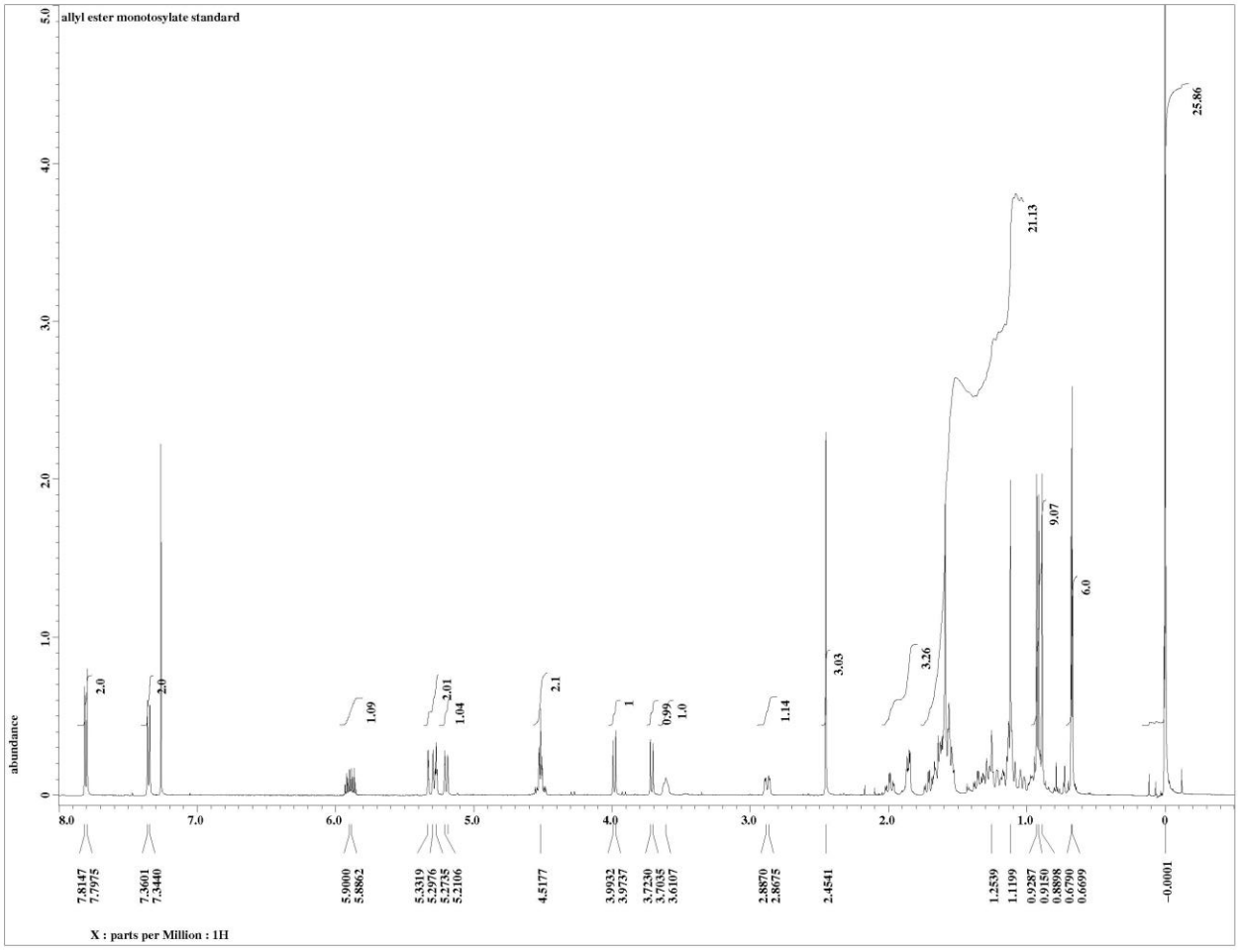

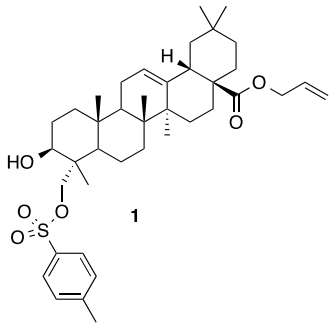

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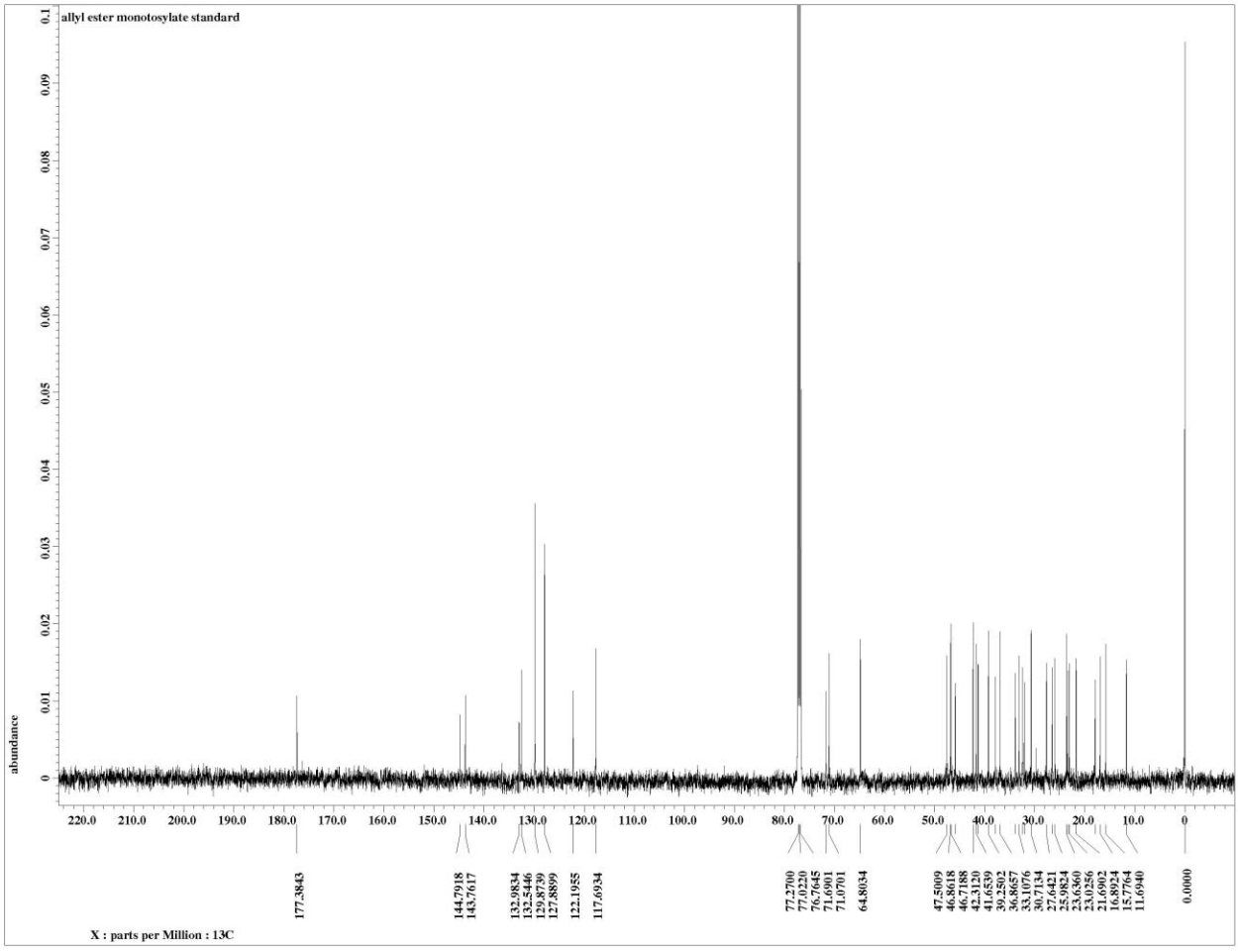

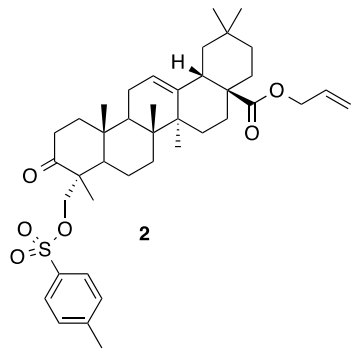

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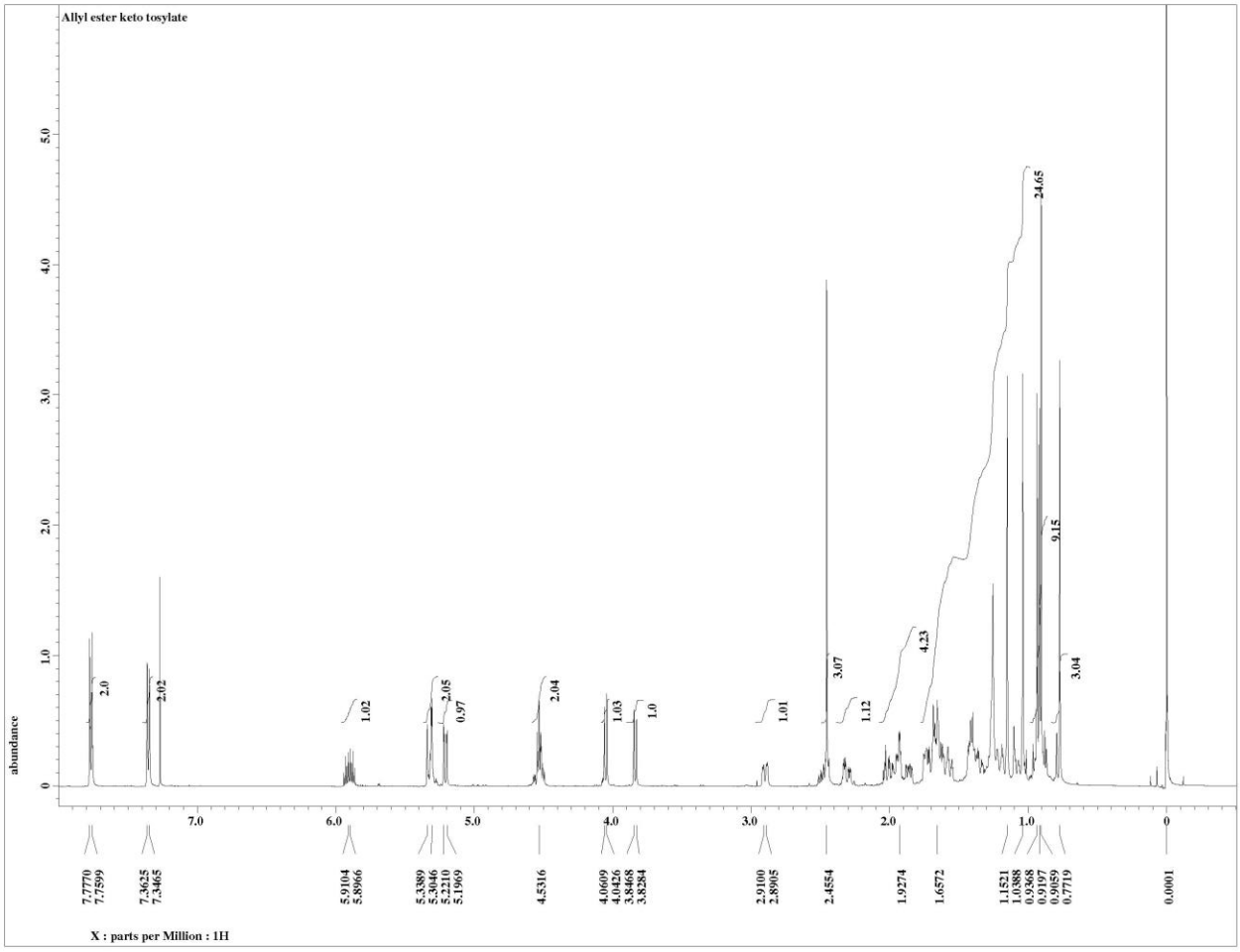

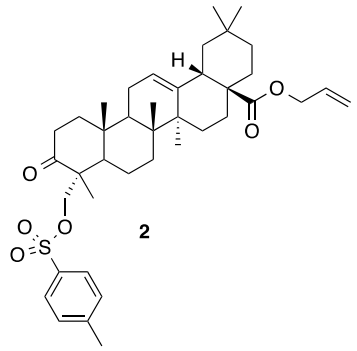

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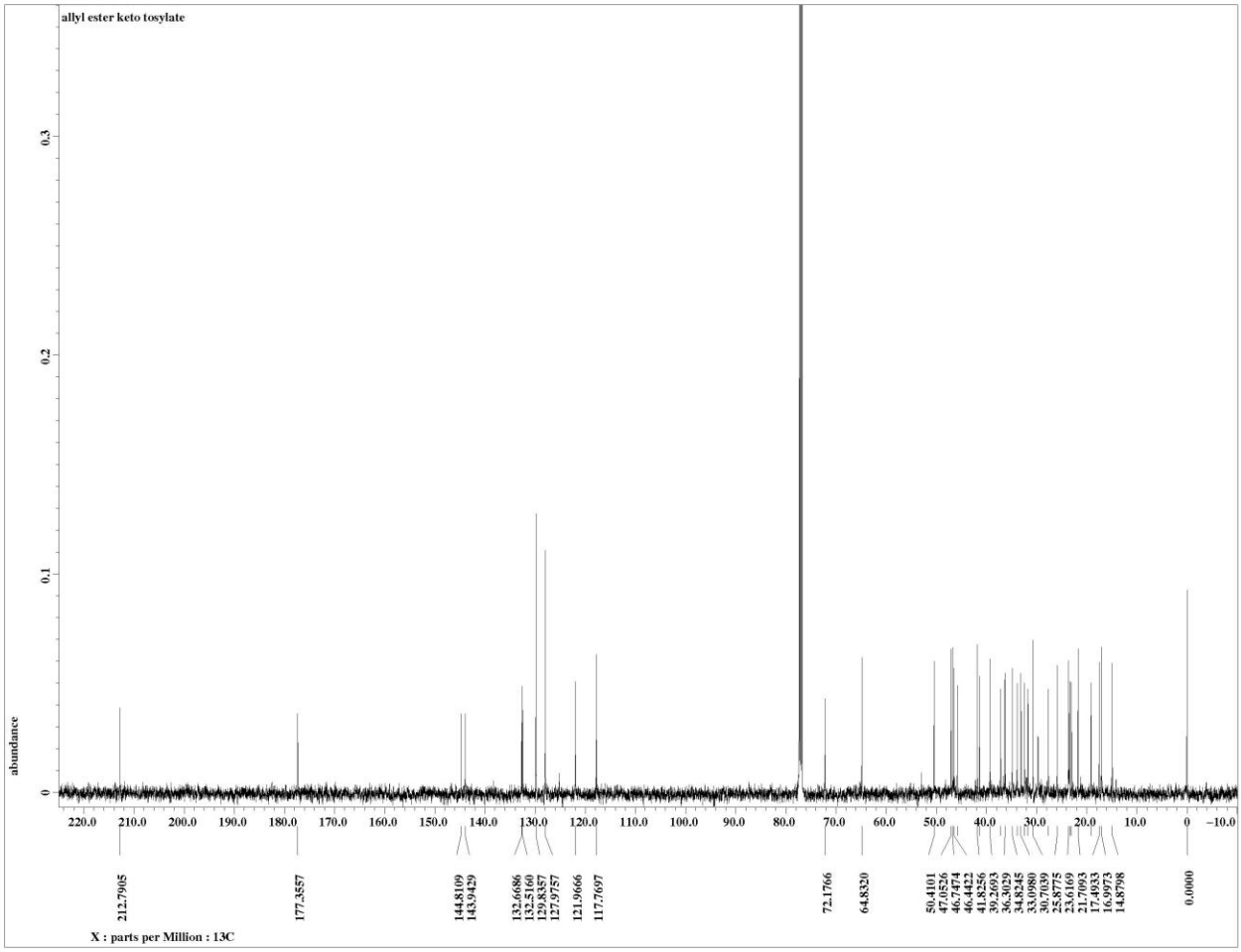

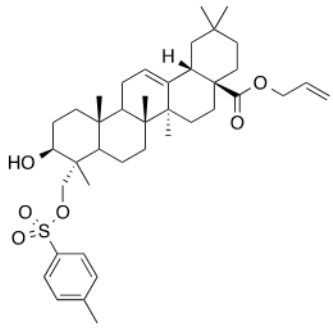

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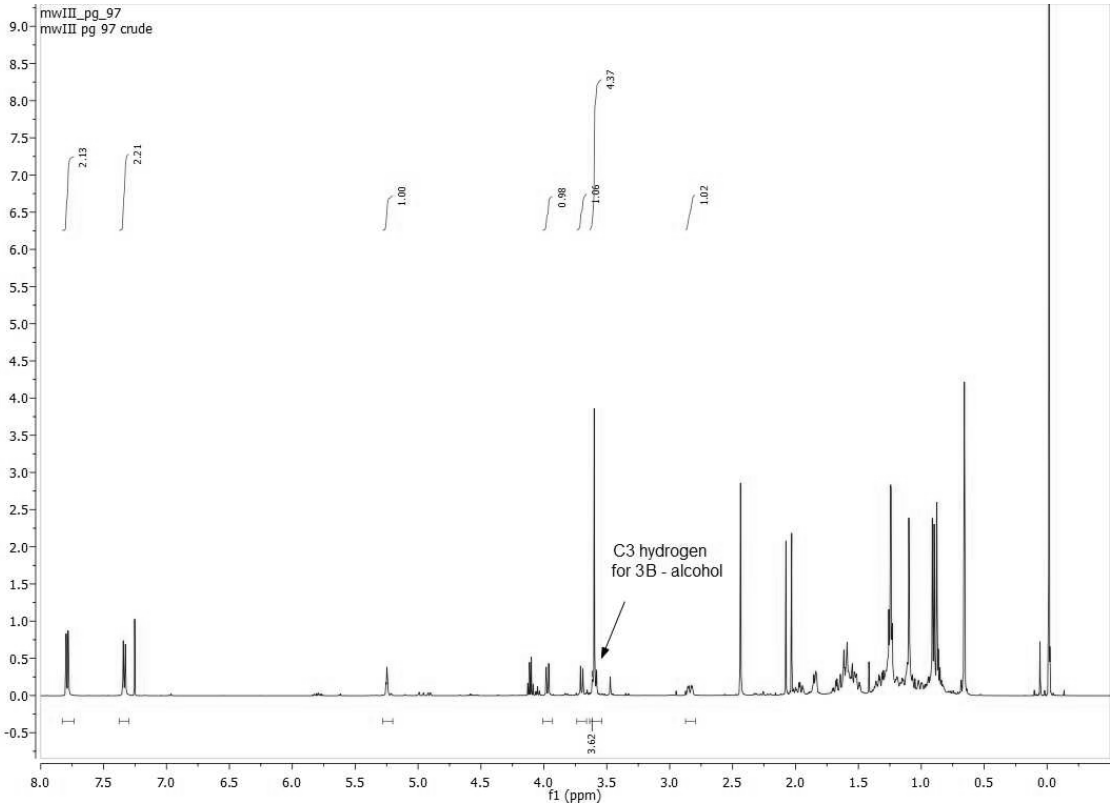

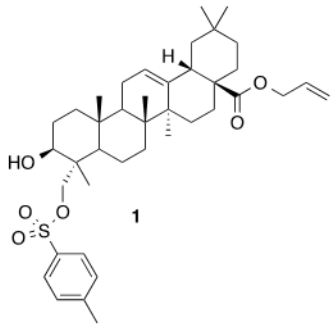

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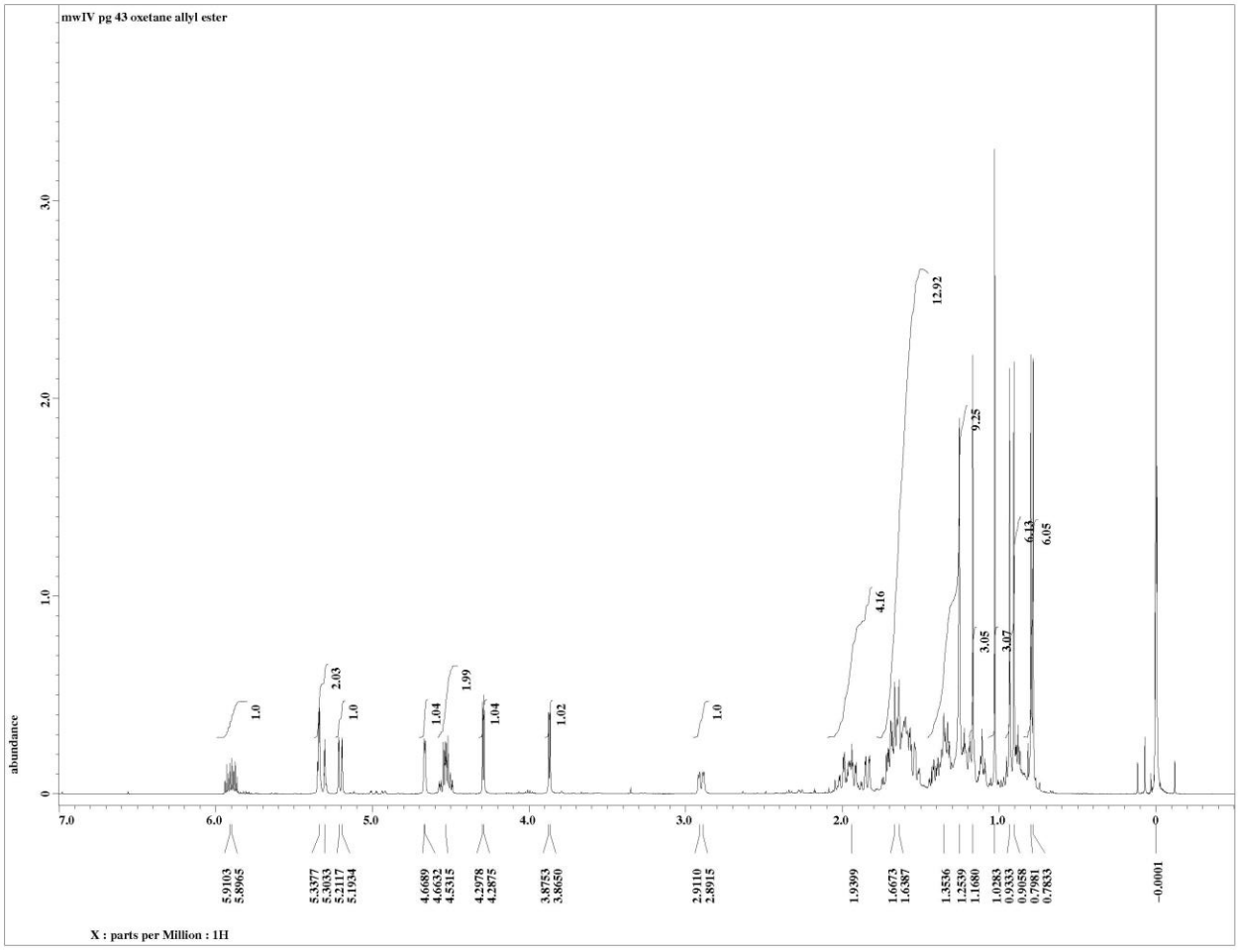

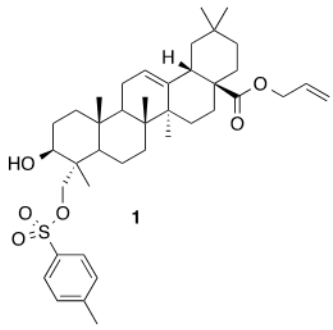

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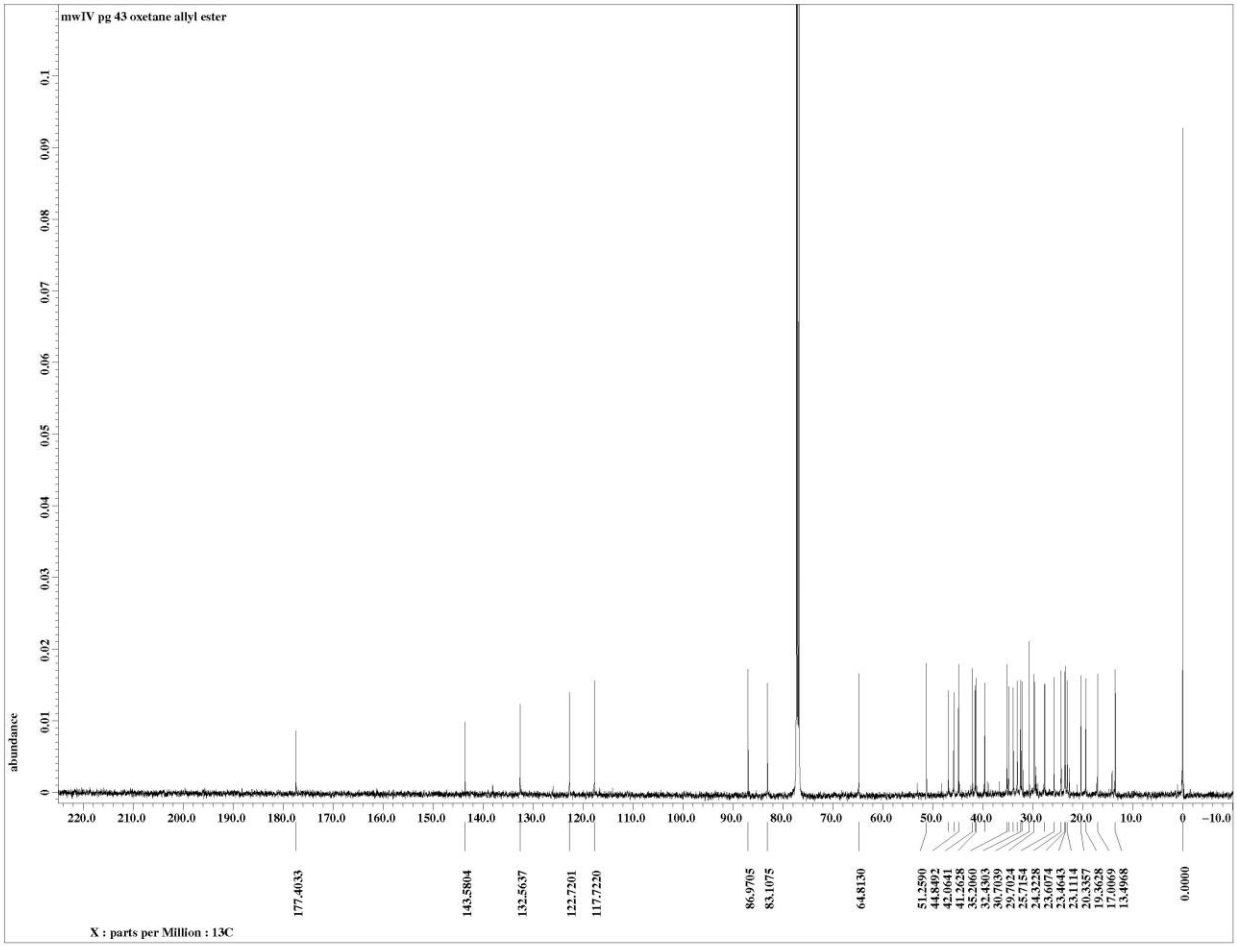
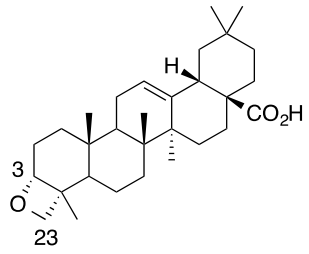
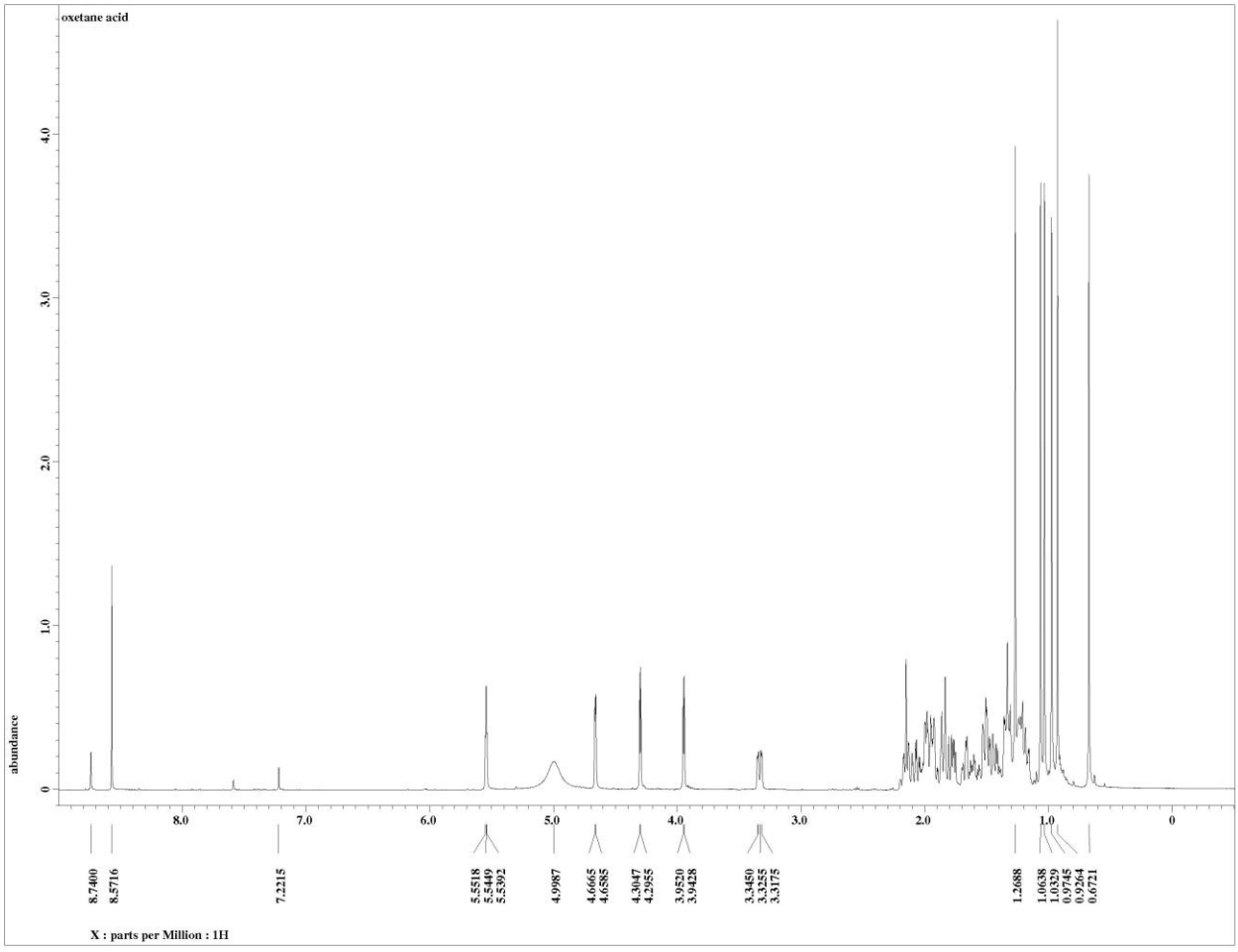
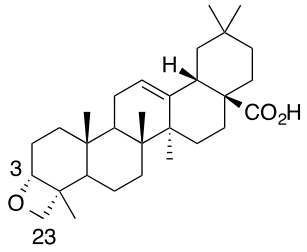




Cyclocaric acid A

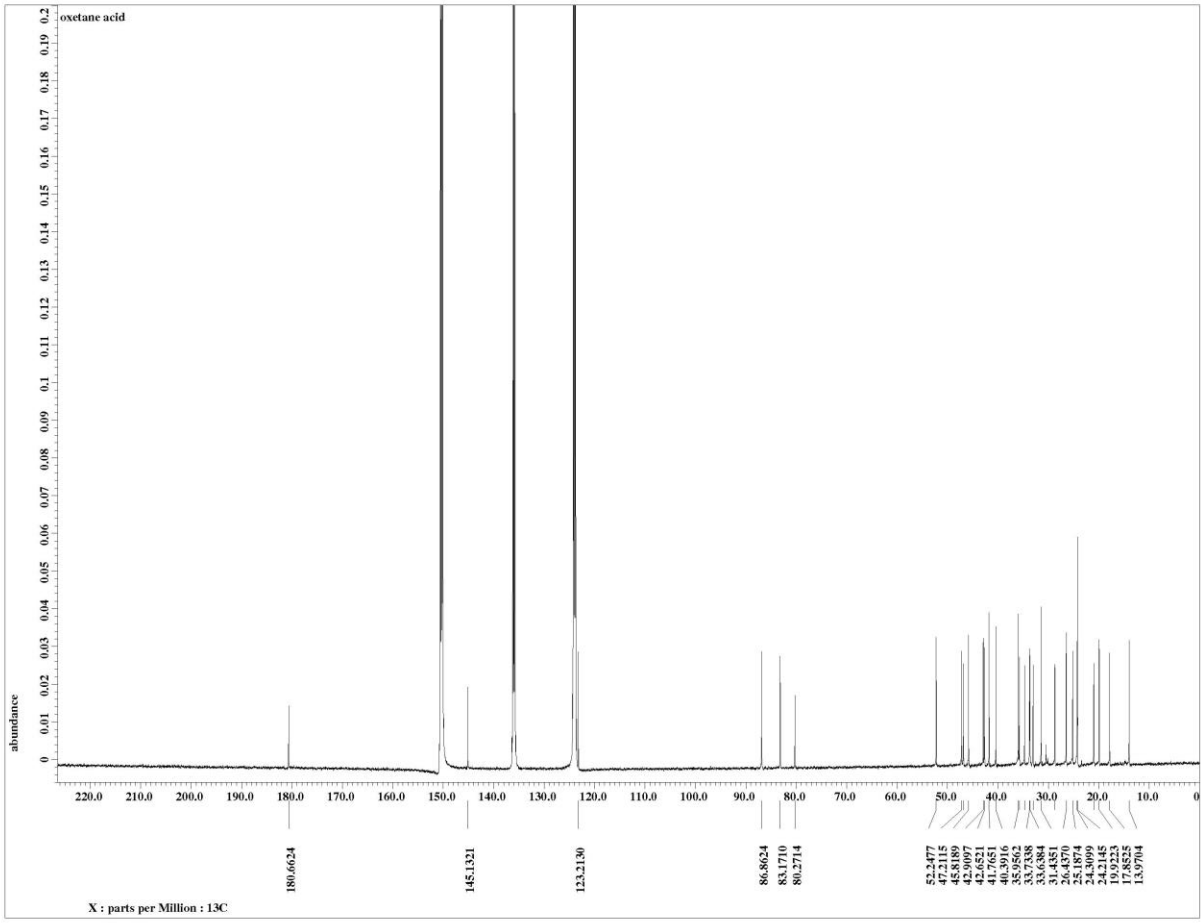
^{1}H NMR (500MHz, D5-pyridine) of cyclocaric acid A

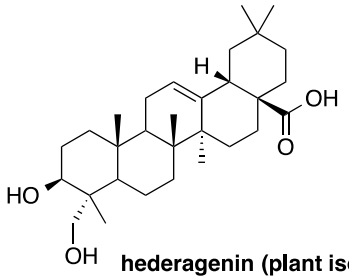




Cyclocaric acid A

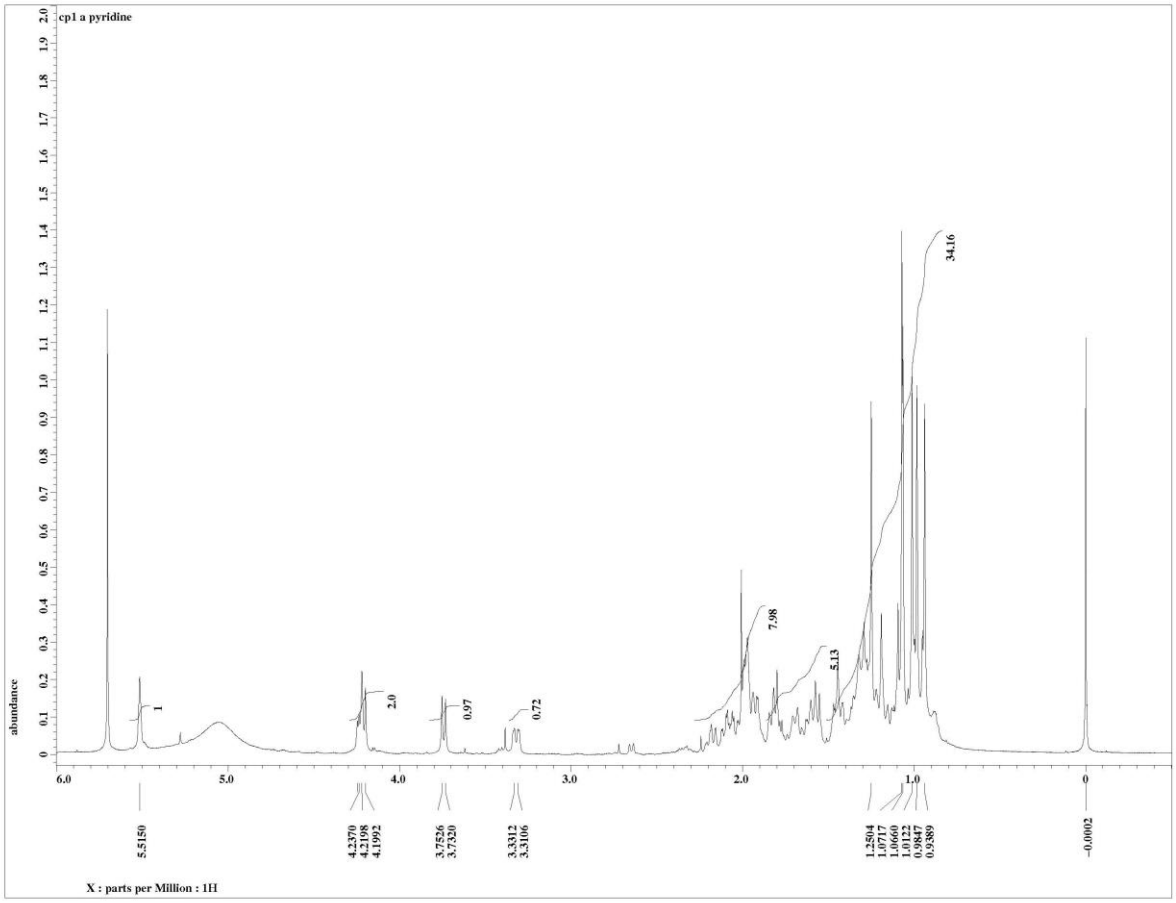
^{13}C NMR (500MHz, D5-pyridine) of cyclocaric acid A

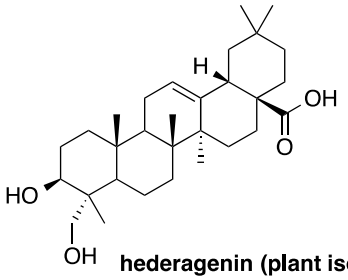




hederagenin (plant isolate)

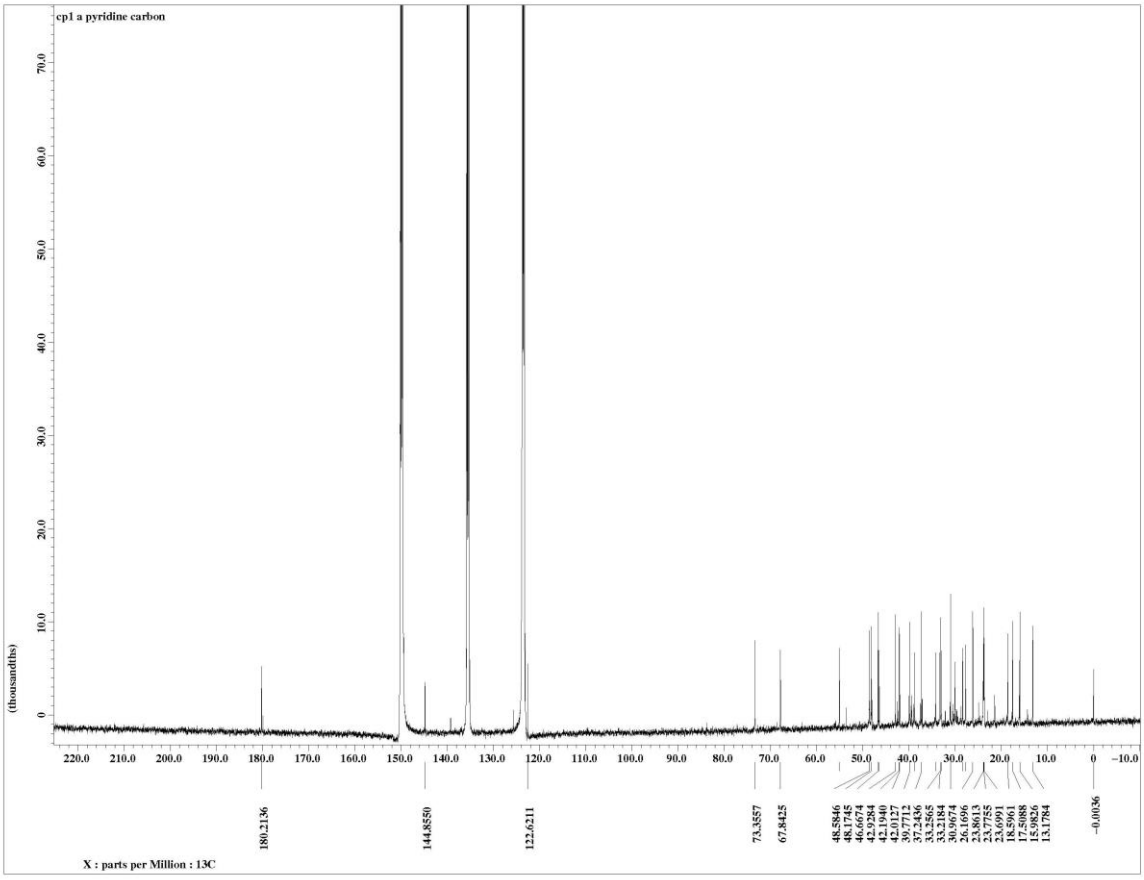
^{1}H NMR (500MHz, D5-pyridine) of (plant isolate)



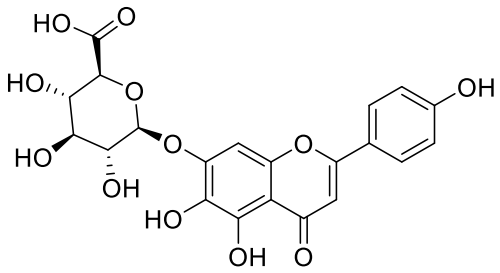


hederagenin (plant isolate)

^{13}C NMR (125MHz, D5-pyridine) of hederagenin (plant isolate)

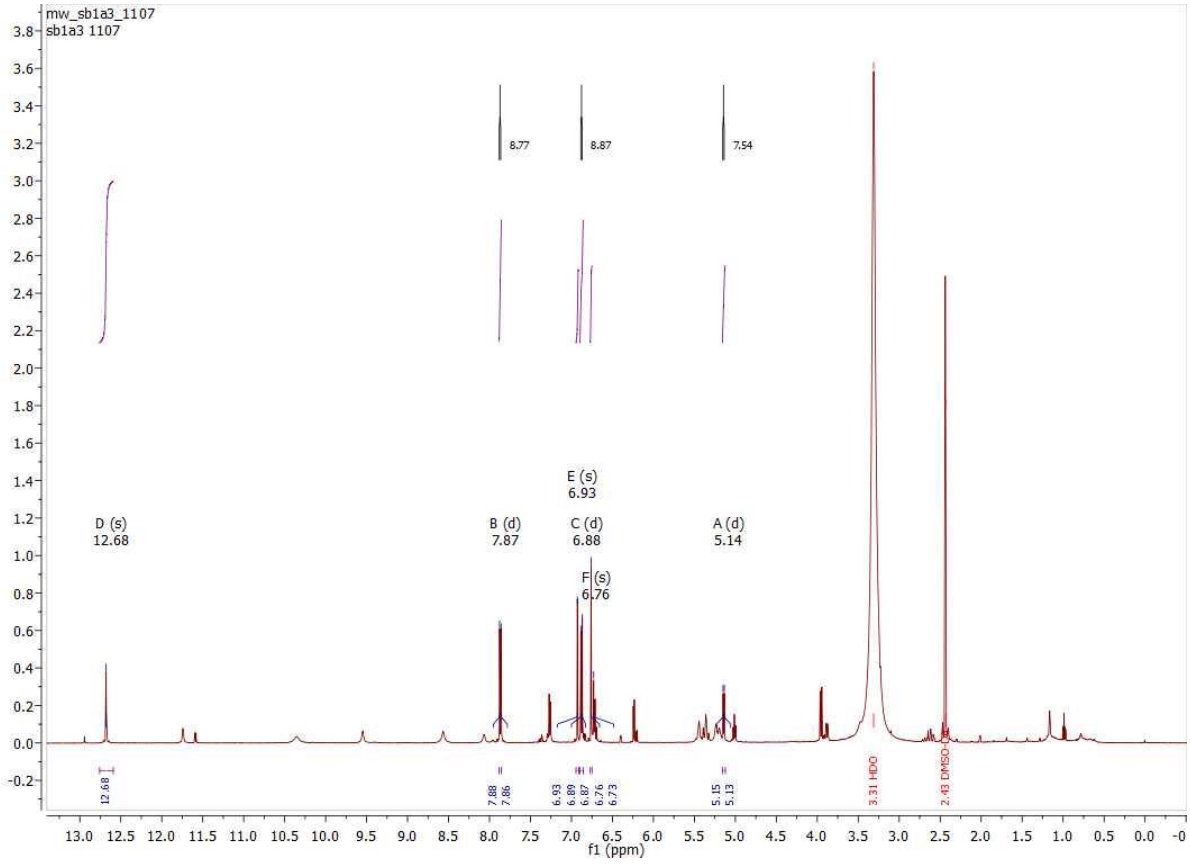


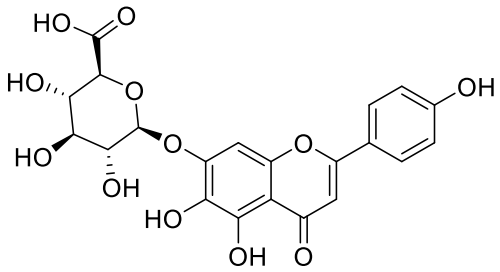
APPENDIX B: SPECTROSCOPY FOR *SCUTELLARIA BAICALENSIS*



scutellarin (Sb1a3)

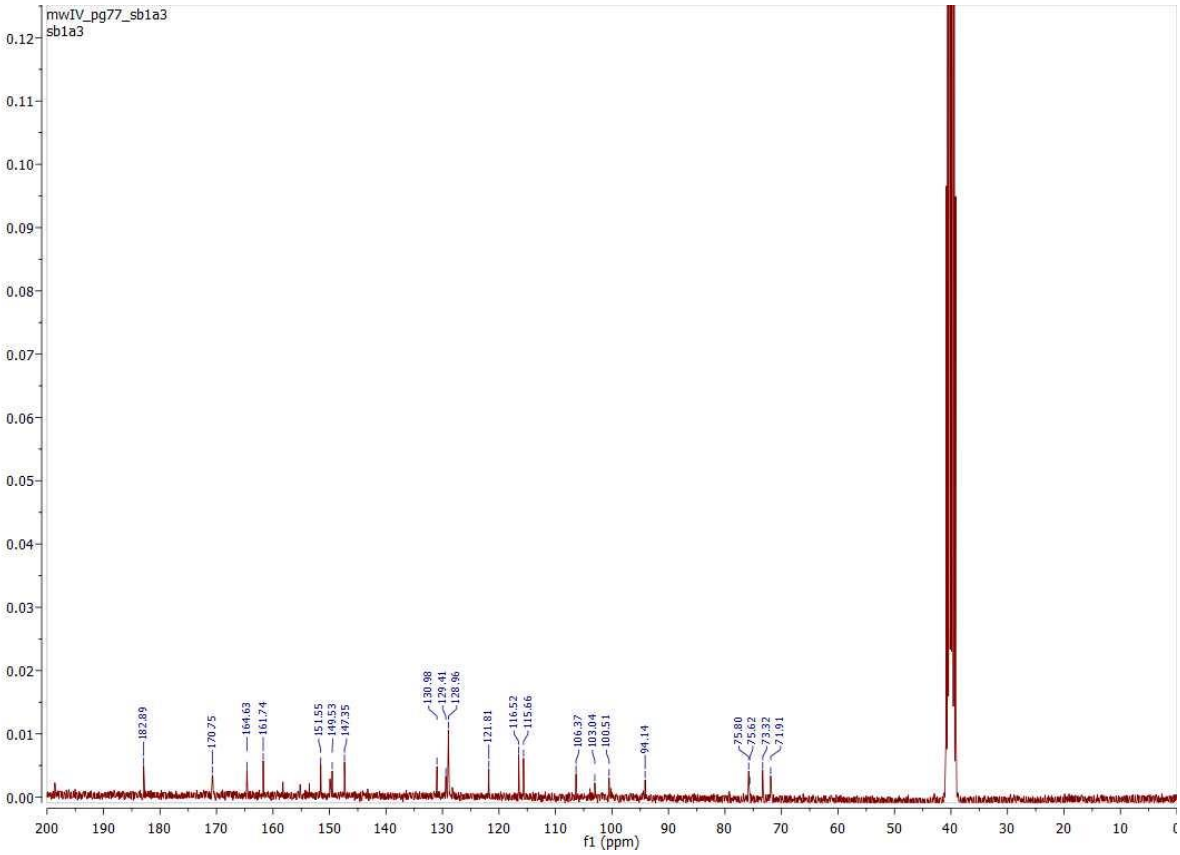
^{1}H NMR (500MHz, DMSO-d_6) of scutellarin

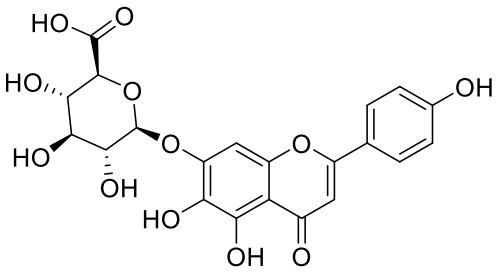




scutellarin (Sb1a3)

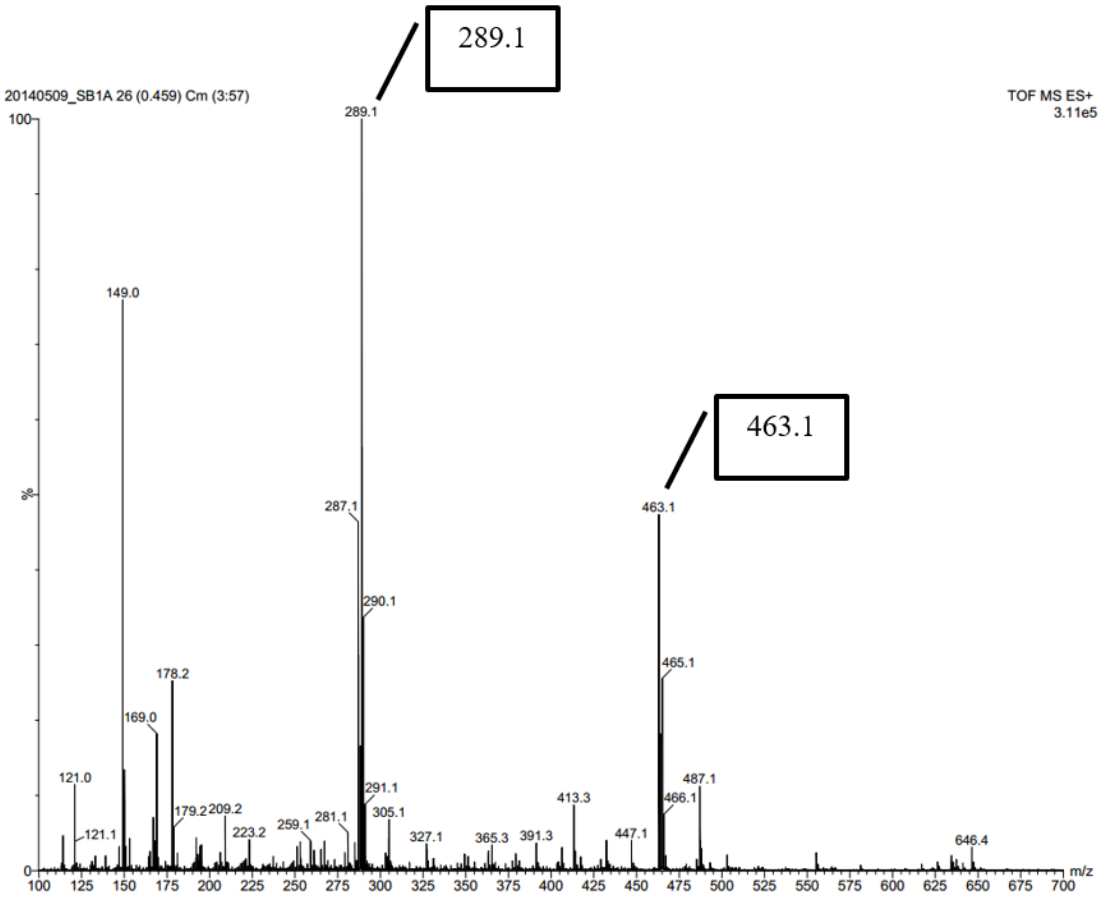
^{13}C NMR (300MHz, DMSO-*d*$_6$) of scutellarin





scutellarin (Sb1a3)

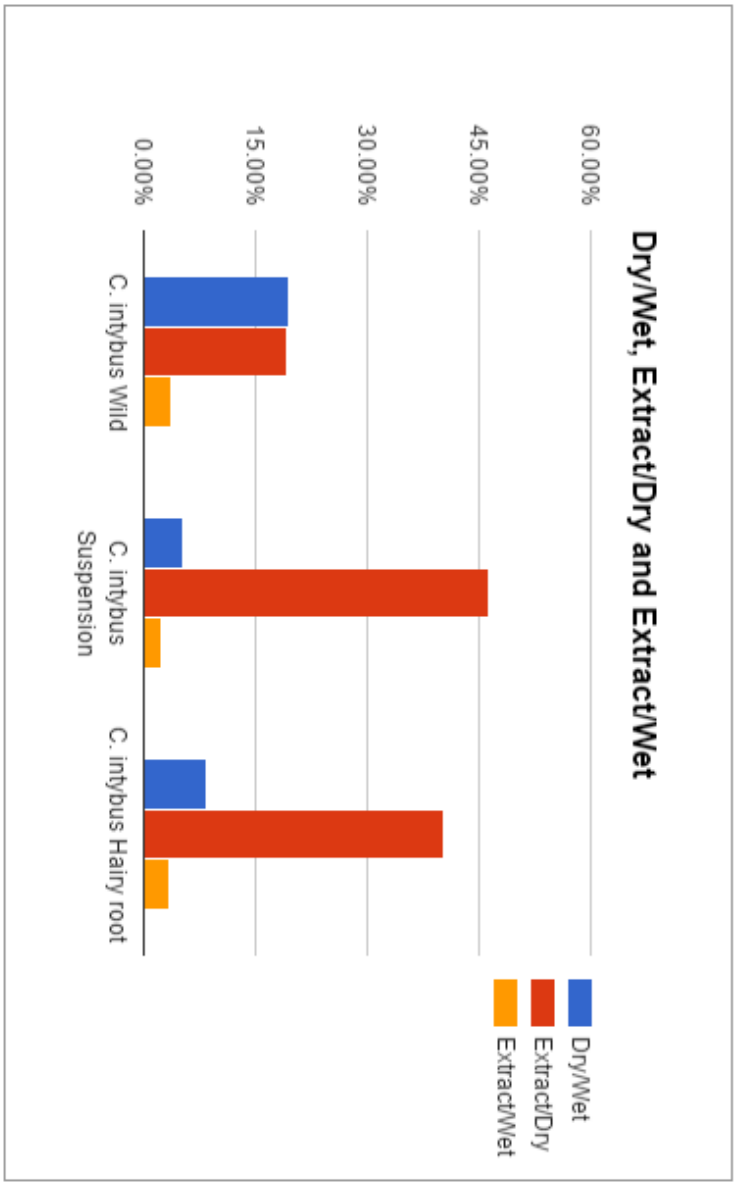
ESIMS of scutellarin



APPENDIX C: DATA FOR *CICHORIUM INTYBUS*

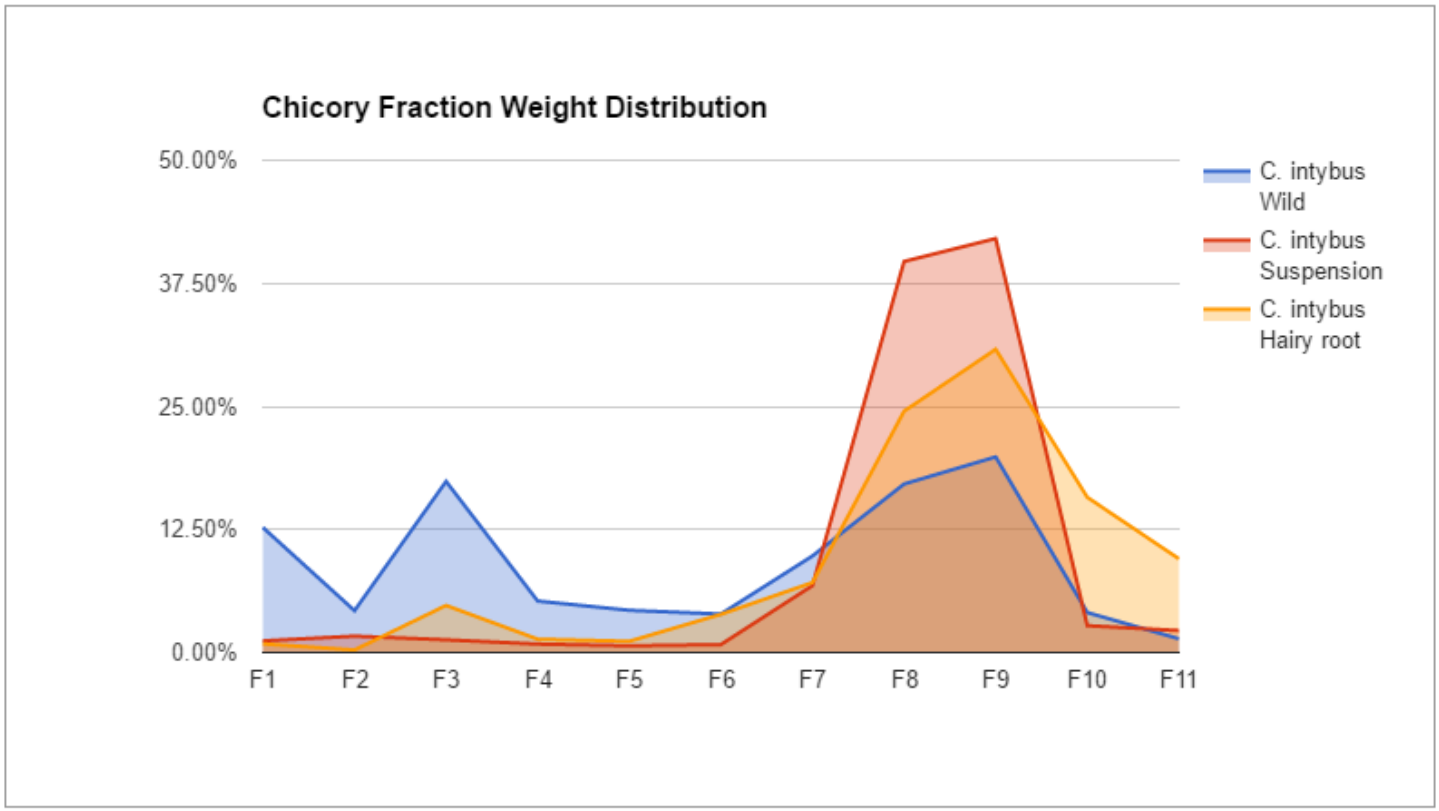
Biomass and Extract Ratios

	Dry/Wet	Extract/Dry	Extract/Wet
***C. intybus* Wild**	19.53%	19.33%	3.78%
***C. intybus* Suspension**	5.37%	46.43%	2.50%
***C. intybus* Hairy root**	8.42%	40.27%	3.39%



Percent of Total Extract Weight

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
***C. intybus* Wild**	12.70%	4.25%	17.38%	5.23%	4.29%	3.91%	9.81%	17.11%	19.87%	4.06%	1.40%
***C. intybus* Suspension**	1.18%	1.66%	1.29%	0.86%	0.68%	0.81%	6.79%	39.72%	42.05%	2.73%	2.23%
***C. intybus* Hairy root**	0.88%	0.26%	4.78%	1.36%	1.14%	3.88%	7.11%	24.51%	30.80%	15.77%	9.52%



Percent of Total Extract Weight

	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
***C. intybus*-Wild**	12.70%	4.25%	17.38%	5.23%	4.29%	3.91%	9.81%	17.11%	19.87%	4.06%	1.40%

F6-F11 percent of total weight **56.15%**

***C. intybus*-CC-E**	1.18%	1.66%	1.29%	0.86%	0.68%	0.81%	6.79%	39.72%	42.05%	2.73%	2.23%

F6-F11 percent of total weight **94.32%**