Isolation of apigenin from *Antirrhinum majus*

By

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Abstract

Many adults have been exposed to Herpes Simplex Virus Type 1 (HSV-1), which manifests as a painful cold sore. Currently, Acyclovir is the most commonly used drug to reduce the number and severity of HSV-1 outbreaks. The purpose of this research is to isolate an anti-viral compound from plant material that will treat HSV-1. Snapdragon (*A. majus*) was selected for extractions due to the anti-viral activity found in its crude plant material. Detailed bioassay guided fractionation of *A. majus* plant material resulted in identification of a compound called apigenin. Although apigenin is a common flavonoid with documented anti-viral activity, it has not been reported previously in *A. majus*. A published comparison of EC_{50} values for apigenin and Acyclovir indicates that apigenin could potentially be a replacement drug for the treatment of herpes simplex viruses in Acyclovir-resistant patients.

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1. Introduction

1.1 Impact of Herpes Simplex Virus Type 1

Approximately 90% of adults in the United States have been exposed to Herpes Simplex Virus Type 1 (HSV-1).[1] The virus presents itself in its primary form as a small, painful lesion by the mouth known as a cold sore. During primary infection, HSV-1 inters the body *via* an open cut and infects the adjacent sensory neuron endings. The virus then travels down the neuron until it reaches the sensory ganglia in the spine, where it remains in a latent state. HSV-1 can reactivate and travel back down the sensory neuron to the epithelial tissue and cause another cold sore.[2] The virus itself contains double stranded DNA that codes for DNA polymerase, various binding proteins, and other items necessary for viral replication. It is enclosed in an icosahedral nucleocapsid, surrounded by a tegument and a lipid envelope containing envelope proteins.[3] The general structure of HSV-1 is shown in Figure 1.

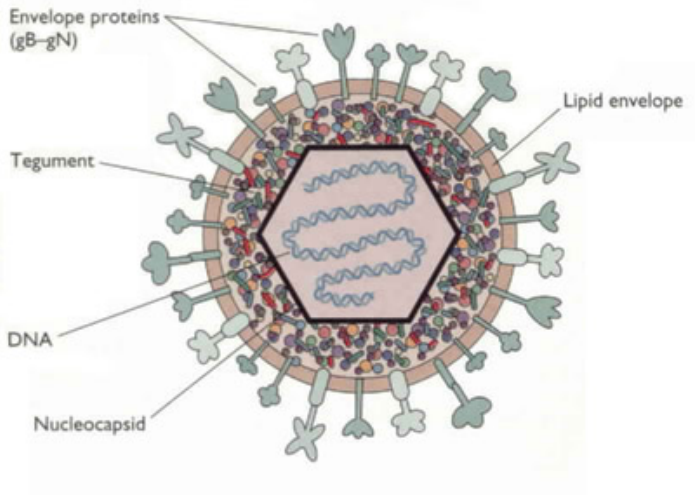

Figure 1. Structure of an HSV-1 particle.[4]

1.2 Treatments Used for HSV-1 Infection

As there is not yet a cure for HSV-1, the goal of current medicines is to reduce the number of outbreaks. Acycloguanosine (acyclovir), an acyclic nucleoside derivative of guanosine, shown in Figure 2, was synthesized in 1978 by Gertrude B. Elion and is still the most commonly used drug for the treatment of herpes viruses, shown in Figure 2.

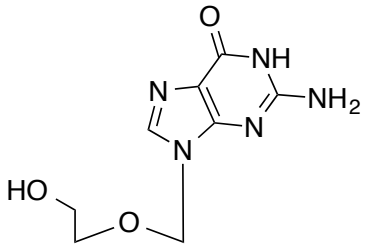

Figure 2. Structure of acyclovir.

Acyclovir's mechanism of action includes activation by phosphorylation *via* a thymidine kinase specific to herpes viruses.[5] Once mono-phosphorylated at the hydroxyl, acycloguanosine is then further phosphorylated to the triphosphate form, which inhibits viral DNA polymerase. The triphosphate form is incorporated into the growing DNA strand and blocks further replication. Due to the specificity of the viral thymidine kinase, acyclovir does not affect healthy cells. Therefore, acyclovir has a high inhibition of viral DNA polymerase and low cytotoxicity. The general mechanism of phosphorylation, common to all nucleotides, is illustrated in Figure 3.

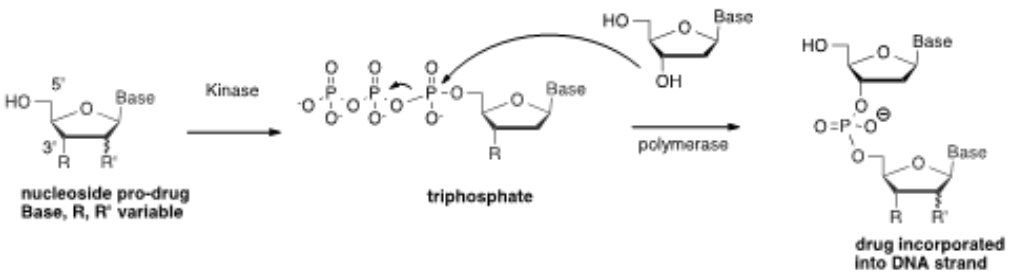

Figure 3. Nucleoside activation mechanism by phosphorylation.

1.3 Natural Product Isolation for Antiviral Drugs

The major focus of modern antiviral medicine has been on synthetic drugs. Only recently have research efforts been devoted to finding natural products as drugs, which involves isolating bioactive compounds from plants. For example, crude extracts of Brazilian medicinal plants traditionally used to treat diarrhea were screened for antiviral activity against rotavirus in 2012. Four of the fourteen plant species studied exhibited potential antiviral activity against rotavirus.[6]

1.4 Mechanisms of Action of Known HSV Drugs

The HSV life cycle consists of viral entry into the cell, dissociation of its tegument, transport of capsids to nuclear pore, and release of viral DNA into the host nucleus. Transcription and DNA replication (catalyzed by DNA polymerase) then occur followed by capsid assembly and DNA packaging. After initial infection, the virus can establish latency in the nervous system, which can lead to a repeat in the viral replication cycle and induction of another infection when stimulated. Several natural products have been shown target the viral attachment and entry stage. These types of compounds account for 73.4% of about 400 total reports.[7]

Most of these inhibit HSV attachment and fusion by targeting two or more glycoproteins, unlike synthetic drugs, which target DNA polymerase.[7]

All of the current classes of drugs licensed for HSV treatment target viral DNA replication, which is an important step for virus infection. For example, acyclovir, the most commonly used HSV drug, inhibits HSV-encoded DNA polymerase in the drug's active triphosphate form.

1.5 Natural Product Research Targeting HSV-1

With the renewed interest in natural products in drug discovery, plants used in traditional medicine have yielded many novel drug leads over the past 10-20 years. A total of 241 plants and microorganism extracts have been identified as having inhibitory activity against HSV.[8] Of those extracts, 162 contained activity in fractions obtained from highly polar solvents such as water, methanol, ethanol, and acetic acid.[8] Thus, many known anti-HSV molecules are polar, including polyphenols and flavones with multiple hydroxyl groups. According to reports from 2000 to 2013, 38.8% of fractions were identified as terpenoids and volatile oils, 24.7% as saccharides, and 20.0% as phenols and polyphenols.[8]

Many natural product classes known to exhibit various bioactivities include terpenes, terpenoids, alkaloids, and flavonoids. Examples of compounds in these classes are shown in Figure 4.

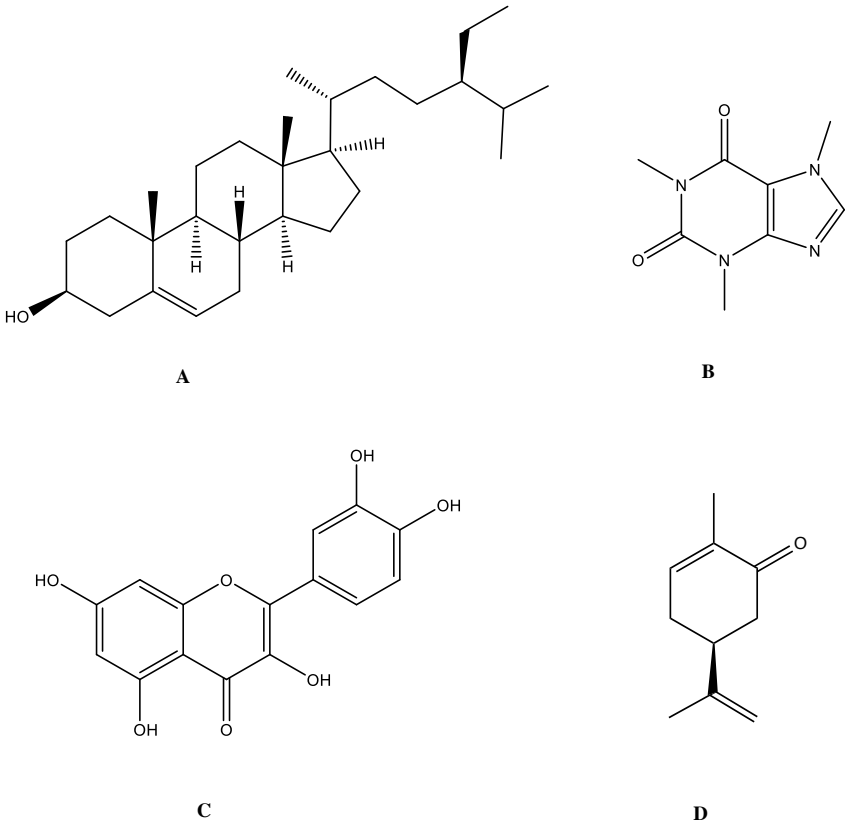

Figure 4. Examples of compounds from classes of natural products.

A 2006 study explained that S-carvone (Figure 4D), a terpene isolated from *Mentha spicata* (spearmint), exhibited high antioxidant activity.[9] The terpene β-sitosterol (Figure 4A), displayed anti-inflammatory activity in a 2009 study.[10] A study done in 2012 indicated that the alkaloid caffeine (Figure 4B) exhibited antioxidant activity.[11] Quercetin (Figure 4C), a common flavonoid, possesses many bioactivities, including antibacterial, anti-inflammatory, and anticancer bioactivities.[12-13]

Flavonoids, included in the class of phenols and polyphenols, are abundant in plant seeds, citrus fruits, olive oil, tea, and red wine. Reports indicate that free

hydroxyl groups of flavonoids are required for anti-HSV activity.[8] The basic structure of a flavonoid is shown in Figure 5.

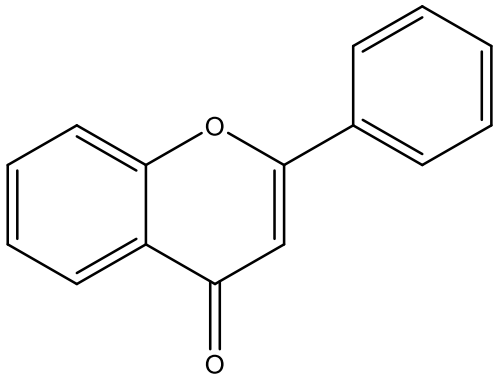

Figure 5. Flavonoid parent structure: most have hydroxyls on the aromatic rings.

At MTSU, we have access to plants used in traditional Chinese medicine through the Tennessee Center for Botanical Medicine Research (TCBMR). Crude extracts are typically prepared by heating dried plant material in a specific solvent. Over one hundred crude extracts from China have been screened at MTSU for activity against HSV-1. One of the extracts sent from China that showed antiviral activity was *Antirrhinum majus*, or snapdragon (Figure 6).

*Figure 6. Antirrhinum majus (*snapdragon) flowers.[14]

A literature search for antiviral activity of *A. majus* found no references. However, other identified bioactivities of *A. majus* includes antibacterial, antifungal, and antioxidant activities.

According to a 2013 study, extracts of snapdragon exhibited considerable antimicrobial activity against four bacterial and four fungal strains. In this study, it is reported that the hexanes fraction showed the least activity, and the ethyl acetate fraction showed the most activity compared to the other fractions that they tested, including the methanol, butanol, and chloroform fractions. [15]

Another 2013 study tested the antioxidant effects of snapdragon extracts against hydrogen peroxide. According to this study, snapdragon provided protection against damage in plasmid pBR322 DNA and was concluded to be a possible source of natural antioxidants.[16]

1.6 Statement of Project Goals

Although *A. majus* has been reported to possess bioactivities such as antibacterial, antifungal, and antioxidant activities, there have been no previous reports of antiviral activity in *A. majus*. Preliminary testing of the crude extracts of *A. majus* sent to MTSU from China exhibited antiviral activity against HSV-1. The goal of this Project was to use bioassay-guided fractionation of *A. majus* plants to identify the individual compound or compounds responsible for the antiviral activity against HSV-1.

2. Materials & Methods

Crude extracts of many different plants from China were screened at MTSU for bioactive properties such as anti-cancer, anti-viral, anti-protozoan, anti-inflammatory activity. Due to its anti-viral activity, *A. majus* (snapdragon) was chosen for further investigation and isolation of the compounds responsible. Activity was found in the hexanes fraction. Steps included initial extraction of dried plant material, leaves and stems, with methanol to give a crude extract. Partitioning by liquid-liquid extraction with solvents of increasing polarity afforded five extracts, one of which is the hexanes fraction. The active fractions were further purified by gravity column chromatography to give seven fractions. Those active fractions were then purified further by flash column chromatography. That step was repeated until flash column chromatography yielded an active semi-pure compound. Figure 7 illustrates the specific fractionation procedure.

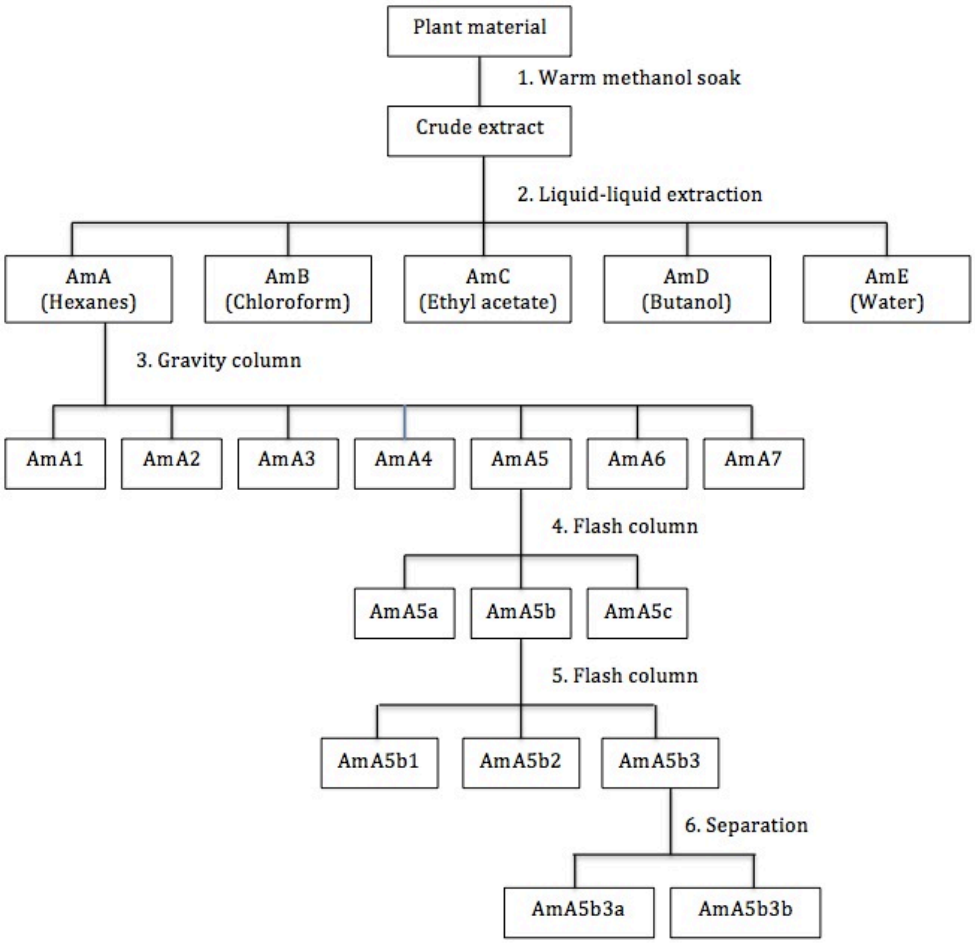

Figure 7. Flow chart of fractionation process of *A. majus* (Am).

2.1 Instruments, Materials, and Reagents

Twelve flats of snapdragons were purchased from a local nursery, Martin's Home and Garden, Murfreesboro, TN. Ethyl acetate, hexanes, and methanol were purchased from Fisher Scientific, (Pittsburgh, PA). Deutero-acetone (CD_3COCD_3) was purchased from Norell, Inc., (Landisville, NJ). Solvent evaporation was achieved using a Buchi rotary evaporator (Model RII, Buchi, Switzerland).

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were collected using a 500 MHz FT-NMR model ECA-500 JEOL (Peabody, MA). Chemical shifts are reported in parts per million using tetramethylsilane (TMS) as an internal reference. Splitting patterns are reported as following: s (singlet), d (doublet), t (triplet), m (multiplet), and dd (doublet of doublets). Compounds were dissolved in deutero-acetone. High-resolution electrospray ionization-mass spectrometry (ESI-MS) was performed at Notre Dame University, Indiana.

Thin layer chromatography (TLC) was performed on glass plates coated with silica gel and UV-active backing purchased from Fisher Scientific, Pittsburgh, PA. The plates were analyzed under UV light (254 nm) and stained with phosphomolybdic acid (PMA) purchased from Aldrich, Milwaukee, WI. Gravity column chromatography was performed using silica gel, 63-200 micron 70-230 mesh ASTM (reagent grade, Aldrich, Milwaukee, WI). Flash column chromatography was performed using silica gel, 60 Å 230-400 mesh ASTM, reagent grade, Fisher Scientific, Pittsburgh, PA.

2.2 Isolation and Purification Methods

After purchasing twelve flats of snapdragons, the leaves and stems were washed and dried completely, resulting in 221.6 g of dried plant material. The dried plant material underwent three 40 °C methanol soaks overnight (for three subsequent days), followed by solvent evaporation, to obtain 109.4 g of a crude extract, labeled “Am” for *A. majus*. Sequential liquid-liquid extractions were then performed on the crude extract using hexanes, chloroform, ethyl acetate, 1-butanol,

and water. The initial extraction of 109.4g afforded the hexanes fraction (labeled AmA) and an aqueous fraction. Extraction of the aqueous fraction with chloroform afforded the chloroform fraction (AmB) and a new aqueous fraction. That aqueous fraction was extracted with ethyl acetate to give an ethyl acetate fraction (AmC) and a new aqueous fraction. Final extraction of that aqueous fraction with 1-butanol gave the last two fractions, the 1-butanol fraction (AmD) and the final aqueous fraction (AmE). Table 1 illustrates the solvent used and the weights of the resulting fractions; active fractions are marked in bold.

Table 1. Initial liquid-liquid extraction information

Fraction number	Name	Weight (g)	Solvent
1	**AmA**	**6.070**	**Hexanes**
2	AmB	0.100	Chloroform
3	AmC	0.327	Ethyl Acetate
4	AmD	2.780	1-Butanol
5	AmE	Not weighed	Water

The hexanes fraction (AmA) was purified further by gravity column chromatography eluting with a gradient of 100% hexanes (AmA1) to 100% ethyl acetate (AmA7). Using 1.0 g of the hexanes fraction (AmA), seven subfractions were obtained, as shown in Table 2.

Table 2. Gravity column chromatography information

Fraction number	Name	Weight (g)	Solvent ratio (EA/hex)
1	AmA1	0.460	0:100
2	AmA2	0.120	15:85
3	AmA3	0.142	30:70
4	AmA4	0.059	45:55
5	**AmA5**	**0.025**	**60:40**
6	AmA6	0.024	75:25
7	AmA7	0.017	100:0

Of each of these subfractions, samples (5-9 mg) were sent to Dr. Stephen Wright's biology research lab for antiviral testing.

Activity against HSV-1 was tested using Vero cells and MacIntyre strain, human herpesvirus 1. Cells were treated using 100 μg/mL of extract and HSV at a multiplicity of infection of 0.1. Inhibition of the virus was determined using IC_{50} values, the concentration of the extract where the virus is reduced by half.

Subfraction AmA5 showed antiviral activity. A flash column was then performed on 15 mg of subfraction AmA5 using a gradient of hexanes to ethyl acetate and then an ethyl acetate and methanol flush, resulting in 3 more subfractions, denoted AmA5a, AmA5b, and AmA5c, as shown in Table 3.

Table 3. First flash column chromatography information

Fraction number	Name	Weight (g)	Solvent ratio
1	AmA5a	0.004	1:10 EA/hex
2	**AmA5b**	**0.031**	**10:1 EA/hex**
3	AmA5c	0.013	1:1 EA/MeOH

These weights may be incorrect due to improper evaporation of solvent. A sample containing 4-8 mg of each subfraction was sent for antiviral testing, and AmA5b exhibited antiviral activity. A flash column was then performed on 23.6 mg of AmA5b, resulting in four subfractions, labeled AmA5b1, AmA4b2, and AmA5b3, as shown in Table 4.

Table 4. Second flash column chromatography information

Fraction number	Name	Weight (g)	Solvent ratio (EA/hex)
1	AmA5b1	0.0038	1:10
2	AmA5b2	0.0008	1:5
3	**AmA5b3**	**0.0082**	**1:1**

Analysis of ^{1}H-NMR spectra indicated AmA5b1 and AmA4b2 had no identifiable signals. From the NMR deuterated chloroform ($CDCl_3$) solution of AMA5b3, a precipitate formed. The chloroform soluble portion was labeled AmA5b3A, and the precipitate, which was soluble in acetone, was labeled AmA5b3B. These subfractions were sent to for antiviral testing. Results indicated that the chloroform precipitate, AmA5b3B exhibited antiviral activity.

2.3 Spectroscopic data for AmA5b3B (apigenin)

Analysis of AmA5b3B spectroscopic data indicated that it is the flavonoid apigenin. ^{1}H-NMR (300 MHz acetone-d): δ 7.93(d, 2H, J=8.94 Hz), δ 7.01 (t, 2H, J=8.58), δ 6.62 (s, 1H, J=8.58), δ 6.53 (d, 1H, J=2.04), δ 6.24 (d, 1H, J=2.16). ^{13}C-NMR (125 MHz acetone-d): δ 182, 165, 164, 162, 160, 157, 129, 127, 124, 116, 105, 103, 99, 94. MS (ESI), *m/z* ($C_{15}H_{11}O_5$) calculated for 271.0601, found 271.0578 (M^+ + H).

3. Results & Discussion

3.1 Identification of Apigenin

In this study, the isolation and identification of potential antiviral compounds from snapdragon resulted in identification of a compound called apigenin, shown in Figure 8.

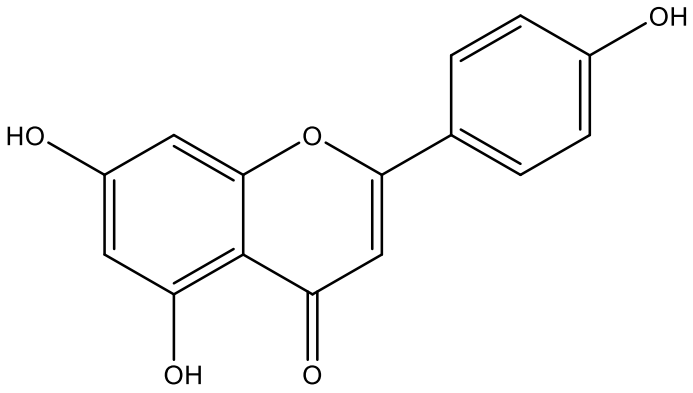

Figure 8. Structure of apigenin.

Apigenin has not been previously isolated from *A. majus*, but is a common flavonoid. It is most abundant in propolis, a glue-like material that bees use to protect their hives. Propolis is used as a health food because of its antibacterial, anti-inflammatory, and antiviral activities. Studies show that these biological

activities are due to propolis's rich composition of flavonoids.[17] Apigenin is also a commonly found flavonoid in the plant kingdom.[18]

The fraction identified as AmA5b3B was a white solid, and both TLC and NMR indicated that it was a pure compound.

Analysis of the ^{1}H-NMR spectrum (Figure 9) indicated that there were only aromatic signals, which rules out the possibility of a steroid. The two doublets at δ 7.93 and δ 7.01 indicated a para-disubstituted aryl ring. The ^{13}C-NMR, shown in Figure 10, indicated the presence of a ketone at δ 182. This analysis clearly indicated that the compound is a flavonoid, which would show only aromatic and vinyl hydrogen signals in the δ 5-8 region of the ^{1}H-NMR spectrum, as well as signals of a ketone carbonyl in the δ 170-190 region of the ^{13}C-NMR spectrum. Upon conducting a literature search, comparison of flavonoids containing para-disubstituted aromatic rings showed that AmA5b3B is apigenin. A comparison of the ^{1}H-NMR spectra of AmA4b3B and purchased apigenin was performed, shown in Figure 11. Mass spectrometry (Figure 12) also confirmed that the compound had the correct molecular weight of apigenin. This was evidence by the signal at 271.0578 m/z for M + H of apigenin ($C_{15}H_{10}O_5$) as seen in Figure 12.

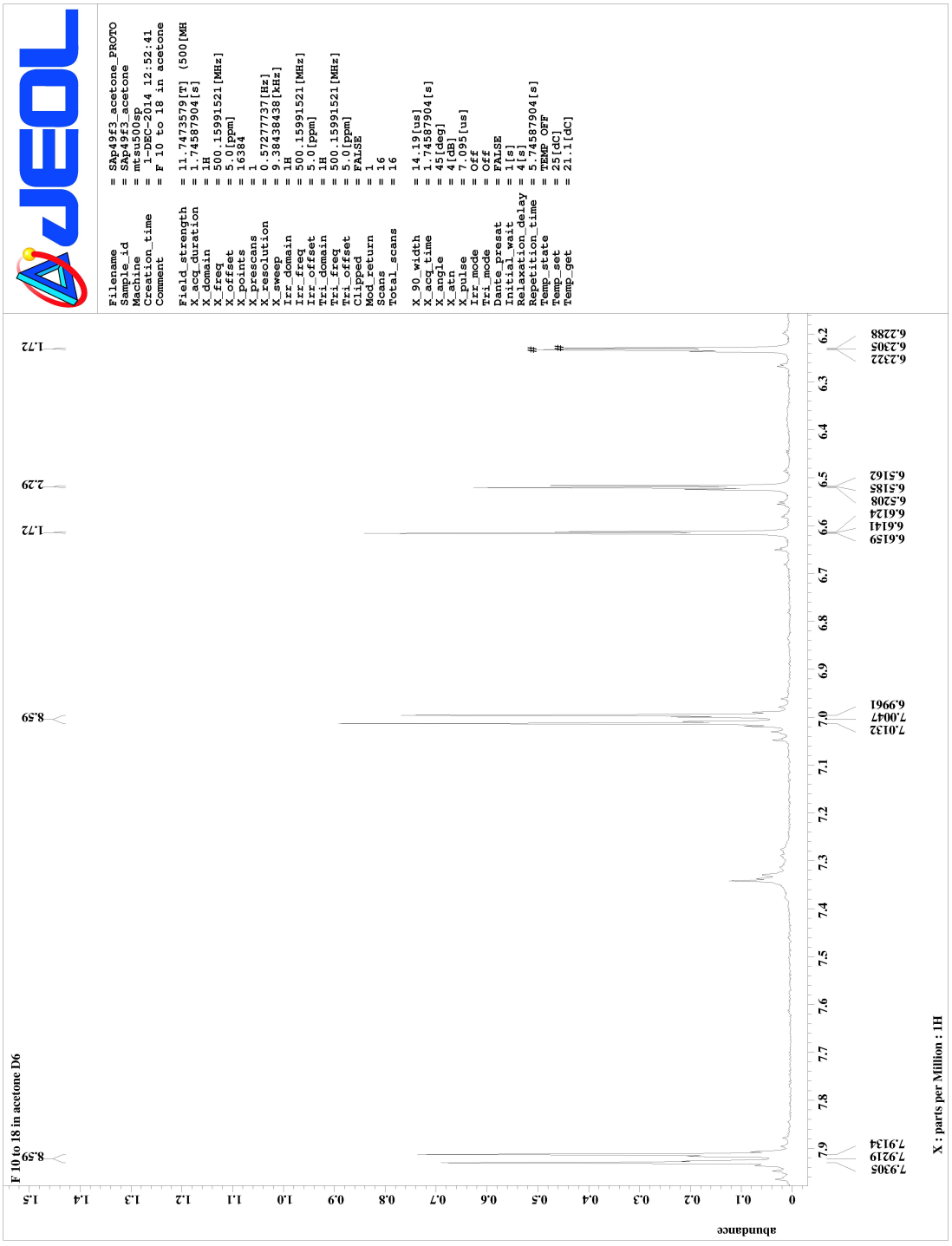

Figure 9. ^{1}H-NMR (500 MHz) of apigenin isolated from snapdragon.

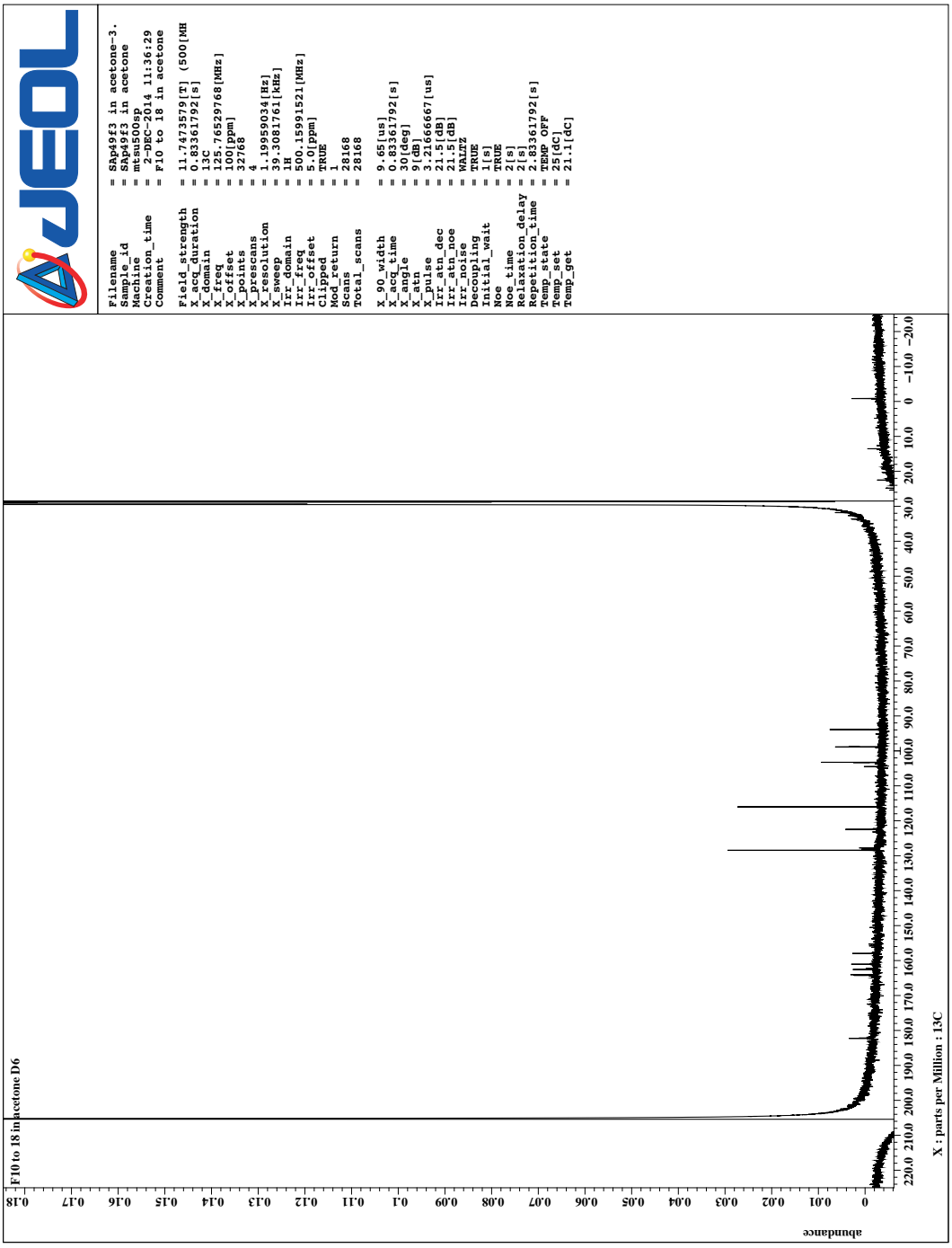

Figure 10. ^{13}C-NMR (125 MHz) of apigenin isolated from snapdragon.

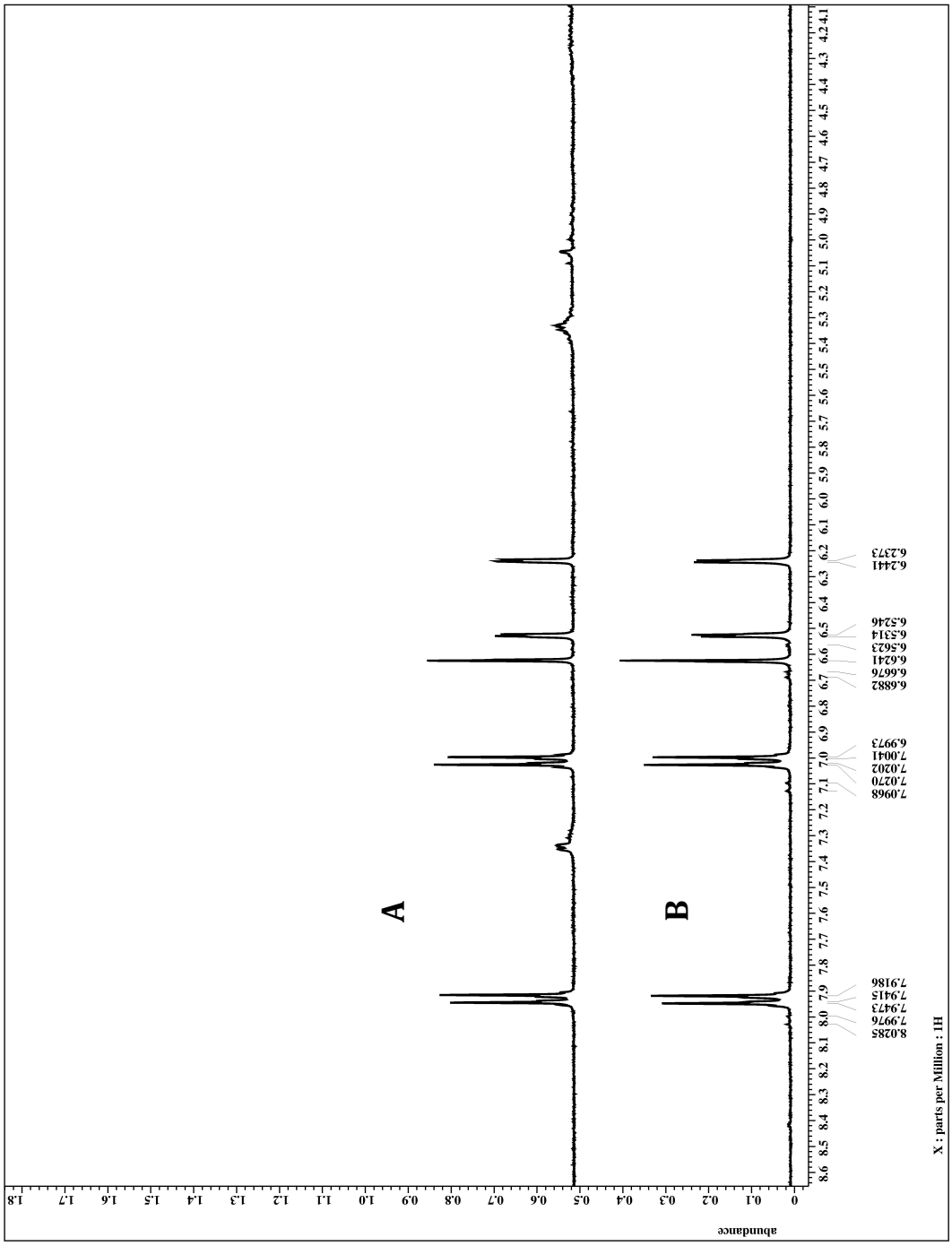

Figure 11. Overlay of ^{1}H-NMR spectra of A) the isolated compound and B) purchased apigenin.

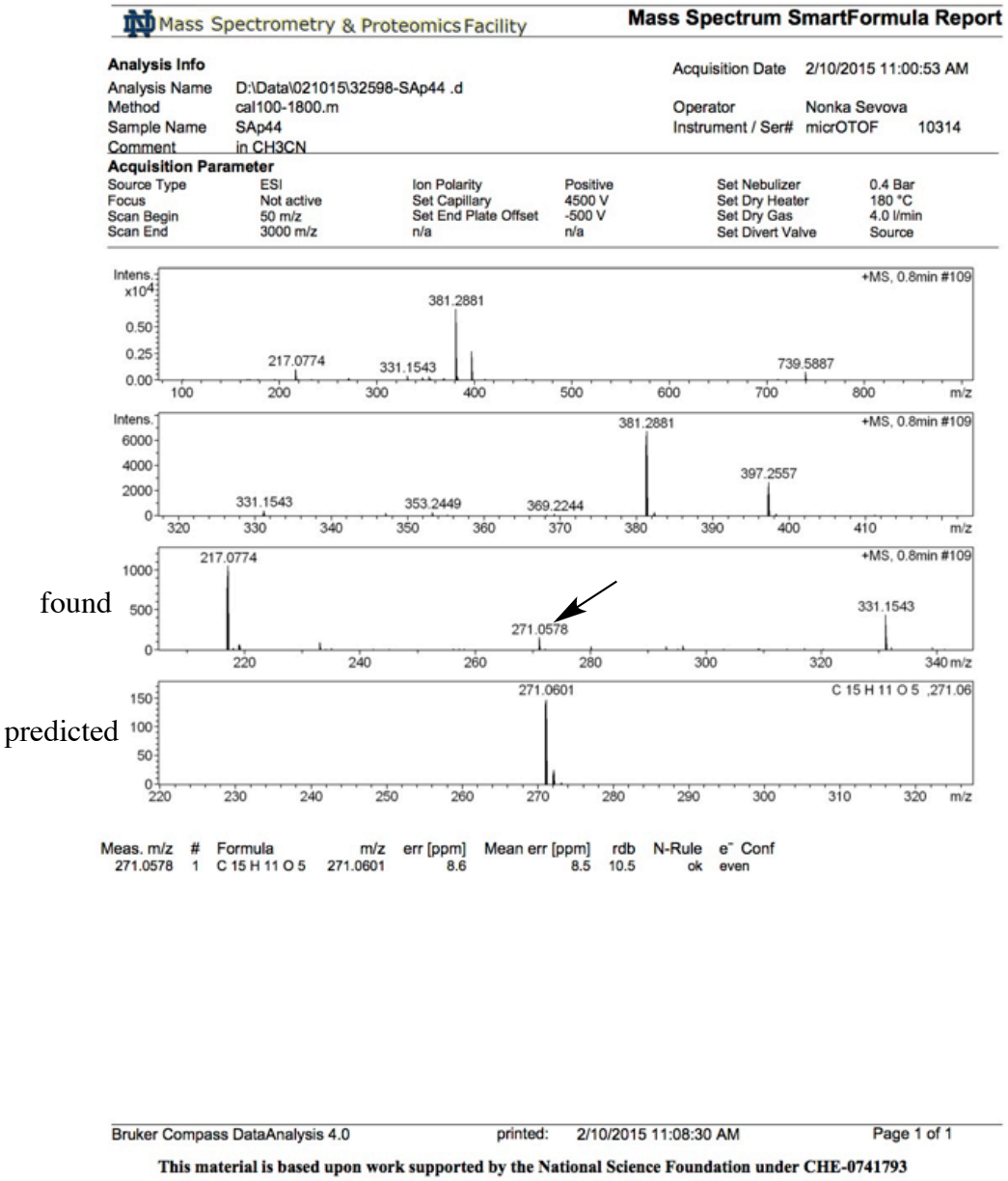

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Figure 12. Mass spectrometry of apigenin isolated from snapdragon. The third spectrum (labeled found) is the region from 210-345 *m/z* where the molecular ion for apigenin is found (indicated by the arrow). The fourth spectrum (labeled predicted) is the predicted high-resolution mass for apigenin. The first two spectra are the expanded regions showing more impurities.

3.2 Known activity of Apigenin

Flavonoids have been shown exhibit bioactivities such as anti-cancer, anti-inflammatory, anti-bacterial, anti-oxidation, and anti-viral activities.

In a study done in China in 2008, apigenin displayed significant anti-influenza virus activity by inhibiting neuraminidase activities. Neuraminidase is an enzyme that catalyzes the cleavage of sialic acid, the unit prior to viral budding from the cell. Drugs such as Tamiflu and Relenza are neuraminidase inhibitors. This study sought to identify the structure-activity relationship of flavonoids on neuraminidase inhibitory effect. Apigenin and similar flavonoids that differed in substitution at one or more carbon were tested to locate the individual structure or structures that play a role in neuraminidase inhibitory activity.[19]

A study done in 2005 concluded that IC_{50} values against HSV of apigenin were lower than those of acyclovir, but EC_{50} values (in a living system) of apigenin and acyclovir were similar. This result suggests that apigenin could potentially be a replacement drug in the treatment of herpes simplex viruses in acyclovir-resistant patients.[20]

A 2008 study found sage extracts to possess antiviral activity against HSV-1 and HSV-2. Apigenin and luteolin glucuronides were isolated and determined the cause of this antiviral activity. When Herpes Simplex Viruses Type 1 and Type 2 were treated with the plant extracts for one hour prior to infection of the host cell, plaque formation was significantly reduced. This suggests that the extracts interact with structures of the viral envelope, which are necessary for entry into the host

cell. This study concluded that flavonoids are able to inactivate herpes simplex viruses by blocking receptors on the surface of the virus and host cells.[21]

A 1992 journal article suggests the possibility of synergy between two or more compounds. Antiviral assays performed on apigenin showed that it was inactive against avian herpes, but the combination of quercetin and apigenin was more active than each individual compound alone. Other combinations of flavones also produced the same result. The study proposes the theory that only drugs with different modes of action could exhibit synergism.[22]

Although apigenin is a known compound with previously reported antiviral activity, it has not previously been reported as a component of *A. majus*. One area for further study would be to investigate structure-activity relationships of derivatives of apigenin. Another would be to investigate the mechanism of action of apigenin's anti-HSV activity.

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