Characterization of Flavonoids from *Artemisia annua*

A Major Qualifying Project Report

Submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements

for the Degree of Bachelor of Science

By

Megan Barriga

April 25, 2013

Approved:

Dr. Pamela J. Weathers, Advisor

Table of Contents

Table of Figures

Table of Tables

Acknowledgements

I would like to thank Dr. Pamela Weathers for providing me guidance for my project.

I would also like to thank Dr. John MacDonald for all the help and advice on my extractions and thin layer chromatography experiments.

I would like to thank Dr. Marion Emmert and Dr. Zhou's lab for providing me solvents.

I would like to thank Dr. Robert Connors for getting my lab equipment out of a tight situation.

I would like to thank Dr. Melissa Towler for TLC advice and help in the lab.

I would like to thank the graduate students Ying Yang and Liwen Fei for their help in the lab and ideas for my project.

I would like to thank Prof. James Dittami for helping me understand previously documented methods.

I would like to thank Andrew Butler for his help with the NMR analyses.

I would like to acknowledge the NIH Grant (2R15 GM069562-03).

I would like to thank Christopher Molica and my parents for their support.

Abstract

Malaria is a malicious disease that still prevails in the world. Efforts to find a treatment have been in progress for decades, especially since the plasmodia parasites have begun to build a resistance against present antimalarials, specifically artemisinin, a sesquiterpene lactone. This project aimed to advance the search for some of the synergistic compounds present in the plant, namely flavonoids. To verify the identities of several flavonoids of interest, chrysosplenol-D, chrysoplenetin, and cirsilineol, various TLC mobile and solid phases were compared and then used to analyze fresh plant extracts from both plant shoot tip extracts and mature leaves. To profile the many flavonoids and terpenes in this species the best TLC method used was the mobile phase 36:9:5, benzene-pyridine-formic acid with silica gel as the stationary phase. Analysis of the two major plant extracts showed there were at least six flavonoids and six terpenes present. These results will improve our understanding of the changes in these compounds as plants grow to maturity and will help further the work on using this plant for treatment of malaria.

1.0 Introduction

The original objective of this Major Qualifying Project was to extract, isolate, and verify the identities of three specific flavonoids, chrysoplenetin (CRY), chrysosplenol-D (CRYD) and cirsilineol (CRS) from the plant *Artemisia annua*. Flavonoids, a subclass of polyphenols, were found to act synergistically with artemisinin (AN), a sesquiterpene lactone found in *A. annua*, giving greater aid in the treatment of malaria than when AN was administered alone. The challenge of this project included the limited journal articles that specified a procedure to extract only those three aforementioned flavonoids, and the process itself of separating the three flavonoids of strikingly similar structures from the 46 known flavonoids discovered in *A. annua*. This project was foreseen to advance the search for a treatment for malaria and cancer, as there were, to our knowledge at the time, no standards for the three desired flavonoids, nor any studies that tested their action in combination with AN against diseases other than malaria. Now we know that the standards for the three desired flavonoids are obtainable; however, they are very expensive.

Key for Terpene Abbreviations	
AA	Artemisinic acid
AB	Arteannuin B
DHAA	Dihydroartemisinic acid
AN	Artemisinin

Compound	Abbrev.
Artemetin	ART
Artemisinin	AN
Artemisitene	AT
Casticin	CAS
Chrysoplenetin	CRY
Chrysosplenol-D	CRYD
Cirsilineol	CRS
Eupatorin	EUP
Kaempferol	K
Myricetin	M
Quercetin	Q
Scopoletin	SCP

2.0 Background

2.1 Malaria

Protozoan plasmodia parasites cause malaria, a vector-borne infectious disease (de Ridder *et al*., 2008). The five types of *Plasmodium* species are *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale,* and *P. knowlesi* (de Ridder *et al*. 2008; Alonso *et al*., 2011). The main species of malaria existing in sub-Saharan Africa is *P. falciparum* (Breman and Brandling-Bennett, 2011). While *P. vivax* is often the most prevalent malaria infection in tropical and subtropical areas, *P. falciparum* is responsible for most malaria-induced deaths, and is thus the greater focus of research (Clemente and Corigliano, 2012).

2.1.1 Life Cycle of Malaria Parasite

The life cycle of the malaria parasite is complicated to the extent that procuring a diagnosis and preventing the disease can be difficult, especially during the initial diagnostic period, when the four *Plasmodium* species, *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malaria*, have analogous clinical presentations (Bledsoe, 2005). Initially, the *Anopheles* mosquito feeds on an infected human, taking in male and female *Plasmodium* gametocytes, which combine sexually and reproduce countless sporozoites in the stomach lining of the mosquito (Bledsoe, 2005). The sporozoites can be transferred to healthy humans and infect them through the salivary glands of the mosquito, when they drink human blood (Bledsoe, 2005). After entering the blood stream, the sporozoites journey to the liver and asexually reproduce, inducing a dormant stage that continues for about eight to thirty days (Bledsoe, 2005). Then merozoites form in the liver, are released into the bloodstream, and invade erythrocytes, red blood cells, where they develop into trophozoites, then schizonts (Bledsoe, 2005; Perez-Jorge and Herchline, 2012). Erythrocytes lyse after 48-72 hours, causing signs of fever and the release of merozoites,

which then infect healthy red blood cells and spread the infection, resulting in a repeat of the life cycle (Bledsoe, 2005; Perez-Jorge and Herchline, 2012).

2.1.2 Statistics and Groups Most Affected

According to Alonso *et al*. (2011), about one-fifth of the world's population in 2009 was living in regions that were at high risk for transmission of malaria. The 81 out of 106 countries where malaria transmission was reported were focusing their sights on control, whereas 25 were in pre-elimination, elimination, and prevention of re-introductory phases (Alonso *et al*., 2011). Of estimated deaths due to malaria worldwide in 2010, about 86 percent (655,000) were children under five years old infected with *P. falciparum* in Africa (WHO, 2011). Malaria can be detrimental to women who are pregnant, as well as to the fetus they are carrying, since some of the immunity built up by adults after repeated infections does not transfer to the fetus (Greenwood and Mutabingwa, 2002). If women are living in an area of low transmission, malaria can lead to an abortion or stillbirth, while in areas of high malarial transmission, the placenta may become infected with *P. falciparum*, which may impair placental function, resulting in low birth weight of the baby, as well as increased infant mortality (Greenwood and Mutabingwa, 2002).

2.1.3 Taking Action against Malaria

Finding a cure or a way to eliminate malaria is a daunting feat, as the five *Plasmodium* species are transmitted by over 30 different female Anopheline mosquito species with various breeding and feeding habits, and result in a variety clinical presentations in different population target groups and epidemiological settings (Alonso *et al*., 2011). Other barriers to the elimination of malaria include differences in parasite, vector, human, social, and environmental factors, underperforming health services, lack of political will, insufficient financial, social, and human resources, as well as insufficient tools, such as drugs or vaccines, to interrupt

transmission given an especially high level of transmission (Alonso *et al*., 2011). Some of the measures taken to prevent malaria have been the distribution of insecticide-treated nets, intermittent preventive treatments during pregnancy, and insecticide residual spraying (Breman and Brandling-Bennett, 2011).

Quinine, an alkaloid found in *Cinchona* bark, was a popular antimalarial of the past; however, the *P. falciparum* parasite built up resistance to not only this drug, but to its synthetic analogs, such as chloroquine (Brown, 2010; Smith *et al*., 2010). The resistance to these antimalarials led to the use of AN, a sesquiterpene lactone extracted from the plant *Artemisia annua*, which was later recommended by the World Health Organization (WHO) to be used in combination with another effective blood schizontocide, for instance mefloquine, and is referred to as artemisinin-based combination therapy (ACT). ACT is documented, by WHO in the World Malaria Report 2010 to have been one of the factors, along with long-lasting insecticide-treated nets, to have helped decrease malaria cases in numerous African countries since 2000 (Smith *et al*., 2010; Breman and Brandling-Bennett, 2011).

2.2 *Artemisia annua*

Artemisia annua L. is also referred to by the English common names sweet annie, sweet wormwood, and annual wormwood, and by the Chinese name *qing hao*, which translates closely to 'green herb' (de Ridder *et al*. 2008; Ferreira and Janick, 2009). There are between 200 and 400 species of the large, diversified *Artemisia* genus that are members of the Asteraceae family, which is the second largest flowering plant family in the world (de Ridder *et al*. 2008). *A. annua* is a sturdy, annual herb, native of Asia, naturally growing in the northern regions of China's Chahar and Suiyuan provinces as part of steppe vegetation. It is also now presently cultivated in other countries, such as Russia, Brazil, the United States, Africa, and India (de Ridder *et al*.

2008; Ferreira and Janick, 2009). This aromatic herb prefers to grow in temperate climates and can grow over 2.0 m tall, with its single stem, recurring branches from the bottom of the stem to the tip, and alternating, aromatic leaves (de Ridder *et al*. 2008; Ferreira and Janick, 2009). *A. annua* is pollinated "by insects, self-pollination, and wind distribution" (de Ridder *et al*. 2008). *A. annua* is an herb found beneficial to many facets of life. Not only has this plant been proposed for use by the livestock industry due to its antioxidant, anti-bacterial, and anti-protozoal properties, but also for human medicinal purposes (Ferreira and Janick, 2009). *A. annua* is the only known source of artemisinin, a drug administered to treat malaria and used as an infusion tea in China for over 2,000 years (Brown, 2010).

2.3 Artemisinin

Artemisinin (AN) is a secondary metabolite of *A. annua* and a sesquiterpene lactone, with an endoperoxide bridge, as depicted in Fig. 1, which is responsible for its activity against malaria (Smith *et al*., 2010; Ferreira and Janick, 1996).

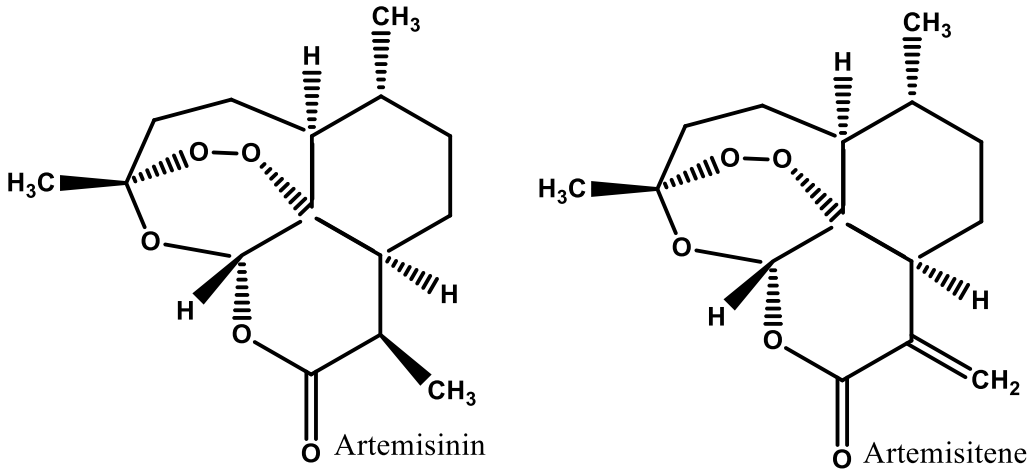

Fig. 1. Chemical structure of artemisinin and artemisitene.

A. annua stores much of its AN content in the glandular trichomes of its leaves, floral buds, and flowers (de Ridder *et al*. 2008; Weathers *et al*., 2010). The sesquiterpene lactones and monoterpenes found in the fluid of the glandular trichomes probably function as the plant's mode of protection against hungry herbivores in the environment (Ferreira and Janick, 1995). Depending on the amount of dry leaf material used for extraction, environmental conditions, extraction methods, and the cultivation strain, between 0.01 and 1.4 % (w/w) AN can be extracted (de Ridder *et al*. 2008; Brown, 2010).

2.3.1 History of Artemisinin and its Medicinal Uses

During the Vietnam War (1965-1975), the Chinese government jumped to the aid of the Vietnamese army by investing in a program aimed at discovering antimalarial drugs in plants (de Ridder *et al*. 2008). Not until 1972, after Prof. Tu's observation that cold ethereal extracts of *A. annua* showed activity against the malarial parasite in mice, was AN finally isolated and determined to be the active constituent in the herb (Brown, 2010). Artemisinin was given the name "Qinghaosu", which means "principle from Qinghao" (Brown, 2010). Before the identification of AN in *A. annua*, the leaves of the plant were brewed in a tea for aiding the treatment of chills, lice, fever, and wounds; the plant was also prescribed as a food supplement (de Ridder *et al*. 2008). *A. annua* also helped to relieve low-grade fever and summer heat stroke, due to its ability to subdue "heat" syndromes (Brown, 2010). Since recent research has resolved that AN is nearly insoluble in water, the past success in using *A. annua* for the treatment of various ailments may be attributed either to the other herbs added to the concoction or to other constituents in *A. annua*, which strengthened the therapeutic effects of AN against such illnesses as malaria (Brown, 2010). Because of AN's low insolubility in water and oil, semi-synthetic derivatives of AN—namely artemether, arteether, and artesunate (Fig. 2)—were developed that

unveiled greater therapeutic and pharmacological potential, in addition to enhanced solubility (Brown, 2010).

2.4 Resistance to Antimalarials

Over the years, evidence has emerged of the resistance of *P. falciparum* to various antimalarials employed today, including chloroquine, mefloquine, quinine, amodiaquine, and sulfadoxine-pyrimethamine; the appearance of the parasite's resistance to AN has also

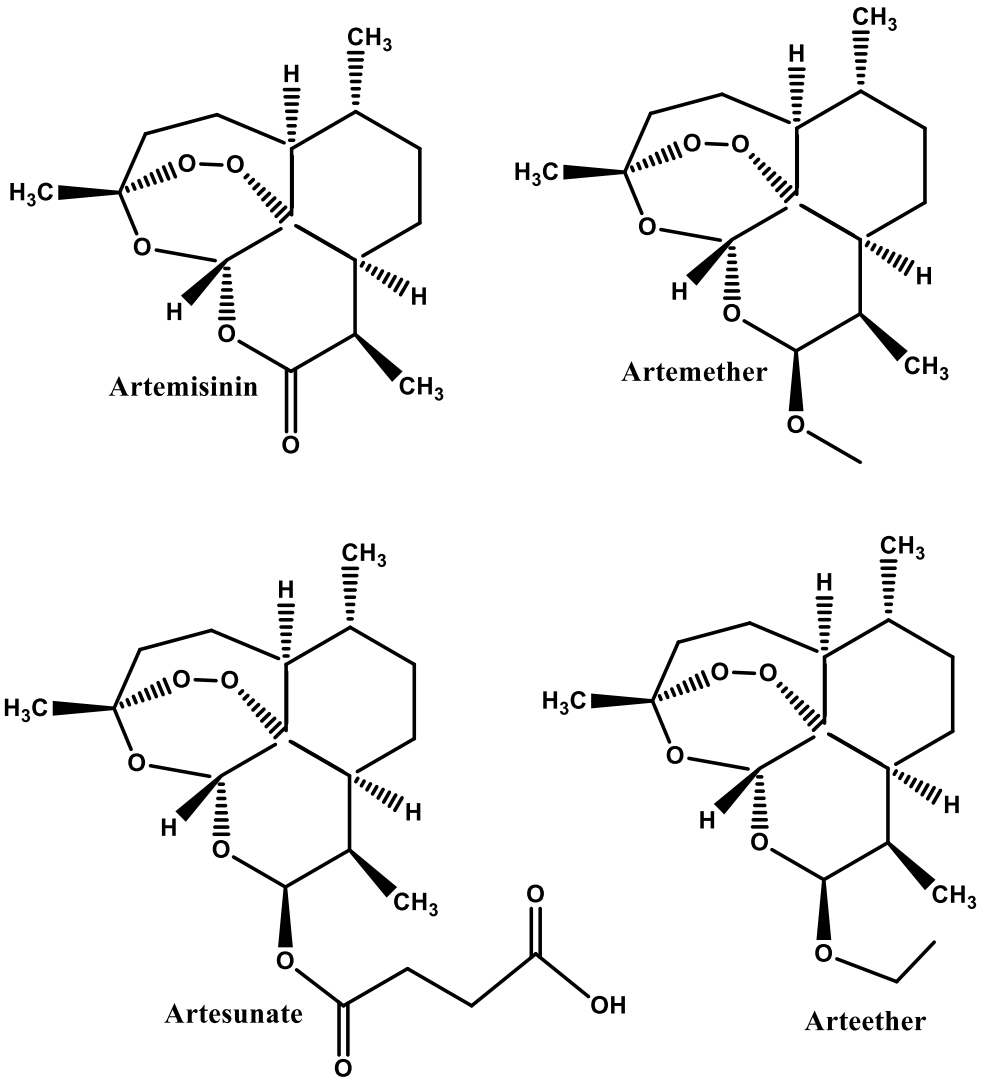

Fig. 2. Artemisinin and its semi-synthetic derivatives.

now occurred in Cambodia (de Ridder *et al*. 2008; Rasoanaivo *et al*., 2011). The gradual increase in resistance to AN may have arisen due to use of a single purified chemical entity and also to its mechanism of action involving the endoperoxide bridge (Brown, 2010). Pandey *et al.* (1999) and Wiwanitkit (2010) posited the drug's mechanism of action. When the parasite ingests hemoglobin inside its digestive vacuole, after entering the red blood cell, "free heme, an iron-porphyrin complex", is released. The heme or iron cleaves the endoperoxide bridge of AN, converting it to an unstable radical, which spurs a series of reactions, "selective alkylation of malarial proteins", and ultimately destroys the parasite. Artemisinins are fast-acting, imposing their effects within an hour after use, which may be attributed to inhibition of hemoglobin decomposition, heme polymerization, and direct interaction of the antimalarial with the malarial pigment within the digestive vacuole (Pandey *et al.*, 1999). The high rate of recrudescence that results when administering AN as a monotherapy may be connected to its short half-life (Rasoanaivo *et al*., 2011). An alternative to monotherapy is administering combinations of antimalarials, which, although increasing cost, may increase efficacy, shorten duration of treatment, and decrease the risk of parasites developing resistance through mutation during treatment (Kremsner and Krishna, 2004).

2.5 Implementation of ACT

In 2006 WHO strongly encouraged the administration of AN along with a second antimalarial drug to help combat malaria more fiercely and beat down the threat of resistance of the malarial parasite to monotherapies. This combination of AN plus an older antimalarial drug is ACT, artemisinin combination therapy. ACT is defined as taking two independently acting drugs, with differing biochemical targets in the malarial parasite, to improve efficacy and to delay emergence of resistance to either drug. If one of the drugs happens to be resisted by the

parasite, then the other drug will kill the lethal invader. WHO (2001) lists some of the following benefits of implementing ACTs:

- Capability of AN derivatives to quickly treat and exterminate parasites
- Chance to delay resistance to available antimalarials that are inexpensive and efficient
- Incompetent first-line treatments presently used in many countries

The ensuing disadvantages mentioned by WHO (2001) repressed the immediate support of ACTs:

- Little is known about ACT with or without AN derivatives
- Potential drug misuse
- The effort and cost required to change the treatment policy
- Higher cost of AN derivatives

Being able to attain the benefits of using an effective, low-cost ACT, without the disadvantages, would be worthwhile and one step closer toward achieving global eradication of malaria.

2.6 Poverty and the Challenges in Treating Malaria

Today, treatment of malaria without sufficiently adequate ACTs is a struggle for much of the poor, malaria-stricken population of Africa and Asia. Africa in particular lacks a strong health-care infrastructure, and essential medicines are also limited (de Ridder *et al*., 2008). Households often do not have the luxury of paying for febrile illnesses and must treat themselves at home with over-the-counter drugs (de Ridder *et al*., 2008). For a family to provide their child one full treatment, they would lose approximately 10 % of their monthly income and rural families, where the disease is most prevalent are especially hard hit (de Ridder *et al*., 2008). Clearly, better and cheaper antimalarials are urgently needed. The cost of introducing ACTs into

societies infected with malaria are more than ten times that of the monotherapies used in Africa (WHO, 2001). The limited supply and availability of AN may continue to keep the cost high in the future (de Ridder *et al*., 2008). Finding a low-cost means to administer AN in combination with flavonoids has been a research pursuit of particular interest; flavonoids, while not as effective against malaria as AN, are present in *A. annua* and exhibit the potential to synergize the therapeutic activity of AN (Ferreira *et al.*, 2010).

2.7 Implications and Synergistic Effects of Flavonoids when Combined with Artemisinin

Liu *et al.* (1992) reported the IC_{50} values of six flavonoids, artemetin, casticin, eupatorin, cirsilineol, chrysoplenetin, and chrysosplenol-D, tested alone and in combination with AN (See Fig. 3 and Table I). The IC_{50} is a measurement of the concentration required to produce 50% maximum pathogen inhibition.

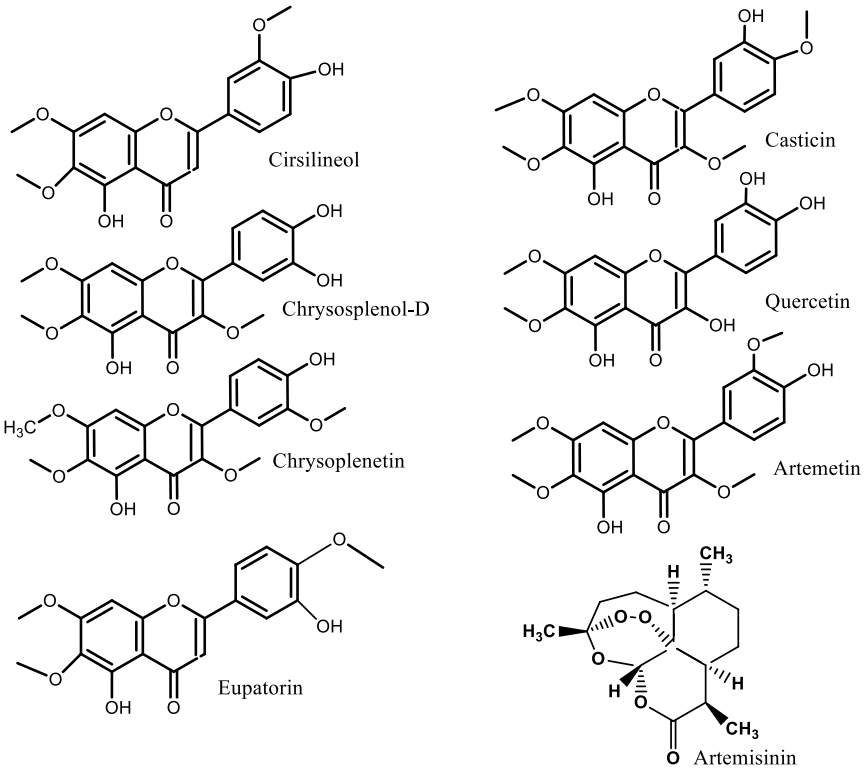

Fig. 3. Artemisinin and flavonoids of interest.

Table I: The in vitro antiplasmodial activity of compounds present in *A. annua* (from Liu *et al.*, 1992).

Compound	IC_{50}	
	Flavonoid alone (μM)	**Artemisinin (μM) + flavonoid (5 μM)**
Artemisinin	—	0.033
Artemetin	26	0.026
Casticin	24	0.026
Chrysoplenetin	23	0.0225
Chrysosplenol-D	32	0.015
Cirsilineol	36	0.016
Eupatorin	65	0.030

The flavonoids tested were ART, CAS, CRS, CRY, and CRYD; their ability to improve AN's antiplasmodial activity *in vitro* was confirmed by preventing [^{3}H]-hypoxanthine from being incorporated into *P. falciparum*. Bilia *et al*. (2002) deduced from the data obtained from UV and HPLC/DAD/MS analysis that flavonoids can promote and enhance the reaction of AN with hemin. The results reported by Bilia *et al.* (2002) revealed that "hemin and [AN] slowly react to give rise to supramolecular adducts." According to the HPLC/DAD/MS technique, free hemin disappears and AN/hemin adducts form, while the UV-Vis method indicated that upon reaction of hemin and AN, the Soret band of hemin gradually vanishes (Bilia *et al.,* 2002). When tested individually at 5.0 μM, weak antiplasmodial activity *in vitro* resulted for CRYD, CRS, or CRY. However, when they were used at 5 μM in conjunction with AN, the IC_{50} of AN decreased from 0.033 μM to 0.015 μM, 0.016 μM and 0.0225 μM AN, respectively, demonstrating an enhancement in the antiplasmodial activity of AN (Liu *et al*., 1992). In addition, flavonoids enhanced the effects of AN on *P. falciparum*. Much research on detecting the specific structural aspects of flavonoids that potentiate the activity of AN against *P. falciparum* is on the horizon, as there remain many flavonoids to be biologically tested (Liu *et al*., 1992). The *in vitro* studies of

Elford *et al*. (1987) revealed that in the presence of methoxylated flavones, such as CAS and ART, the antimalarial activity of AN was noticeably enhanced, but no significant antimalarial activity was observed when these two flavones were used alone. There was a 3 to 5 fold reduction in the ID_{50} value, which is the concentration needed to achieve 50 % inhibition of an infection, for AN, when CAS was present; however, ART was not as effective in enhancing the antimalarial activity of AN (Elford *et al*., 1987; Fung *et al*., 1992). Not only have the antimalarial activity of flavonoids and ART been examined *in vitro*, research has also been done *in vivo* on the bioavailability of AN and flavonoids in tea infusions and mice.

2.7.1 Administration of Artemisinin and Flavonoids

Currently there has been a resurging interest in the use of tea for treating malaria, because of the simplicity and low cost of its use, and the reported synergistic effects of flavonoids from *in vitro* studies (Elford *et al*., 1987; Liu *et al*., 1992). However, according to Weathers and Towler (2012), resorting to tea may prove a vain attempt to treat malaria. Following a tea infusion protocol, Weathers and Towler (2012) discovered that although AN was efficiently extracted from an *A. annua* tea infusion, up to almost 93%, and stable at room temperature for 24 hr, "the flavonoids casticin and artemetin were poorly extracted" and became unstable when stored at room temperature. These results indicated that a tea infusion may lose synergistic potential, and thus its capacity to treat malaria. Weathers *et al.* (2011) proposed administering compacted dried leaves of *A. annua* in a capsule. The results from the mouse study of Weathers *et al.* (2011) showed that more AN was detected in the blood of mice from the *in planta* form than from > 40 times more pure AN administered to the mice. After administering 1,400 µg of pure AN to mice, only 0.074 mg L^{-1} remained in the blood after an hour, whereas, 30 minutes after feeding the mice ground *A. annua* leaves, containing only 31 µg AN, serum levels reached nearly the same level, 0.087 mg AN L^{-1} (Weathers *et al*., 2011). This suggested that other chemical

constituents in *A. annua* enhanced the bioavailability of AN, further encouraging more research into the use of *A. annua* as an ACT in the treatment of malaria, as well as cancer, and various other ailments such as *Pneumocystis carinii*, *Schistosoma*, and *Herpes simplex* viruses (Efferth, 2007). In another study reported by Elfawal *et al.* (2012), the oral delivery of dried leaves of whole plant *A. annua* to mice infected with *Plasmodium chabaudi* proved more effective than an equal dose of pure AN. The efficiency of whole plant *A. annua* in treating malaria may be attributed to the synergy between flavonoids present in *A. annua* and AN (Elfawal *et al.*, 2012). Although the synergistic potential achieved when administering flavonoids in combination with other antimalarials had not been realized until several decades ago, natural medicinal remedies that contain flavonoids have been used for thousands of years in various regions of the world.

2.8 Flavonoids

2.8.1 History of Flavonoids

Centuries ago, healers prepared medicine composed of flavonoids and administered them to the sick. For instance, in Ancient Greece, Hippocrates recommended propolis to those suffering from sores and ulcers (Cushine and Lamb, 2005). In China, the herbal medicine *Scutellaria baicalensis*, containing baicalein, the flavone responsible for its antimicrobial activity, was long applied to infected oral wounds and periodontal diseases (Cushine and Lamb, 2005). Europeans used plants as traditional folk medicines that are thought to have contained flavonoids holding therapeutic activity (Jäger and Saaby, 2011). Apigenin was the flavone confirmed as the constituent accountable for the calming effect of camomile flowers; quercetin provided the nerve calming effect from heather; the combined application of quercetin and kaempferol produced a sedative effect when Linden flowers were used (Jäger and Saaby, 2011). The use of tea was first documented in 2700 BC, while Eisai, a Japanese monk, first recorded its therapeutic effects in 1211 (*Principles*, 2011). Tea extracts were taken by 16th century European

adventurers to ease "fever, headache, stomach ache, and joint pain" (*Principles*, 2011). Antioxidants, polyphenols, such as catechins and flavones, are thought to be the elements in tea responsible for its therapeutic potential (*Principles*, 2011). In the 1930s, Albert Szent-Györgyi was the first to isolate rutin, a flavonoid glycoside, from oranges, which he discovered gave strength to capillary walls by a means that vitamin C could not; flavonoids were originally called vitamin P, but due to the diversity of flavonoids found in nature, they could not be labeled as a single vitamin (Kale *et al*., 2008; *Principles*, 2011).

2.8.2 Overview of Flavonoids

Probably every plant species contains flavonoids—including vegetables, fruits, and flowers—flavonoids occur in every part of plants—stems, seeds, leaves, roots—as complex mixtures of a wide assortment of biochemicals (Tsimogiannis *et al*., 2007). Their general aglycone structure is shown in Fig. 4.

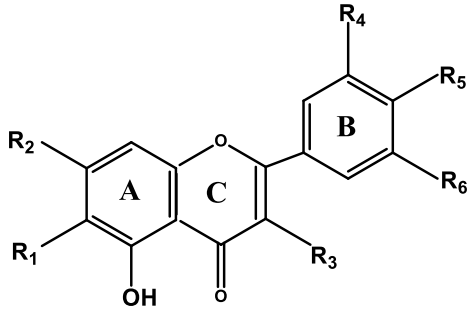

Fig. 4. Generic structure of flavonoids.

Often they are found in plant tissues and vacuoles (Croteau et al., 2000). They are frequently found attached to sugars in the leaves of plants as water-soluble glycosides in the vacuoles of epidermal cells, and less so in the upper surface of leaves as aglycones, free flavonoids, without sugars attached, where they can provide protection from UV-B radiation, or are converted to aglycosides after the process of digestion (Harborne and Williams, 2000; Hollman and Arts,

2000). They can also exist as conjugated forms with aliphatic acids, isoprenyl, methoxyl, and methylenedioxy substituents (Ferreira *et al*., 2010). In leaves, flavonoids are masked by the green chlorophylls, but are attractively visible as colorful pigments in flower petals (Harborne and Williams, 2000) as a means of attracting pollinators and seed dispersers (Croteau *et al*., 2000). For example, the pelargonidins give plants their orange, salmon, pink, and red hue; the cyandins attribute to magenta and crimson pigments; the delphinidins contribute to the purple, blue, and mauve colors of flowers (Croteau *et al*., 2000). Flavonoids occur in wine, tea, honey, and propolis, which is a resin mixed with wax that honeybees collect from plants and use to seal open spaces to keep out unwanted visitors, and furthermore, to smooth out surfaces in their hives (Park *et al*., 2002; Cushine and Lamb, 2005). In the US, a few hundred to a thousand milligrams of mixtures of flavonoids per person are consumed daily (Cushine and Lamb, 2005). Flavonoids are referred to as phytonutrients, nontraditional nutrients, and phytochemicals—bioactive compounds designated as pharmacologically safe (Beecher, 2003; Kale *et al*., 2008). Since flavonoids are consumed on a daily basis in a large array of plants and beverages, and have been incorporated into medicine for hundreds of years, there is little chance that they are toxic, if at all, however further research needs to be pursued to evaluate the toxicity of individual flavonoids (Cushine and Lamb, 2005). In addition, flavonoids are not considered traditional nutrients or vitamins because their removal from diets does not induce deficiency diseases (Beecher, 2003). On the other hand, flavonoids that are classified into separate subgroups, are well recognized as health-promoting anti-oxidants (Pietta, 2000).

2.8.3 Classification of Flavonoids

More than 4,500 flavonoids have been separated and identified, with over 40 present in *A. annua* (Ferreira *et al*., 2010; Rasoanaivo *et al*., 2011). Flavonoids are a subclass of polyphenols, which are further grouped under phenolics, phenolic phytochemicals. Flavonoids

are subdivided into flavones, flavonols, flavanols or catechins, dihydroflavonols, chalcones, aurones, isoflavonoids, bioflavonoids, and anthocyanins (Ferreira et al., 2010). The three flavonoids of interest in this research endeavor are CRY and CRYD, both of which are flavonols, and CRS, a flavone. The general structures of a few of the aforementioned flavonoid subclasses are shown in Fig. 5.

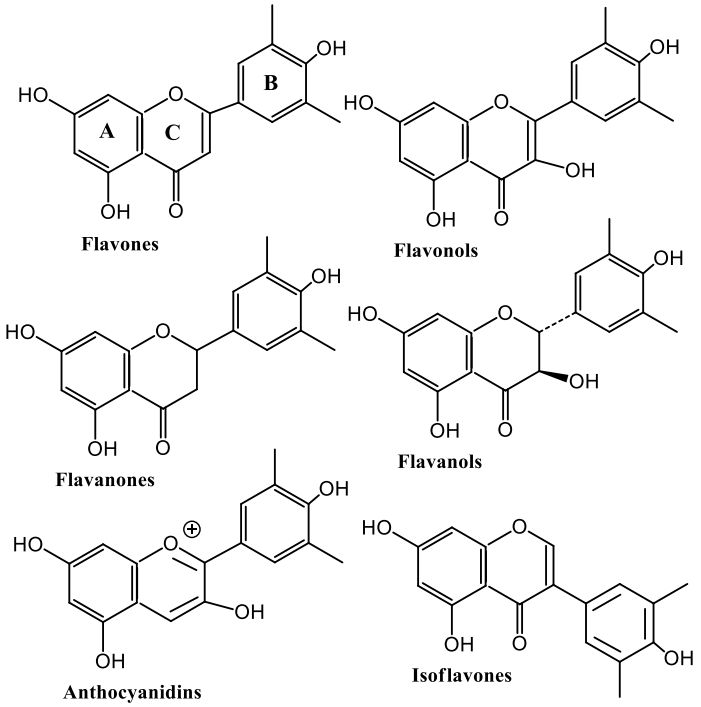

Fig. 5. Subclasses of flavonoids based on their structural variations.

2.8.4 Generic Structure of Flavonoids and the Three of Interest to This Project

Flavonoids have the structural characteristic of an aromatic ring and a benzopyran ring with a phenyl group attached; they also have at least one hydroxyl group as a substituent of the aromatic ring and phenyl group (See Fig. 4). The structures of CRS, CRY, and CRYD are shown in Fig. 6. All three are methoxylated at R1 and R2. However, CRS lacks the methoxyl group at position R3, as well as a substituent at R6, found in CRY and CRYD. Chrysoplenetin and CRYD differ in that CRYD has a hydroxyl group at R6, whereas CRY has a methoxyl group

at R6. The closely related structures and their similarities in polarity make isolation and identification, including association of their observed activity, of the three targeted flavonoids of

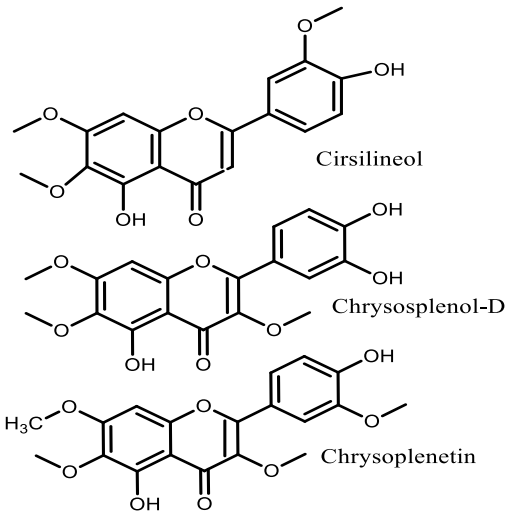

Fig. 6. Structures of chrysoplenetin, cirsilineol, and chrysosplenol-D.

this project a challenge (Tsimogiannis *et al*., 2007). Flavonoids are named on the basis of their structure using various systems of nomenclature.

2.8.5 Nomenclature of Flavonoids

In addition to the oxidation state and substituents of the C ring, flavonoids are divided into subclasses based on the connection of the B ring to the C ring, as seen in Fig. 4 (Beecher, 2003). They are also classified and identified individually based on hydroxylation and conjugation patters of rings A, B, and C (Beecher, 2003). Flavonoids can be given trivial names, which can designate their class or plant source; for example, the suffix 'etin' implies a flavonol, and the compound hypolaetin was elicited from plants of the genus *Hypolaena* (Cushine and Lamb, 2005). They can be named semi-systematically based on trivial names, for example, chalcone as the parent structure in 3,3',4',5,7-pentahydroxyflavone (Cushine and Lamb, 2005). A third, inconvenient method of naming flavonoids is to appoint them systematic chemical names, like 3,4-dihydro-2-phenyl-2H-benzopyran for flavan (Cushine and Lamb, 2005). The

synonyms of the three flavonoids of interest are in Appendix I. The biosynthesis of the various subclasses of flavonoids occurs through an intricate pathway catalyzed by specific enzymes.

2.8.6 Biosynthesis of Flavonols and Flavones

After general phenylpropanoid metabolism occurs, the first step leading to the flavonoid subgroups is catalyzed by chalcone synthase (CHS), requiring 4-coumaroyl CoA and malonyl CoA as substrates (Winkel-Shirley, 2001). The resulting chalcone is then isomerized by chalcone isomerase (CHI), to a flavanone, which through one mechanism can transform into a dihydroflavonol by the enzyme flavanone 3' hydroxylase (F3H), which then can be converted into flavonols, when catalyzed by flavonol synthase (FLS) (Winkel-Shirley, 2001). Through an alternative mechanism, the flavanone is catalyzed into a flavone through flavone synthase, which occurs in two isoforms (FSI, FSII) (Winkel-Shirley, 2001). The biosynthetic pathway of flavonols and flavones is summarized in Fig. 7.

2.8.7 Previous Methods of Extraction and Isolation of Flavonoids

Methods of extraction and isolation of the three flavonoids of interest to this project from dried *A. annua* plant material and other plant species have been reported in the past, but most were too vague to replicate. Those reports are first organized by flavonoid and briefly described, then the key literature reports are described more in depth. Table II contains the chromatography methods suitable for profiling CAS, CRYD, CRS, and ART, as reported in the literature.

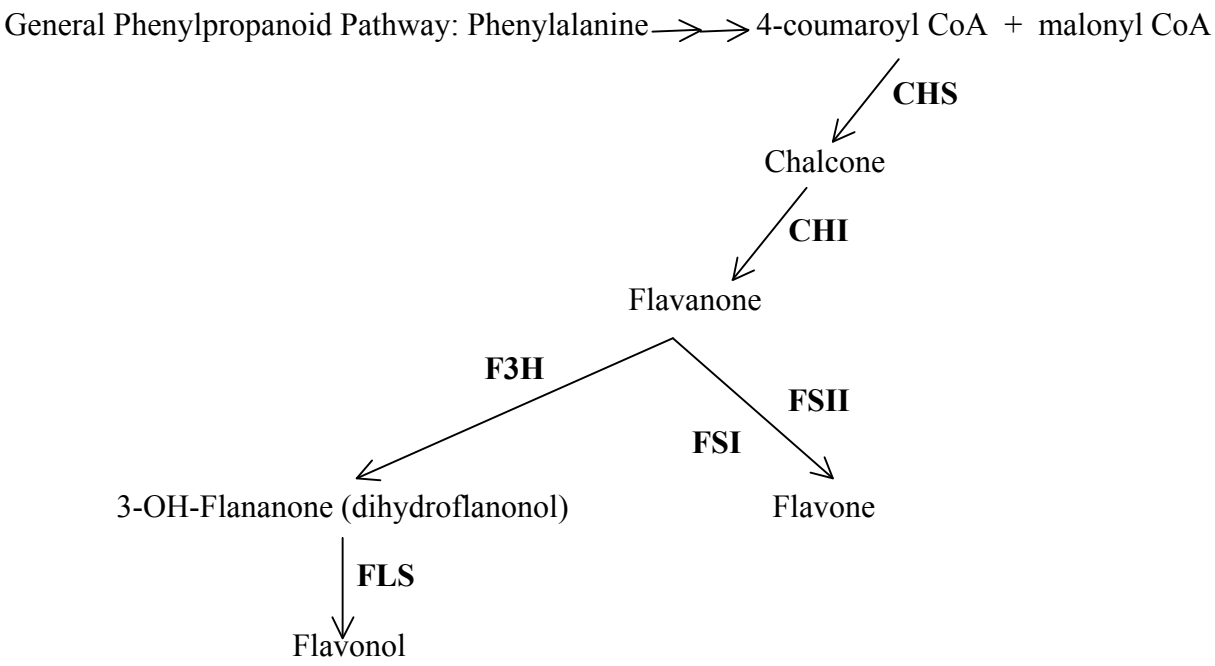

Fig. 7. Biosynthetic pathway of flavonols and flavones. The following abbreviations for the enzymes are given: flavanone 3' hydroxylase (F3H), flavonol synthase (FLS), and flavone synthase isoforms (FSI and FSII) (Winkel-Shirley, 2001).

2.8.7.1 Extraction and Isolation of Chrysosplenol-D (CRYD)

Chrysosplenol-D was isolated from the plants *Brickellia veronicaefolia* and *A. hispanica*, as reported by Roberts *et al*. (1980) and Marco *et al*. (1988), respectively. A purified form of CRYD was also isolated from *A. annua* L. as reported by Liu *et al*. (1992) and Shilin *et al*. (1989). The isolation procedures reported by the aforementioned authors involved using MeOH for extraction of the plant material, successive partitioning between organic solvents, and running through a series of columns (polyclar, Sephadex LH-20, silica gel, polyamide).

Table II: Chromatography methods suitable for profiling casticin, kaempferol, eupatorin, myricetin, chrysosplenol D, cirsilineol, chrysoplenetin, and artemetin in *Artemisia annua* as reported by other literary works.

Compound	Sample suspension solvent	Stationary phase	Mobile phase	R_f	Reference
ART	1:1, $CHCl_3$-MeOH	Paper Whatman 3MM	3:1:1, t-BuOH-H_2O-HOAc	0.82	Roberts et al. 1980
CRYD	CH_2Cl_2			0.82	
ART	1:1, $CHCl_3$-MeOH	TLC Cellulose 300	15 % HOAc	0.16	
CRYD	CH_2Cl_2			0.18	
CAS	1:1, $CHCl_3$-MeOH	TLC Si gel GF_{254}	2:1, Toluene-Ethyl Acetate	0.26	Hoberg, E. 1999
CRS	CH_2Cl_2		5 % MeOH in $CHCl_3$	0.44	Grayer *et al.* 1996
CAS	1:1, $CHCl_3$-MeOH		36:9:5, benzene-pyridine-formic acid	0.69	Saleh *et al.* 1987
CRS	CH_2Cl_2			0.60	
CAS	1:1, $CHCl_3$-MeOH	TLC Polyamide	60:60:7:7, benzene-petrol (60-80) methyl ethyl ketone-methanol	0.45	
CRS	CH_2Cl_2			0.26	

2.8.7.2 Extraction and Isolation of Cirsilineol (CRS)

Saleh *et al*. (1987) reported that CRS was detectable in *A. herba-alba*, *A. mesatlantica*, *A. monosperma*, and *A. capillaris*, and isolated CRS from *A. herba-alba*. Marco *et al*. (1988) isolated CRS from *A. hispanica*, whereas Liu *et al*. (1992) and Shilin *et al*. (1989) isolated CRS from *A. annua* L. The methods of extraction reported by Liu *et al*. (1992), Marco *et al*. (1988), and Shilin *et al*. (1989) used MeOH extracts that were eluted through a series of columns (Sephadex LH-20, silica gel, and polyamide), while the procedure reported by Saleh *et al*. (1987) involved crude extraction of the plant material using EtOH, followed by elution through a series

of columns (polyamide and Sephadex LH-20). Greenham *et al*. (2003) reported analyzing the flavones, one of which was CRS, in their lab using HPLC, TLC, and UV-Vis techniques.

2.8.7.3 Extraction and Isolation of Chrysoplenetin (CRY)

Chrysoplenetin was isolated from *A. hispanica* as reported by Marco *et al*. (1988), whereas Liu *et al*. (1992) and Shilin *et al*. (1989) isolated CRY from *A. annua* L. The reported methodology described for isolating CRY included extraction using MeOH and elution of the extracts through a series of columns (Sephadex LH-20, silica gel, polyamide, Polyclar AT).

2.8.7.4 Roberts et al. (1980)

Ground leaf material (80 g) of *B. veronicaefolia* was extracted (x5) with 6 L 80 % and 6 L 50% aq. MeOH (Roberts *et al*., 1980). The extracts were combined and dried at room temperature until only water remained (Roberts *et al*., 1980). The aqueous layer was successively extracted with n-hexane (2 L), $CHCl_3$ (3 L) , and finally EtOAc (3 L). The remaining aqueous layer was dried to a volume of 200 mL. The hexane and $CHCl_3$ extracts were combined, however, since the three flavonoids of interest to this project were not found in that combined extract, further procedures for that specific fraction are not described here. The EtOAc extract (20 g) was first eluted through a polyclar column (7.5 x 100 cm, 500 g) with $CHCl_3$-MeOH-EtOAc-2,4-pentanedione (20:10:5:1), then with $CHCl_3$-MeOH-EtOAc-2,4-pentanedione (10:10:5:1), increasing the polarity of the solvent until the column was finally eluted with MeOH. CRYD (15 mg), the only flavonoid of interest to this project, was isolated using this column, but the volume of the fractions collected was not specified.

2.8.7.5 Marco et al. (1988)

Plant material of *A. hispanica* (1 kg) was extracted at room temperature with 80 % MeOH (10 L, 5 days) and 50 % MeOH (10 L, 5 days). The extracts were combined, dried *in vacuo* to 2 L, and extracted successively with hexane (3 x 2 L), Et_2O (4 x 2 L), and EtOAc (4 x 2

L). Since the hexane extract contained mostly waxes and essential oils, it was not further studied.

The Et_2O extract (6.7 g) was chromatographed on a polyamide column (50 x 60 cm) with toluene-MeOH mixtures of increasing MeOH percentage. Three main fractions were made, corresponding to percentages of up to 15 % MeOH (E-1), 15 to 25 % MeOH (E-2) and 25 % MeOH (E-3). The three main fractions were made after TLC inspection, the exact method of which was not described.

Fraction E-1 was re-chromatographed on silica gel with $CHCl_3$- Et_2O mixtures of increasing Et_2O percentage. After inspection by TLC, fractions E-11 to E-13 were collected. None of the three flavonoids of interest to this project were reported to be fraction E-11. Therefore, further procedures for separation and isolation of components in E-11 are not described here. Fraction E-12 was also re-chromatographed on silica gel, eluting with hexane-Et_2O mixtures, giving CRY, which was further purified by column chromatography on Sephadex LH-20 (elution with MeOH), yielding 25 mg of CRY (Marco *et al*., 1988). Fraction E-13 was re-chromatographed on silica gel (elution with hexane- Et_2O mixtures). The flavonoid fractions were submitted to column chromatography on polyamide, eluting with toluene-MeOH mixtures, then Sephadex LH-20 (MeOH or 80 % MeOH), giving arteanoflavone (6 mg), penduletin (7 mg), CRS (46 mg), and more CRY (12 mg).

Fraction E-2 was re-chromatographed on polyamide, using the eluent toluene-MeOH, 10:1. Two main fractions, E-21 and E-22, were collected after TLC inspection, the exact method of which was not mentioned. Fraction E-21 was subjected to column chromatography on Sephadex LH-20 (elution with MeOH) to give two fractions, one of which contained CRYD (20

mg). The other fraction, which was not identified, was purified by prep TLC on polyamide and then by percolation through Sephadex LH-20 (elution with MeOH) to give methyl caffeate (160 mg) and a second fraction, which was submitted to paper partition chromatography (35 % HOAc). The main bands were eluted with MeOH and percolated through Sephadex LH-20 (MeOH) to give more CRYD (5 mg) and 5,3',4'-trihydroxy-6,7,5'-trimethoxyflavone (3 mg). Since Fraction E-3 did not contain the three flavonoids of interest to this project, further chromatographic and separation procedures for this fraction are not described here. Column dimensions and fraction volumes were not provided.

2.8.7.6 Liu et al. (1992)

Seeds of *A. annua* L. were extracted with $CHCl_3$ and subjected to column chromatography on Sephadex LH-20, using MeOH as the eluent. Column dimensions and fraction volumes were not provided. Fraction 3 of the five fractions obtained contained flavonoids as major constituents; this fraction was further separated on a silica gel column (silica gel 5-25 μm particle size) under N_2 gas at 725 μPa, eluting with $CHCl_3$, and gradually increasing the relative concentration of MeOH from 1-50 %. The fractions eluted from the column with $CHCl_3$ were observed to contain flavonoids. These mixtures of flavonoids were purified using HPLC, after which CRY (0.06 % DW), CRYD (0.06 % DW), and CRS (0.05 % DW) were collected. The unspecified flavonoids were subjected to TLC using silica gel GF_{254} plates, using $CHCl_3$-MeOH (9:1) as the mobile phase and NH_3 for the detection of flavonoids.

2.8.7.7 Shilin et al. (1989)

Plant material (19 kg) of *A. annua* L. was extracted with MeOH. The amount of MeOH used for extraction was not indicated, nor was the length of time the material extracted with MeOH. The solution of MeOH and organic compounds was concentrated, and 970 g of extract were obtained. The residue was partitioned between water and the series of solvents, n-hexane,

$CHCl_3$, EtOAc, and lastly, n-BuOH. The residues obtained from each extraction were 172 g, 224 g, 21.2 g, and 288 g, respectively.

The hexane extract was subjected to column chromatography (CC) on polyclar AT; the size of the column was not denoted. Elution was with $CHCl_3$, and gradually introduced MeOH to 100 %. CRY and CAS were collected from this column. However, Shilin *et al*. (1989) did not indicate the fractions from the column that CAS and CRY were collected from nor the amount of volume collected in each fraction. Likewise, the column dimensions were not provided. These flavonoids were further purified using prep TLC on silica gel in $CHCL_3$-MeOH (9:1).

CRYD, CRY, and CRS were eluted from the $CHCl_3$ extract using the same method applied to the hexane extract. The amount of CRYD, CRY, and CRS collected from the fractions was 12 mg, 35 mg, and 2 mg, respectively.

2.8.7.8 Saleh et al. (1987)

The procedure for extraction and isolation detailed by Saleh *et al*. (1987) was especially ambiguous. Neither the elution solvents used nor the resulting amount of flavonoids collected was reported. Based on other previously established works, CRS is detected in *A. herba-alba*, *A. mesatlantica*, *A. monosperma*, and *A. capillaris*. Plant material from *A.herba-alba* was extracted with 70% EtOH and the extracts were concentrated and eluted through a polyamide column. The flavonoid glycoside fractions were further purified on Sephadex LH-20, and the aglycones fractions were subjected to prep TLC on polyamide plates, then separated on Sephadex LH-20. A TLC method was included that claimed that on polyamide TLC plates, in a mobile phase of benzene-petrol (60-80) methyl ethyl ketone (MEK)-MeOH (60:60:7:7), CAS ran at an R_f of 0.45, and CRS at 0.26. Another TLC method maintained that CAS had an R_f of 0.69, and CRS had an

R_f of 0.60 when run on a silica gel TLC plate in the solvent system benzene-pyridine-formic acid (36:9:5). These TLC methods were considered potentially useful to the project in determining the presence of CRS in *A. annua*.

2.8.7.9 Greenham et al. (2003)

Greenham *et al*. (2003) investigated the possible value of HPLC in combination with TLC and UV spectral analysis as a means of identifying the lipophilic flavones in their laboratory. An HPLC with diode array detection, using a Waters Bondapak phenyl C_{18} reverse-phase column (300 x 3.9 mm i.d.) at 25°C was used for analysis. The R_f values of CRS in three different solvent systems on two stationary phases were reported:

1) Silica gel layer eluted with toluene: acetic acid (4:1). R_f: 0.37

2) Microcrystalline cellulose layer eluted with acetic acid: water (3:10). R_f: 0.25

3) Microcrystalline cellulose layer eluted with acetic acid: water (1:1). R_f: 0.63

When using UV-light (365 nm) to detect CRS, it is visible as a dark spot on a UV-irradiated TLC plate (Greenham *et al*., 2003).

2.8.8 Previous NMR Analyses of Flavonoids

The chemical shift values of CRY, CRYD and CRS in certain NMR solvents and conditions have been documented. These NMR values may be useful for future reference to compare the purity of the fractions collected by column chromatography to that of the flavonoid standards, as well as the purity of the flavonoid standards that were obtained from outside sources. Calcagno-Pissarelli *et al*. (2010) reported chemical shift values for the ^{1}H and ^{13}C NMR spectra of CRY obtained, using $CDCl_3$ and DMSO-d_6 as the NMR solvent. Kraus and Roy

(2008) reported the ^{1}H NMR chemical shift values of CRYD, using $CDCl_3$ as the solvent. Ono *et al*. (2000) also reported the ^{1}H and ^{13}C NMR chemical shift values for CRYD, using DMSO-d_6 as the solvent. Hammoud *et al*. (2012) reported the chemical shift values of CRS, from the ^{1}H and ^{13}C NMR spectra obtained, using MeOH-d_4 as the solvent.

3.0 Hypothesis

If CRYD, CRY, and CRS, can be successfully and efficiently isolated with their identities confirmed, then further research can be undertaken to track these flavonoids in *A. annua* extracts to determine how these flavonoids are produced in tandem with AN.

4.0 Objectives

Because pure standards were obtained in early 2013 for CRY and CRYD, the original objectives for this project aimed at reaching the desired result of collecting separate, pure samples of CRY, CRS, and CRYD from *A. annua* were modified as follows:

1. To test various solvent systems and determine which best extracts flavonoids, while extracting the least amount of chlorophyll

2. To determine how many successive extractions are sufficient to remove greater than or equal to 90 percent of flavonoids from plant samples, using the least volume of solvent

3. To determine what solvent systems are best suited to extract each flavonoid separately using TLC

4. To validate the presence of CRY in the SAM cultivar of *A. annua*

5. To compare the TLC profiles of compounds extracted from fresh SAM shoot tip and fresh SAM main stem leaves by visualizing the TLC plates with UV-light and 2 % $AlCl_3$ in MeOH for flavonoids, and with an anisaldehyde solution for AN and terpenoids

5.0 Methods

The plant material used in this project was that of finely ground, dried leaves of *A. annua* L., clone SAM or FLV5; the former is a WPI isolated clone and the latter is from a seed propagated cultivar from Brazil. Fresh shoot tips and leaves of SAM were also harvested and extracted. The standards CRY (5 mg) and CRYD (5 mg) were obtained at great expense and after a long wait, from a source in China, while a source for CRS has only recently been identified and purchase is pending. All TLC plates were photographically recorded using a 3.2 megapixel camera.

5.1 Preliminary Extraction and TLC Experiments

The chromatography methods suitable for profiling CAS, Q, K, EUP, M, CRYD, CRS, CRY, ART, AN, and AT as reported by experiments in this report can be found in Table III. The standards were resuspended in one of several suitable solvent systems as indicated in Table III, spotting from 1-5 µg of the stocks on silica gel 60 F_{254} TLC plates (EMD Chemicals Inc., Catalog No. M57357), polyamide (Sorbent Technologies, Catalog No. 3522126), or on cellulose 300 TLC plates (Sorbent Technologies, Catalog No. 0322126) depending on the experiment. All dried TLC plates were viewed under UV-light (366 nm). The standards were run in various solvent systems because CRY and CRD were similar in structure to M, while CRS was similar to that of EUP. Therefore, if sufficient separation between the standards and movement along the TLC plate could be achieved, then the conjecture was made that the three flavonoids of interest to this project would run in a similar manner, and could be separated using the designated solvent system. Also, with the addition of CRY and CRYD to the collection of standards, a small amount of both would be tested using the most efficient TLC solvent system for separating the flavonoids of SAM as empirically determined.

Table III: Chromatography methods suitable for profiling casticin, kaempferol, eupatorin, myricetin, chrysosplenol D, cirsilineol, chrysoplenetin, and artemetin in *Artemisia annua* as documented in this report.

Compound	Sample suspension solvent	Stationary phase	Mobile phase	R_f	Color under UV 366 nm
ART	1:1, $CHCl_3$-MeOH	TLC Cellulose 300	3:1:1, t-BuOH-H_2O-HOAc	0.93	Dark
CAS	1:1, $CHCl_3$-MeOH		15 % HOAc	0.17	
K	1:1, $CHCl_3$-MeOH	TLC Si gel GF_{254}	2:1, Toluene-Ethyl Acetate	0.20	Yellow
EUP				0.16	
ART				0.31	
M				0.03	
M			1:2, Toluene-EtOAc	0.08	
M			Acetonitrile	0.30	
EUP				0.67	
CAS			4:1, Toluene- EtOAc	0.14	
K				0.08	Yellow
EUP				0.08	Dark
M				0	
ART				0.18	
CAS			1:2, Toluene-ACN	0.70	
CAS			36:9:5, benzene-pyridine-formic acid	0.61	
CRY	$CHCl_3$			0.61	
CRYD	Acetone			0.33	
AN	MeOH			0.98	Not visible
AT				*	
CAS	1:1, $CHCl_3$-MeOH	TLC Polyamide	60:60:7:7, benzene-petrol (35-60) methyl ethyl ketone-methanol	0.44	Dark
Q	EtOH			Origin	Yellow

*AT was never visible under any TLC condition per this study.

5.1.1 CH_2Cl_2 as the Extraction Solvent

To observe which solvent system extracted flavonoids best with the corresponding least amount of chlorophyll, *A. annua* cultivar FLV5 (0.5 g DW) was extracted with 10 mL MeOH (test tube E-1) in a sonicating water bath at room temperature for 30 minutes. The solution was filtered from the plant material using a vacuum filtration apparatus, then dried under N_2 using an N-Evap. The remaining solids were re-extracted two more times, so that a comparison could be made between the three extracts. FLV5 (0.5 g DW) was also thrice extracted, using 10 mL CH_2Cl_2 (x3) to compare which solvent was more efficient in extracting flavonoids from the dried

plant material. To observe the results of three successive extractions and the effectiveness of MeOH versus CH_2Cl_2, each of the three dried extracts was resuspended in 200 µL of extraction solvent, 10 µL of which was spotted on a silica gel TLC plate, using 2:1, toluene-EtOAc as the mobile phase. The extraction was repeated using less biomass to determine if extracting 0.1 g DW of FLV5 in CH_2Cl_2 (x3) would be sufficient, so that the maximum amount of flavonoids could be extracted using the least amount of solvent.

Three successive extractions of 0.1 g DW SAM in CH_2Cl_2 were performed as described above to detect the compounds present in that cultivar that differed from or coincided with the spots observed from the three FLV5 extractions in CH_2Cl_2 and determine if a third extraction was necessary. The residue collected in the three test tubes was resuspended in 200 µL of CH_2Cl_2, 20 µL of which was spotted on a silica gel TLC plate, using 2:1, toluene-EtOAc as the eluent.

5.1.2 Extraction of Dried SAM Whole Plant Material (February 2010 Lab Harvest) using Hexanes, Et_2O, and EtOAc

Extraction of 0.1 g dried SAM clone (February 2010 Harvest) with CH_2Cl_2 (10 mL) following the separate extraction using hexanes (10 mL), Et_2O (10 mL), and EtOAc (10 mL), in comparison to extraction using solely CH_2Cl_2 (10 mL) was analyzed using TLC, to determine if hexanes, Et_2O, and EtOAc were selective for extracting specific compounds. The second extraction with CH_2Cl_2 indicated what compounds remained after the first extraction with one of the three solvents, as well as how well the starting solvent extracted the compounds in SAM. After sonicating the solution of 0.1 g DW of SAM in each of the four solvents in separate glass test tubes for 30 minutes, the solutions were filtered from the plant material using a vacuum filtration apparatus, then dried under N_2. The plant material remaining was transferred to four new test tubes, and the procedure was repeated using CH_2Cl_2 (10 mL) for the plant material that

was first extracted with hexanes, Et_2O, and EtOAc. The plant residue collected from the extractions, which involved the seven different combinations of solvents, was analyzed by TLC. The plant residue collected in each of the seven test tubes was resuspended in 100 µL of the following solvents, 10 µL of which was spotted on a silica gel TLC plate (20 cm x 8.5 cm), and run in the mobile phase 2:1, toluene-EtOAc:

Table IV: Two extraction protocols for extracting flavonoids.

Test tube (1st Extraction solvent)	Resuspension Solvent (100 µL)
Hexanes	Hexanes
EtOAc	EtOAc
Et_2O	Et_2O
CH_2Cl_2	CH_2Cl_2

Test tube (2nd Extraction solvent)	Resuspension Solvent (100 µL)
Hexanes, then CH_2Cl_2	CH_2Cl_2
EtOAc, then CH_2Cl_2	CH_2Cl_2
Et_2O, then CH_2Cl_2	CH_2Cl_2

5.1.3 Extraction of Fresh SAM Shoot Tips

Subsequently, fresh SAM shoot tips collected from field-grown plants (2012) were extracted to compare and contrast the different compounds extracted from fresh and dried extracts. Fresh tips of SAM, approximately 1.5 cm down, were pinched off and placed in a jar filled with enough CH_2Cl_2 so that the tips were covered (Fig. 8). The solution of SAM plant tips in 10 mL CH_2Cl_2 was sonicated, covered, for 30 minutes, then filtered from the tips using vacuum filtration.

Fig. 8. Example of fresh SAM shoot tip extracted.

The solution was dried to concentration under N_2 using an N-Evap. This process was repeated twice more. The approximately 2.9 g of dark green and brown-colored, tar-textured residue collected was pooled and stored in the freezer.

5.1.4 TLC and GC/MS Analysis of SAM Tip vs. CAS, K, and ART in 2:1, Toluene-EtOAc

The dried residue was resuspended in 2 mL CH_2Cl_2, 1 µL of which (1.4 mg) was spotted on a silica gel plate, using 2:1, toluene-EtOAc as the mobile phase. The standards ART (5 µg), K (5 µg), and CAS (5 µg) were separately spotted along with SAM, in the order: CAS, SAM, K, ART. Spots 1-5 were recovered from the TLC plate into separate centrifuge tubes, using 2:1, toluene-EtOAc (3 mL) as the solvent, sonicating the solution containing the recovered spots for 30 minutes, then centrifuging the solution for 5 minutes. The solvent was filtered from the silica gel powder, dried under N_2, and later transferred to GC/MS sample vials. The samples were re-suspended in 100 µL pentane and submitted to GC/MS. GC/MS analysis of any eluted TLC

spots or any plant extracts was done according to Weathers and Towler (2012) and spectra of spots 1-5 are shown in the Results.

5.2 Detection of CRYD in SAM Tip through TLC Analysis

The detection and separation of CRYD from other compounds in fresh SAM shoot tip was pursued using the methodology of Roberts *et al.* (1980), which allowed the use of ART standard as a reference point. CAS was also used as a standard flavonoid and solvent systems other than that reported by Roberts *et al.* (1980) were experimented used.

5.2.1 TLC of SAM Tip to Compare R_f of CAS, ART, and Q in Solvent Systems Reported by Roberts *et al.* (1980)

The procedure reported by Roberts *et al.* (1980) was followed, but cellulose 300 TLC plates were used instead of Whatman 3MM paper. To determine the amount of SAM tip to spot on the TLC plate that would result in resolved spots, 2.87 g residue of SAM tip extracts were resuspended in 2.5 mL CH_2Cl_2, spotting1 µL (1.15 mg) residue, 2 µL (2.3 mg), and 3 µL (3.4 mg) of the solution, along with two spots of 5 µg ART on a cellulose 300 TLC plate (11 cm x 11 cm). The plate was developed in the solvent system 3:1:1,t-BuOH-HOAc-H_2O (Roberts *et al*., 1980).

A 2-D TLC experiment was run in similar conditions to those described by Roberts *et al*. (1980). For the first dimension, 2.5 µg ART and approximately 230 µg SAM tip extract residue were spotted on the same position of a cellulose 300 TLC plate (20 cm x 20 cm) and run in 3:1:1,t-BuOH-HOAc-H_2O. After the plate had developed and dried, the plate was rotated 90 degrees clockwise to develop the second dimension. To provide an ART standard for the second dimension, ART (2 µg) was spotted above the solvent front, after running the first dimension. The plate was placed into the TLC developing chamber in the second solvent system, 15 % HOAc. The R_f of ART (2 µg) on an 11cm x 3 cm cellulose TLC plate developed in 15 % HOAc

was also measured. In another run also using 15 % HOAc, the standards Q (3 µg) and CAS (3 µg) were spotted in the same position on either side of the line of SAM tip extract (5,750 µg) on a cellulose TLC plate (20 cm x 20 cm) in the mobile phase 15 % HOAc.

5.2.2 TLC of SAM Tip to Compare to R_f of CAS, ART, and Q in TLC Conditions other than Those Reported by Roberts *et al.* (1980)

ART (2.5 µg) and CAS (3 µg) were spotted on a cellulose 300 TLC plate (12.5 cm x 3 cm) and developed in the mobile phase 1:2, toluene-ACN. The standards CAS (3 µg) and ART (3 µg) were spotted on a cellulose TLC plate (15.5 cm x 3.5 cm) and the plate was developed in the solvent system 3:1:1:5, t-BuOH-HOAc-H_2O-Et_2O.

The standards CAS (1.5 µg), Q (1.5 µg), and SAM tip extract (approx. 77 µg) were developed on three different stationary phases (silica gel, cellulose, and polyamide) in three different mobile phases (2:1, toluene-EtOAc, 3:1:1, t-BuOH-HOAc-H_2O, and 1:4, ACN-CH_2Cl_2). The residue of fresh SAM tip extract (approx. 77 µg) and 1 µg Q were applied to a silica gel TLC plate (9 cm x 3 cm). The TLC plate was developed in the mobile phase 1:1:3, ACN-HOAc-H_2O.

5.3 Detection of CRS in SAM Tip through TLC Analysis

The TLC conditions of conditions reported by Voirin *et al*. (1999), Grayer *et al*. (1996), and Saleh *et al*. (1987) were repeated in the experiments documented here.

5.3.1 TLC of SAM Tip Extract to Compare to R_f of CAS and Q using the TLC Conditions Reported by Voirin *et al.* (1999) and Grayer *et al.* (1996)

To observe how CAS and Q would move in comparison to compounds from SAM tip extract, using the TLC conditions reported by Voirin *et al*. (1999), CAS (1 µg), Q (1 µg), and SAM tip extract (77 µg) were spotted on a cellulose TLC plate (9 cm x 3 cm). The plate was developed in the mobile phase 4:6, HOAc-H_2O. The TLC conditions reported by Grayer *et al*.

(1996) were also followed. The standards CAS (1 µg) and Q (1 µg) were developed along with SAM tip extract (77 µg) in the mobile phase 5 % MeOH in $CHCl_3$ on a silica gel TLC plate (9 cm x 3 cm).

5.3.2 TLC of SAM Tip Extract to Compare to R_f of CAS, Q, ART and AN using the TLC Conditions Reported by Saleh *et al*. (1987) and Various TLC Visualization Reagents

CAS (1 µg), and Q (1 µg) were used as an added reference, and SAM tip extract (77 µg) were either applied onto a polyamide TLC plate and developed in the solvent system (12:5:3) toluene-MEK-MeOH or (60:60:7:7) benzene-petroleum ether (b.pt. 35-60°C)-MEK-MeOH, or the compounds were applied onto a silica gel TLC plate and developed in the mobile phase (36:9:5) benzene-pyridine-formic acid. Q (1 µg), ART (1 µg), and AN (1-2 µg) were also spotted with Q and SAM tip to determine their R_f values in the solvent system (36:9:5) benzene-pyridine-formic acid on silica gel TLC plates to determine their R_f values under those TLC conditions. Plates were sprayed with either 2 % $AlCl_3$ in MeOH to visualize additional flavonoids beyond those observed under UV-light, after allowing the plate to dry, or with the *p*-anisaldehyde solution (50:1:0.5, glacial acetic acid-sulfuric acid-*p*-anisaldehyde) to visualize AN. The protocols for preparing the *p*-anisaldehyde and $AlCl_3$ solutions can be found in Appendix III. After spraying the plate with the *p*-anisaldehyde solution, the TLC plate had to be heated at 105°C for approximately 10 minutes in order for the stained spots to appear (Pras *et al*., 1991).

5.4 Development of Acquired Standards CRY and CRYD on Silica Gel in (36:9:5) Benzene-Pyridine-Formic Acid (Saleh *et al*., 1987)

Fresh SAM tip extract (77 µg), CAS (1 µg), CRY (1 µg), and CRYD (1 µg) were applied to a silica gel TLC plate (14.5 cm x 3 cm). The TLC plate was developed in the solvent system 36:9:5, benzene-pyridine-formic acid.

5.5 TLC Analysis and Visualization of AN and AT, as Compared to SAM Tip Extract

Using the method of Saleh *et al*. (1987), 1 µg AN, 1 µg CAS, 2-10 µg AT, and 154-385 µg SAM tip extract were applied to silica gel TLC plates and run in the solvent system (36:9:5), benzene-pyridine-formic acid (Saleh *et al*.,1987). The plates were then sprayed with the *p*-anisaldehyde solution.

5.6 TLC Analysis and Visualization of Fresh SAM Leaves Extract vs. Fresh SAM Tip Extract

The aim here was to make a qualitative comparison between the compounds (flavonoids and terpenoids) in fresh SAM shoot tip and fresh mature SAM leaves. First, leaves from the main stem of SAM were sonicated in about 50 mL CH_2Cl_2 for 30 minutes, then filtered from solution using a vacuum filtration apparatus. The residue was dried under N_2. A tar-like, dark green residue (approx. 0.22 g), similar to the residue obtained from fresh SAM shoot tip, was obtained after drying under N_2. The SAM leaf residue was resuspended in 948 µL CH_2Cl_2. A 5 µL aliquot was transferred to a smaller, clean test tube, and 10 µL CH_2Cl_2 was added to that tube. Then, 2 µL (154 µg) of that 15 µL (1.15 mg) total solution SAM leaf extract and 154 µg SAM tip extract were spotted on two silica gel TLC plates (9.5 cm x 10 cm). In addition, 2 µg AN was spotted on one TLC plate, and 1 µg CAS on the other. The compounds were developed in the mobile phase 36:9:5, benzene-pyridine-formic acid (Saleh *et al*.,1987). The compounds were applied on the TLC plates from left to right in the same sequence to obviate extract effects on mobility. After the TLC plates dried, both were viewed and photographed under UV-light, then the plate containing CAS was sprayed with the $AlCl_3$ solution and visualized under UV-light, while the other containing AN was sprayed with the *p*-anisaldehyde solution.

5.7 NMR Analyses of Crude SAM Shoot Tip Extract, CRY, CRYD, and CAS

The standards CAS (unknown amount, ~ 5.62 mg), CRY (~0.8 mg), CRYD (~0.76 mg), and the SAM tip plant extract (~51.9 mg) were dissolved in 600 µL $CDCl_3$ at room temperature. The ^{1}H and ^{13}C NMR spectra were collected at 300 K on a Bruker Avance AvIII spectrometer, operating at 11.75T, using a 5 mm Brucker PA BBO 500S2 BB-H-D-05 Z probe with z-gradients.

6.0 Results

6.1 Crude Extraction of Flavonoids from Plant Material

The first objective of this project was to determine what solvent system best extracted the greatest amount of flavonoids from *Artemisia annua* with minimum chlorophyll content and waste of solvent. A TLC analysis, using 2:1, toluene-EtOAc and a silica gel TLC plate, of 0.1 g DW of FLV5 in 10 mL CH_2Cl_2 (x3) showed that only a small amount of biomass (0.1 mg/10 µL CH_2Cl_2) was needed to observe the diversity of compounds that clearly separated out of the extract, and with the least amount of chlorophyll. As shown in Fig. 9, the compounds in the 0.5 g DW FLV5 extract, which used a single volume of 30 mL CH_2Cl_2 (C3), contained more chlorophyll than the 0.1 g DW FLV5 extracted with only 10 mL CH_2Cl_2 (L1). Extraction with other solvents, such as hexanes, Et_2O, and EtOAc, did not prove as effective as CH_2Cl_2, which extracted the most compounds from dried whole plant SAM (See Fig. 10). Extraction with hexanes did not yield many flavonoids, and the white fluorescing spot was visible in all but the hexanes extraction. The EtOAc extraction resulted in an overloaded TLC plate, therefore, no spots could be easily defined, only a streak was observed. The ether extraction seemed specific for two spots at R_f 0.26 and 0.39. Ether did not extract all of what comprised the contents of these two spots from the plant material, because the two spots were still evident after the plant material was extracted with the second solvent, CH_2Cl_2. The solvent, CH_2Cl_2, extracted flavonoids the most efficiently, while ether and EtOAc were the second best solvents for extraction (Fig. 10).

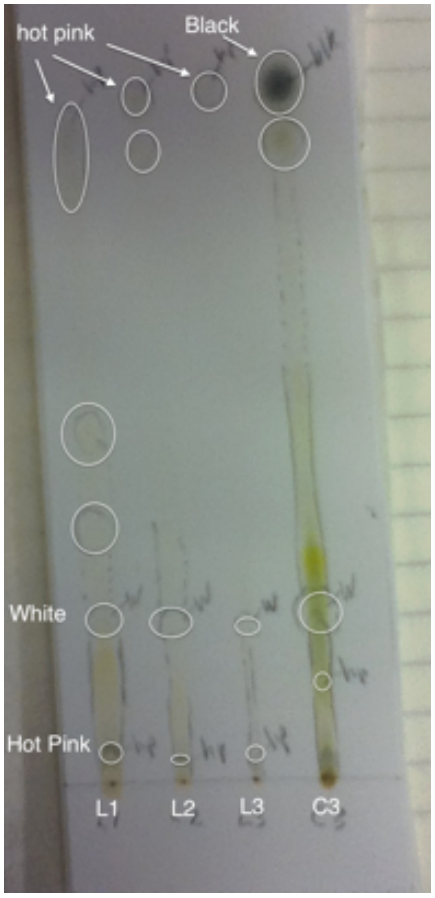

Fig. 9. Comparison of three successive CH_2Cl_2 (10-30 mL) extractions of 0.1 g FLV5 (L1-L3) and a third successive extraction (30 mL) of 0.5 g FLV5.
0.1 g DW of FLV5 was extracted in 10 mL CH_2Cl_2 (x3). The residue of the three successive extractions was collected in separate test tubes, which were resuspended in 200 µL CH_2Cl_2, 20 µL of which was applied to a silica gel TLC plate (17.2 cm x 4.9 cm). The third successive extraction of 0.5 g DW FLV5 was also resuspended in 200 µL CH_2Cl_2, 20 µL of which was applied to the same plate. The mobile phase was 2:1, toluene-EtOAc.

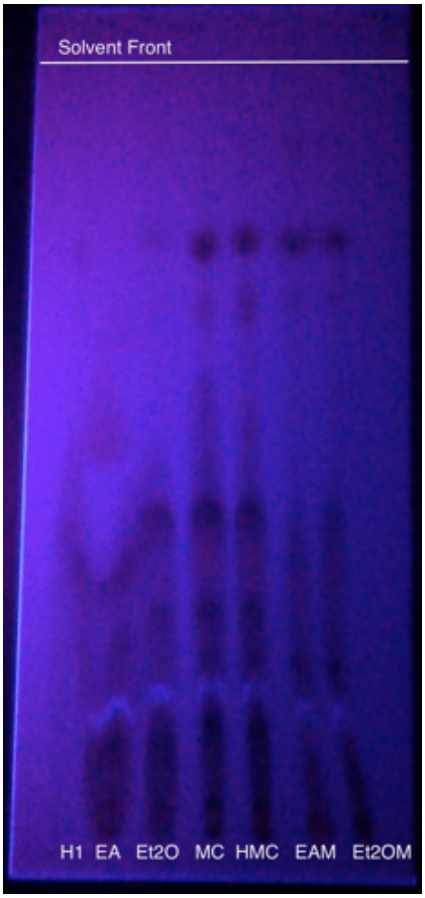

Fig. 10. Comparison of successive extraction of SAM tip with hexanes, Et_2O, and EtOAc, followed by CH_2Cl_2.
SAM tip extracts were spotted on a silica gel TLC plate (17.2 cm x 3.5 cm) and with mobile phase 2:1, toluene-EtOAc. The compounds were spotted on the plate in the order: H1, EA, Et2O, MC, HMC, EAM, and Et2OM. H1, EA, Et2O, and MC refer to the first extraction in hexanes, EtOAc, and Et_2O, and CH_2Cl_2, respectively. HMC, EAM, and Et2OM, refer to the second sequential extraction of the plant material in CH_2Cl_2. (Refer to Section 5.1.2 for details.) The plate was visualized under UV-light, 366 nm.

6.2 Identification of Spots 1-5 from SAM tip Extract using GC/MS

An experiment was performed with the objective of determining the R_f values of the standards in the solvent system, 2:1, toluene-EtOAc, and to record the R_f of the spots that ran in the SAM extract (Fig. 11A and B). As seen in Fig. 11A, spot 2 from the SAM tip extract ran close to, but just below the CAS standard, while spot 3 ran just above the ART standard. Therefore, all spots were recovered from the TLC plate (Fig. 11A) and analyzed by GC/MS to determine whether any of them contained traces of CAS or ART (Fig. 12-16). The GC/MS spectrum of spot 1 showed the presence of casticin, artemetin, and scopoletin (Fig. 12). Spot 2 seemed to be mainly casticin, but its area and therefore amount was less than spot 1 (Fig. 13); scopoletin and artemetin were not present.

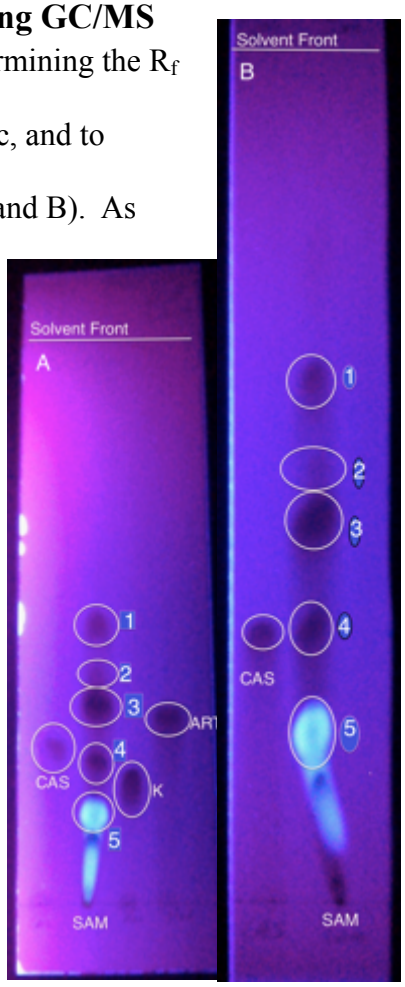

Fig. 11A & B. TLC of SAM tip extract vs. CAS, K, and ART. In Fig. 11A, a 5 µg aliquot of the standards, CAS, K, and ART, and 1.44 mg SAM tip extract were spotted on a silica gel TLC plate (17.2 cm x 4.5 cm) with mobile phase 2:1, toluene-EtOAc. Left to right order: CAS, SAM, K, ART. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: 0.26; K: 0.20; ART: 0.31; SAM: 1, 0.48; 2, 0.39; 3, 0.34; 4, 0.24; 5, 0.15. In Fig. 11B, a 2 µg aliquot of CAS and 1.1 mg SAM tip extract were spotted on a silica gel TLC plate (17.2 cm x 2.6 cm) and with mobile phase 2:1, toluene-EtOAc. Spot order: CAS and SAM. The plate was visualized under UV-light, 366 nm. R_f values for the observed spots observed were: CAS: 0.30; Spot: 1, 0.6; 2, 0.5; 3, 0.44; 4, 0.31; 5, 0.19.

Perhaps casticin and artemetin did not separate well. Spot 2 also had the smallest overall peak heights compared to spots 1, 3, 4, and 5. Spot 3 of the SAM tip extract was observed as a larger spot than ART; therefore GC/MS analysis was performed to determine if more than one compound was present in spot 3 (Fig. 14). Q was not detected in the GC/MS spectra. Other flavonoids of interest could not be identified in the spots, as standards for them had not yet been obtained. Octadecanoic

acid was also detected in spots 1-5. Hexadecanoic acid was detected in spots 2-4. Benzoic acid was present in spot 2. The terpene DHAA was present in spots 3-5. AA and AB were found in spots 4 and 5. Spot 5 also contained AN and deoxyartemisinin. Table V indicates the approximate peak area and concentration of artemetin and casticin in the GC/MS spectra. From GC/MS analyses reported by Weathers and Towler (2012), casticin and artemetin were known to be present in SAM. Therefore, CAS and ART were used to help find the unknown 3 flavonoids, CRY, CRS, and CRYD. AN was also used as a standard in TLC experiments because it was also present in SAM (Weathers and Towler, 2012).

Table V: GCMS determined peak areas and estimates of concentration of artemetin and casticin in spots 1 and 2 (Fig. 12 and 13).

Flavonoid	Spot #	Approx. peak area	Approx. concentration (µg)
ART	1	1.4×10^6	0.56
	2	No peak	0
CAS	1	8.5×10^5	0.25
	2	5.2×10^6	1.49

6.3 TLC Conditions for Separation of *Artemisia annua* Compounds in Attempt to Quantify CRYD and CRS

Another objective of this project was to achieve the best separation between the many compounds in the plant extracts. This solvent system could then potentially be used as the solvent system for separating flavonoids on a larger scale in column chromatography. The separation of compounds in the SAM tip extracts was examined using various solvent systems and stationary phases to determine which combinations gave the best separation with the most definitive spots. In addition, if the any of the spots in SAM tip extract gave the same R_f value for the flavonoids of interest as those reported in the literature, then it would suggest their presence in the extract.

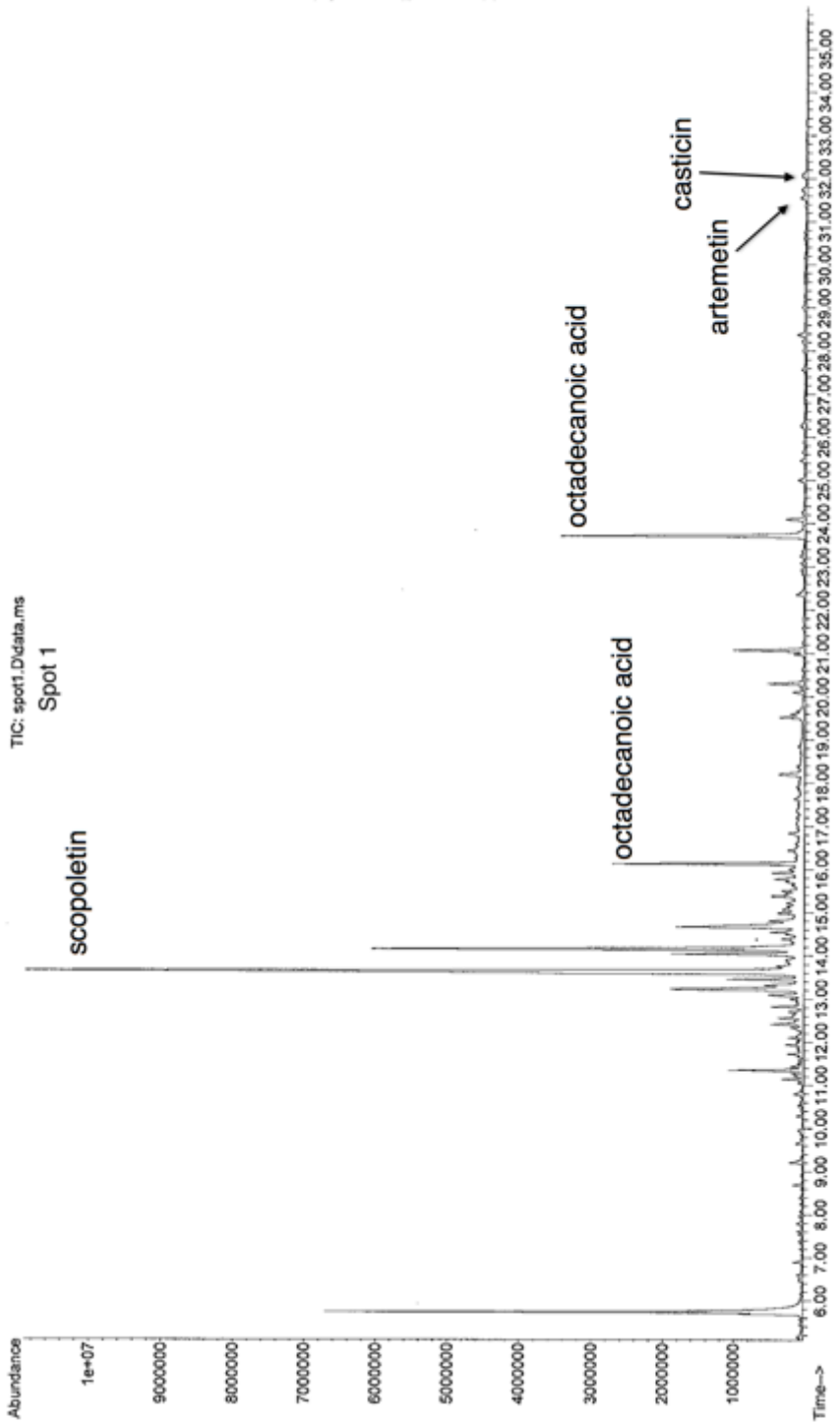

Fig. 12. Spot 1 GC/MS spectrum

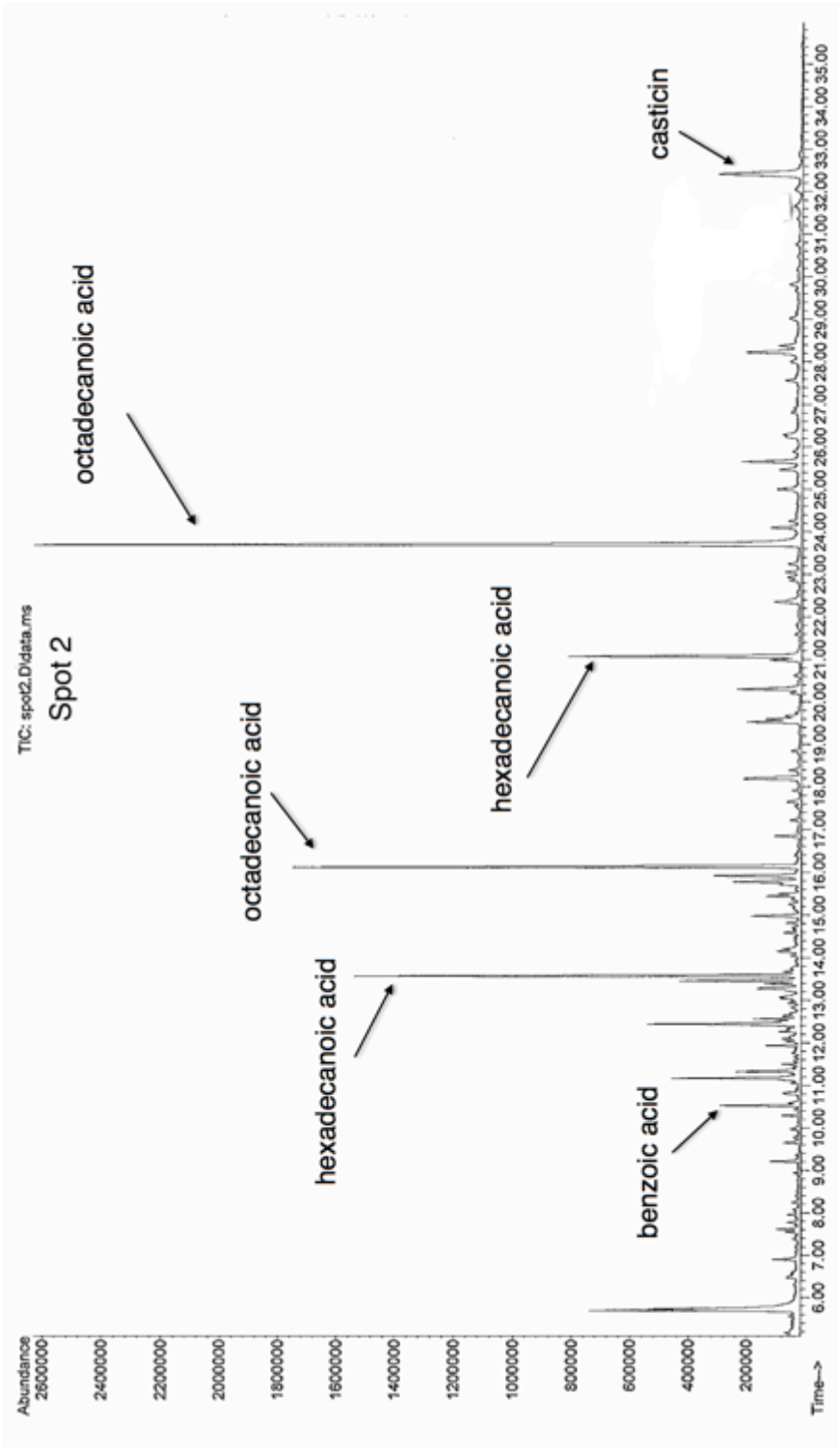

Fig. 13. Spot 2 GC/MS spectrum

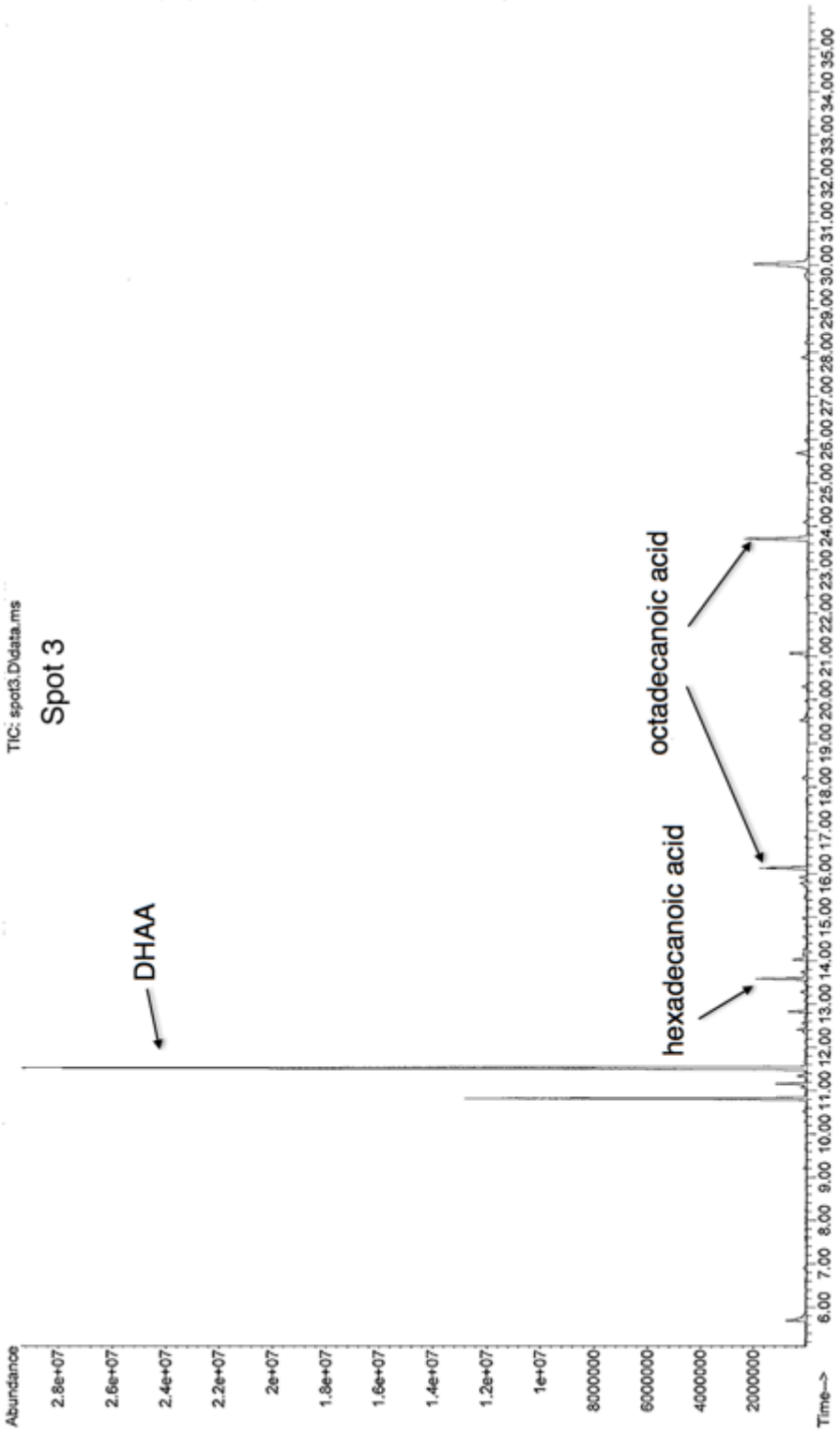

Fig. 14. Spot 3 GC/MS spectrum

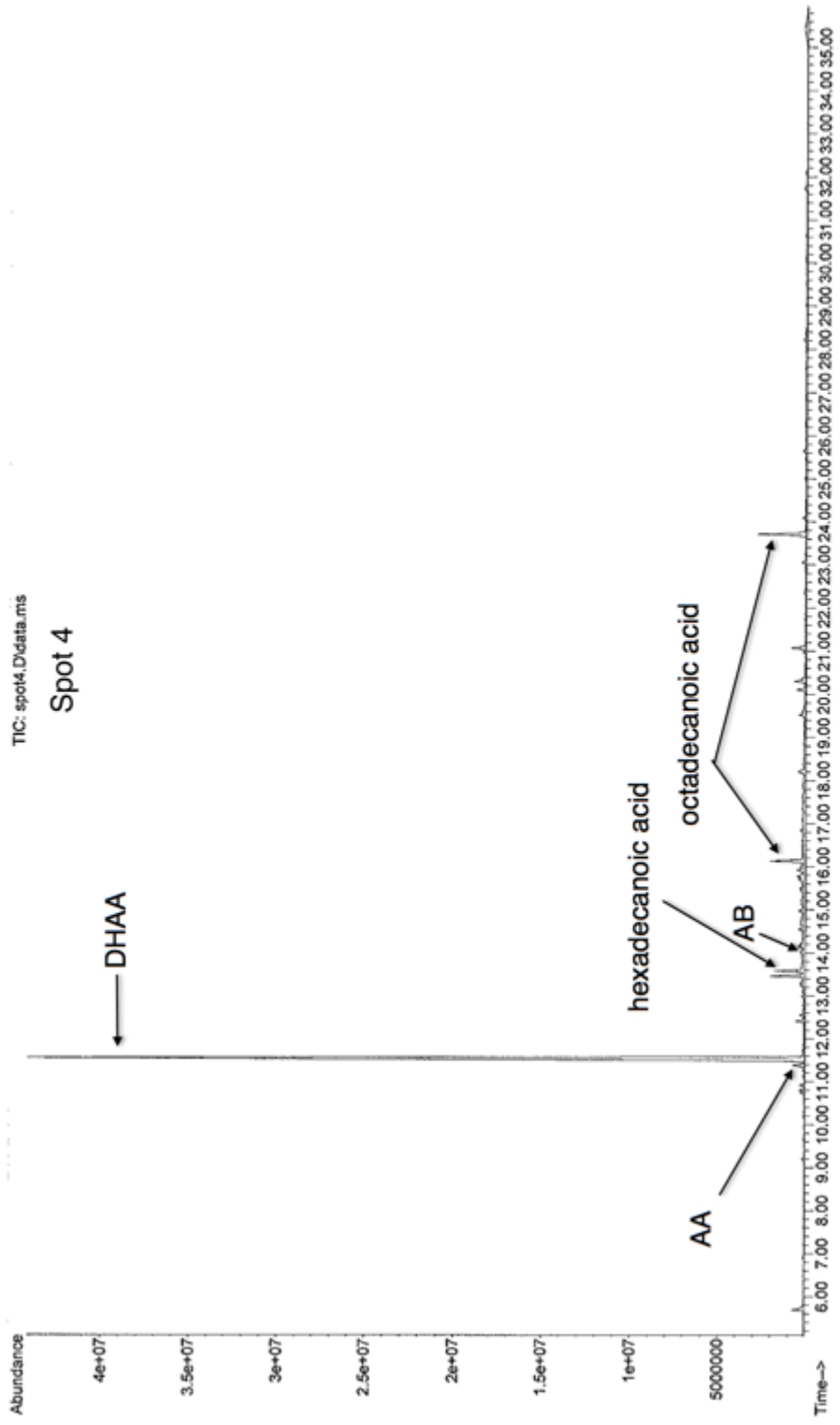

Fig. 15. Spot 4 GC/MS spectrum

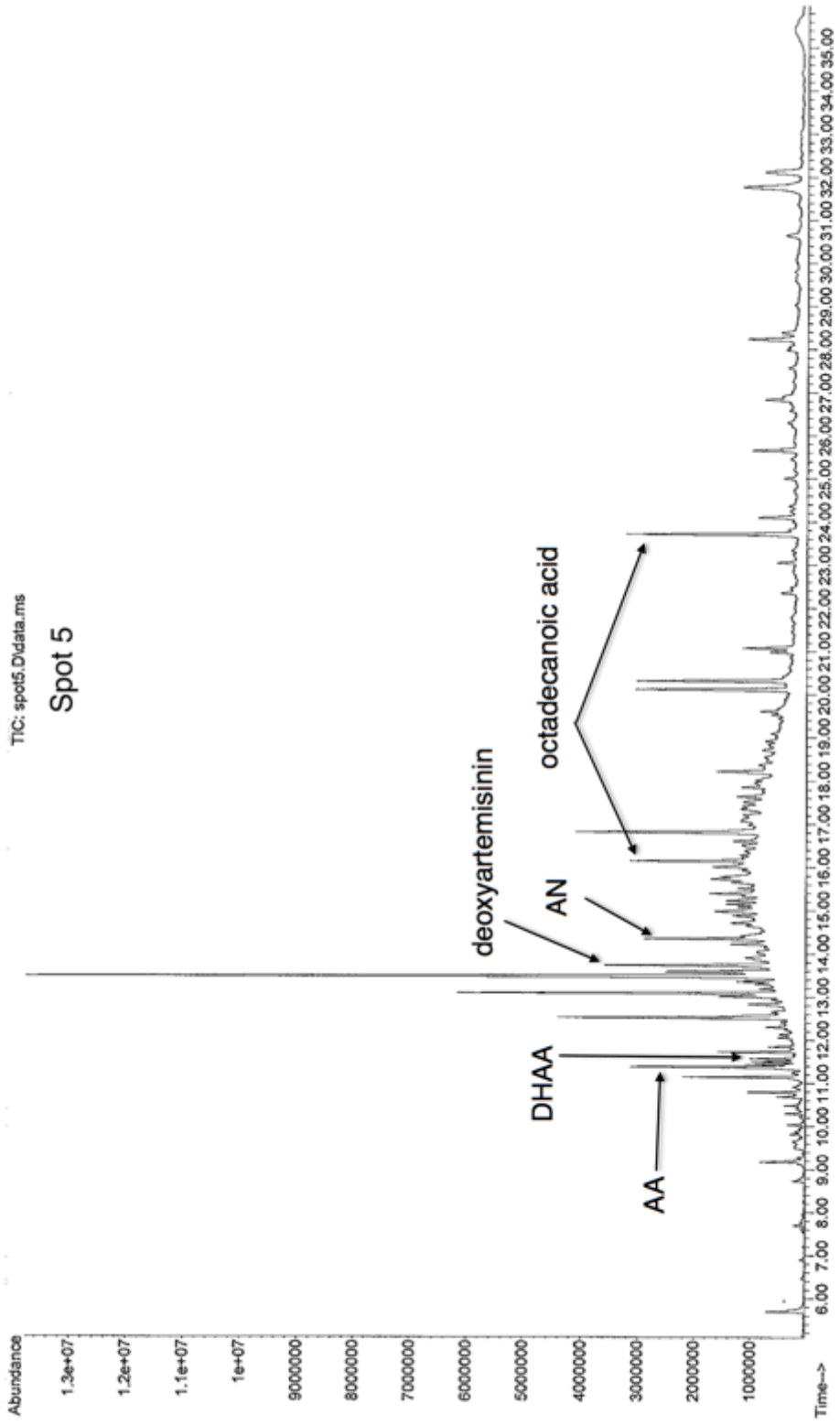

Fig. 16. Spot 5 GC/MS spectrum

First, the amount of SAM tip extract to spot on the TLC plate that would result in resolved spots was determined by following the procedure reported by Roberts *et al.* (1980). Three different amounts of SAM tip extract (1.15 mg, 2.3 mg, and 3.4 mg) were spotted on a cellulose TLC plate, along with 5 µg ART and developed in 3:1:1,t-BuOH-HOAc-H_2O. The resulting TLC plate showed that 2.3 mg gave sufficient resolution of the compounds of the SAM tip extract (Fig.17).

Fig. 17. Comparison of 1 µL (1.15 mg residue), 2 µL (2.3 g), and 3 µL (3.4 g) SAM tip extract and 5 µg ART.
Compounds were spotted on a cellulose 300 TLC plate (11 cm x 11 cm) and with mobile phase 3:1:1, t-BuOH-HOAc-H2O. Spot order: ART, 1 µL SAM, 2 µL SAM, 3 µL SAM, ART. The plate was visualized under UV-light, 366 nm. The R_f value of ART was 0.93.

A 2-D TLC experiment described by Roberts *et al*. (1980) was repeated to determine if ART and a spot from the SAM tip extract, possibly CRYD, would run at an R_f of 0.82 in the first solvent system 3:1:1, t-BuOH-HOAc-H_2O, then afterwards, at R_fs of 0.16 and 0.18, respectively in the second solvent system 15 % HOAc. ART was developed alongside the SAM tip extract in the second dimension (Fig. 18). For the first dimension, a white fluorescing spot with a dark center was observed at an R_f of 0.88. After developing the second dimension, two dark spots of interest a and b had R_f values of 0.06 and 0.14, respectively. Spots b was of interest because it was close to the R_f value of the ART standard of 0.17 (Fig. 18). The presence of CRYD could not be confirmed from this TLC experiment.

Another TLC experiment was performed using only 15 % HOAc as the mobile phase. CAS (3 µg) and 2.5 µg ART, along with approximately 76 µg SAM tip extract were spotted on a 17 cm x 4 cm cellulose TLC plate to determine if allowing the plate to develop in the one solvent system would be sufficient to separate the two dark compounds ('a' and 'b') of SAM, which ran closer to the spot of ART in a previous experiment, to yield smaller, clearer spots (Fig. 19). The resolution of spots a and b did not improve after this, as their R_f values of 0.05 and 0.13, respectively, were not significantly different from their previous R_f values of 0.06 and 0.14. These spots ran below CAS and ART, both of which had an R_f value of 0.18. A prep TLC of SAM extract, CAS, and Q was run in 15 % HOAc on a full sheet of cellulose (20 cm x 20 cm) (See Fig. 20 for details). However, much streaking was observed and confirmations of their identities could not be made.

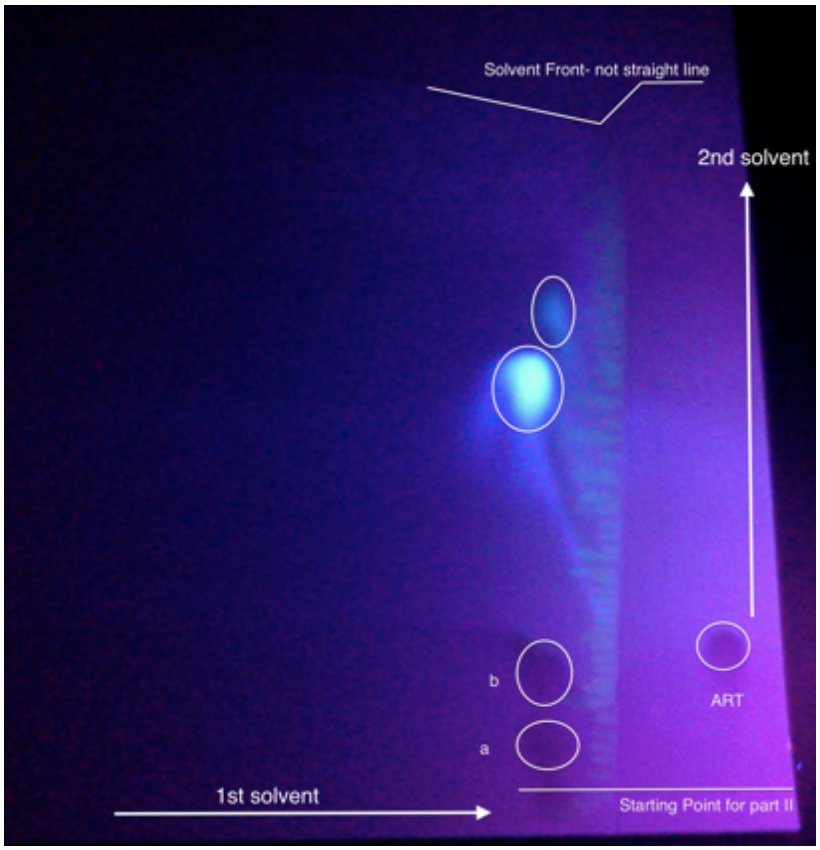

Fig. 18. Second dimension of 2-D TLC experiment: 230 µg SAM tip extract + 2.5 µg ART vs. 2 µg ART.
The first dimension was developed SAM tip (230 µg) and ART (2 µg) were spotted on a cellulose 300 TLC plate (20 cm x 20 cm) with the mobile phase 15 % HOAc. Spot order: SAM + ART and ART. The plate was visualized under UV-light, 366 nm. The R_f values for the observed spots were: a, 0.06; b, 0.14; ART: 0.17.

Fig. 19. CAS, SAM tip extract, and ART in 15 % HOAC.
CAS (3 µg), SAM tip extract (76 µg), and ART (2.5 µg) were spotted, respectively, on a cellulose 300 TLC plate (17 cm x 4 cm) with mobile phase 15 % HOAc. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: 0.18; a, 0.05; b, 0.13; ART: 0.18

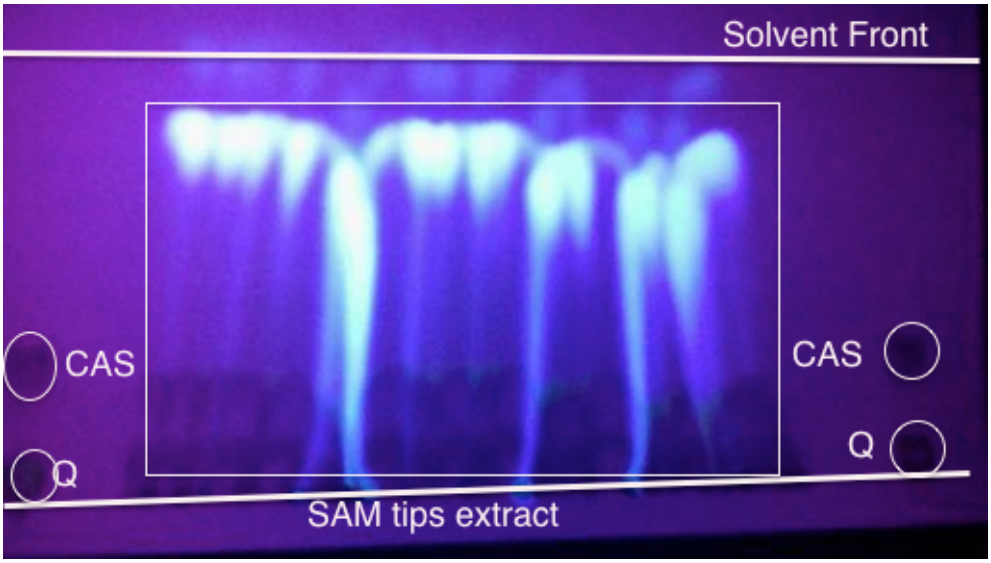

Fig. 20. Prep TLC of SAM tip extract in 15 % HOAc.
CAS (6 µg total) and Q (6 µg total) were spotted on either side of a line of SAM tip extract (5,750 µg) on a cellulose TLC plate (20 cm x 20 cm) with mobile phase 15 % HOAc. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: 0.19; Q: 0.03.

R_f values of CAS (5 µg) and ART (5 µg) were calculated after allowing them to develop in the solvent systems 3:1:1, t-BuOH-HOAc-H_2O and 15 % HOAc on cellulose TLC plates (Fig. 21A and B) (Roberts *et al*., 1980). The logic of this experiment was that spot 'b', which was observed in Fig. 19, could be identified as CAS if the resulting R_f values of CAS in this experiment were the same as those of spot 'b' in the aforementioned experiment. The resulting Rf values of CAS and ART were 0.91 and 0.93, respectively in 3:1:1, t-BuOH-HOAc-H_2O and 0.17 in 15 % HOAc. Therefore, the identity of neither spot 'a' nor spot 'b' as CAS could be confirmed.

To achieve greater separation between CAS and ART in the first solvent system, the mobile phase 1:2, toluene-ACN was used instead of the 3:1:1, t-BuOH-HOAc-H_2O mobile phase reported by Roberts *et al.* (1980). This mobile phase 1:2, toluene-ACN would have been used to separate CRYD from the other compounds in the SAM tip extract if it provided better resolution than 3:1:1, t-BuOH-HOAc-H_2O; however, the separation between spots did not improve as the R_f values of CAS and ART after developing in 1:2, toluene-ACN were 0.95 and 0.96, respectively (Fig. 21C). The mobile phase 1:2, toluene-ACN also did not improve the resolution of the spots so as to aid in identification of either CAS or ART via the R_f values.

The mobile phase reported by Roberts *et al.* (1980), 3:1:1, t-BuOH-HOAc-H_2O, is relatively polar, having a polarity index, P', of approximately 5.5. The polarity index was calculated using the following formula (Cazes, 2005): $P' = \Sigma i Pi' \phi i$, where *Pi'* is the polarity index of solvent *i* and ϕi is the volume fraction of solvent *i*. Since the R_f values of CAS and ART were relatively close to the solvent front (Fig. 21A), another solvent system with a polarity index lower (less polar) than that of 3:1:1, t-BuOH-HOAc-H_2O (5.5) was tested.

The nonpolar solvent Et_2O was added to the mobile phase 3:1:1, t-BuOH-HOAc-H_2O in another attempt to achieve separation between CAS and ART. The solvent system tested was 3:1:1:5, t-BuOH-HOAc-H_2O-Et_2O, which consisted of a polarity index of approximately 4.15 (Fig. 21D). The goal was also to achieve lower R_f values for the standards, as they were seen to readily move up the plate due to their weak interaction with the stationary phase, which indicated their nonpolar character; unfortunately, their R_f values remained near the solvent front.

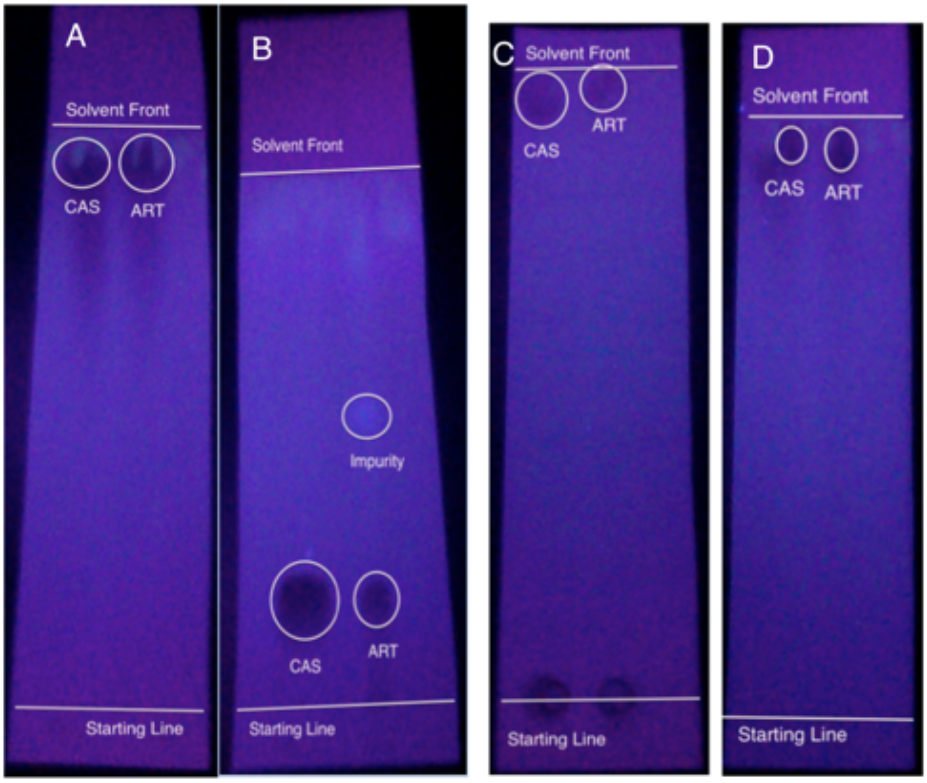

Fig. 21A-D. CAS and ART in 3:1:1,t-BuOH-HOAc-H_2O (A), 15 % HOAc (B) 1:2, toluene-ACN (C), and 3:1:1:5, t-BuOH-HOAc-H_2O-Et_2O (D).
In A and B, CAS (5 µg) and ART (5 µg) were spotted on either a 15.7 x 4 cm and 15.9 x 4 cm cellulose 300 TLC plate with mobile phases 3:1:1, t-BuOH-HOAc-H_2O and 15 % HOAc, respectively. The plates were visualized under UV-light, 366 nm. R_f values were: (A) CAS:0.91 and ART: 0.93; (B) CAS and ART: 0.17. Touching the plate may have caused the impurity. In C, CAS (3 µg) and ART (2.5 µg) were spotted on a cellulose 300 TLC plate (12.5 cm x 3 cm) with mobile phase 1:2, toluene-ACN. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: 0.95; ART: 0.96. In D, CAS (3 µg) and ART (3 µg) were spotted on a cellulose 300 TLC plate (15.5 cm x 3.5 cm) and allowed to develop in the mobile phase 3:1:1:5, t-BuOH-HOAc-H_2O-Et_2O. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: 0.96; ART: 0.94

The SAM tip extract, CAS, and Q were also applied to three different solid adsorbents and developed in three different solvent systems to observe their separation and R_f values in various chromatographic conditions (Fig. 22-24). Up to these experiments, the solvent systems 2:1, toluene-EtOAc and 1:4, ACN-CH_2Cl_2 were determined as the best in separating the component compounds of the SAM tip extract. In addition, since CRYD was similar in structure to Q, and Q streaked in prior TLC experiments (Fig. 22-24), further experiments were done to increase the R_f value of Q and to observe whether the spot of Q could be sharpened. The solvent system, 1:1:3, ACN-HOAc-H_2O, was successful in condensing Q and moving it further up the plate (Fig. 25).

Fig. 25. Q vs. SAM tip extract in 1:1:3, ACN-HOAc-H_2O.
Q (1 µg) and SAM tip extract (approx. 77 µg) were applied to a silica gel TLC plate (9 cm x 3 cm) and with mobile phase 1:1:3, ACN-HOAc-H_2O. The plate was visualized under UV-light, 366 nm. The R_f value of CAS was 0.7. Addition of ACN to the mobile phase helped condense Q and move it further up the plate.

Efforts to detect and separate CRS from the other components of fresh SAM shoot tip were made by using a variety of TLC conditions reported by Voirin *et al*. (1999), Grayer *et al*. (1996), and Saleh *et al*. (1987). The standards CAS, Q, ART, and AN were used in these TLC experiments as references for the SAM tip extract spots. Some of the TLC plates were stained with *p*-anisaldehyde in glacial acetic acid and sulfuric acid (0.5:50:1) to visualize terpenoids (Pras *et al*., 1991). AN showed up as a dark pink spot after staining, and other unidentified terpenoids were shades of dark purple to pink. By using 2 % $AlCl_3$ in MeOH, other flavonoids that may not have been initially visible under UV-light also appeared (Sharon *et al*., 1992).

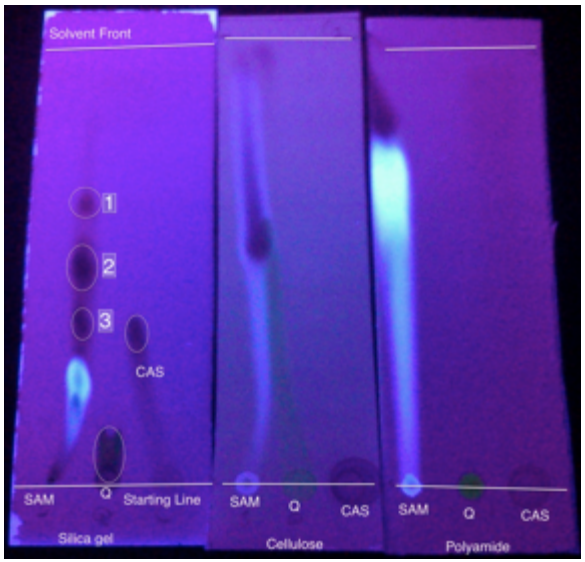

Fig. 22. SAM tip extract, CAS, and Q in 2:1, toluene-EtOAc on silica gel, cellulose, and polyamide TLC plates, respectively.
SAM tip extract (77 µg), Q (1.5 µg), and CAS (1.5 µg) were spotted on silica gel, cellulose, and polyamide TLC plates (9 cm x 3 cm) with mobile phase 2:1, toluene-EtOAc. The plates were visualized under UV-light, 366 nm. R_f values were: (left to right) SAM: 1, 0.61; 2, 0.47; 3, 0.33; Q: 0.05; CAS: 0.31. Spot 3 may be CAS. Compounds of SAM extract on the cellulose and polyamide TLC plates were streaked with white fluorescence. Neither CAS nor Q moved on either the cellulose or polyamide plates.

Fig. 23. SAM tip extract, CAS, and Q in 3:1:1, t-BuOH-HOAc-H_2O on silica gel, cellulose, and polyamide TLC plates, respectively.
Same conditions used as Fig. 22 but with mobile phase 3:1:1, t-BuOH-HOAc-H_2O. The plates were visualized under UV-light, 366 nm. R_f values on silica gel were: SAM (top to bottom): white to dark to pink fluorescing streak; Q: yellow, 0.87; CAS: dark, 0.82. On cellulose, R_fs were: SAM (bottom to top): white to dark, then orange/pink fluorescing streak; Q: yellow, 0.83; CAS: dark, 0.96. On polyamide: SAM (bottom to top): pink streak to yellow, white, dark, and pink fluorescence; Q: yellow, 0.16; CAS: dark, 0.84.

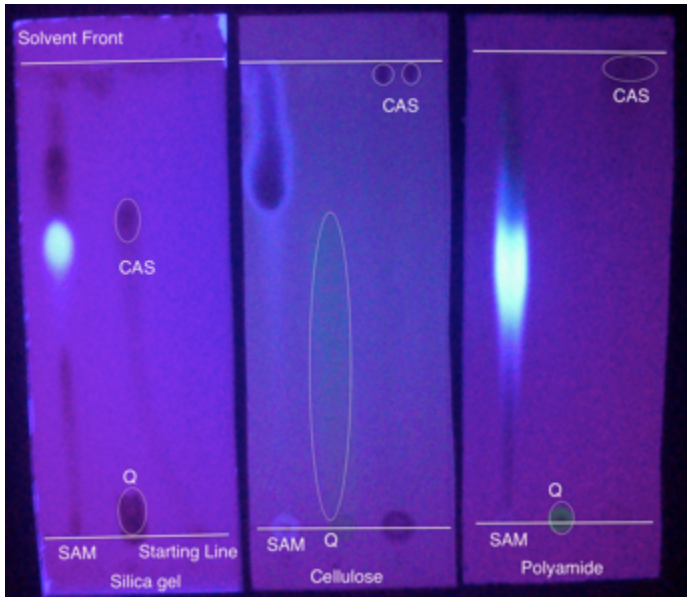

Fig. 24. SAM tip extract, CAS, and Q in 1:4, ACN-CH_2Cl_2 on silica gel, cellulose, and polyamide TLC plates, respectively.
Same conditions as Fig. 22 with mobile phase 1:4, ACN-CH_2Cl_2. Plates were visualized under UV-light, 366 nm. R_f values on the silica gel TLC plate were: SAM (bottom to top): orange to dark, white, dark, then orange streak; Q: yellow, 0.07; CAS: dark, 0.68. On cellulose, R_fs were: SAM (bottom to top): white to dark inside white streak; Q: yellow, 0.35; CAS: dark, 0.97. On polyamide: SAM (bottom to top): white to yellow fluorescing streak; Q: yellow, at origin; CAS: dark, 0.96.

According to Sharon *et al.* (1992), $AlCl_3$ interacts with the hydroxyl groups of a flavonoid, resulting in fluorescence. If flavonoids are present, then fluorescent spots will appear after spraying a TLC plate with 1 % $AlCl_3$ in ethanol. Chang *et al*. (2002) further elaborated that $AlCl_3$ "forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols." Fluorescence of additional flavonoids became apparent after spraying selected TLC plates with $AlCl_3$ (Fig. 44 and 47).

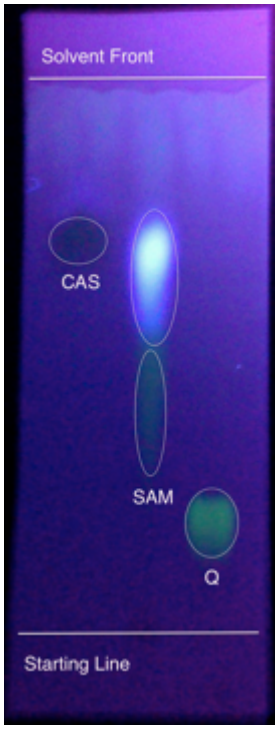

Fig. 26. CAS and Q vs. SAM tip extract in 4:6, HOAc-H_2O. CAS (1 µg), SAM tip extract (approx. 77 µg), and Q (1 µg) were applied to a cellulose TLC plate (9 cm x 3 cm) and with mobile phase 4:6, HOAc-H_2O. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: dark yellow-brown, 0.68; SAM (bottom to top): yellow to white streak, 0.63 and 0.69; Q: bright yellow, 0.19.

Voirin *et al*. (1999) reported that CRS had an R_f value of 0.5 in the solvent system HOAc-H_2O (4:6) on a cellulose TLC plate. The former TLC conditions were repeated to observe whether a compound from the chromatographic separation of the SAM tip extract would have the same R_f. The standards CAS and Q were also run under those conditions, with neither the standards, nor the SAM tip extract showing the literature R_f value (See Fig. 26).

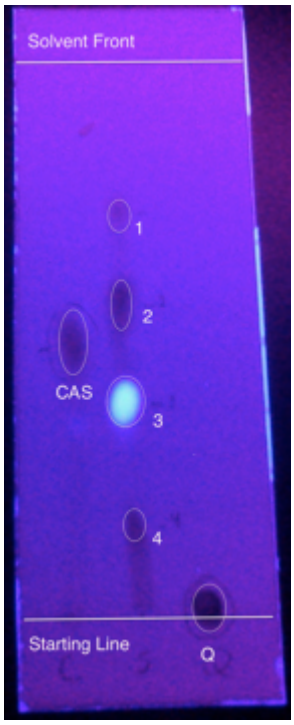

Fig. 27. CAS and Q vs. SAM tip extract in 5 % MeOH in $CHCl_3$. CAS (1 µg), SAM tip extract (approx. 77 µg), and Q (1 µg) were applied to a silica gel TLC plate (9 cm x 3 cm) with mobile phase 5 % MeOH in $CHCl_3$. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: dark, 0.45; SAM (top to bottom): 1, dark, 0.7; 2, dark, 0.53; 3, white, 0.45; 4, dark, 0.35; Q: yellow, at origin.

The TLC conditions of Grayer *et al*. (1996) (5 % MeOH in $CHCl_3$; silica gel TLC plate) were used to ascertain whether the same R_f value of 0.44 could be obtained for one of the spots of SAM tip extract. If a spot from the SAM tip extract had an R_f value of 0.44, then there would be supporting evidence that CRS was present. Spot 3 of the SAM tip extract and CAS showed an R_f of 0.45, but CRS could not be quantified without further analysis (Fig. 27).

The TLC methods reported by Saleh *et al*. (1987) were repeated to determine if CAS and one of the spots developed from the fresh SAM tip extract, possibly CRS, would have the same literature R_f values. The literature R_f values of CAS and CRS were 0.83 and 0.76 in (12:5:3) toluene-MEK-MeOH on polyamide TLC plates, 0.45 and 0.26, respectively, in (60:60:7:7) benzene-petroleum ether (b.pt. 60-80°C)-MEK-MeOH on polyamide TLC plates and 0.69 and 0.6 in (36:9:5) benzene-pyridine-formic acid (BPF) on silica gel TLC plates. TLC plates run in those conditions are shown in Fig. 28, 29A and B, and 30, respectively. Another plate was run on polyamide with the mobile phase 36:9:5, BPF to observe the separation of compounds in the SAM tip extract (Fig. 31). However, the SAM tip extract only separated into two spots. Application of the SAM tip extract on silica gel TLC plates and development in 36:9:5, BPF was determined as the best TLC method for separating at least 7 different UV-fluorescent compounds (Fig. 30). Condensed, dark, and fluorescent spots were observed. Unfortunately, most of the SAM tip extract spots did not show the same R_f values as those reported in the literature. Silica gel TLC plates were used instead of polyamide plates with the mobile phase 36:9:5, BPF, as a greater amount of resolved spots resulted when using silica gel plates.

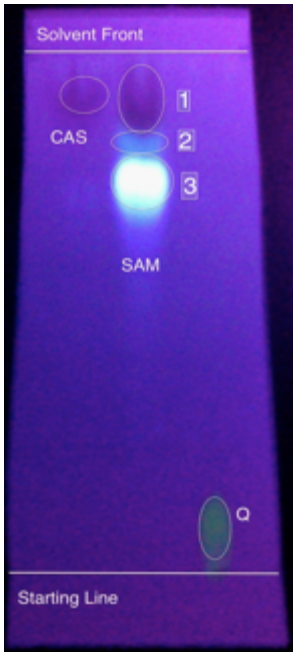

Fig. 28. CAS and Q vs. SAM tip extract in 12:5:3, toluene-MEK-MeOH.
CAS (1 µg), SAM tip extract (approx. 77 µg), and Q (1 µg) were applied to a polyamide TLC plate (9 cm x 3 cm) with mobile phase 12:5:3, toluene-MEK-MeOH. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: dark, 0.85; SAM (bottom to top): 1, dark, 0.85; 2, green, 0.79; 3, white, 0.72; Q: yellow, 0.06.

Fig. 29A & B. CAS and Q vs. SAM tip extraction on polyamide in (60:60:7:7) benzene-petroleum ether (b.pt. 35-60°C)-methyl ethyl ketone-MeOH.
Same conditions as Fig. 28, but with mobile phase (60:60:7:7) benzene-petroleum ether (b.pt. 35-60°C)-MEK-MeOH. The plate was visualized under UV-light, 366 nm. R_f values were: CAS-dark, 0.44; SAM (bottom to top): 1, white, 0.21; 2, blue/green, 0.27; 3, dark, 0.33; 4, dark, 0.44; 5, dark, 0.88; Q: yellow, at origin. Spot 2 may be identified as CAS.

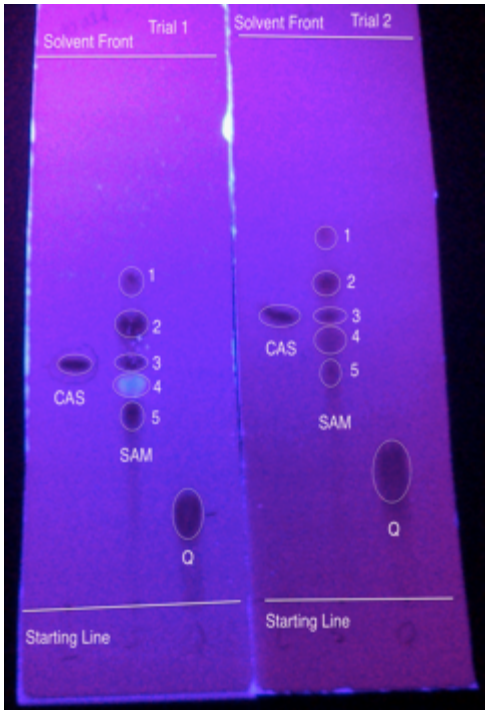

Fig. 30. Two trials of CAS and Q vs. SAM tip extract on silica gel with 36:9:5, benzene-pyridine-formic acid.
Same as Fig. 28, but with 36:9:5,BPF on silica gel. The plate was visualized under UV-light, 366 nm. R_f values for Trial 1 were: CAS: dark, 0.40; SAM consisted of all dark spots with a pink fluorescence to them: 1, 0.54; 2, 0.47; 3, 0.40; 4, 0.36; 5, 0.31; Q: dark, 0.14. R_f values in Trial 2 were: CAS: 0.47; SAM: 1, 0.62; 2 ,0.53; 3, 0.47; 4, 0.43; 5, 0.36; Q: 0.21. This solvent helped move Q and also improved the resolution.

6.4 Possible Interactions between Flavonoids from the Crude Fresh SAM Tip Extract

A curiosity about Fig. 11B was explored, of whether if allowed to develop alone on a clean TLC plate, spot #1 would have an R_f value closer to that of the CAS standard. After recovering spot #1 and running it under the previous conditions (silica gel TLC plate; 2:1, toluene-EtOAc) alongside CAS, the R_f value revealed for CAS was approximately 0.22, while spot #1 was 0.20. Therefore, perhaps other flavonoids in the SAM tip extract are possibly associating with or binding to CAS in spot #1. Interestingly, however, spot #1 ran closer to CAS, when it was run alone after recovery, as compared to when it was run on the TLC plate as part of the entire SAM tip extract (See Fig. 32A). Further experimentation was done to determine if spot #1 could be separated into any additional component compounds, by running spot #1 and CAS in a more polar mobile phase 1:2, toluene-ACN on silica gel. However, only CAS and spot #1 were observed with R_f values of 0.7 and 0.57, respectively. Although spot 1 was not separated into additional components, better resolution between both spot #1 and CAS was achieved (Fig. 32B)

Fig. 31. CAS and Q vs. SAM tip extract on polyamide with 36:9:5, benzene-pyridine-formic acid. CAS (1 µg), SAM tip extract (approx. 77 µg), and Q (1 µg) were applied to a polyamide TLC plate (9 cm x 3 cm) with mobile phase (36:9:5) benzene-pyridine-formic acid. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: dark, 0.92; SAM (top to bottom): 1, white, 0.92; 2, white, 0.75; Q: orange, 0.28.

6.5 NMR analysis of Crude Plant Extract (Fresh SAM Tip), CAS, CRY, and CRYD

The possibility that the compounds in *A. annua* extracts have an effect on each other's R_f values was further supported by the comparison of NMR spectra of the SAM extract, CRY, CRYD, and CAS. The NMR spectrum of the SAM extract was different from that of the standards CRY, CRYD, and CAS in that the chemical shift values were altered to an extent that

no similarities between the spectra could be concluded (See Fig. 33). The presence of the standards in SAM could not be confirmed by NMR analysis. In addition, the standard of CRYD obtained was found to be impure as it did not go into solution ($CDCl_3$) and peaks other than those reported by Kraus and Roy (2008) were present in the proton NMR spectrum, which were labeled as impurities, "imp" or as a dot above a peak, in Fig. 33. Also note that in Fig. 33, "Ar" refers to an aromatic ring of the compound. The more detailed and individual ^{1}H and ^{13}C NMR spectra of the plant extract and standards (Fig. 34-41) can be found in Appendix IV.

Fig. 32A & B. CAS vs. Spot 1 of SAM tip extract in 2:1, toluene-EtOAc and in 1:2, toluene-ACN
In Fig. 32A (left), the approx. 2 µg CAS and 0.22 mg Spot 1 were spotted on a silica gel TLC plate (17.2 cm x 2.6 cm) with the mobile phase 2:1, toluene-EtOAc. Spot order: CAS and Spot 1. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: 0.22; Spot 1: 0.20. In Fig. 32B (right), the approx. 2 µg CAS and 0.22 mg Spot 1 were spotted on a silica gel TLC plate (17.2 cm x 3.5 cm) with mobile phase 1:2, toluene-ACN. Spot order: CAS and Spot 1. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: 0.70; Spot 1: 0.57

Fig. 33. Comparison of proton NMR spectra of CRYD, CRY, CAS, and Plant Extract

6.6 Staining TLC Plates for Analysis of Flavonoids and Terpenoids in SAM Tip Extracts

TLC plates were sprayed with the *p*-anisaldehyde solution to observe AN, as well as other terpenoids, in the fresh SAM tip extract. This spray produces a hot pink color for AN and also reacts with other terpenoids to give various hues of mainly purples and blues (Pras *et al*., 1991). From the first TLC plates that were sprayed after developing in 36:9:5, BPF, only two pink spots were observed (Fig. 42A-D, 43, and 44). Fig. 44 was also sprayed with 2 % $AlCl_3$ in MeOH to determine whether flavonoids that were not initially visible under UV-light, would appear after staining. On the third TLC plate in Fig. 44, where SAM 2 was applied, more spots for flavonoids in positions other than the bright green dashes were seen after spraying with $AlCl_3$. This indicated that other flavonoids were present in the SAM tip extract than the ones usually observed after running in 36:9:5, BPF. As seen in Fig. 44, terpenoids were evident after spraying SAM 1 with the *p*-anisaldehyde solution, some of which were located in positions other than the bright green dashes.

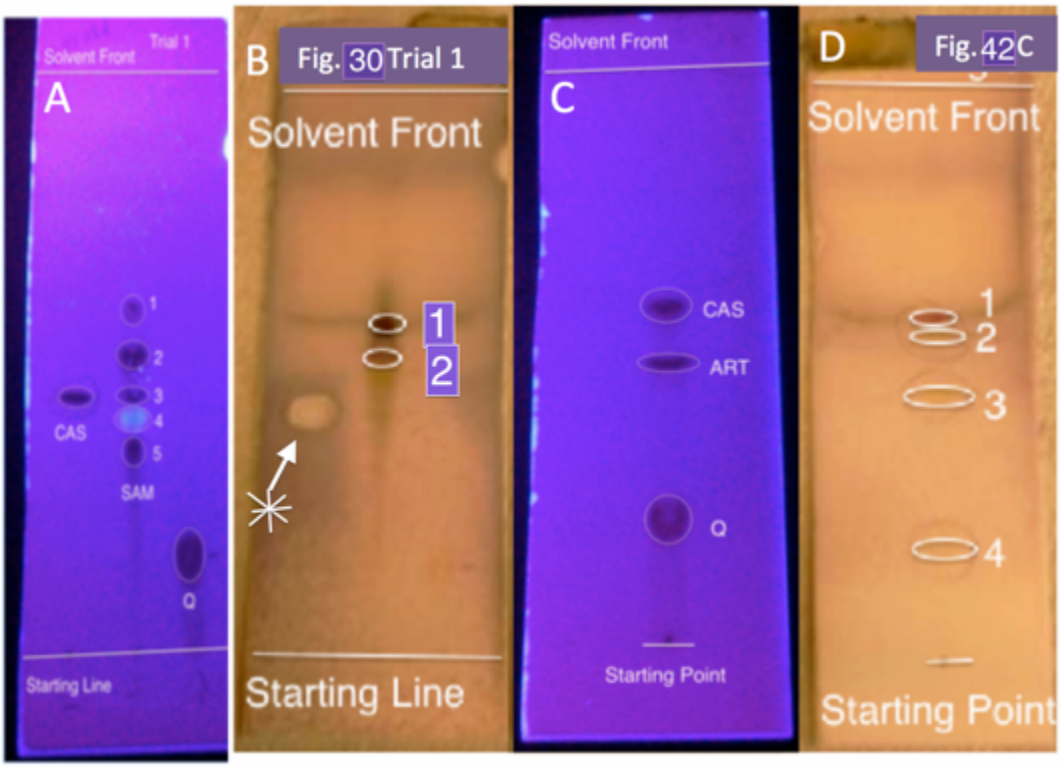

Fig. 42A-D. ART, AN, CAS, SAM tip extract and Q in 36:9:5, benzene-pyridine-formic acid on silica gel and trial staining with *p*-anisaldehyde solution.
A is the plate from Fig. 30 trial 1 under UV-light, 366 nm. R_f values for A were: CAS: dark, 0.40; SAM consisted of all dark spots with a pink fluorescence to them: 1,0.54; 2,0.47; 3,0.40; 4,0.36; 5,0.31; Q: dark, 0.14. B is a trial staining of plate A with *p*-anisaldehyde solution. The stained plates were visualized under visual light. The pink spot labeled 1 on B had an R_f of 0.56, while the lighter pink 2 had an R_f of about 0.5. Yellow and blue spots were also observed. The starred spot indicates it was recovered from the plate. C consisted of CAS (1 µg), AN (1 µg), ART (1 µg), and Q (1 µg) applied to a silica gel TLC plate (9 cm x 2.5 cm) with mobile phase (36:9:5) BPF. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: 0.55; ART: 0.44; Q: 0.19. D, which is the plate from Fig. 41C sprayed with *p*-anisaldehyde solution, AN, spot 1 (pink) had an R_f of about 0.58. The yellow spots 2, 3, and 4 (or CAS, ART, and Q) had R_f values of 0.55, 0.45, and 0.19, respectively.

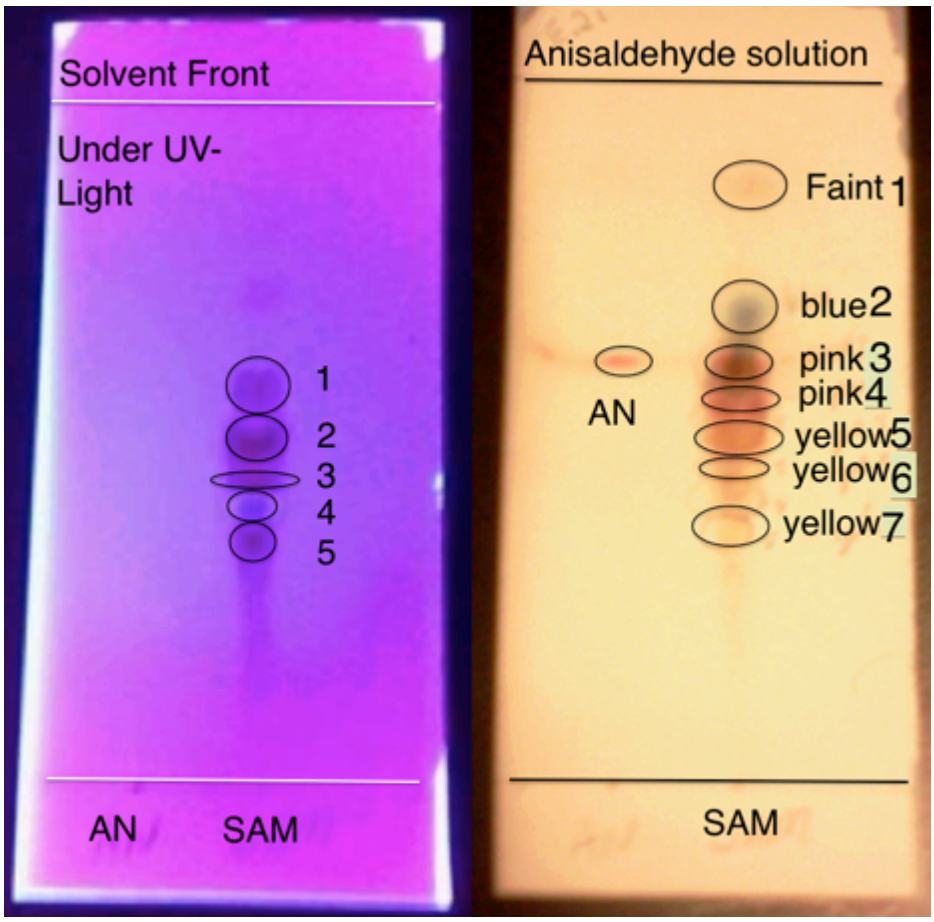

Fig. 43. AN and SAM tip extract on silica gel in 36:9:5, benzene-pyridine-formic acid and stained with *p*-anisaldehyde solution.

AN (1 µg) and SAM (77 µg) were applied to a silica gel TLC plate (9 cm x 4 cm) with mobile phase (36:9:5) benzene-pyridine-formic acid. The plate was viewed first under UV-light (left), and then sprayed with *p*-anisaldehyde solution (right). UV-visible R_f values of SAM were: 1, dark, 0.59; 2, dark, 0.49; 3, dark, 0.43; 4, white, 0.39; 5, dark, 0.34. Spots that appeared after staining had the R_f values: AN: 0.58; 1, faint, 0.84; 2, blue, 0.67; 3, pink, 0.59; 4, pink, 0.53; 5, yellow, 0.49; 6, yellow, 0.41; 7, yellow, 0.34.

Fig. 44. Development of SAM tip extract in 36:9:5, benzene-pyridine-formic acid, followed by staining with *p*-anisaldehyde solution and 2 % $AlCl_3$ in MeOH.

SAM tip extract (77 µg, x2) was applied to a 14.5 cm x 4 cm silica gel TLC plate with mobile phase (36:9:5) benzene-pyridine-formic acid. The plate was visualized under UV-light, 366 nm, then cut in half. R_f values for the 7 spots visible under UV-light were: 1, dark, 0.62; 2, dark, 0.58; 3, dark, 0.48; 4, dark with pink center, 0.46; 5, hot pink, 0.45; 6, dark, 0.42; 7, dark, 0.37. SAM 1 was stained with *p*-anisaldehyde solution and observed under visible light. R_f values of spots in SAM1 were: dark pink 1, 0.69; light pink 2, 0.54; yellow 3, 0.47; green 4, 0.45. SAM2 was stained with 2 % $AlCl_3$ in MeOH and then viewed under UV-light. R_f values of spots in SAM2 were: yellow 1, 0.62; yellow 2, 0.47; white 3, 0.40. The bright green dashes indicate where spots were first visible under only UV-light were located.

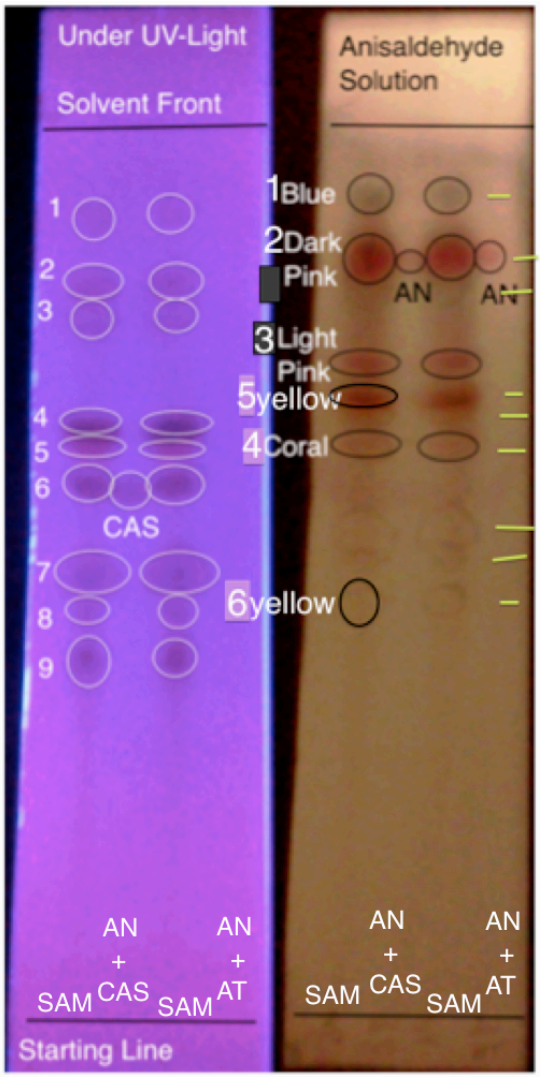

Fig. 45. Development of SAM tip extract, AN, CAS, and AT on silica gel in 36:9:5, benzene-pyridine-formic acid, followed by staining with *p*-anisaldehyde.
The compounds were spotted on a 20 cm x 4.2 cm silica gel TLC plate in the order: SAM (154 µg); AN (1 µg) + CAS (1 µg); SAM (385 µg); AN (1 µg) + AT (10 µg), with mobile phase (36:9:5) benzene-pyridine-formic acid. The plate first viewed under UV-light, then sprayed with *p*-anisaldehyde and viewed in visible light. R_f values under UV-light were: CAS: 0.6; SAM: 1, orange, 0.9; 2, dark, 0.82; 3, dark, 0.78; 4, dark, 0.67; 5, hot pink, 0.65; 6, dark, 0.61; 7, dark, 0.52; 8, dark, 0.48; 9, dark, 0.43. Spot 6 is CAS. Spots appearing after staining had R_f values: AN: 0.83; 1, blue, 0.91; 2, dark pink, 0.83; 3, light pink, 0.71; 4, coral, 0.62; 5, yellow, 0.67; 6, yellow, 0.43. Spot 2 is AN. The bright green dashes indicate where spots were first visible under only UV-light were located.

In a later TLC experiment, three pink spots were noticed in the SAM tip extract, although the presence of AN in each of the spots could not be concluded without further analyses (Fig. 45). In Fig. 43 and Fig. 45, AN and one of the pink spots from SAM tip extract had a similar R_f value, which indicated that those spots are AN. However, the other pink spots, such SAM spot 4, coral from Fig. 45, may consist of AN, as well, since NMR spectra and the experiment with spot 1 running separate from the SAM tip extract indicated that interactions between the compounds may have an affect on one another's R_f and chemical shift values.

Fig. 46. AT and AN on silica gel in 36:9:5, benzene-pyridine-formic acid with *p*-anisaldehyde. AT (2 µg) and AN (1 µg) were applied to a silica gel TLC plate (14.5 cm x 3 cm) with mobile phase (36:9:5) benzene-pyridine-formic acid. The plate was stained with *p*-anisaldehyde solution, then visualized in visible light. The R_f value of AN was approximately 0.75. AT did not appear after staining. Not enough may have been applied.

The three pink spots visible after spraying the plates with *p*-anisaldehyde brought about the question as to whether one could be identified as AT, since AT is similar in structure to AN. AT has a double bond where AN has a methyl group. AT was applied to the plates in Fig. 45 and 46. However, AT did not appear on either plate after spraying with *p*-anisaldehyde. This may have occurred if the amount of AT on the stock test tube was incorrectly labeled, and therefore, not enough was applied to the TLC plate.

A large change in the R_f values of the compounds in the SAM tip extract occurred when a 20 cm x 12 cm and 20 x 7.8 cm TLC plate were used in the same solvent system of 36:9:5, BPF. The spots moved from their usual position in the middle of the plate to closer to the solvent front. This may have been caused by too much solvent evaporating off the plate or not enough filter paper lining the developing chamber. A smaller plate that was developed afterwards confirmed that the solvent system was not the cause of the change in R_f

values. Therefore, smaller TLC plates were used in the TLC analyses of the fresh SAM leaves extract.

6.7 Fresh SAM Leaf Extract vs. Fresh SAM Tip Extract

The compounds from fresh leaves of SAM were extracted to observe how they differed qualitatively and quantitatively from the fresh SAM shoot tip extract. The fresh SAM leaf extract applied to the silica gel TLC plate, showed fewer compounds and lower levels than the fresh SAM tip extract (Fig. 47). This shows that after tips expand and develop into mature leaves, some compounds decline in content and/or disappear. Table VI contains R_f values for the plates. The SAM leaf and tip extracts showed the same spots on plate 2 after spraying with *p*-anisaldehyde. Overall, the SAM tip extract seemed to have more flavonoids and terpenoids per milligram than the SAM leaf extract. After staining with $AlCl_3$, three flavonoids, T2, T3, and T5, were present in the SAM tip extract that were not present in the SAM leaf extract. The flavonoids in the SAM leaf extract that correlated with those in the tip extract were: L2 and T1; L3 and T4; L4 and T6; L5 and T7; L6 and T8.

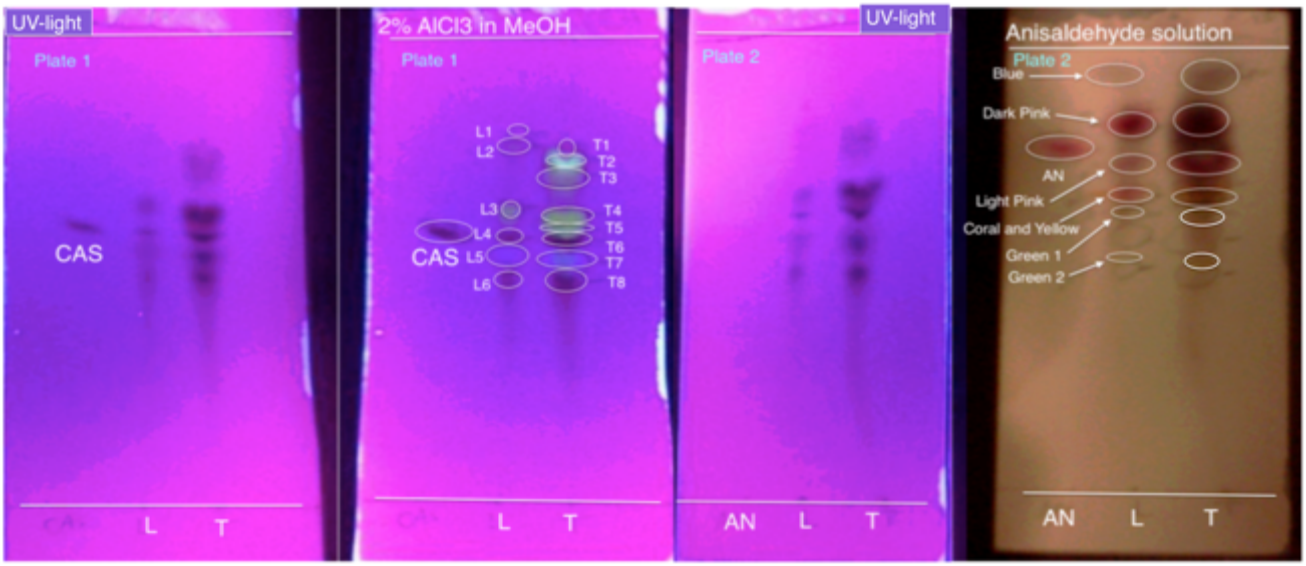

Fig. 47. Comparison of SAM tip and leaf extract, and AN (plate 2) and CAS (plate 1) in 36:9:5, benzene-pyridine-formic acid, followed by staining of plate 2 with *p*-anisaldehyde and of plate 1 with $AlCl_3$.

The three compounds each were spotted from left to right on two 9 cm x 4 cm silica gel TLC plates in the order: AN (2 µg); SAM leaf extract, L (154 µg); SAM tip extract, T (154 µg); CAS (1 µg); L (154 µg); T (154 µg), run in mobile phase (36:9:5) benzene-pyridine-formic acid, and then viewed under UV-light. Plate 1 was then sprayed with $AlCl_3$ and viewed again under UV-light. Plate 2 was sprayed with *p*-anisaldehyde and then viewed in visible light. R_f values of the spots observed on plate 1 under UV-light were the same as the colored spots seen after spraying with $AlCl_3$ (See Table VI). Also see Table VI for the spots observed on plate 2 under UV-light and after staining with the anisaldehyde solution. Some of the colored spots in the SAM leaves and tip extracts were difficult to discern, such as green and yellow.

Table VI: R_f values of AN, CAS, and compounds in SAM leaf and tip extracts.

R_f values and appearance of spots were observed under UV-light and visible light, after developing plates 1 and 2 in (36:9:5) benzene-pyridine-formic acid, spraying plate 1 with $AlCl_3$ and plate 2 with the anisaldehyde solution (See Fig. 47 for TLC plates).

Leaves		Tips		Leaves		Tips		Leaves and Tips	
Plate 1 under UV-light and after $AlCl_3$	R_f value	Plate 1 under UV-light and after $AlCl_3$	R_f value	Plate 2 under UV-light: (all dark spots)	R_f value	Plate 2 under UV-light: (all dark spots)	R_f value	Plate 2 after anisaldehyde solution	R_f value
L1-orange	0.81	T1-orange	0.78	1	0.85	1	0.85	1-Blue	0.92
L2-yellow	0.78	T2-yellow	0.77	2	0.81	2	0.8	2-Dark Pink	0.82
L3-yellow	0.64	T3-yellow	0.72	3	0.65	3	0.76	3-Light Pink	0.73
L4-dark	0.58	T4-yellow	0.64	4	0.61	4	0.68	4-Coral and Yellow	0.66
L5-white	0.53	T5-yellow	0.59	5	0.55	5	0.65	5-Green 1	0.62
L6-orange	0.47	T6-dark	0.57	6	0.49	6	0.62	6-Green 2	0.5
		T7-white	0.53			7	0.55	AN standard-dark pink	0.77
		T8-orange	0.49			8	0.47		
		CAS standard-dark yellow	0.57						

- L2=T1
- L3=T4
- L4=T6
- L5=T7
- L6=T8

- 3 new/different flavonoids in T vs. L (T2, T3, T5)

- All the same spots, but very different amounts of each
- Seems that tips have a lot more per gram

6.8 TLC Analysis of CRY and CRYD

When standards for CRY and CRYD were finally acquired, they were analyzed using the TLC conditions reported by Saleh *et al*. (1987), silica gel in (36:9:5) benzene-pyridine-formic acid. Standards CRY and CRYD showed different mobilities. CRY exhibited the same R_f value as CAS, but unfortunately, this did not confirm the presence of CRY in the SAM tip extract (Fig. 48). Further GC/MS and NMR analysis of CRY from the SAM tip extract needs to be done to quantify it in the plant.

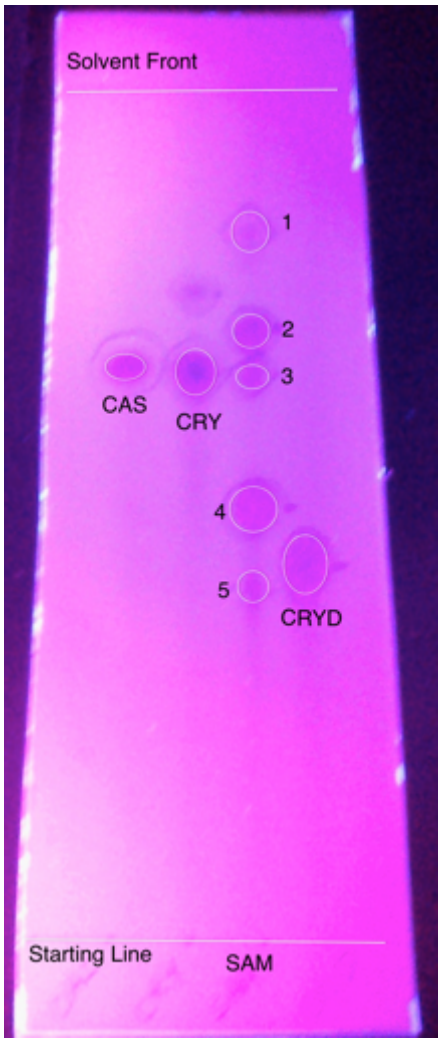

Fig. 48. CAS, CRY, SAM tip extract, and CRYD on silica gel in 36:9:5, benzene-pyridine-formic acid under UV-light.
CAS (1 µg), CRY (1 µg), SAM (77 µg), and CRYD (1 µg) were run on a silica gel TLC plate (14.5 cm x 3 cm) with mobile phase (36:9:5) benzene-pyridine-formic acid. The plate was visualized under UV-light, 366 nm. R_f values were: CAS: 0.61; CRY: 0.61; SAM: 1, 0.79; 2, 0.66; 3, 0.60; 4, 0.45; 5, 0.35; CRYD: 0.33.

7.0 Discussion

Some researchers, such as Shilin *et al*. (1989) reported using MeOH and extracting with MeOH for 5 days (Marco *et al*., 1988). However, MeOH extracted more chlorophyll than methylene chloride and was therefore not as proficient an extraction solvent as CH_2Cl_2. Bilia *et al*. (2006) also analyzed infusions, decoctions, an n-hexane extract, and ethanol tinctures of *A. annua* L. for extraction efficiency of AN and flavonoids. A 60 % v/v tincture with ethanol was the most proficient, having an extraction efficiency of 40 % for AN and 29.5 % for flavonoids (Bilia *et al*., 2006). Future analyses may be performed with the SAM tip extract to determine the extraction efficiency of ethanol and how the results compare to those reported by Bilia *et al*. (2006).

In this project, the appearance and R_f values of compounds of the flavonoids of interest, CRY and CRYD, and the standards available in the lab, K, Q, CAS, AN, M, AT, EUP, and ART in various mobile phases and stationary phases were recorded (Table II and III). Often times, the experimental R_f values did not match the literature values. In addition, there were many gaps in the methodologies reported by researchers, which made attempts to replicate their experiments unfeasible. Previous reports declared extracting the flavonoids of interest in mixtures, along with other flavonoids, which implied the difficulty in separating these flavonoids that are very similar in structure.

The NMR spectra of the crude extract versus CRY, CRYD, and CAS indicated that further purification of the crude extract may help verify the presence of the flavonoids of interest. The chemical shift values of the crude plant extract did not correlate with those of the standards, which implied that the flavonoids are most likely interacting with each other in such a strong way that they affected each other's chemical shift values. However, a TLC analysis of CRY did

suggest that it is present in at least the SAM tip extract. The confirmation of the presence of CRY in the SAM leaf extract is yet to be done.

In the comparison of SAM tip and leaf extracts, more flavonoids and terpenoids were observed on a per gram basis in the tips than the leaves. The SAM tip extract also contained three other flavonoids that the leaf extract did not (Fig. 47). Although to our knowledge, no analyses have been done specifically on the tips and mature leaves of *A. annua* to make an absolute comparison, Baraldi *et al*. (2008) distinguished the production of AN and flavonoids, namely eupatin, ART, and an inseparable mixture of CAS and CRY, in various aerial parts of *A. annua* L. at the pre-flowering, full bloom, and post-flowering stages. Baraldi *et al*. (2008) did not analyze the leaves during only the vegetative stage, which is prior to reproduction (flowering). Both leaves and flowers had the highest content of flavonoids at full bloom; AN, on the other hand, was highest in the leaves than in the flowers at full bloom (Baraldi *et al*., 2008). An interesting endeavor would be to study the inflorescences further using TLC and column chromatography.

The original objective of this project changed from isolating and identifying CRY, CRS, and CRYD to clarifying the compounds found in fresh SAM tip and leaf extracts, and maximizing the number of compounds that could be separated and visualized from them. Having a limited supply of the expensive standard flavonoids of interest to this project near the end also restricted the experiments where they could be used in, such as column chromatography. Although the original objectives were not possible to attain, new information profiling the constituents found in young growing shoot tips and mature leaves was achieved.

References:

Alonso, P. L., Brown, G., Arevalo-Herrera, M., *et al*. (2011). A research agenda to underpin malaria eradication.PLos Medicine *8*(1): 481-504.

Baraldi, R., Isacchi, B., Predieri, S., Marconi, G., Vincieri, F., Bilia, A. (2008). Distribution of artemisinin and bioactive flavonoids from *Artemisia annua* L. during plant growth. Biochemical Systematics and Ecology *36*: 340-348.

Beecher, G. (2003). Overview of dietary flavonoids: nomenclature, occurrence and intake. *The* Journal of Nutrition: 3248-3254.

Bilia, A., Lazari, D., Messori, L., Tagliolia, V., Temperini, C., Vincieri, F. (2002). Simple and rapid physio-chemical methods to examine action of antimalarial drugs with hemin. Its application to *Artemisia annua* constituents. Life Sciences *70*: 769-778.

Bilia, A., Gabriele, C., Bergonzi, M., de Malgalhaes, P., Vincieri, F. (2006). Variation in artemisinin and flavonoid content in different extracts of *Artemisia annua* L. Natural Product Communications *0*(0): 1-5.

Bledsoe, G. H. (2005). Malaria primer for clinicians in the United States. Southern Medical Journal *98*(12): 1197-1204. Retrieved September 3, 2012, from http://journals.lww.com/smajournalonline/Fulltext/2005/12000/Malaria_Primer_for_Clinicians_in_the_United_States.12.aspx

Breman, J.G., Brandling-Bennett, A.D. (2011). The challenge of malaria eradication in the twenty-first century: research linked to operations is the key. Vaccine 2011. http://dx.doi.org/10.1016/ j.vaccine.2011.12.003

Brown, G. D. (2010). The biosynthesis of artemisinin (*qinghaosu*) and the phytochemistry of *Artemisia annua* L. (*qinghao*). Molecules *15*: 7603-7698. Retrieved May 31, 2012, from http://www.mdpi.com/1420-3049/15/11/7603

Calcagno-Pissarelli, M., Alonso-Amelot, M., Mora, R., Nunez, D. (2010). Foliar exudates of *Blakiella barsiifolia* (s.f. blake) cuatrec. (asteraceae, astereae). A preliminary study of the chemical composition. Avances en Quimica *5*(3): 161-166.

Cazes, J. (2005). Eluotropic series of solvents for TLC. In *Encycolopedia of chromatography*, 2nd ed. Taylor & Francis Group. Boca Raton, FL. Pp. 548-552.

Chang, C., Yang, M., Wen, H., Chern, J. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of Food and Drug Analysis *10*(3): 178-182.

Clemente, M., Corigliano, M.G. (2012). Overview of plant-made vaccine antigens against malaria. Journal of Biomedicine and Biotechnology *2012*: 1-8.

Croteau, R., Kutchan, T., Lewis, N. (2000). Natural Products (Secondary Metabolites). Buchanan, B., Gruissem, W., Jones, R. (Eds.). Biochemistry & Molecular Biology of Plants: 1250-1318.

Cushine, T., Lamb, A. (2005). Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents *26*: 343-356.

de Ridder, S., van der Kooy, F., Verpoorte, R., (2008). *Artemisia annua* as a self-reliant treatment for malaria in developing countries. J Ethnopharmacol *120*: 302–314.

Elfawal, M., Towler, M., Reich, N., Golenbock, D., Weathers, P., *et al.* (2012). Dried whole plant *Artemisia annua* as an antimalarial therapy. PLOS ONE *7*(12): 1-7.

Efferth, T. (2007). Willmar schwabe award 2006: antiplasmodial and antitumor activity of artemisinin- from bench to bedside. Planta Med *73*(4): 299-309. Retrieved August 20, 2012, from https://www.thieme-connect.com/ejournals/html/10.1055/s-2007-967138

Elford, B. C., Roberts, M. F., Phillipson, J. D., Wilson, R. J. M. (1987). Potentiation of the antimalarial activity of qinghaosu by methoxylated flavones. T Roy Soc Trop Med H *81*: 434-436.

Ferreira, J. F. S. and Janick, J. (1995). Floral morphology of *Artemisia annua* with special reference to trichomes. Int J Plant Sci *156*: 807–815. Retrieved June 5, 2012, from http://www.jstor.org/stable/2475112?seq=2

Ferreira, J. F. S. and Janick, J. (1996). Distribution of artemisinin in *Artemisia annua*. ASHS Press. 579-584. Retrieved September 7, 2012, from http://www.hort.purdue.edu/newcrop/proceedings1996/v3-578.html#*

Ferreira J. and Janick J. (2009). Annual wormwood (*Artemisia annua* L.) Retrieved September 20, 2012, from http://www.hort.purdue.edu/newcrop/cropfactsheets/artemisia.pdf

Ferreira, J. F. S., Luthria, D.L., Sasaki, T., Heyerick, A. (2010). Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. Molecules *15*: 3135-3170.

Fung, M., Sun. C., Gordon, W., Liou, R., Chang, T., Sun, W., Daar, E., Ho, D. (1992). Identification and characterization of a neutralization site within the second variable region of human immunodeficiency virus type 1 gp120. J. Virol. *66*(2): 848-856.

Grayer, R., Bryan, S., Veitch, N., Goldstone, F., Paton, A., Wollenweber, E. (1996). External flavones in sweet basil, *ocium basilicumi*, and related taxa. Phytochemistry *43* (5): 1041-1047.

Greenham, J., Harborne, J. B., Williams, C. A. (2003). Identification of lipophilic flavones and flavonols by comparative HPLC, TLC and UV spectral analysis. Phytochem. Anal. *14*: 100-118.

Greenwood, B., and Mutabingwa, T. (2002). Malaria in 2002. *Nature 415*: 670-672. Retrieved July 30, 2012, from http://geneplot.plasmodb.org/publications/naturev419_2002/intro.pdf

Hammoud, L., Seghiri, R., Benayache, S., Mosset, P., Lobstein, A., Chaabi, M., Leon, F., Brouard, I., Bermejo, J., Benayache, F. (2012). A new flavonoid and other constituents from *Centaurea nicaeensis* All. Var. walliana M. Nat Prod Res *26* (3): 203-208.

Harborne, J., Williams, C. (2000). Advances in flavonoid research since 1992. Phytochemistry *55*: 481-504.

Hoberg, E. (1999). Phytochemical and analytical investigation of Vitex agnus- castus L. Swiss Federal Institute of Technology. Zurich, Switzerland.

Hollman, P., Arts, I. (2000). Flavonols, flavones and flavanols – nature, occurrence and dietary burden. Journal of the Science of Food and Agriculture: 1081-1093.

Jäger, A., Saaby, L. (2011). Flavonoids and the CNS. Molecules *16*: 1471-1485.

Kale, A., Gawande, S. Kotwal, S. (2008). Cancer phytotherapeutics: role for flavonoids at the cellular level. Phytother. Res. *22*: 567–577.

Kraus, G., Roy, S. (2008). Direct synthesis of chrysosplenol d. J. Nat. Prod. *71*: 1961-1962.

Kremsner, P. G. and Krishna, S. (2004). Antimalarial combinations. Lancet *364*(9430): 285-294. Retrieved on October 9, 2012, from http://www.sciencedirect.com/science/article/pii/S0140673604166804#

Liu, K.C.-S.; Yang, S.-L.; Roberts, M.E.; Elford, B.C.; Phillipson, J.D. (1992). Antimalarial activity of *Artemisia annua* flavonoids from whole plants and cell cultures. Plant Cell Rep *11*: 637-640. Retrieved August 20, 2012, from http://www.springerlink.com/content/9dbedbe6e6a886c0/fulltext.pdf

Marco, J. A., Barbera, O., Rodriguez, S., Domingo, C., Adell, J. (1988). Flavonoids and other phenolics from *Artemisia hispanica*. Phytochemistry *27*: 3155-3159.

Nijkamp, F., Parnham, M. (2011). Phenolic compounds as immunodulators. Pp. 463 in *Principles of immunopharmacology*, 3rd, revised and extended ed. Basel: Birkhäuser.

Ono, M., Masuoka, C., Odake, Y., Ikegashira, S., Ito, Y., Nohara, T. (2000). Antioxidative constituents from *Tessaria integrifolia*. Food Sci. Technol. Res. *6* (2): 106-114.

Pandey, A. V., Tekwani, B. L., Singh, R. L., Chauhan, V. S. (1999). Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification

systems in malarial parasite. J. Biol. Chem *274*(27): 19383-19388. Retrieved June 4, 2012, from http://www.jbc.org/content/274/27/19383.full.pdf

Park, Y., Alencar, S., Aguiar, C. (2002). Botanical origin and chemical composition of brazilian propolis. J. Agric. Food Chem. *50*: 2502-2506.

Perez-Jorge, E.V. and Herchline, E. T. (2012, June 28). Malaria. Medscape Reference: Drugs, Diseases, & Procedures. Retrieved September 10, 2012, from http://emedicine.medscape.com/article/221134-overview

Pietta, P.-G. (2000). Flavonoids as antioxidants. J. Nat. Prod. *63*: 1035-1042.

Pras, N., Visser, J., Batterman, S., Woerdenbag, H., Malingre, T. (1991). Laboratory selection of *Artemisia annua* L. for high artemisinin yielding types. Phytochem Analysis *2*: 80-83.

Rasoanaivo, P., Wright, C. W., Willcox, M. L., Gilbert, B. (2011). Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions. Malaria Journal *10* (Suppl 1): S4. Retrieved May 21, 2012, from http://www.biomedcentral.com/content/pdf/1475-2875-10-s1-s4.pdf

Roberts, M. F., Timmermann, B. N., Mabry, T. J. (1980). 6-Methoxyflavonols from *Brickellia veronicaefolia (compositae)*. Phytochemistry *19*: 127-129.

Saleh, N. A. M, El-Negoumy, S., Abou-Zaid, M. M. (1987). Flavonoids of *Artemisia judaica, A. monosperma, and A. herba-alba*. Phytochemistry *26*: 3059-3064.

Sharon, A., Ghirlando, R., Gressel, J. (1992). Isolation, purification, and identification of 2-(*p*-Hydroxyphenoxy)-5,7-dihydroxychromone: A fungal-induced phytoalexin from *Cassaia obtusifolia*. Plant Physiol. *98*: 303-308.

Shilin, Y., Roberts, M. F., Phillipson, J. D. (1989). Methoxylated flavones and coumarins from *Artemisia annua*. Phytochemistry *28*: 1509-1511.

Smith, L. M. J., Bentley, S., Jones, H., Burns, C., Aroo, R. R. J., and Woolley, J.G. (2010). Developing an alternative UK industrial crop *Artemisia annua*, for the extraction of artemisinin to treat multi-drug resistant malaria. Aspects of Applied Biology *101*: 99-106. Retrieved June 7, 2012, from http://www.cabdirect.org/abstracts/20123173393.html

Tsimogiannis, D., Samiotaki, M., Panayotou, G., Oreopoulou, V. (2007). Characterization of flavonoid subgroups and hydroxy substitution by HPLC-MS/MS, Molecules *12*: 593-606.

Voirin, B. (1983). UV spectral differentiation of 5-hydroxy- and 5-hydroxy-3-methoxyflavonse with mono-(4';), di-(3',4') or tri-(3',4',5')-substituted b rings. Phytochemistry *22*: 2107-2145.

Voirin, B., Bayet, C., Faure, O., Jullien, F. (1999). Free flavonoid aglycones as markers of parentage in *Mentha aquatic, M. citrate, M. spicata* and *M.* x *piperita*. Phytochemistry *50*: 1189-1193.

Weathers, P. J., Arsenault, P. R., Covello, P. S., McMickelle, A., Teoh, K. H., Reed, D. W. (2011). Artemisinin production in *Artemisia annua*: studies *in planta* and results of a novel delivery method for treating malaria and neglected diseases. Phytochem Rev. *10*: 173-183.

Weathers, P. J., Towler, M. J. (2012). The flavonoids casticin and artemetin are poorly extracted and unstable in an *Artemisia annua* tea infusion. Planta Med *78*: 1024-1026.

WHO (2001). Antimalarial drug combination therapy: report of a WHO technical consultation. April 4-5. Geneva, Switzerland. Retrieved June 20, 2012, from http://whqlibdoc .who.int/hq/2001/WHO_CDS_RBM_2001.35.pdf

WHO (2011). World malaria report 2011. Geneva, Switzerland. Retrieved October 7, 2012, from http://www.who.int/malaria/world_malaria_report_2011/ WMR2011_noprofiles_lowres.pdf

Winkel-Shirley, B. (2001). Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology. Plant Physiol. *126*: 485-493.

Wiwanitkit, V. (2010). New emerging drug-resistant malaria. Int. J. Gen. Med. *3*: 327-329. Retrieved June 7, 2012, from www.dovepress.com/getfile.php?fileID=7893

Appendix I

Synonyms of chrysoplenetin, chrysosplenol-D, and cirsilineol

Chrysoplenetin/ Chrysosplenetin [1]	**Chrysosplenol-D/ Chrysoplenol D [2]**	**Cirsilineol [3]**
5,4'-Dihydroxy-3,6,7,3'-tetramethoxy flavone Quercetagetin-3,6-7,3'-tetramethyl ether Chrysosplenetin B	Chrysosplel D CHRYSOSPLENOLD chrysophenol D 3,6,7-Trimethoxy-3',4',5-trihydroxyflavone 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,6,7-trimethoxy-4H-1-benzopyran-4-one 5,3',4'-Trihydroxy-3,6,7-trimethoxyflavone Quercetagetin 3,6,7-Trimethyl ether	3',4',5,6,7-Pentahydroxyflavone 3',6,7-tri methylether 4',5-Dihydroxy-3',6,7-trimethoxyflavone 3',4',5,6,7-Pentahydroxyflavone 6,7-dimethyl ether 3',4',5-Trihydroxy-6,7-dimethoxyflavone Anisomelin 5,4'-Dihydroxy-6,7,3'-trimethoxyflavone 5-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-6,7-dimethoxy-4H-1-benzopyran-4-one 2-(4-Hydroxy-3-methoxyphenyl)-5-hydroxy-6,7-dimethoxy-4H-1-benzopyran-4-one 4',5-Dihydroxy-3',6,7-trimethoxyflavone

[1]Source: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=12216

[2]Sources: http://www.chemicalbook.com/ChemicalProductProperty_EN_CB81404010 .htm; http://biolod.org/PosMed/search?actionType=searchexec&condition=GeneIds&geneIds1=KID:C00004692&keyword=plant+WHERE+synapse&posSelectMode=false&rankScore=squ

[3]Sources: Brown, 2010; Saleh et al., 1987; http://www.chemicalbook.com/ChemicalProductProperty_EN_CB91387604.htm; http://www.chemicalbook .com/ProductChemicalPropertiesCB91387604_EN.htm

Appendix II

Other TLC Data

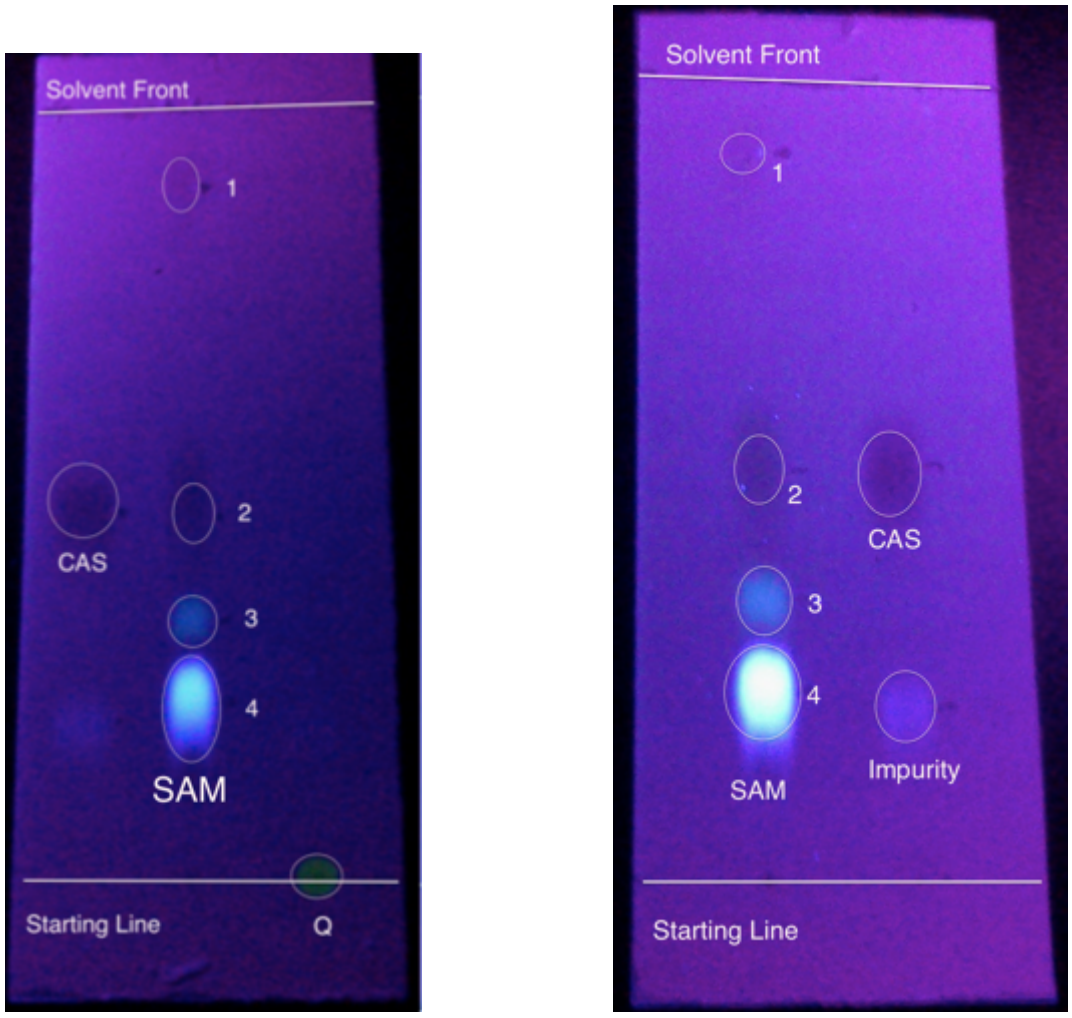

Fig. 49A and B. CAS and Q vs. SAM tip extract in 60:60:7:7) benzene-petroleum ether (b.pt. 35-60°C)-methyl ethyl ketone-MeOH on polyamide.

CAS (1 µg), SAM tip extract (approx. 77 µg), and Q (1 µg) were applied to both polyamide TLC plates (9 cm x 3 cm) with mobile phase (60:60:7:7) benzene-petroleum ether (b.pt. 35-60°C)-methyl ethyl ketone-MeOH. Plates were visualized under UV-light, 366 nm. R_f values of plate A (left) were: CAS: dark, 0.43; SAM (top to bottom): 1, dark, 0.88; 2, dark, 0.49; 3, dark, 0.40; 4, blue/green, 0.35; 5, white, 0.24; Q: yellow, origin. R_f values of plate B (right) were: CAS: dark, 0.48; SAM: 1, dark, 0.89; 2, dark, 0.48; 3, green, 0.31; 4, white, 0.22. For unknown reasons, spot 3 as seen in Fig. 29A and B was not present on Fig. 49A and B.

Appendix III

Preparation of TLC visualization reagents

p-Anisaldehyde Solution:

1. For a 50 mL *p*-anisaldehyde solution, 50 mL of glacial acetic acid was added to a 100 mL beaker.
2. Sulfuric acid (1 mL) was slowly added to the beaker.
3. *p*-Anisaldehyde (0.5 mL) was stirred as it was added to the beaker, using a glass rod.
4. After spraying the plate with the *p*-anisaldehyde solution, the TLC plate was heated at 105°C for approximately 10 minutes in order for the stains to appear (Pras *et al*., 1991).

$AlCl_3$ Solution:

1. 10 g $AlCl_3$ was slowly added to MeOH (500 mL) in a fume hood.
2. The solution was stored in a tightly sealed bottle.

Caution: Exothermic reaction occurs. Take extra precaution.

Appendix IV

^{1}H and ^{13}C NMR spectra of plant extract, CAS, CRY, and CRYD

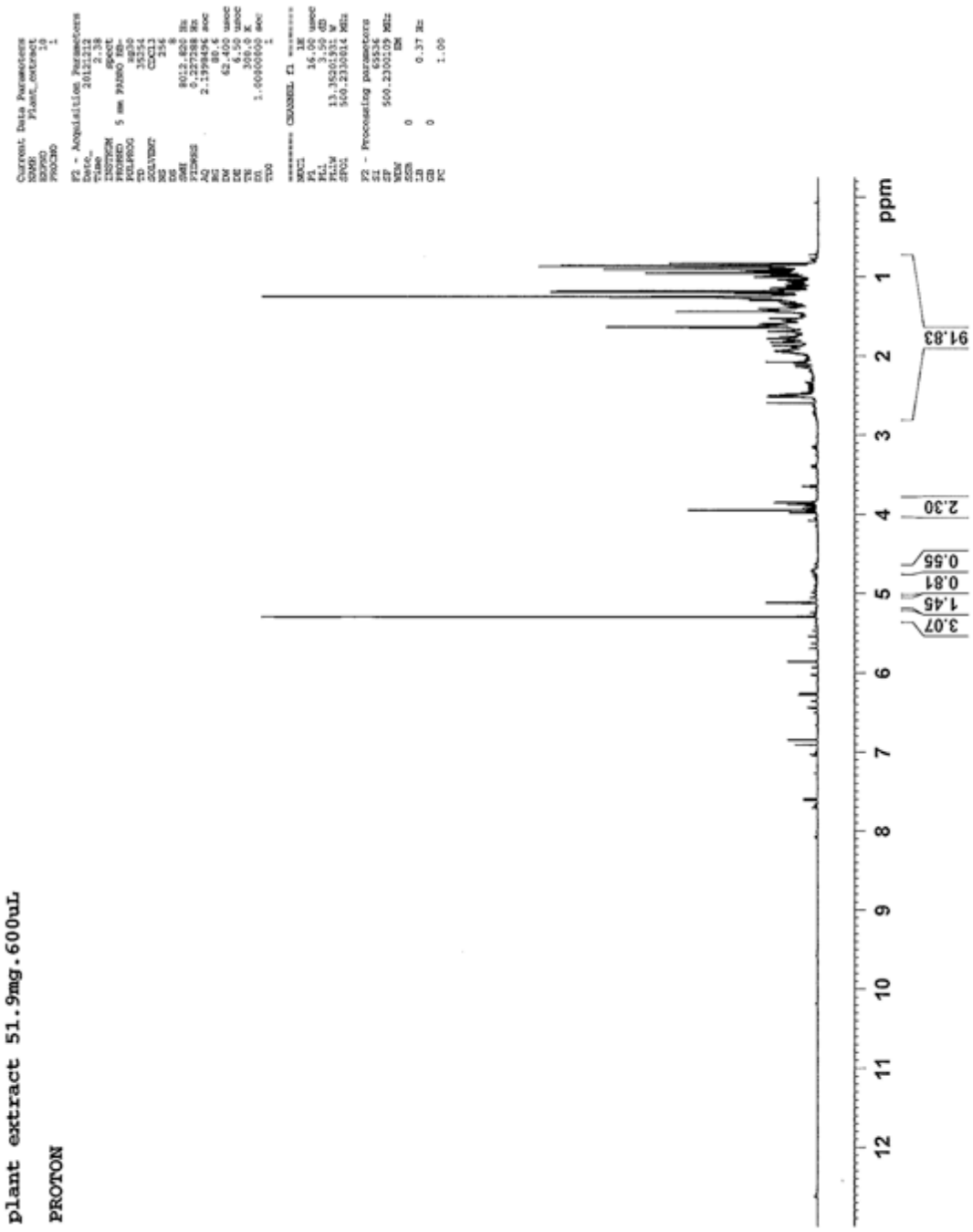

Fig. 34. Proton NMR spectrum of Plant Extract (fresh SAM tip).

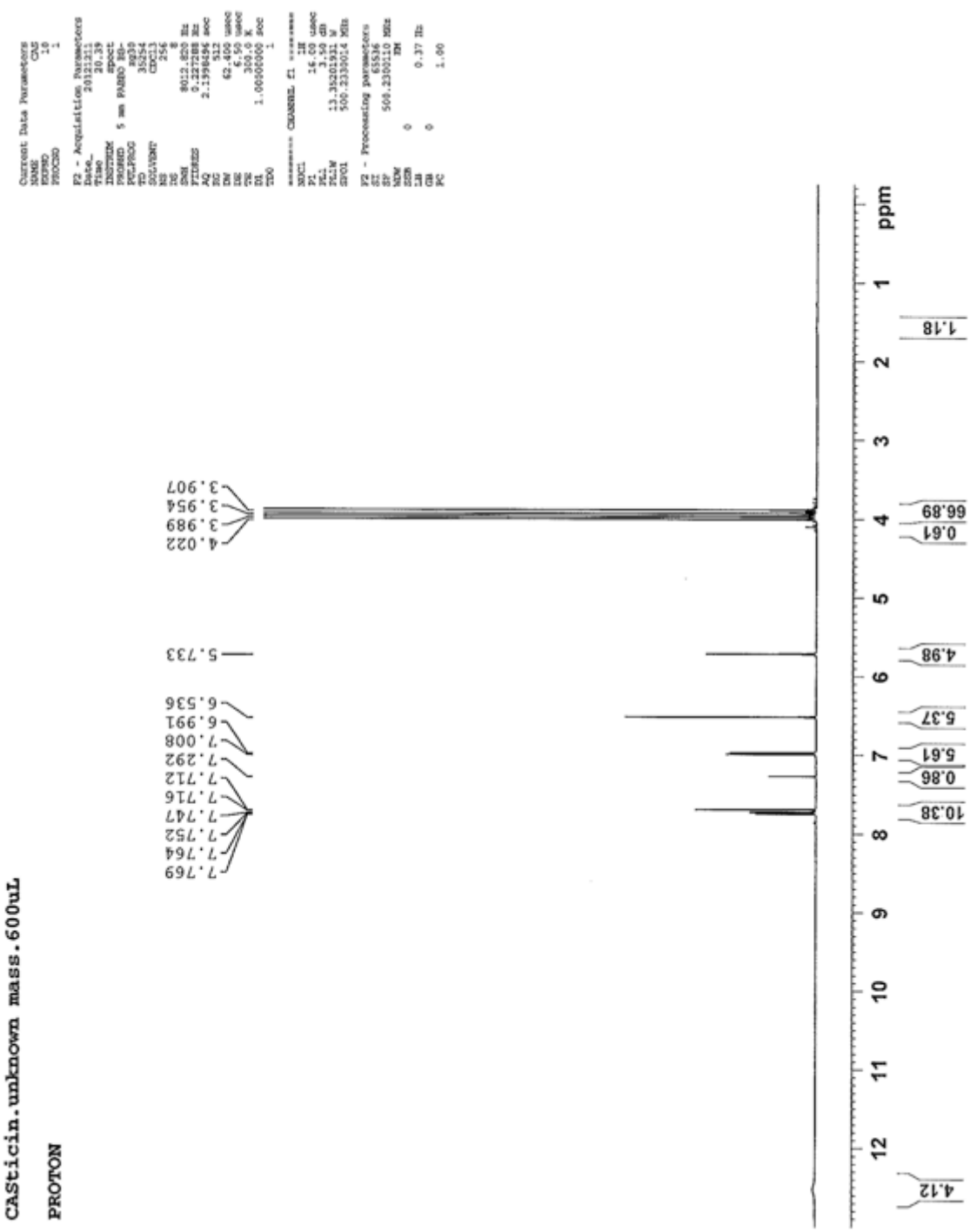

Fig. 35. Proton NMR spectrum of CAS.

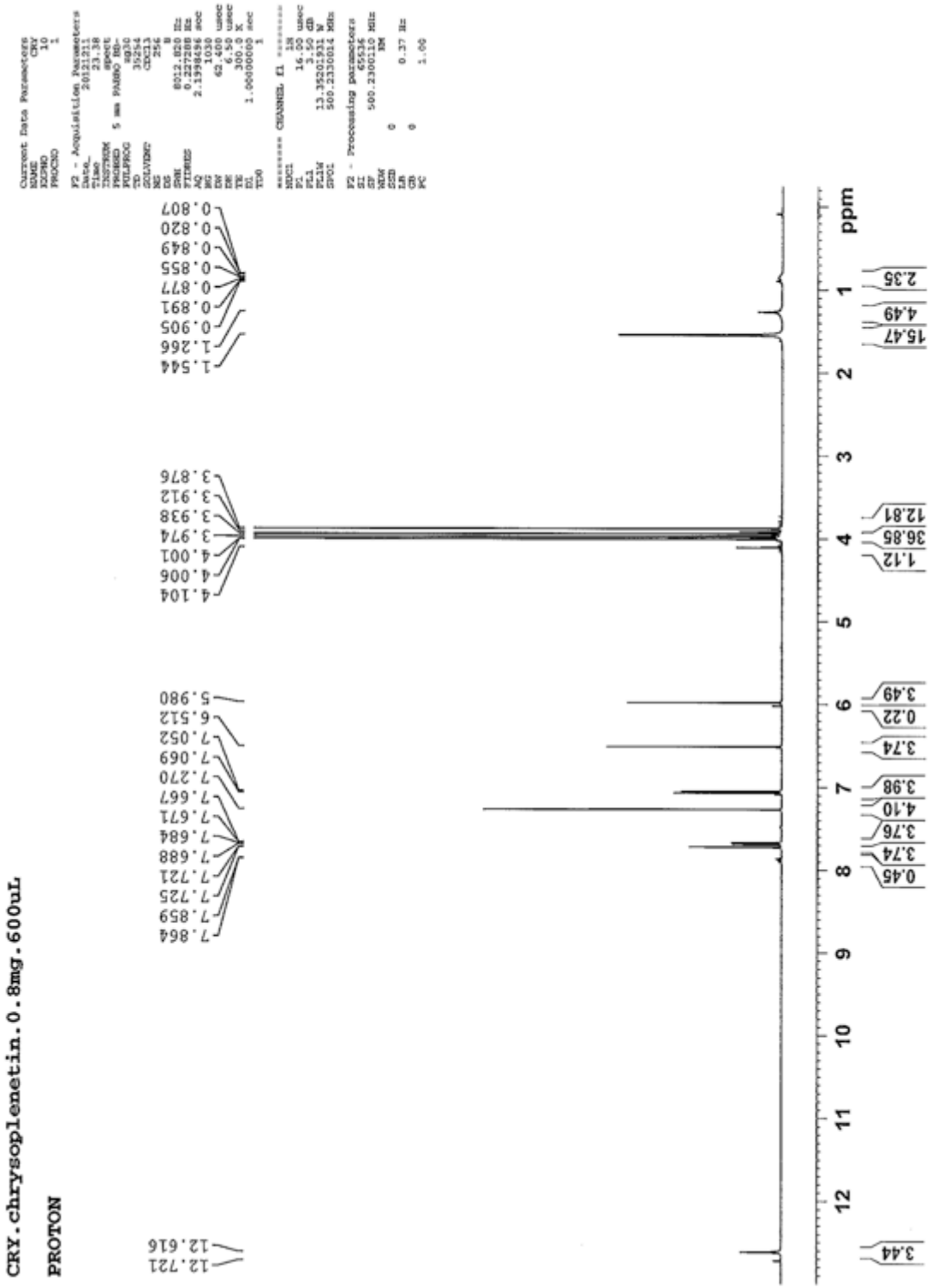

Fig. 36. Proton NMR spectrum of CRY.

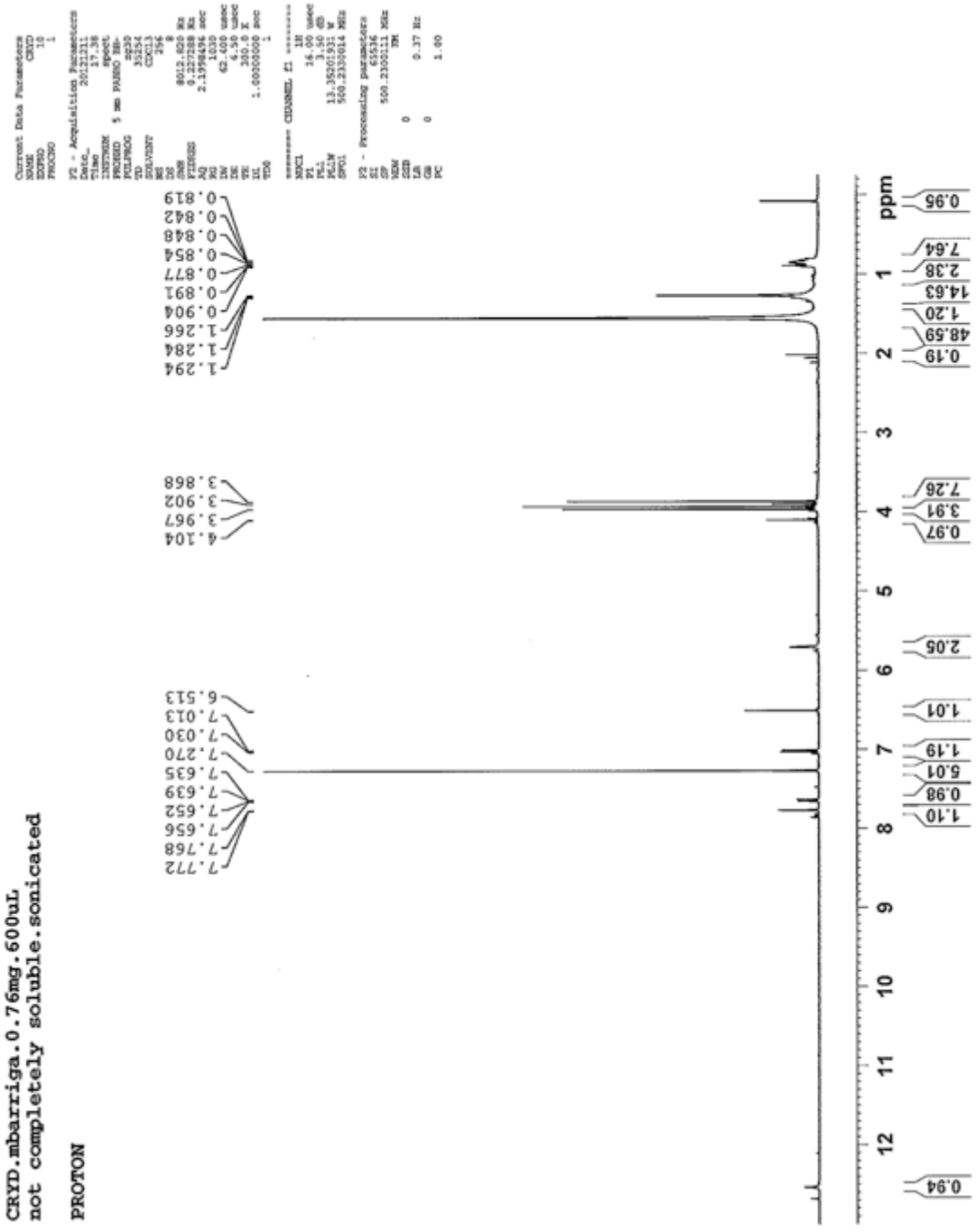

Fig. 37. Proton NMR spectrum of CRYD.

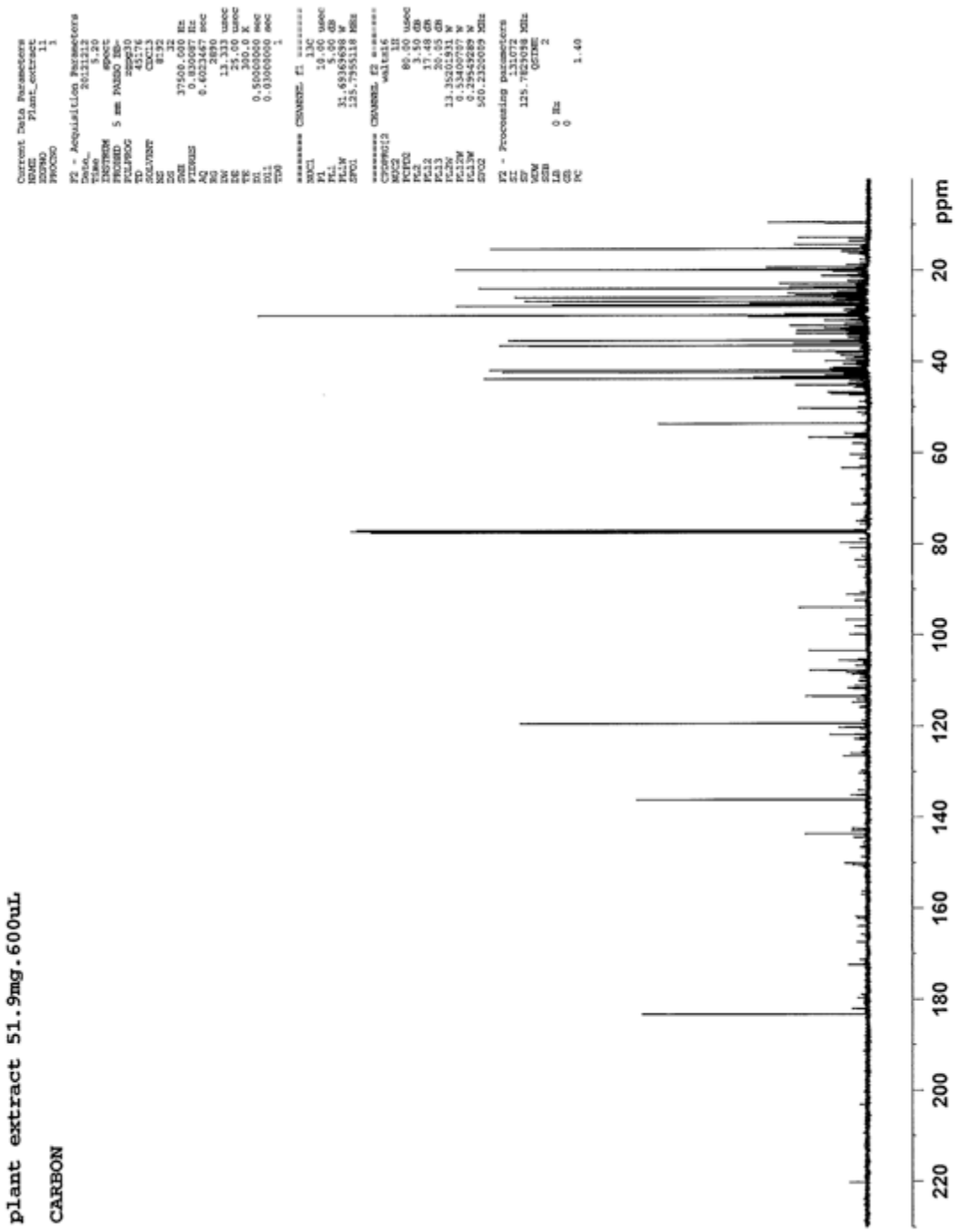

Fig. 38. Carbon NMR spectrum of Plant Extract (fresh SAM tip).

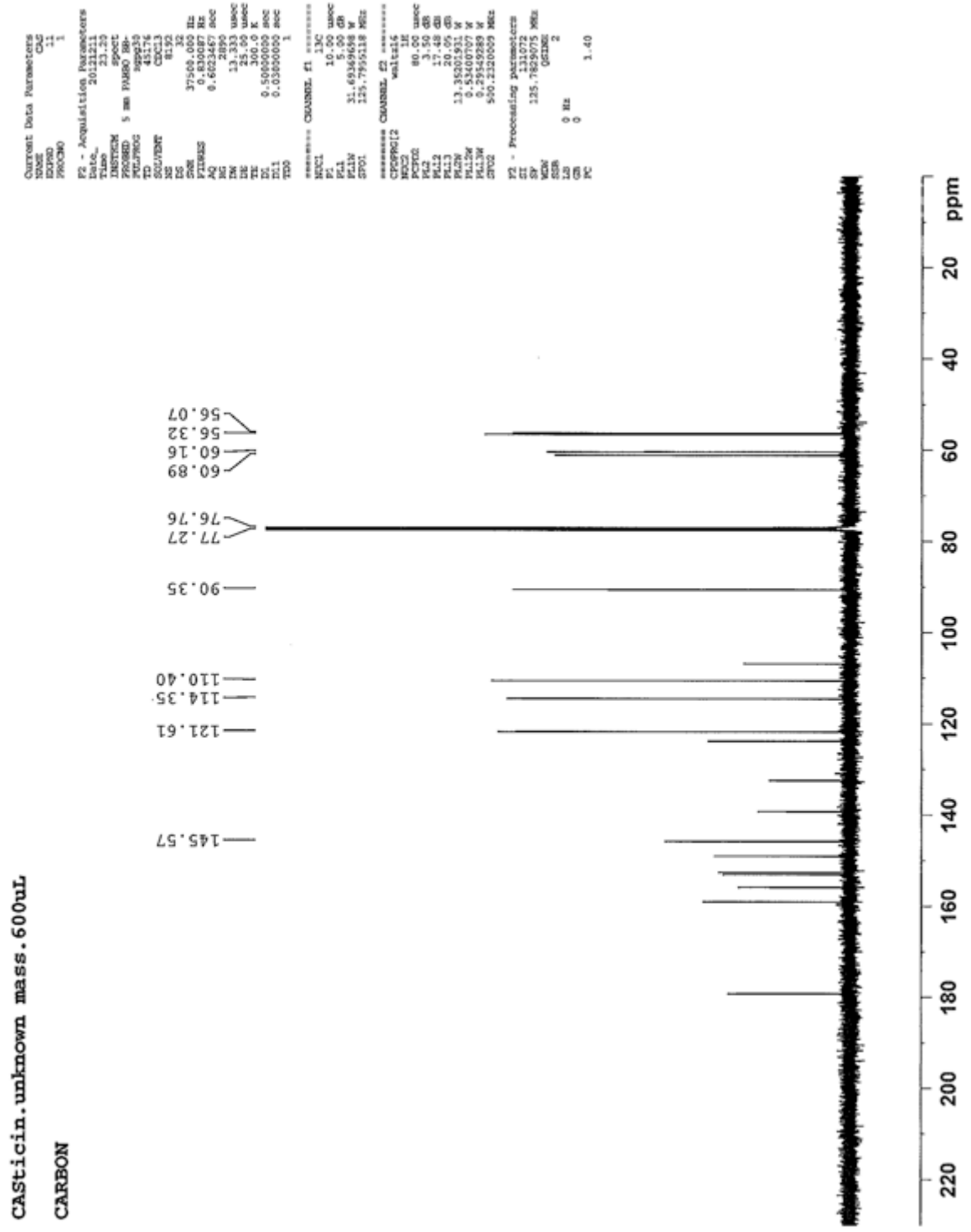

Fig. 39. Carbon NMR spectrum of CAS.

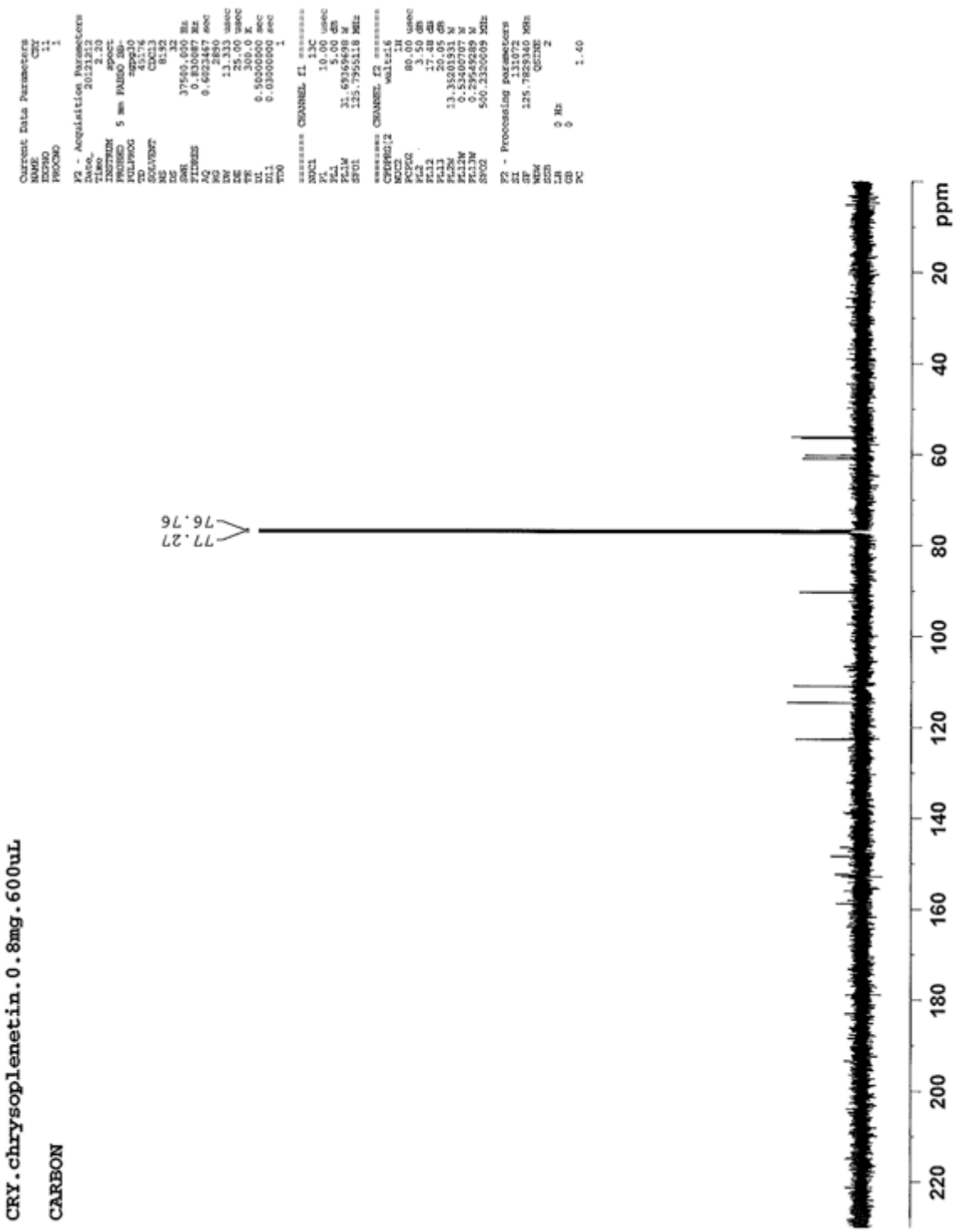

Fig. 40. Carbon NMR spectrum of CRY.

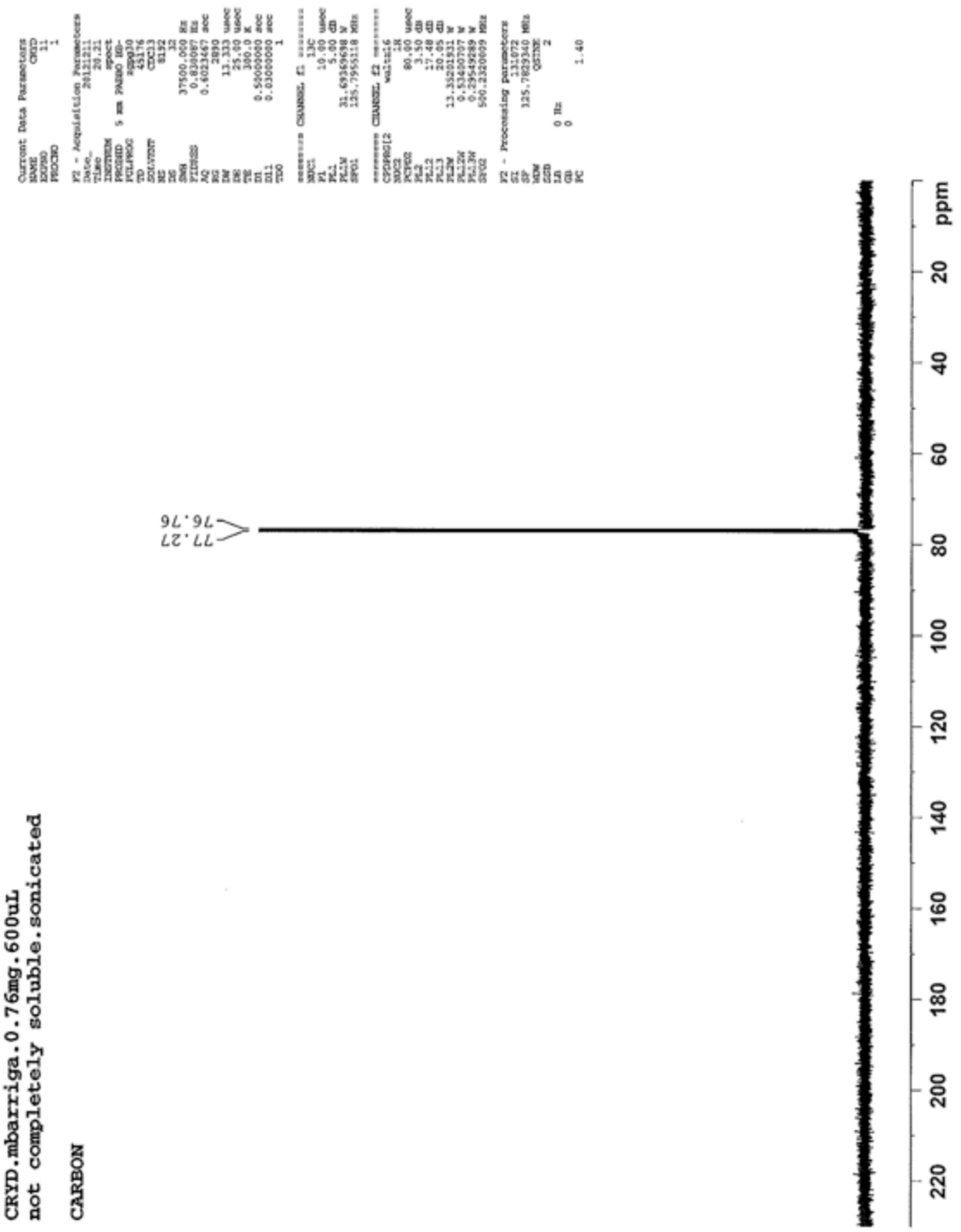

Fig. 41. Carbon NMR spectrum of CRYD.