# AN INVESTIGATION INTO THE BIOACTIVE COMPONENTS OF *EUPHORBIA HIRTA* AND *PANDANUS TECTORIUS*

by

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## ABSTRACT

### AN INVESTIGATION INTO THE BIOACTIVE COMPONENTS OF *EUPHORBIA HIRTA* AND *PANDANUS TECTORIUS*

For centuries, natural products have served as a major source of medicinally relevant biological activity. From over-the-counter cold medicines to chemotherapeutic agents, many drugs today are primarily composed of bioactive naturally occurring compounds. For this reason, natural products serve as key resource for research seeking to discover new methods of treatment for an array of illnesses and ailments including cancer, viral infections, parasite infections, and inflammatory responses. Ethno-pharmacology, such as Traditional Chinese Medicine (TCM), has advocated the usage of plant and herbal extracts as medicinal remedies for thousands of years. For this reason, drug discovery research involving natural products has focused on these claims by attempting to isolate the source of bioactivity in the reportedly bioactive extracts. While some of these investigations have been successful, the healing nature of many ancient remedies remains a mystery. Described herein is an investigation into the bioactive components of two plants used in TCM: *Euphorbia hirta* and *Pandanus tectorius*. Using a bioassay guided approach, crude extractions and subsequent purifications were performed, leading to the isolation and identification of two pure compounds: (±)-lariciresinol and (+)-4,5'-(1S,3aR,4S,6aR)-hexahydrofuro[3,4-c]furan-1,4-diyl)bis(2-methoxyphenol.

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# CHAPTER I: INTRODUCTION

## Background

For centuries, naturally occurring compounds have had a significant impact on the treatment and cure of disease. Even before the implementation of modern medicine and pharmacology, ancient cultures relied on herbs and plant preparations to treat an array of illnesses. Traditional Chinese Medicine (TCM) is thought to be the oldest of these ethno-medicines, originating before 2800 B.C. Practitioners of TCM have long believed that the components of certain natural products, such as ginseng possess medicinal properties.[1] A type of historical pharmaceutical reference book, the *Chinese Materia Medica*, lists ancient herbal formulas as treatments for specific symptoms. Ginseng, for example, is said to "cool fire in the lung".[2] A closer look at the specific compounds composing some of the TCM remedies has led to the discovery of well-known natural products, such as the alkaloid ephedrine. Isolated from the Chinese plant *Ephedra sinica*, ephedrine gained popularity for its therapeutic effects around 1924.[3,4] Since this time, it has been used as a stimulant and treatment for asthma. TCM, however, promoted the usage of the *Ephedra* herb, *Má huáng,* for these for centuries, even before the source of its effects had been identified.[5] The structure of ephedrine is shown in Figure 1.

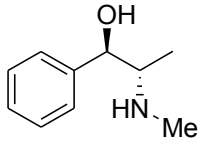



**Figure 1. Structure of ephedrine**

Research into the plant extracts of TCM has been invaluable to the discovery of treatments for worldwide epidemics such as malaria. The drug artemisinin, referred to as *Qinghaosu* in Mandarin was first isolated from the Chinese plant *Artemisia annua* in 1979 and is now a standard treatment option against the malaria-causing parasite *Plasmodium falciparum*.[6] The structure of artemisinin is shown in Figure 2.

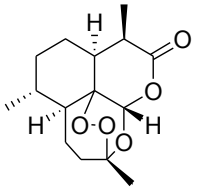



**Figure 2. Structure of artemisinin**

Febrifugine is another compound with high *P. falciparum* inhibition. It was identified as the active component of the Chinese plant *Dichroa febrifuga*, (*Cháng Shan* in Chinese) in 1948, after TCM had advocated the usage of the herb for malarial fevers for centuries.[7] In 1999, it was determined that certain synthetic febrifugine derivatives would inhibit strains resistant to another anti-malarial drug, chloroquine.[8] The structure of naturally occurring febrifugine is shown in Figure 3.

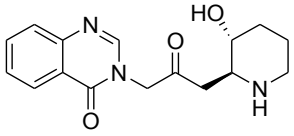



**Figure 3. Structure of febrifugine**

The discovery of drugs such as febrifugine and artemisinin demonstrate the significance of continued research in the field of TCM, particularly as the problem of drug resistance escalates worldwide. Unfortunately, very few other TCM remedies have led to the approval of novel pharmaceuticals. Many of these remedies call for a formula of specific natural products, so research in this area has required that numerous compounds be isolated and identified in order to determine the source of bioactivity. Despite its ancient history and growing popularity, scientific evidence on the effectiveness of TCM remains limited, requiring more research into the chemical constituents of its natural product remedies.

Other worldwide interests in natural product research have had prolific effects on health care. The opiate morphine, identified in 1804 by the German chemist Friedrich Sertürner, was the first botanical bioactive product to be isolated and identified.[9] Produced in the opium of the plant *Papaver somniferum*, morphine has served the world as a potent analgesic since it was first distributed in 1827. There are now more than 250 synthetic derivatives of morphine, many of which maintain the same pentacyclic alkaloid, (or nitrogen containing), structure as morphine and also serve as potent analgesics. Codeine is one such example. Although it is also a naturally occurring opiate from *Papaver somniferum*, it can be easily synthesized from morphine by replacing one of morphine's hydroxyl (-OH) groups with a methoxy ($-OCH_3$) group.[10] The similarity between these two structures is depicted in Figure 4 with the differences highlighted in red.

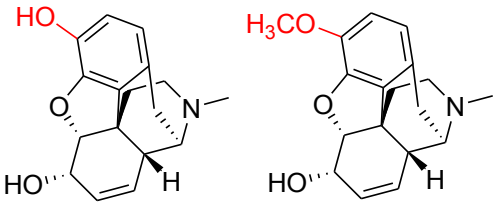



**Figure 4. Structure of morphine (left) and codeine (right)**

## Current Research

Today, natural products comprise the vast majority of preventative and clinical treatments options available worldwide. According to a study published in the *Journal of Natural Products* in 2012, 70% of the drugs approved for human clinical trials, known as new chemical entities (NCE), by the United States Food and Drug Administration (FDA) between 1981 and 2010, were either naturally occurring, naturally derived, or mimicked naturally occurring compounds.[11] This comprised 77% of the anti-cancer agents, 78% of the anti-parasitic agents, and 80% of anti-viral agents identified during this 30-year period.[10,11] Such data demonstrate that natural products are of invaluable medicinal significance. However, the continued existence of many diseases worldwide for which there is no treatment or cure requires that further research be conducted in this area.

It is suspected that only a small percentage of the world's natural product resources have been tested for bioactivity. Approximately 250,000 species of plants are thought to exist, but few have actually been tested as pharmacological agents.[12] This idea, combined with the fact that nearly 40% of the most popular prescription drugs on the market are naturally derived, establishes the urgent need for the continued discovery of new medicinal natural compounds. Natural products from TCM and other cultures

serve as target molecules in many drug discovery laboratories. The World Health Organization (WHO) advocated such research in a 2002 report entitled *Protection and Promotion of Traditional Medicine*.[13] In this report, WHO encouraged research focusing on bioactive natural products. In particular, they stressed the importance of natural product research relating to the discovery of treatments and cures for fatal diseases like malaria, human immunodeficiency virus, and others. The discovery of such compounds could benefit poor, disadvantaged communities in India, Africa, and other parts of the world by providing scientific evidence for the traditional treatments that serve, in many communities, as the only treatment option available.[13]

**Botanical Medicine**

While animals, microbial organisms, and marine life can all serve as sources of natural products, many natural compounds used today are derived from plants. In fact, 25% of all medications administered worldwide and 11% of the drugs considered by the WHO to be basic and essential contain botanical components.[13, 14] The large majority of plant derived bioactive compounds are secondary metabolites. They are referred to as such because they are not required in order to sustain the life of the plant, but rather exist to support the plant's secondary metabolism by participating in a defense mechanism, for example. Of the secondary metabolites, most bioactive naturally occurring compounds can be broadly classified into one of three categories. They are terpenes, phenylpropanoids, and alkaloids.[15, 16]

***Terpenes***

Terpenes, also referred to as isoprenoids or terpenoids, are derived from isoprene and are further classified into six sub-categories based on the number of isoprene units they contain. The prefixes mono-, sesqui-, di-, sester-, tri-, and caro- are given to terpenes possessing 2, 3, 4, 5, 6, and 7 isoprene units respectively. Monoterpenes are volatile oils that may be responsible for a plant's aroma while diterpenes may play a role in the plant's defense mechanisms as well as other biosynthetic pathways.[15] Several terpene structures are provided in Figure 5 with the isoprene units highlighted in red and blue. S-Carvone, a monoterpene, is the most abundant compound in spearmint oil.[17] The diterpene forskolin has been used as an over-the-counter dietary supplement and as a treatment for glaucoma.[18] Oleanane is a triterpene found in flowering plants.[19]

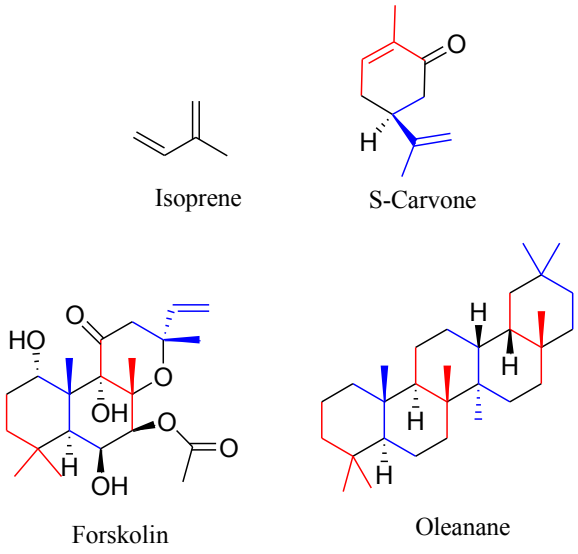
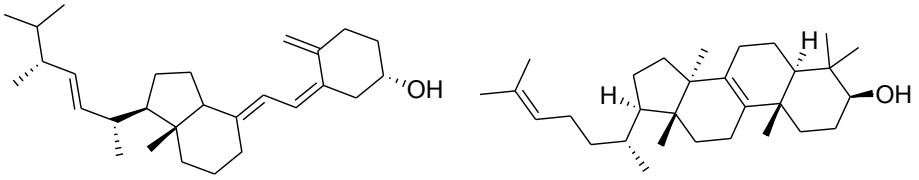



**Figure 5. Examples of terpenes**

The triterpenoid class is comprised of phytohormones and phytosterols such as lanosterol and Vitamin D depicted in Figure 6. The Chinese plant *Panax ginseng*, more commonly referred to as simply ginseng, has been studied for bioactivity of its triterpenoid compounds.[20] Ginseng is thought to have many medicinally relevant uses in TCM including the treatment of diabetes.[21] The anti-malarial drug artemisinin discussed previously and shown in Figure 1 is an example of a sesquiterpene.[6]




Vitamin D Lanosterol

**Figure 6. Examples of triterpenoids**

***Phenylpropanoids***

Phenylpropanoids, another class of naturally occurring compounds, are classified based on their derivation from the amino acid phenylalanine shown in Figure 7. They are composed of a phenyl group attached to a three-carbon chain.

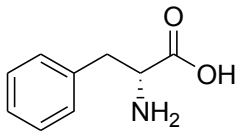



**Figure 7. Structure of phenylalanine**

The phenylpropanoid class of compounds includes the cinnamates, flavonoids, natural phenols, and polyphenols, which are generally characterized by a tricyclic system.[15] The flavonoids are responsible for the pigmentation in plants and the bitter taste of some fruit, among other things. The compound quercetin, shown in Figure 8, falls into this category and has been used in combination with other compounds as a Chinese remedy for cardiovascular disease.[22]

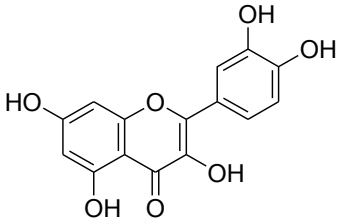



**Figure 8. Structure of quercetin**

Some phenylpropanoids are known for their cytotoxic effects. Podophyllotoxin, isolated from the Chinese plant *Podophyllum versipellis*, is one example of a cytotoxic phenylpropanoid.[23] The structure of podophyllotoxin is given in Figure 9.

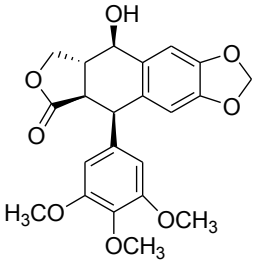
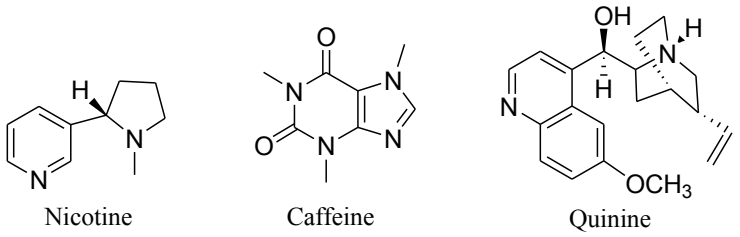



**Figure 9. Structure of podophyllotoxin**

***Alkaloids***

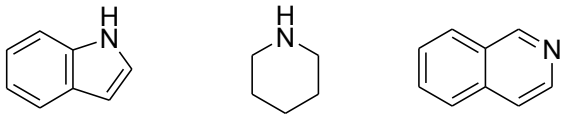
The class of compounds characterized as alkaloids are perhaps the most biologically active. They are classified based the presence of a nitrogen-containing group and include stimulants such as caffeine and nicotine as well as the potent analgesic morphine, discussed previously.[9, 16] The chemotherapeutic agent quinine is another example of a popular alkaloid. Many of these nitrogen-containing compounds contain neuroactive properties as a result of their structures. The structures of nicotine, caffeine, and quinine are given in Figure 10.




Nicotine Caffeine Quinine

**Figure 10. Examples of alkaloids**

The alkaloids are generally classified into sub-categories based on the structure of their nitrogen-containing ring. These sub-categories are indole, piperidine, and isoquinoline.[15] These heterocyclic structures are depicted in Figure 11.




Indole Piperidine Isoquinoline

**Figure 11. Heterocyclic structures**

The indole compounds are derived from the amino acid tryptophan. Compounds such as the psychoactive drug reserpine and the poison strychnine belong to this class. Their structures are shown in Figure 12.

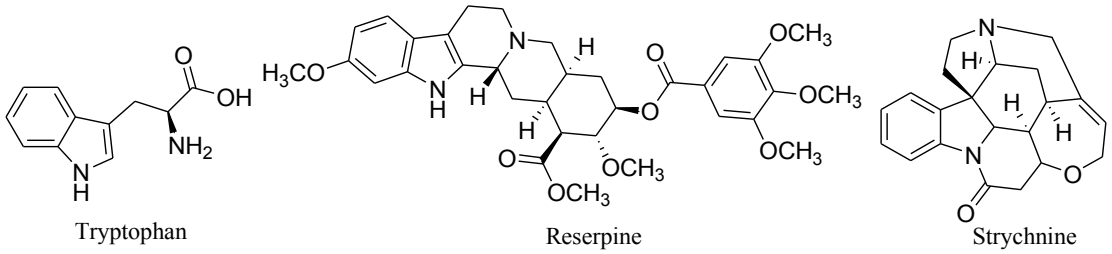



**Figure 12. Examples of indoles**

The highly addictive, psychoactive drug cocaine is a member of the piperidine class. First isolated from the coca plant *Erythroxylan coca*, cocaine is deemed a "triple reuptake inhibitor" because it simultaneously blocks the actions of the serotonin, norepinephrine, and dopamine transporters. Much more dangerous than any approved central nervous stimulant, cocaine can be fatal in even small doses.[24, 25] Similar to the phenylpropanoids, some of the isoquinoline compounds are derived from the amino acid phenylalanine, shown in Figure 7, while others are derived from the amino acid tyrosine. Many of the compounds in this class, such as morphine, shown in Figure 4, have been studied in an effort to understand the addictiveness that accompanies their analgesic properties.[25] The structures of tyrosine and cocaine are shown in Figure 13.

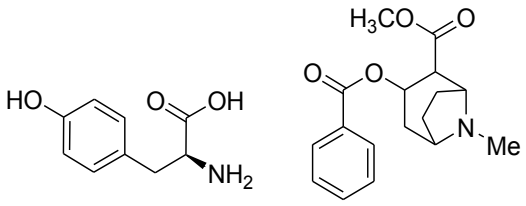



**Figure 13. Structure of tyrosine (left) and cocaine (right)**

## Procedural Background

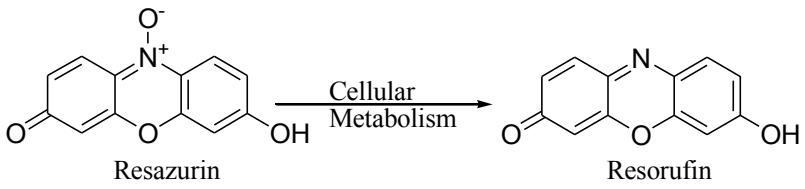
The isolation and identification of bioactive compounds from crude plant material is a stepwise process that begins with the testing of the crude material for a particular activity or inhibitory effect. For example, the extract may be tested for inhibition against a breast cancer cell line, virally infected eukaryotic cells, or as an anti-inflammatory agent. Once it is established that a crude extract possesses the bioactivity of interest, various procedures can be utilized for isolating the sources of the activity. Because the crude extract contains many different compounds, this isolation process typically requires several steps and various different methods of purification. Such methods include different types of chromatography, distillation, and solid or liquid phase extraction.[26] Regardless of the method, each step in the isolation procedure can produce several fractions composed of one or more compounds. Each fraction can then be tested for activity using bioassays. The isolation procedure then continues until each fraction is only composed of one compound. Identification of the isolated compounds can then be achieved using various spectroscopic methods, in addition to x-ray crystallography.

***Bioassay for Cytotoxicity***

*In vitro* cell viability bioassays provide quantitative data on the amount of living cells present in a system. For this reason, they are effective research tools for the isolation of bioactive compounds. From the quantitative results of these assays, one can determine a substance's effectiveness at inhibiting a particular pathogen, or even its ability to distinguish between cancerous and non-cancerous cells.[26] Bioassays can be performed as many times as necessary throughout the isolation process.

The fluorometric bioassay method is performed using a multi-well cell plate. Mammalian cells from live tissue culture are added to each well along with appropriate media. The live pathogen, such as a bacteria, protozoan, or virus is also added to each well in addition to the plant extract, but not necessarily in that order. The order of this addition depends on the goal of each individual experiment. After incubating for a pre-determined amount a time, usually 24-48 hours, a solution containing the compound rezasurin is added to each well. The multi-well plate is then allowed to incubate for a short amount of time before a fluorometric reading is performed.[27]

The fluorometric assay technique is a reliable method because it depends on the mammalian cell's biochemical processes to convert blue, non-fluorescent rezasurin to pink, fluorescent resorufin. A living cell is able to perform the necessary chemical reaction that reduces resazurin to resorufin.[28] This reaction is given in Figure 14 below.




Resazurin Resorufin

**Figure 14. Reduction of resazurin to resorufin**

Once placed in the fluorometer, the fluorescence of each well is measured quantitatively and compared against control wells in the same multi-well plate. From this data, one can calculate the percentage of living cells in each well or in the plate overall, as well as the percent to which a compound or extract inhibited a particular pathogen. This procedure can be performed as many times as necessary during the isolation procedure, because it works as a guide for determining the location of a bioactive substance among many fractions.[28] Isolation of a bioactive compound using this procedure is an example of "bio-assay guided isolation".[15]

***Extraction and Isolation***

The extraction and isolation procedure begins by grinding up the dry plant components including the roots, leaves, bark, and stems. An extraction is then performed on this material using a soxhlet apparatus, which allows the components of the crude plant material to be dissolved in a liquid solvent. During this procedure, the dry plant material is placed in the thimble. Solvent is placed in the round bottom flask and flows through the soxhlet extractor in the manner shown by the dotted arrows in Figure 15.

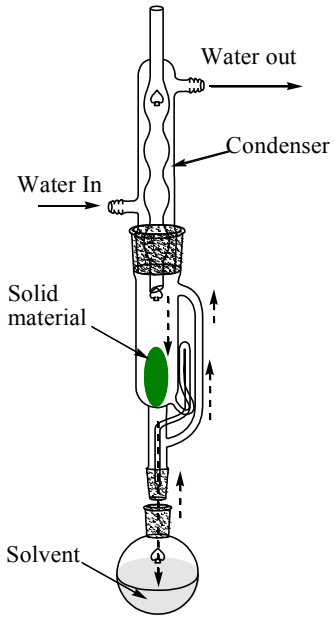



**Figure 15. Soxhlet Apparatus**

For approximately 24-48 hours, a distillation occurs in which the heated solvent runs into the thimble containing the crude material and back down into the original flask. Any vapors resulting from this are immediately condensed. As the solvent passes through the thimble, the components of the crude plant material slowly dissolve, traveling with the solvent back down to the flask. At the end of this extraction, the crude extract components are contained in the solvent flask and the material left in the thimble is waste. A crude extraction at this level can also be performed by heating the dry plant material for several days in methanol and then filtering out the impurities that did not dissolve. [29]

Once this initial extraction is complete, the crude extract, containing many different compounds, can then be further fractionated. This usually begins with a liquid-liquid extraction. During this procedure, the chemical constituents of the extract are

separated based on their polarity. This procedure typically results in a total of five fractions from the initial extract. Solvents used in this process can include: hexane, chloroform, ethyl acetate, 1-butanol, and water. Each resulting fraction is subsequently analyzed using a bioassay method such as the one described above. Based on the results of the bioassay, it is then decided which fractions to further purify. Subsequent isolation techniques are performed using column chromatography and high-performance liquid chromatography (HPLC), during which the components of the fractions are again separated based on their polarity. Between each fractionation, a bioassay can be performed to guide the researcher in isolating the bioactive component(s) of the plant.[27-29]

Each fractionation following the liquid-liquid extraction can result in ten or more fractions depending on the fraction that is being analyzed. Hexane, for example, is typically the first solvent used in the liquid-liquid extraction. Due to its hydrophobicity, it will extract a large number of compounds from the crude fraction, such as large fats and long hydrocarbon chains. This will result in a larger number of fractions during the isolation procedure. The hexane extract can contain up to 95% of the weight after a liquid-liquid extraction. Bioactive compounds are rarely found in the hexane fraction so it is typically used as a filter to remove compounds that are not of significant interest. Ethyl acetate, however, is a more polar solvent and will typically only extract approximately 5% of the components of the crude fraction. Purification of an ethyl acetate fraction can therefore be accomplished in fewer steps following the initial liquid extraction.

During the purification process, the components of each fraction can be analyzed using Thin Layer Chromatography (TLC).[15] This is a useful tool in determining the

purity of a fraction, as different compounds will theoretically show up as different spots on the TLC plate as depicted in Figure 16.

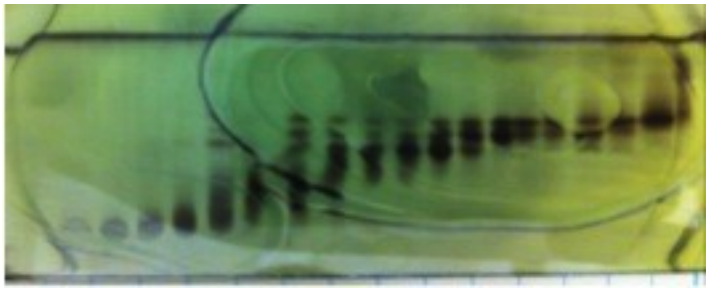


**Figure 16. Example of Thin Layer Chromatography[30]**

***Compound Identification***

Upon isolating a pure compound, several methods can be employed for identification purposes. One-dimensional Nuclear Magnetic Resonance Spectroscopy (NMR) methods such as $^{1}$H-NMR and $^{13}$C-NMR provide information on the nature and relationship of hydrogen and carbon atoms. Other two-dimensional spectroscopy methods provide information on the relationships and interactions between different atoms in the molecule. Correlation Spectroscopy (COSY) and Nuclear Overhauser Effect Spectroscopy (NOESY) analyze the relationship between different hydrogen atoms in the molecule. Heteronuclear Multiple Quantum Coherence (HMQC) allows for the determination of direct hydrogen-carbon bonds, while Heteronuclear Multiple Bond Coherence (HMBC) allows for the determination of hydrogen-carbon relationships across multiple bonds as well as carbon-carbon bonds. Distortionless Enhancement by Polarization Transfer (DEPT) analysis displays the presence of primary, secondary, and tertiary carbon atoms. Mass Spectrometry is also used to verify the molecular formula of

the compound and crystallization procedures can be used to verify its structure. In addition to this, optical rotation can verify the enantiomeric purity of a compound.[31]

***Procedural Limitations***

The isolation procedure described above presents several limitations and disadvantages. Generally, a large amount of crude product is required in order to isolate enough of the pure compound for analysis or identification. A typical isolation will yield <0.1% of the final product from the crude plant. For this reason, it is less expensive and time consuming to identify the compound and devise a synthesis for it rather than continue to isolate it. Chemical laboratories investigating new bioactive natural products have frequently encountered this limitation.

Additionally, the results of a bioassay using crude fractions can be easily misinterpreted for several reasons. This is further complicated by the fact that small amounts of otherwise bioactive compounds may not give significant results in a bioassay when tested as part of a crude fraction, but do give significant results when tested individually. These compounds, for example, may only exhibit significant pathogen inhibition alone or in higher concentrations, but not in the presence of other compounds in the fraction, or in low concentrations.

Furthermore, all plants contain a multitude of compounds that, upon fractionation, exhibit different colors. After being exposed to solvents and fractionation, a dry, crude plant material that was once brown can produce fractions that are bright green, yellow, red, or orange. This can create technical problems when conducting a fluorescence bioassay, particularly of a fairly crude fraction. The pigments in the plant extract may

cause a higher fluorescence reading and could therefore be mistakenly interpreted as a living cell on the bioassay. For this reason, it is preferred to remove compounds such as chlorophyll from the extract before performing the bioassay. Mixing activated charcoal with the liquid fraction can achieve this, however only minute amounts of charcoal should be used, as any more can absorb compounds of interest as well.

All plants contain compounds of very large molecular weight, such as polyphenolic tannins and fatty acids that need to be removed during the isolation process. Most of them can be removed using liquid-liquid extraction except for some fatty acids and phenolic compounds that will not elute in hexane. Size exclusion chromatography can be used to remove these compounds.

**Research Focus**

The goal of this research has focused on the isolation and identification of the bioactive compounds present in two plants, *Euphorbia hirta* and *Pandanus tectorius*, commonly used in the practice of Traditional Chinese Medicine. These two plants were chosen as the focus of further research due to the bioactivity of their crude extracts and because the major source of this bioactivity appears to be unknown.

***Euphorbia hirta***

More commonly referred to as the Asthma Weed or Garden Spurge, *Euphorbia hirta*, is a plant that has been used in TCM to primarily treat skin infections and disorders.[32] Others uses for this plant include the treatment of parasitic infections,

respiratory illnesses and gastrointestinal illnesses.[33,34] *E. hirta* is found in warmer climates and is native to China and parts of India.[35]

Scientific research using bioassays for cytotoxicity has demonstrated that components of *E. hirta* can potentially serve an array of medicinal purposes including anti-malarial, anti-inflammatory, anti-bacterial, anti-viral, and anti-oxidation.[33, 34, 36] Further bioactivity reported for the crude extract has included anti-pyretic and tranquilizing effects.[33] Most recent research has identified the crude ethanol extract as having significant inhibition on lipopolysaccharide-induced and *Mycobacterium tuberculli*-induced arthritis in mice, although the source of this bioactivity has not been identified.[37] In addition to this, others have reported that the crude ethanol and petroleum ether extracts demonstrate anti-diabetic activity.[38] The compounds responsible for most of the activity described in the crude extracts have not been reported.

A study published in a 2009 issue of *In Vivo* reported that compounds in the 50% methanol extract of *E. hirta* exhibited activity against human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2) and against simian immunodeficiency virus (SIV).[33] Upon subjecting this extract to liquid extraction with dichloromethane and ethyl acetate, the inhibitory effect of the extract remained in the 50% methanol fraction. Based on this observation, the researchers concluded that tannins were responsible for the bioactivity, however their identities, as with many other bioactive compounds from this plant, were not reported.

Compounds isolated and identified from *E. hirta* to date include the flavonoids quercetrin and myricitrin.[32, 39] Quercetrin, not to be confused with the flavone quercetin discussed previously, is currently being investigated as a chemotherapeutic agent.[40]

Myricetrin, which has been isolated from other plants in addition to *E. hirta*, has been investigated as an HIV-1 integrase inhibitor along with the other flavones quercetin and quercetrin. The structures of these three compounds have been used as models for the design of other HIV-1 integrase inhibitors.[41] The structures of myricetrin and quercetrin are provided in Figure 17. The structure of quercetin is given in Figure 8.

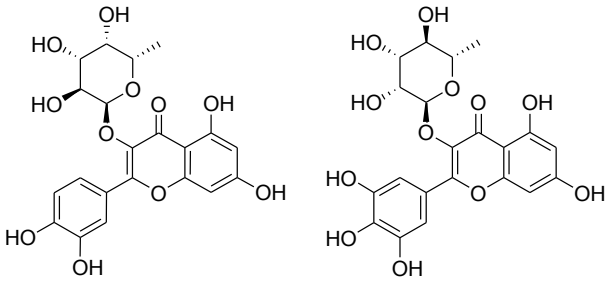



**Figure 17. Structure of quercetrin (left) and myricitrin (right)**

Other recently isolated compounds include the triterpenes β-amyrin and taraxerone, the monoterpene camphor, and the phenylpropanoid, rutin.[39, 40] β-Amyrin is also found in dandelions and exhibits anti-inflammatory activity. Taraxerone is currently being investigated for its inhibition of the bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus*. TCM promotes the usage of rutin in combination with the compound quercetin for the treatment of cardiovascular disease and camphor exhibits anti-microbial activity.[20,32,33] The structures of camphor, taxarone, and rutin are given in Figure 18.[22, 34, 35]

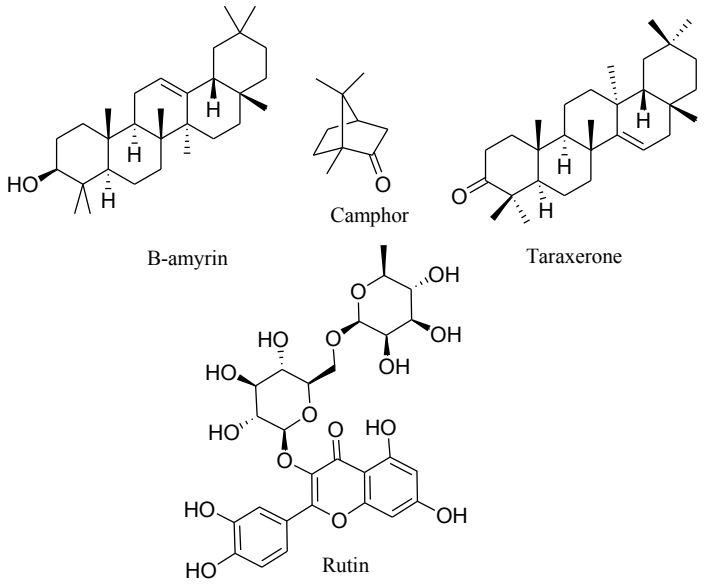



**Figure 18. Examples of compounds isolated from *E. hirta***

***Pandanus tectorius***

*Pandanus tectorius* is native to areas of southern China. For years, the natives of this area have ingested its pineapple-like fruit for its anti-hyperlipidemic effects, believing that it aided in lowering unhealthy levels of cholesterol.[43] Although it is a plant of Chinese origin, it is also common to Hawaii, Malaysia, and the Philippines. [44]

Little medicinal research has been conducted on *Pandanus tectorius* compared to *Euphorbia hirta.* Much of the research conducted on the different 600 *Pandanus* species, has focused on species such as *P. amaryllifolius* or *P. simplex* and not on *P. tectorius*. Of the research published on the bioactivity of *P. tectorius* plant extracts, properties reported include anti-oxidation, anti-inflammatory, anti-hyperglycemia.[45,46] One study published in 2008 in the *Journal of Natural Medicines* describes the isolation of a novel triterpene, 24,24-dimethyl -5β-tirucall-9(11),25-dien-3-one, from *Pandanus*

*tectorius* that reportedly inhibits the growth of the tuberculosis-causing bacteria *Mycobacterium tuberculosis*.[47] The structure of this compound is given in Figure 19.

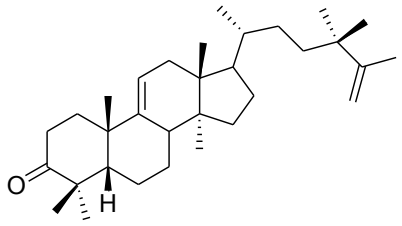



**Figure 19. Structure of an antitubercular triterpene isolated from *P. tectorius***

Additional research on the fruit of *Pandanus tectorius* has shown that it is a significant source of Vitamin A and other carotenoids. A recent study published in the *Journal of Medicinal Plants Research* in 2012 listed fifteen phenylpropanoid compounds isolated from the fruit of *Pandanus tectorius*. Of the compounds isolated, none were identified as novel, however it was the first time any of the fifteen compounds had been isolated in the *Pandanus* genus.[48] Five of the fifteen compounds had bioactivity that been previously reported in the literature. These compounds are: vanillin (anti-oxidant), *trans*-ethyl caffeate (anti-inflammatory), tangeretin (cholesterol lowering), chrysin (aromatase inhibitor), and naringenin (anti-viral).[48] The structures of these compounds are given in Figure 20.

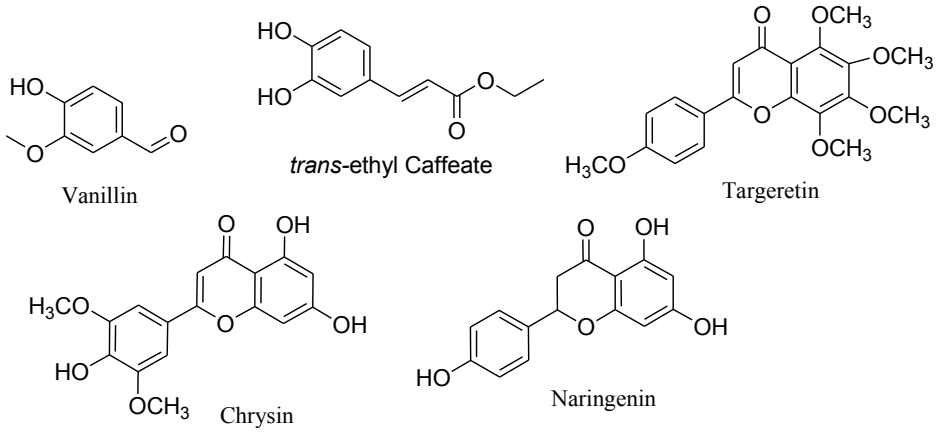



**Figure 20. Structures of bioactive compounds isolated from *P. tectorius***

Another study published in 2013 reported the isolation of two novel phenolic compounds, identified as pandanusphenol A and pandanusphenol B (ficusol), from the fruit *Pandanus tectorius*.[49] The bioactivity of these compounds is currently unknown. Their structures are given in Figure 21.

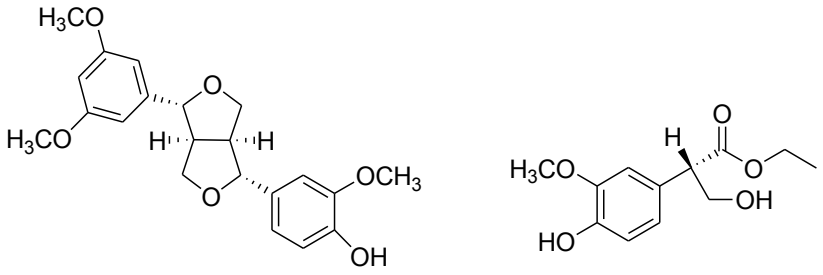


**Figure 21. Structure of pandanusphenol A (left) and pandanusphenol B (ficusol) (right)**

**Objective**

This research will focus on the isolation of natural products for medicinal purposes with the ultimate objective of isolating and identifying major sources of bioactivity in two crude plant extracts, *Euphorbia hirta* and *Pandanus tectorius*. Since the target of this isolation is an unknown bioactive compound, biological assays will serve as a guide for the step-wise fractionation and purification procedure. Fractions will be tested for anti-viral, anti-cancer, anti-protozoan, or anti-inflammatory activity as appropriate during the isolation process. It is expected that this method will ultimately lead to one or more pure bioactive compounds. The procedure will begin with the extraction of crude plant material from the dry plant material of *Euphorbia hirta* and *Pandanus tectorius*. Isolation and identification of the bioactive compounds will then be performed using the methods described previously. It is anticipated that novel compounds can be identified for the treatment of cancer, viral infections, parasitic infections, and inflammatory responses. Additionally, it is likely that new activity could be observed for previously discovered compounds.

# CHAPTER II: MATERIALS AND METHODS

Two plants, *Euphorbia hirta* and *Pandanus tectorius*, were chosen for examination of their bioactive properties based on the results of bio-screens that identified them as possessing potential active compounds. Over 140 extracts used in Traditional Chinese Medicine (TCM) were screened for anti-viral (herpes simplex virus 1) and anti-cancer (BT20 and A549) activity. Both *E. hirta* and *P. tectorius* ethyl acetate fractions exhibited inhibition of BT20 (breast cancer) and A549 (lung cancer) but only the *P. tectorius* extract inhibited HSV-1. The two crude plant extracts were subjected to extraction and isolation procedures in an effort to identify the compounds responsible for their bioactivity. Figure 22 provides a basic summary of this isolation procedure.

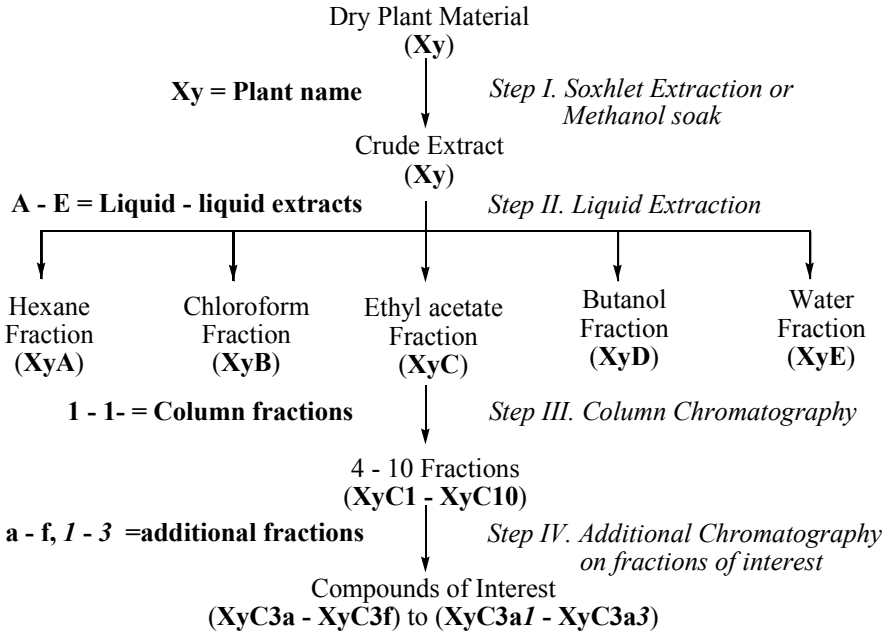



**Figure 22. Summary of isolation procedure and naming scheme**

## *Euphorbia hirta* Extractions

A total of five separate extractions were performed on the crude *Euphorbia hirta* extract. The first three extractions were performed on the dry plant material obtained from Tropilab Inc. and the last two were performed on a crude *Euphorbia hirta* extract obtained from the Guangxi Botanical Garden of Medicinal Plants (GBGMP) in China. During the first step of the first extraction, a mortar and pestle were used to grind up the dry plant material, which was then subject to Soxhlet extraction with ethanol. Due to a very low yield of crude extract using this method, the first step of the next two extractions involved heating and soaking the plant material in methanol. The last two methods skipped this first step entirely since the material obtained from China was already in the form of a crude ethanol extract. In those last two extractions, the first step was to separate the crude extract into fractions using gravity column chromatography.

Based on the results of the initial screening assays, the ethyl acetate fraction of *E. hirta* was chosen as a main focus. Significant anti-viral activity was observed in this fraction during the testing of the initial crude extracts; however as the purity of the fractions increased, their anti-viral properties became less consistent. Removal of polyphenolic compounds from the crude extracts using a brine wash as well as size exclusion chromatography did not appear to affect this inconsistency.

Although *E. hirta* was chosen for further examination based on its anti-viral activity, it was also tested for anti-cancer properties. Fractions in the third stage of purification in the third extraction procedure appeared to inhibit the growth of breast and lung cancer. The source of this bioactivity has not yet been identified.

***Pandanus tectorius* Extractions**

A total of seven separate extractions were performed on the *Pandanus tectorius* ethyl acetate extract leading to the isolation of two pure compounds. A combination of the first five extractions was successful in isolating compound **1** on four separate occasions. The methods used in the isolation of compound **1** included liquid-liquid extraction, gravity column chromatography, flash column chromatography with varying solvent systems, and high performance liquid chromatography. The last two extractions were successful in isolating compound **2**. The methods used in this isolation were flash column chromatography with varying solvent systems and high performance liquid chromatography. Both compounds were identified using the same spectroscopy and spectrometry methods of: $^{1}$H-NMR, $^{13}$C-NMR, COSY, DEPT, HMQC, HMBC, HSQC, NOESY and direct probe mass spectrometry using electron ionization. None of the isolation methods performed on this plant extract required an initial extraction such as a Soxhlet extraction to produce the crude ethanol or methanol fraction because it was obtained as an abstract from GBGMP in China.

Based on the analysis of spectroscopic and spectrometric data, the first compound isolated from the *P.* t*ectorius* ethyl acetate fraction was positively identified as a racemic mixture of lariciresinol, shown in Figure 23. Lariciresinol was tested for anti-viral, anti-cancer, anti-protozoan, and anti-inflammatory activity. Its anti-cancer properties appeared to have decreased as the fraction was purified. It did not demonstrate substantial anti-inflammatory activity and its activity regarding anti-viral and anti-protozoan properties is inconclusive at this time.

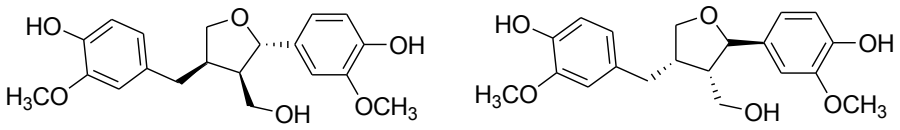



**Figure 23. Structure of (+)-lariciresinol (left) and (-)-lariciresinol (right)**

The structure of the second compound isolated from *P. tectorius* has not yet been established for certain; however, it is very similar to (+) pinoresinol. The structure of (+)-pinoresinol is provided in Figure 24 next to a proposed structure for compound **2**. The difference between the two structures is highlighted in red.

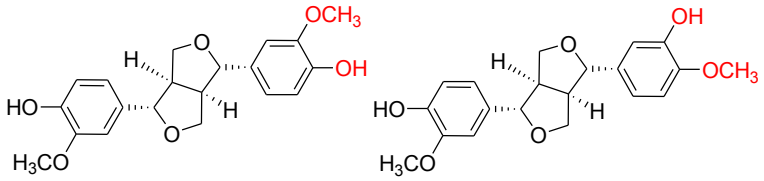



**Figure 24. Structure of (+)-pinoresinol (left) and proposed compound 2 (right)**

## Instruments, Materials, and Reagents

NMR data were obtained using a 500 MHz FT-NMR model ECA-500 JEOL (Peabody, MA) purchased with funding provided by the National Science Foundation through the NSF-MRI program (#0321211). Chemical shifts are reported in parts per million using tetramethylsilane (TMS) as an internal reference. Splitting patterns are designated by the following: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and dd (doublet of doublets). Coupling constants (J values) are recorded in Hertz (Hz). Polarimetry was performed using an Autopol III polarimeter from Rudolf Research,

(Fairfield, NJ). High resolution electrospray ionization-mass spectrometry (ESI-MS) was performed at Notre Dame University, Notre Dame, Indiana. High resolution direct probe mass spectrometry with electron ionization (EI) was performed at the University of South Carolina, Columbia, South Carolina.

Thin layer chromatograpy (TLC) was performed on glass plates coated with silica gel and UV active backing purchased from Fisher Scientific, Pittsburgh, PA. The TLC plates were analyzed with a short wavelength (254 nm) UV light and subsequently stained with phosphomolybdic acid (reagent grade, Aldrich, Milwaukee, WI) prepared as a 10% solution in ethanol. Gravity column chromatography was performed with silica gel, 63-200 micron 70-230 mesh ASTM (reagent grade, Fisher Scientific, Pittsburgh, PA). Flash column chromatography was performed with silica gel, 60 Å 230-400 mesh ASTM, reagent grade, Fisher Scientific, (Pittsburgh, PA) and where indicated on an ISCO CombiFlash $R_f$ 200 Teledyne ISCO, (Lincoln, NE) using a Teledyne ISCO cartridge pre-loaded with 2.5g of normal phase silica and a Teledyne ISCO pre-loaded 4g flash column. HPLC was performed using a Breeze Waters System with a normal phase Waters Spherisorb column having 10 x 250 mm dimensions and a 2487 Dual λ Absorbance Detector (Milford, MA). HPLC grade ethyl acetate and hexane were purchased from Acros Organic, (New Jersey, USA).

Methylene chloride, methanol, acetone, ethyl acetate, ethanol, and hexanes were purchased from Fisher Scientific, (Pittsburgh, PA). Chloroform was purchased reagent grade from Acros Organic, (New Jersey, USA). Deutero-chloroform ($CDCl_3$) and deutero-methanol ($CD_3OD$) were purchased from Aldrich, (Milwaukee, WI) and Cambridge Isotope Laboratories, Inc., (Andover, MA). Deutero-acetone ($CD_3COCD_3$)

was purchased from Norell, Inc., (Landisville, NJ). Solvent extractions were performed using hexane, ethyl acetate, methylene chloride, and acetone, and when indicated washed with brine (reagent grade, Fisher Scientific, Pittsburgh, PA). Organic fractions were tested for polyphenols using a ferric chloride solution. The organic layers resulting from liquid-liquid extraction were dried with magnesium sulfate (Fisher Scientific, Pittsburgh, PA) and filtered. Evaporation of solvents was achieved using a Buchi rotary evaporator (Model RII, Buchi, Switzerland).

The *Euphorbia hirta* plant extract was obtained from Tropilab Inc. (St. Petersburg, FL) and through the Guangxi Botanical Garden of Medicinal Plants (GBGMP) in China when indicated. The *Pandanus tectorius* extract was obtained through the Guangxi Botanical Garden of Medicinal Plants (GBGMP). All research was done through funding provided by the Middle Tennessee State University (MTSU) Tennessee Center for Botanical Medicine Research (TCBMR), (Murfreesboro, TN). Students and faculty in the Biology Department at MTSU conducted all bioassays. Dr. Stephen Wright and Megan House conducted the anti-viral assays using herpes simplex virus 1 (HSV-1) and African Green Monkey Kidney (Vero) tissue cultures. Dr. Anthony Newsome and Jeannie Stubblefield conducted the anti-protozoan assays. Dr. Ying Gao conducted the anti-cancer assays and Hyo Sim Park conducted the anti-inflammatory bioassays.

**Isolation and Purification Methods: *Euphorbia hirta* (Eh)**

A total of five extractions are summarized herein. The first three extractions began with dry plant material obtained from Tropilab Inc. The last two began with

extract obtained from GBGMP in China, the preparation of which is uncertain. Some reported weights may be high due to hygroscopic properties or oil-like composition. Fractions marked with the superscript *v* were submitted for anti-viral testing but the results have not been received yet. Fractions marked with the superscript $\rho$ were submitted for anti-protozoan testing but the results have not been received yet.

***(Eh) Extraction 1*:** (12.0 g, Tropilab plant material)

I. Soxhlet extraction (500 mL ethanol, 60°C, 24 hours) yields 610 mg (5.1%) **Eh**

II. Eh (610 mg) liquid – liquid extraction (yields):

   A. Hexane: 281.9 mg (46.2%)

   B. Chloroform: 4.1 mg (2.3%)

   C. Ethyl acetate: 52.3 mg (8.6%) (anti-viral)[50] [**EhC**]

   D. (D-E) Butanol, methanol, water: 270 mg (44.3%)

III. EhC (26.2 mg) brine wash (100 mL) yields 17.2 mg (32.9%) (anti-viral) [**EhC**]

IV. **EhC** gravity column (70-230 mesh silica, methanol/ methylene chloride gradient) yields:

   1) 7.4 mg (43.0%)$^{v}$

   2) 8.1 mg (47.1%)$^{v}$

   3) 0.1 mg (0.6%)$^{v}$

   4) 0.6 mg (3.5%)$^{v}$

***(Eh) Extraction 2*:** (200.0 g, Tropilab plant material)

I. Warm methanol soak (600 mL, 45°C, 2 hours) yields 6.4 g (3.2%) **Eh**

II. **Eh** (6.4 g) liquid – liquid extraction yields:

A. Hexane: 2.41 g (37.7%)$^{\rho}$

B. Chloroform: 470 mg (7.3%)$^{\rho}$

C. Ethyl acetate: 370 mg (5.8%)$^{\rho}$ [**EhC**]

III. **EhC** (370 mg) gravity column (70-230 mesh silica, methanol/ ethyl acetate/ hexane gradient) yields nine fractions. The first two demonstrate anti-viral activity so they are combined to give **EhC1**.[50] Fraction yields are:

1) 167.0 mg (45.1%) (anti-viral)[50] [**EhC1**]
2) 17.1 mg (4.6%) (anti-viral)[50] [**EhC1**]
3) 32.3 mg (8.7%)
4) 8.6 mg (2.3%)
5) 5.2 mg (1.4%)
6) 14.2 mg (3.8%)
7) 27.1 mg (7.3%)
8) 7.1 mg (1.9%)
9) 5.7 mg (1.5%).

IV. **EhC1** flash column (230-400 mesh silica, ethyl acetate/ hexane gradient) yields:

a) 88.7 mg (50.7%)$^{v}$
b) 18.1 mg (10.3%)$^{v}$

c) 6.8 mg (3.9%)[v]

d) 6.5 mg (3.7%)[v]

e) 5.1 mg (2.9%)[v]

f) 11.6 mg (6.6%)[v]

g) 7.0 mg (4.0%)[v]

h) 18.4 mg (10.5%)[v]

***(Eh) Extraction 3.*** (350.0 g, Tropilab plant material)

Note: In step II, an alternate to liquid – liquid extraction is used.

I. Warm methanol soak (600 mL, 45°C, 2 hours) yields 8.7 g (2.5%) **Eh**

II. **Eh** (2.2 g) de-fat (200 mL ethyl acetate, 800 mL hexane, 12 hours) yields 277.2 mg (3.2%) of precipitate [**EhC**]

III. **EhC** flash column (230-400 mesh silica, acetone/ ethyl acetate/ hexane gradient) yields 11 fractions with the first nine exhibiting anti-cancer activity. **EhC6** is the most pure of these.[51] Fraction yields are:

1) 17.7 mg (6.4%)

2) 1.3 mg (0.5%)

3) 2.4 mg (0.8%)

4) 3.2 mg (1.1%)

5) 1.1 mg (0.4%)

6) 14.6 mg (5.3%) (99% tumor inhibition)[51] [**EhC6**]

7) 14.5 mg (5.2%)

8) 2.0 mg (0.7%)

9) 30.1 mg (10.9%)

10) 115 mg (41.5%)

11) 9.3 mg (3.4%)

IV. EhC6 flash column (230-400 mesh silica, acetone/ methylene chloride gradient) yields two major fractions, both with anti-tumor activity:[51]

a) 2.0 mg (15.4%) (99% tumor inhibition)[51] [**EhC6a**]

b) 7.4 mg (56.9%) (100% tumor inhibition)[51] [**EhC6b**]

***(Eh) Extraction 4.*** (87.5 mg, GBGMP extract)

I. Initial extraction is not necessary. Extract from GBGMP is already in the crude form and labeled as **EhC** (ethyl acetate extract).

II. Liquid – liquid extraction attempts demonstrate that GBGMP crude sample may be **EhA** (hexane extract) rather than **EhC** (ethyl acetate extract) as 95% of the crude extract remains in **EhA**.

III. **EhA** (87.5 mg) gravity column (70-230 mesh silica, ethyl acetate/ hexane gradient) yields nine fractions. The first two contain the majority of the weight and are combined.

1) 17.0 mg (19.4%) [**EhA1**]

2) 23.5 mg (40.9%) [**EhA1**]

IV. **EhA1** (40.5 mg) CombiFlash column (ethyl acetate/ hexane gradient) yields:

a) 0.9 mg (2.2%)[v]

b) 30.1 mg (74.3%)[v]

c) 1.6 mg (4.0%)[v]

***(Eh) Extraction 5.*** (3.1 g, GBGMP extract) (Steps I and II not performed)

III. GBGMP extract assumed to be **EhA**. Gravity column on **EhA** (3.1 g) yields:

1) 18.6 mg (6.0%)
2) 1.0 g (32.3%)
3) 197.6 mg (6.4%)
4) 337.8 mg (10.9%)
5) 452.8 mg (14.6%)
6) 82.4 mg (2.7%)
7) 118.8 mg (3.8%)

**Isolation and Purification Methods: *Pandanus tectorius* (Pt)**

A total of seven extractions leading to the isolation of two pure compounds are summarized herein. All extractions began with crude extract obtained from GBGMP in China, the preparation of which is uncertain. An initial Soxhlet extraction, or otherwise initial crude extraction, was not required in these procedures. Instead, Step I will outline a liquid – liquid or defatting procedure. Some reported weights may be high due to hygroscopic properties or oil-like composition. Fractions marked with the superscript $v$ were submitted for anti-viral testing but the results were never received. Fractions marked with the superscript $\rho$ were submitted for anti-protozoan testing but the results have not been received yet.

***(Pt) Extraction 1.*** (1.0 g, GBGMP extract labeled as **PtC** (ethyl acetate extract))

I. Pt (1.0 g) liquid – liquid extraction yields:

A. Hexane: 393.4 mg (39.3%)

B. Chloroform: not obtained

C. Ethyl acetate: 259.7 mg (26.0%)$^{\rho}$ (anti-cancer)[51] [**PtC**]

D. (D-E) Butanol, water: 135.3 mg (13.5%).

II. **PtC** (210 mg) gravity column (70-230 mesh silica, acetone/ methylene chloride gradient) yields six major fractions with > 90% tumor inhibition.[51] **PtC2** was the most pure.

1) 36.4 mg (17.3%)
2) 25.8 mg (12.3) [**PtC2**]
3) 28.9 mg (13.8%) [**PtC3**]
4) 23.4 mg (11.1%)
5) 15.7 mg (7.5%)
6) 13.4 mg (6.4%)
7) 8.0 mg (3.8%)

***(Pt) Extraction 2.*** (10.0 g, GBBMP extract labeled as **PtC** (ethyl acetate extract))

I. **Pt** de-fat (200 mL ethyl acetate, 600 mL hexane, 12 hours) yields 6.11 g (61.1%) precipitate [**PtC**].

II. **PtC** (600 mg) flash column (230-400 mesh, acetone/ methylene chloride gradient) yields:

1) 102.9 mg (17.2%)
2) 146.6 mg (24.4%) [**PtC2**]
3) 287.0 mg (47.8%).

III. Combine **PtC2** fractions (168.9 mg). Two consecutive flash columns (230-400 mesh silica, acetone/ methylene chloride gradient) yields one fraction of 54.7 mg (32.4%) of one fraction showing two spots on TLC with $R_f$ (1:2 acetone/ methylene chloride) values:

   a) $R_f$ = 0.64 [**PtC2a**]

   b) $R_f$ = 0.45 [**PtC2b**]

IV. Perform two consecutive **PtC2** flash columns (as in Step III) to yield 7.3 mg (19.4%) of one fraction showing one spot on TLC with $R_f$ (1:2 acetone : methylene chloride) 0.45. This is taken to be **PtC2b**.

***(Pt) Extraction 3.*** (1.8 g, GBBMP extract labeled as **PtC** (ethyl acetate extract))

I. Repeat defat procedure as in Extraction 2 yielding 860 mg (47.8%) precipitate [**PtC**].

II. Repeat (1) flash column procedure as done in Extraction 2 yielding 48.4 mg (6.7%) of **PtC2**.

III. **PtC2** flash column (230-400 mesh silica, ethyl acetate/ hexane gradient) yields:

   a) 7.5 mg (15.5%)

   b) 8.6 mg (17.8%) [**PtC2b**]

IV. **PtC2b** fractions combined (15.9 mg) and fractionated by normal phase HPLC (10 x 250 mm dimensions) with 1:1 (ethyl acetate : hexane) holding for four minutes and changing to 9:1 in 12 minutes and holding for 13 minutes, giving

a total run time of 25 minutes with a flow rate of 2.5 mL/min. and a 49:51 ratio between individual peaks, affording two fractions:

*1.* 1.9 mg (11.9%), RT = 14.282 min [**PtC2b*1*]**

*2.* 0.5 mg (3.1%) RT =15.116 min [**PtC2b*2*]**

***(Pt) Extraction 4.*** (1.8 g, GBGMP extract labeled as **PtC** (ethyl acetate extract))

I. Repeat defat procedure as in Extraction 2 yielding 926.9 mg (51.2%) of precipitate [**PtC**].

II. Repeat (1) flash column procedure as done in Extraction 2 yielding 115.2 mg (12.5%) of **PtC2**.

III. Repeat **PtC2** (110 mg) flash column using an ethyl acetate/ hexane gradient as in Extraction 3 to afford:

1) 70.0 mg (63.6%) [**PtC2a**]

2) 25.3 mg (23.0%) [**PtC2b]**

IV. With **PtC2b** (25.3 mg), repeat flash column as in previous step to afford 21.2 mg (83.4%) of pure **PtC2b** based on TLC appearance.

***(Pt) Extraction 5.*** (3.3 g, GBGMP extract labeled as **PtC** (ethyl acetate extract))

I. Repeat defat procedure as in Extraction 2 to yield 1.6 g (48.5%) precipitate [**PtC**].

II. With **PtC** (1.6 g) repeat flash column procedure using ethyl acetate/ hexane gradient as done in Extracton 3 Step III to yield 80 mg (5.0%) of **PtC2**.

III. With **PtC2** (80.0 mg) repeat previous flash column procedure in Step II to yield 65 mg (81.3%) **PtC2b**.

IV. Combine **PtC2b** fractions and repeat previous flash column using acetone/ methylene chloride gradient to yield 24.3 mg (28.6%) **PtC2b*1*,**[p] which is identified as (±) lariciresinol and exhibits <5% tumor inhibition and 28% cytokine inhibition.[51, 52]

***(Pt) Extraction 6.*** (2.0 g, GBGMP extract labeled as **PtC** (ethyl acetate extract))

II. (Skipped Step I). With **Pt** (2.0 g) repeat flash column procedure using ethyl acetate/ hexane gradient as done in Extraction 3 Step III to yield:

1) 258.1 mg (12.9%)
2) 477.6 mg (23.9%) [**PtC2**]
3) 642.2 mg (32.0%) [**PtC3**]
4) 128 mg (6.4%)

***(Pt) Extraction 7.*** (1.2 g, GBGMP extract labeled as **PtC** (ethyl acetate extract))

I. Repeat defat procedure as in Extraction 2 to yield 774.3 mg (64.5%) precipitate [**PtC**].

II. With **PtC** (774.3 mg) repeat flash column procedure using ethyl acetate/ hexane gradient as done in Extraction 6 to yield:

1) 258.1 mg (12.9%)
2) 200.0 mg (25.8%) [**PtC2**]
3) 131.4 mg (17.0%) [**PtC3**]
4) 114 mg (14.7%)

III. Flash column (230-400 mesh silica, acetone/ methylene chloride gradient) on combined **PtC3** fractions (608.5 mg) yields:

a) 223.0 mg (36.6%)

b) 36.1 mg (5.9%)

c) 131.0 mg (21.5%) [**PtC3c**]

d) 121.9 mg (20.0%)

e) 49.0 mg (8.1%)

IV. **PtC3c** (131 mg) purified using CombiFlash to afford one fraction of 103.1 mg (78.6%) showing one major spot on TLC with $R_f$ (0.5 : 3 : 2 methanol : ethyl acetate : hexane) 0.29.

V. **PtC3c** (131 mg) purified using normal phase HPLC with 3:2 (ethyl acetate : hexane) holding for ten minutes and increasing to 19:1 in 20 minutes to give a total run time of 30 minutes with a flow rate of 3.0 mL/min affording one pure compound of 11.0 mg (10.6%) with RT = 13.989 min.

***(Pt) Extraction 1-7 Summary.*** Seven crude extractions, including the isolation of two pure compounds have been summarized. In the first step, liquid extraction primarily afforded two fractions of 100% hexane and 100% ethyl acetate (precipitate). The ethyl acetate fraction (**PtC**) was then subjected to either gravity or flash column chromatography, which afforded 5 – 10 fractions. Based on their bioactivity, two of these fractions were chosen for further purification using flash column chromatography and HPLC. The major compound in **PtC2b** was isolated four times from the crude GBGMP extract to give an overall average yield of 0.71%. The major compound in **PtC3c** was isolated once to produce 11 mg (0.01%) of pure compound.

***Recrystallization of PtC2b1 ((±) lariciresinol).*** Solid PtC4 (30.0 mg) and 1 mL methanol were added to a small test tube and heated for approximately ten minutes (until all PtC4 dissolved in the methanol) using a 40°C water bath. Once PtC4 had dissolved, the test tube was removed from the water bath and allowed to sit in the hood for 15 hours at which point needle-like crystals were visible in the solution. The methanol solution was removed using a pipette and crystals were viewed using a Brightfield microscope. The crystals appeared too small for x-ray crystallography.

**Identification of Pure Compounds**

**(±) 4-[5-(4-Hydroxy-3-methoxyphenyl)-4-(hydroxymethyl)oxolan-3-yl]methyl]-2-methoxyphenol ((±) lariciresinol, PtC2b*1*).** = 0.0° (*c* 0.0059, $CH_3OH$); = 0.0° (*c* 0.0033, $CH_3COCH_3$). The following spectroscopic data was obtained in $CDCl_3$: $^1$H-NMR, $^{13}$C-NMR, COSY, DEPT-90, DEPT-135, HMBC, HMQC, HSQC, NOESY. NMR data is shown in the Appendix. The following spectroscopic data was obtained in $CD_3OD$: $^1$H-NMR, $^{13}$C-NMR. Mass spectrometry was conducted using electron spray ionization (ESI) and direct probe electron ionization. $^1$H-NMR and $^{13}$C-NMR data in both $CDCl_3$ and $CD_3OD$ are provided. $^1$H-NMR (500 MHz $CDCl_3$): δ 6.86 (m, 4 aryl C<u>H</u>), 6.68 (m, 2 aryl C<u>H</u>), 5.50 – 5.57 (d, J = 6.87 Hz, 2 O<u>H</u>), 4.78 (d, J = 6.87 Hz, Ph C<u>H</u>O-), 4.05 (dd, J = 8.6, 6.9 Hz, $\alpha_1$-H, C<u>H</u>$_2$O-), 3.92 (dd, J = 6.87, 5.15 Hz, $\alpha_2$-H, C<u>H</u>$_2$OH), 3.89 (s, C<u>H</u>$_3$O Ph), 3.87 (s, C<u>H</u>$_3$O Ph), 3.75 (m, $\beta_1$-H, $\beta_2$-H, 2C<u>H</u>$_2$O-), 2.91 (dd, J = 13.8, 5.0 Hz, $\alpha_3$-H, C<u>H</u>$_2$Ph), 2.72 (m, C<u>H</u>CH$_2$ Ph), 2.55 (dd, J = 13.5, 10.8 Hz, $\beta_3$-H, C<u>H</u>$_2$ Ph), 2.41 (d, J = 6.87 Hz, C<u>H</u>CH$_2$); $^1$H-NMR (500 MHz $CD_3OD$) δ 6.90 (d, J = 1.1 Hz, aryl C<u>H</u>), 6.75 (d, J = 1.7 Hz, aryl C<u>H</u>), 6.71 (m, aryl 2C<u>H</u>), 6.70 (d, J = 8.0 Hz, aryl

CH), 6.63 (dd, J = 8.0, 2.3, aryl CH), 4.73 (d, J = 6.8 Hz, Ph CHO-), 3.97 (dd, J = 8.0, 6.3 Hz, $\alpha_1$-H, $CH_2O$-), 3.83 (m, $\alpha_2$-H, $CH_2OH$) , 3.81 (s, $2CH_3O$ Ph), 3.71 (dd, J = 8.6, 5.7 Hz, $\beta_1$-H, $CH_2O$), 3.61 (dd, J = 11.1, 6.3 Hz, $\beta_2$-H, $CH_2OH$), 2.91 (dd, J = 13.5, 4.6 Hz, $\alpha_3$-H, $CH_2$ Ph), 2.71 (m, $CHCH_2$ Ph), 2.47 (dd, J = 13.5, 11.4 Hz, $\beta_3$-H, $CH_2$ Ph), 2.35 (m, $CHCH_2$); $^{13}$C-NMR (125 MHz, $CDCl_3$): δ 146.70 (4° aryl CO-), 146.59 (4° aryl CO-), 145.11 (4° aryl CO-), 144.07 (4° aryl CO-), 134.85 (4° aryl C), 132.36 (4° aryl C), 121.28 (aryl CH), 118.85 (aryl CH), 114.48 (aryl CH), 114.24 (aryl CH), 111.25 (aryl CH), 108.34 (aryl CH), 82.91 (Ph CHO-), 73.00 ($CH_2O$-), 61.05 ($CH_2OH$), 56.02 ($CH_3O$ Ph), 52.71 ($CHCH_2$), 42.50 ($CHCH_2$ Ph), 33.43 ($CH_2$ Ph); $^{13}$C-NMR (125 MHz, $CD_3OD$): δ 147.45 (4° aryl CO-), 145.72 (4° aryl CO-), 144.32 (4° aryl CO-), 134.25 (4° aryl CO-), 132.00 (aryl CH), 121.03 (aryl CH), 118.47 (aryl CH), 114.72 (aryl CH), 112.09 (aryl CH), 109.16 (aryl CH), 82.70 (Ph CHO-), 72.07 ($CH_2O$-), 58.00 ($CH_2OH$), 55.10 ($CH_3O$ Ph ), 52.66 ($CHCH_2$), 42.54 ($CHCH_2$ Ph), 32.32 ($CH_2$ Ph); Mass spectrum (direct probe MS with electron ionization) *m/z* ($C_{20}H_{24}O_6$) calculated for 360.1573, found 360.1567.

**4,5'-(1S,3aR,4S,6aR)-Hexahydrofuro[3,4-c]furan-1,4-diyl)bis(2-methoxyphenol), ((+)-compound 2, PtC3c).** = +29.2° (*c* 0.0050, $CH_3OH$). The following data was obtained in $CDCl_3$: $^1$H-NMR, $^{13}$C-NMR, COSY, DEPT-90, DEPT-135, HMBC, HMQC. $^1$H-NMR was also run in $CD_3OD$. NMR data is shown in the Appendix. Mass spectrometry was conducted using direct probe analysis with electron ionization. All $^1$H-NMR and $^{13}$C-NMR data are listed. $^1$H-NMR (500 MHz, $CDCl_3$): δ 6.93 (s, aryl CH), 6.87 – 6.89 (d, J = 8.59 Hz, 4 aryl CH), 6.81 -6.83 (d, J = 8.02 Hz, aryl CH), 6.07 (br. s, Ph OH), 5.63 (br. s, Ph OH), 4.79 (d, J = 5.15 Hz, Ph CHO-), 4.72 (d, J

= 5.15 Hz, Ph C<u>H</u>O-), 4.25 (m, C<u>H</u>$_2$O-), 3.95 (s, C<u>H</u>$_3$O Ph), 3.87 (m, C<u>H</u>$_3$O Ph), 3.81 (m, C<u>H</u>$_2$O-), 3.11 – 3.20 (m, 2 C<u>H</u> Ph); $^1$H-NMR (500 MHz, $CD_3OD$): δ 6.95 (d, J = 11.46 Hz, 2 aryl C<u>H</u>), 6.85 (s, aryl C<u>H</u>), 6.75 (m, 3 aryl C<u>H</u>), 4.73 (d, J= 5.73 Hz, Ph C<u>H</u>O-), 4.67 (d, J = 5.15 Hz, Ph C<u>H</u>O-), 4.22 (m, C<u>H</u>$_2$O-), 3.88 (s, C<u>H</u>$_3$O Ph), 3.83 (s, C<u>H</u>$_3$O Ph), 3.80 (m, C<u>H</u>$_2$O-), 3.11 – 3.20 (m, 2 C<u>H</u> Ph); $^{13}$C-NMR (125 MHz, $CDCl_3$): δ 147.5, 146.8, 145.3, 142.4 (4 4° aryl <u>C</u>O-), 133.0, 132.9 (2 4° aryl <u>C</u>), 124.1 (4° aryl <u>C</u>), 121.0, 119.1, 114.4, 108.6, 108.2 (5 aryl <u>C</u>H), 86.0, 85.9 (2 Ph <u>C</u>H), 71.9, 71.8 (2 <u>C</u>H$_2$O-), 56.3, 56.1 (2 <u>C</u>H$_3$O Ph), 54.3, 54.2 (2 <u>C</u>H Ph); Mass spectrum (direct probe MS with electron ionization) *m/z* ($C_{20}H_{22}O_6$) calculated for 358.1416, found 358.1421.

# CHAPTER III: RESULTS AND DISCUSSION

Natural products, particularly the secondary metabolites of plants, can serve as useful tools in many fields of science, research, and medicine. Plant extracts in the form of teas, ointments, and supplements possess many benefits, but once isolated, the source of their bioactivity can have substantially greater value in the pure form. Thus, considerable research has been dedicated to the isolation, purification, and structure elucidation of bioactive compounds from their crude source. Such a procedure necessitates the pursuit of a target molecule about which little is known. Aside from the approximate polarity of the molecule, few details concerning its properties or structure can be obtained until the molecule is purified. Consequently, a standard isolation procedure cannot be established prior to beginning any crude extraction procedure. The step-wise isolation method used in this study allowed for revisions in each stage of the procedure, providing opportunities for improvement in purity and increased yield.

As in this research, compounds isolated from natural sources generally afford very low yields. Hence, attempts were made during the course of this research to streamline the isolation process, reducing the number of steps required to achieve purity and thereby recovering the largest amount of product possible in a single extraction. Subsequently, the overall yield of lariciresinol afforded from crude *Pandanus tectorius* increased from 0.07% and 0.5% in the second and third extractions, respectively, to 1.2% and 1.4% in the fourth and fifth extractions, respectively. This was achieved primarily by experimenting with different solvent elution systems.

Isolation of the second compound from *Pandanus tectorius* afforded an overall yield of 0.3%. Unlike with the first compound, two crude extractions and one pure isolation procedure were adequate to attain enough weight and sufficient purity for spectroscopic analysis and structure elucidation of **2**. Isolation of each compound was achieved in five or six steps.

**Discussion of Extraction Procedures: *Euphorbia hirta* (Eh)**

Of the five *Euphorbia hirta* extractions performed, the first three began with the dry plant leaves and stems obtained from Tropilab Inc. Beginning with dry plant material rather than an extract required that the crude extract be obtained. With the exception of the first extraction where a Soxhlet extraction was performed, this was achieved by immersing the plant material in warm methanol for several hours, thereby allowing all the compounds in the plant material to dissolve. The remaining stems and leaves were then filtered out and the methanol evaporated off before continuing to step two. This method afforded an average yield of about 2.6%. Although this is considerably lower than the yield of 5% obtained from the Soxhlet extraction in the first **Eh** extraction, the methanol method can accommodate up to 400 grams of dry plant material in one extraction compared to the 12 grams per thimble of the Soxhlet extractor. The methanol soak method was considered the most effective at obtaining the greatest weight in the least number of steps. For the fourth and fifth **Eh** extractions, an initial crude extraction was not required since the material obtained from GBGMP was already in an extract.

***Brine Wash***

In the first Eh extraction, half of the ethyl acetate fraction (**EhC**) was subjected to a brine wash to remove polyphenolic compounds. Results of the bioassay tests from both ethyl acetate fractions (washed and unwashed) indicated that the brine wash did not significantly alter the outcome of the bioassay. Both fractions demonstrated anti-viral activity with the washed fraction demonstrating 8% vero cell cytotoxicity and 97% HSV-1 inhibition and the unwashed fraction demonstrating 18% vero cell cytotoxicity and 125% HSV-1 inhibition.[50] While it should be noted that the percent cytotoxicity and the percent inhibition were both higher in the unwashed fraction, the differences are not so great as to assume that polyphenols were interfering with the tests. Acceptable cytotoxicity and inhibition was still reported even with the removal of polyphenols. Based on these results, the brine wash was discontinued in subsequent extractions.

***Variations in Procedures***

Based on the results of the initial bioassay screens, the source of bioactivity in Eh was believed to be in the ethyl acetate fraction (EhC). Consequently, this fraction became the main focus of the Eh extraction procedure, which was eventually streamlined for time efficiency and the expectation of greater yields. Step II of the third extraction used a de-fat procedure as an alternative to the liquid – liquid extraction performed in previous extractions. The crude methanol extract obtained in Step I was re-dissolved in a small amount of ethyl acetate and diluted with copious amounts of hexane (about 4 times the amount of ethyl acetate present). The precipitate that formed was filtered out and called **EhC**.

In the next step a flash column was performed on EhC rather than a gravity column. Anti-viral activity had been reported for the first two fractions of the gravity column in the previous extraction, but better separation and more sample was needed in order to properly isolate this bioactivity. Additionally, the majority of the yield from the previous gravity columns had eluted in the first two fractions and produced yields that were not sufficient for further purification. The objective of this method was to streamline the isolation process by minimizing the number of separations, leading to higher yields. By using a flash column, rather than a gravity column, and a slower elution gradient, better separation between fractions was expected.

As anticipated, better separation was achieved with the compounds eluting slowly rather than immediately, affording 11 total fractions. All of the fractions were tested for anti-cancer activity with the first nine exhibiting inhibition greater than 99%. It should be noted that the inhibition values reported for crude fractions, as these were, are not as reliable as those reported for a pure compound. Such high inhibition in a large number of fractions suggests that a component of the extract may have been interfering with the bioassay results. Regardless, the sixth fraction from this column (**EhC6**) was chosen for further examination because it appeared to be most pure based on TLC observations. **EhC6** was chromatographed to yield two fractions (**EhC6a** and **EhC6b**), both of which exhibited greater than 99% cancer inhibition.[51] Unfortunately, due to low product yields and an insufficient amount of crude plant material, the purification and identification of **EhC6a** and **EhC6b** could not be carried forward. An attempt was made to isolate these compounds in the fourth and fifth **Eh** extractions; however differences in the starting materials hindered this investigation as well.

***Hexane Fraction (EhA) Extractions***

The fourth and fifth **Eh** extractions began with crude extract that was obtained from GBGMP in China and labeled as the ethyl acetate fraction (**EhC**). From initial observations of the extract, it did not appear to have similar properties to the former **EhC** fractions. It appeared very oily, sticky, and hygroscopic much like the hexane fractions (**EhA**) from previous extractions. The extraction procedure used at GBGMP to prepare this extract was uncertain so an attempt was made to do a liquid – liquid extraction. It was immediately noted that the crude sample did not dissolve as well in ethyl acetate as the other **EhC** fractions, but did dissolve in a 1:9 mixture of chloroform : hexane. Approximately 1.0 gram of crude extract was dissolved in 200 mL of 1 : 9 chloroform : hexane and a liquid extraction was performed with water. Visual observation of the liquid extraction revealed that upon mixing of the two layers, the aqueous layer remained clear and the organic layer dark and opaque. A back extraction with ethyl acetate was performed on the aqueous layer to yield only 5 mg of the ethyl acetate fraction (**EhC**).

Based on these observations and an extremely low yield of **EhC** from the liquid liquid extraction, it was concluded that the crude extract received from GBGMP was not the ethyl acetate fraction (**EhC**), but instead was the hexane fraction (**EhA**). Since the liquid – liquid extraction did not yield useful results and no precipitation was visible upon interaction with hexane this **Eh** extract, now called **EhA**, was subjected to gravity column chromatography. During the first gravity column of **EhA**, the elution solvent polarity was increased too quickly causing most of the extract to elute in the 1:10 ethyl acetate : hexane fraction. In order to achieve better separation, a second gravity column was performed where a slower gradient was employed, beginning with 1:20 ethyl acetate

: hexane. Even so, a great deal of extract eluted immediately in the first few fractions, particularly the 1:20 and 1:15 ethyl acetate : hexane fractions. Samples from these **EhA** extractions were submitted for anti-viral testing, but results are not complete. Therefore, the bioactivity of **EhA** compared with that of **EhC** is unknown. Figure 25 provides a summary of the extractions performed on *Euphorbia hirta*.

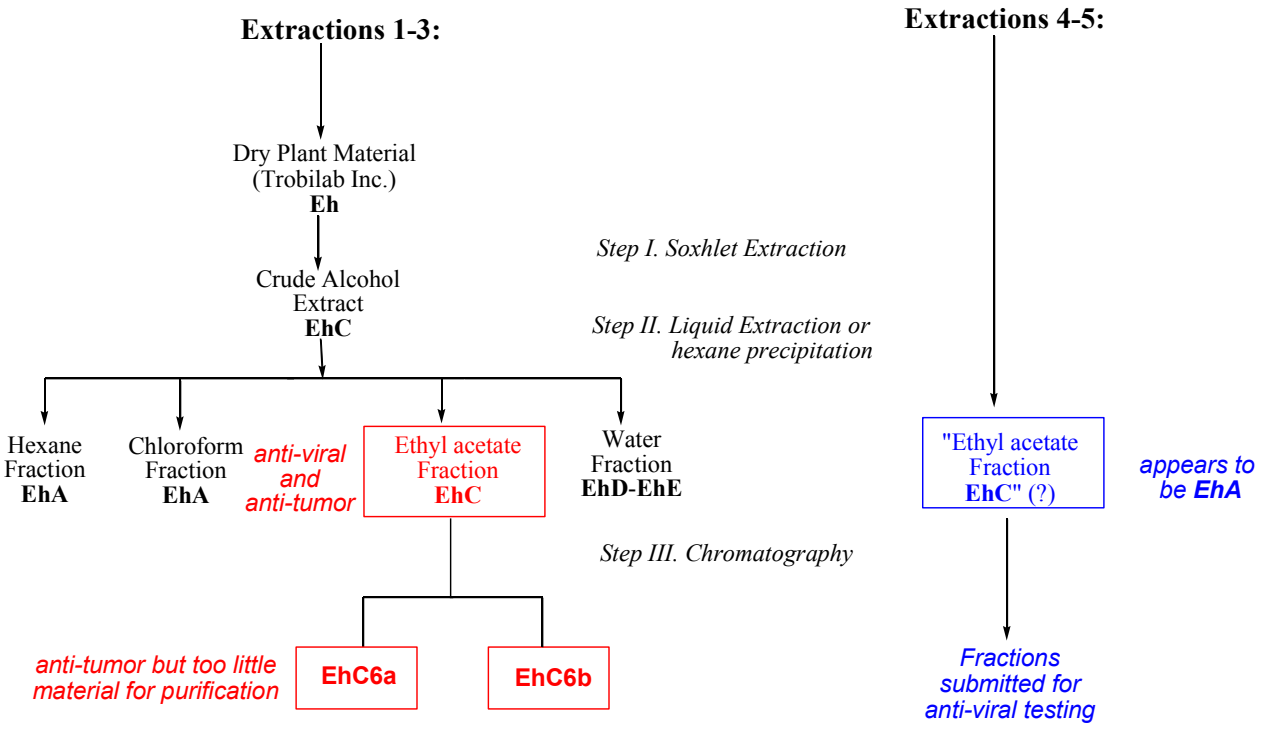



**Figure 25. Summary of extractions performed on *Euphorbia hirta***

**Discussion of Extraction Procedures: *Pandanus tectorius* (Pt)**

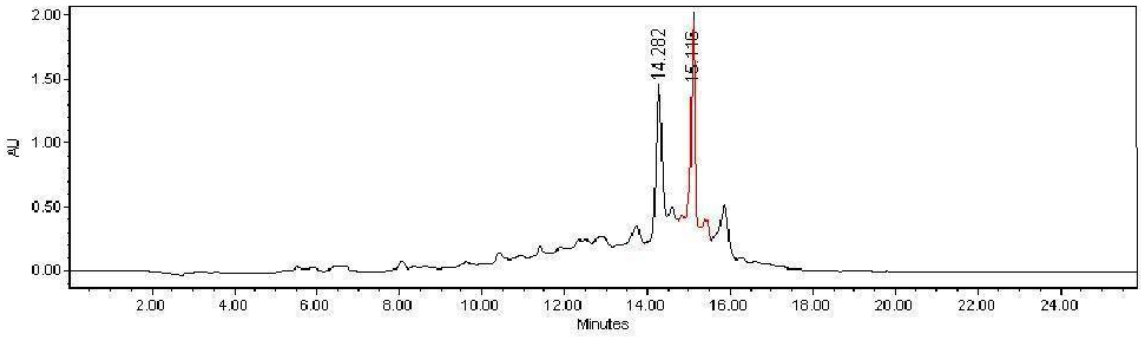
All seven extractions performed on *Pandanus tectorius* (**Pt**) began with the crude ethyl acetate fraction (**PtC**) obtained from GBGMP in China. Due to some uncertainty surrounding the preparation of the extract by GBGMP, all of the **PtC** extracts, except in the sixth extraction, were separated by either liquid – liquid extraction or de-fatting before being chromatographed. In the first extraction, a liquid – liquid extraction was attempted, however, when it became clear that the extract would not dissolve in aqueous methanol, ethyl acetate was added to the mixture. This aided in dissolving the extract and created a separation of the two solvent layers. The ethyl acetate layer was removed and de-fatted with hexane. The collected precipitate was called **PtC**. The hexane, ethyl acetate, and water fractions were submitted for anti-cancer testing. The results verified that the main source of the bioactivity was in the ethyl acetate fraction as was expected based on the results of the initial bioassay screens. The de-fatting procedure was then substituted in place of the liquid – liquid extraction in step II for the rest of the extractions. In the sixth extraction, however, no initial separation or de-fat was performed prior to the flash column. This was done in an effort to streamline the process and create a more time efficient procedure, as the de-fatting method requires an overnight waiting period. Unfortunately, this resulted in the column becoming clogged, affording minimal separation between fractions. Based on this, it was determined that an initial de-fat procedure is preferred prior to performing column chromatography.

***Variations in Procedures***

The first three extractions on *Pandanus tectorius* varied with regards to the procedures used and the solvent elution combinations chosen. When a fraction of interest (**PtC2**) was identified after the first extraction, isolation procedures were modified in an effort to focus specifically on the major compound in that fraction. Omitting the gravity column in the second extraction procedure and running a flash column instead allowed for a streamlined procedure. Based on the results of this column, it was determined that acceptable separation could be achieved without using a gravity column and subsequent gravity columns were deemed unnecessary.

After isolating 7.3 mg of relatively pure compound (**PtC6b**) in the second extraction, the third extraction focused on obtaining more sample for the purposes of bioassay testing and spectroscopic analysis. Also in this extraction, the previously used acetone : methylene chloride solvent system was supplemented with an ethyl acetate : hexane solvent system that was used in the second flash column (Step III). The objective of incorporating two solvent systems into the isolation procedure was to achieve better purification. The acetone : methylene chloride system appeared to provide decent separation of crude fractions, however as the purity increased, TLC of ethyl acetate : hexane showed better separation. The fifth extraction later demonstrated that regardless of the order they were used in, a combination of these two solvent systems in the isolation procedure provided the best purification. Extractions 1 and 2 only used the acetone : methylene chloride solvent system while extraction 4 only used the ethyl acetate : hexane solvent system.

In the third extraction, the supplementation of the second solvent system increased the purity of the fraction as anticipated, allowing for further purification of it using HPLC, shown in Figure 26.



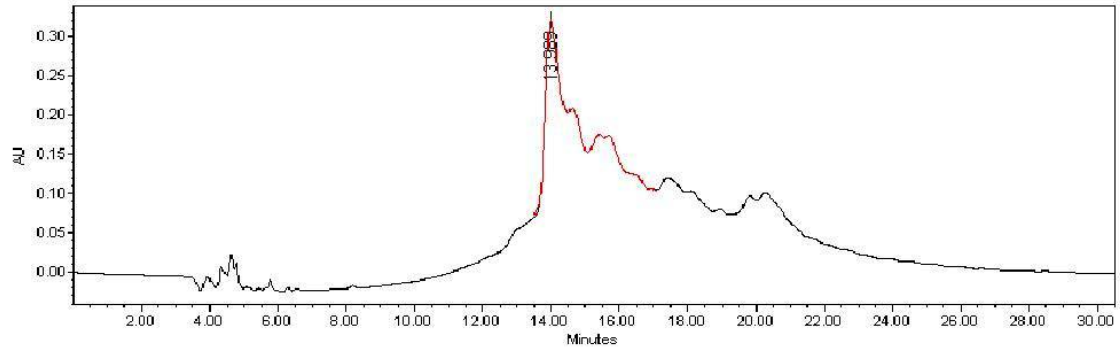

| Retention Time (min) | Peak Type | Area (µV*sec) | % Area | Height (µV) | % Height |
|---|---|---|---|---|---|
| 14.282 | Unknown | 23706681 | 48.41 | 1339721 | 41.57 |
| 15.116 | Unknown | 25263925 | 51.59 | 1883209 | 58.43 |

**Figure 26. HPLC Chromatography of (±)-lariciresinol (RT = 14.282 min.)**

Two peaks were observed as shown in Figure 25. The first peak with a retention time of 14.282 minutes was later determined to be lariciresinol (**PtC2b*1***). The second peak at a retention time of 15.116 minutes was only present in very small amounts affording a total yield of 0.5 mg at the end of the HPLC procedure.

Following the second isolation of lariciresinol (**PtC6b*1***) in the third extraction, the fourth and fifth extractions focused on streamlining the procedure to obtain more weight of the pure compound for bioassay testing and mass spectrometry. This was achieved more efficiently in the fourth and fifth extractions affording overall yields of 1.2% and 1.4% compared to yields of 0.07% and 0.5% in the first two isolations.

The sixth and seventh extractions focused on the isolation of the second bioactive compound reported, **PtC3**. The dual solvent system method was employed again in this extraction. Since the major TLC spot in **PtC3** showed up directly beneath that for **PtC2**, all the fractions leading up to **PtC2** were quickly eluted using a fast gradient, 1:1 ethyl acetate : hexane, because previous extractions had proven that only **PtC2** would elute with this solvent ratio. It was anticipated that **PtC3**, being slightly more polar, would elute at 2:1 ethyl acetate : hexane. As anticipated, **PtC3** did elute with this solvent ratio. It was then further purified using the acetone : methylene chloride gradient and a subsequent CombiFlash. The HPLC chromatogram, shown in Figure 27, provided the purification necessary for spectroscopic analysis. The major peak in this chromatogram (13.989 min.) was later identified as compound **2** (**PtC3c**).




| Retention Time (min) | Peak Type | Area (µV*sec) | % Area | Height (µV) | % Height |
|---|---|---|---|---|---|
| 13.989 | Unknown | 15131152 | 100.00 | 231400 | 100.00 |

**Figure 27. HPLC Chromatograph of compound 2 (RT = 13.989 min.)**

In summary, five extractions were performed in order to isolate and positively identify lariciresinol and two were performed in order to isolate and identify compound **2**.

An illustrated summary of the extractions performed on *Pandanus tectorius* and the isolation of lariciresinol and compound **2** is provided in Figure 28.

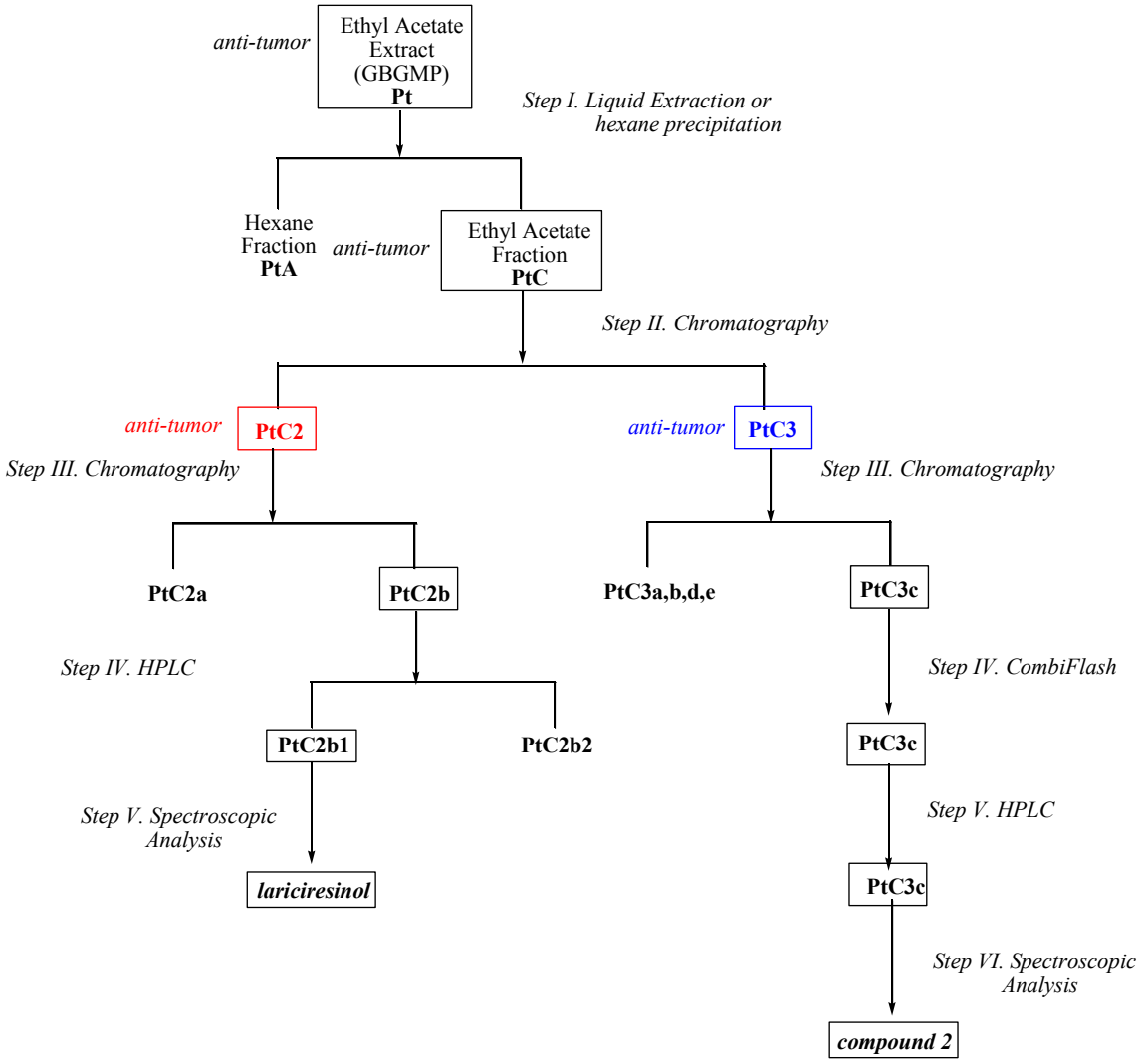



**Figure 28. Summary of extractions performed on *Pandanus tectorius***

Overall, lariciresinol (compound **1**) was isolated on four separate occasions. Due to poor yield in several extractions, this was required to order to obtain enough

compound for spectroscopic analysis and bioactivity testing. Isolation of compound **2** was only required once.

**Structure Elucidation of Pure Compounds**

Structures of the two pure compounds isolated from *Pandanus tectorius* were determined based on spectroscopic data, mass spectrometry, and optical rotation values. The structures of the compounds were elucidated based on the information provided in the spectroscopic analysis. The assembled structures were then researched and determined to match data reported in the literature for lariciresinol and pinoresinol.[53, 54] While the data for the first compound, **PtC2b*1*** (lariciresinol), leaves little room for question regarding its match to the literature data reported for lariciresinol, there are still some questions surrounding the structure of the second compound (**PtC3c**) based on several unexplained $^{13}$C-NMR signals. The ensuing discussion will describe the manner by which the structures of these two compounds were elucidated and offer explanations to the questions surrounding the true structure of compound **2**, (**PtC3c)**.

***Identification of Lariciresinol (compound 1)***

Initial analysis of $^{1}$H-NMR data indicated the presence of an aromatic ring (6.68 – 6.86 ppm, 6H), two methoxy groups (3.87 – 3.89 ppm, 6H), and possibly two hydroxyl groups attached to an aromatic ring (5.50 - 5.57 ppm, 2H). Further analysis of $^{13}$C-NMR, DEPT 90, DEPT 135, and HSQC data indicated the presence of three non-equivalent $CH_2$ groups possessing six non-equivalent hydrogens. Figure 29 shows the presence of these groups on the $^{1}$H-NMR with the proton signals paired accordingly.

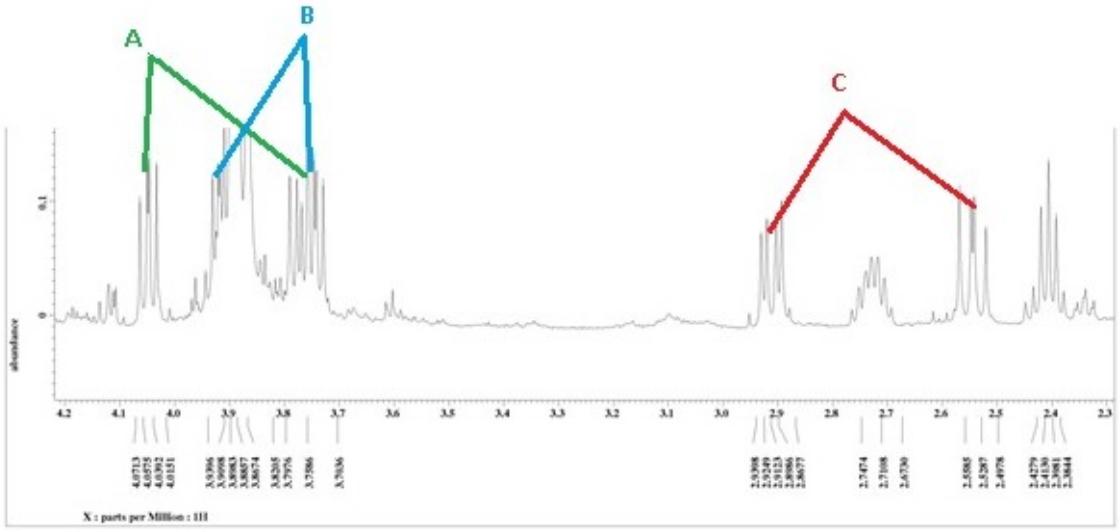



**Figure 29. $^{1}$H-NMR peaks for (±)-lariciresinol**

The peaks resulting from $CH_2$ groups A, B, and C were originally believed to be separate CH groups based on $^{1}$H-NMR integration, however analysis of the HSQC data confirmed that peaks A, B, and C were coupled to carbon signals at 73.0, 61.0, and 33.4 ppm respectively, all of which presented on the DEPT 135 spectrum as $CH_2$ groups. This indicated that three of these carbons possessed diastereotopic protons in which one of the protons was more deshielded than the others. It was anticipated that such a pattern was the result of possibly two more oxygen-containing groups in addition to a strained ring causing the carbon and hydrogen signals associated with peaks A to shift further down field than B and C. This geminal coupling pattern is demonstrated in the HSQC spectrum in Figure 30.

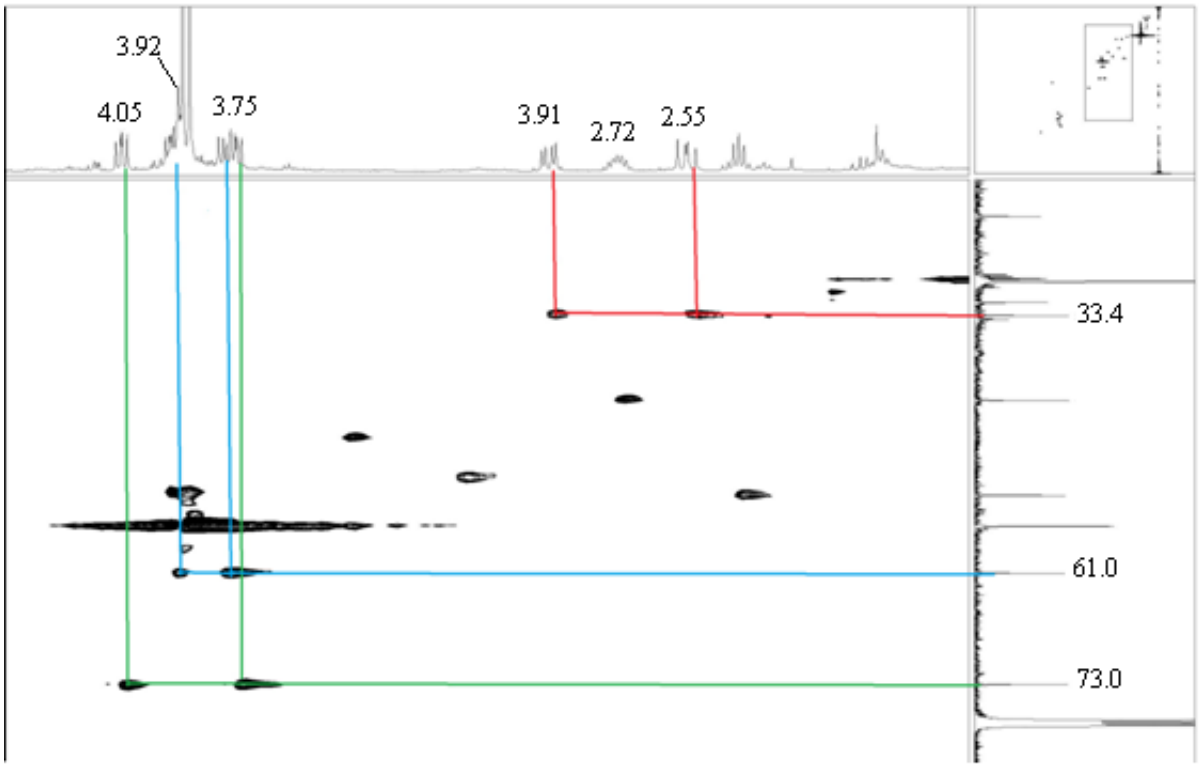



**Figure 30. HSQC showing proton coupling to carbon signals**

Analysis of spectroscopic data also indicated the presence of three CH groups in addition to six quaternary aromatic carbons and six tertiary carbons. $^{1}$H-NMR signals are listed in Table 1 below with the corresponding $^{13}$C-NMR signals based on HSQC data. The number of hydrogens assigned to each carbon signal is indicated based on DEPT 135 and DEPT 90 data. Hydrogen signal integrations are also indicated. All signals are given in ppm.

**Table 1. Summary of Spectroscopy Data for Lariciresinol (ppm) ($CDCl_3$)**

| δ $^{13}$C (ppm) | DEPT | δ $^{1}$H (ppm) | $^{1}$H Integration |
|---|---|---|---|
| 146.7 | C- | | |
| 146.6 | C- | | |
| 145.1 | C- | | |
| 144.1 | C- | | |
| 134.8 | C- | | |
| 132.4 | C- | | |
| 121.3 | CH | 6.68 | 1H |
| 118.8 | CH | 6.86 | 1H |
| 114.5 | CH | 6.86 | 1H |
| 114.2 | CH | 6.86 | 1H |
| 111.2 | CH | 6.68 | 1H |
| 108.3 | CH | 6.86 | 1H |
| 82.9 | CH | 4.78 | 1H |
| 73.0 | CH2 | 3.75, 4.05 | 2H, 1H |
| 61.0 | CH2 | 3.75, 3.92 | 2H, 1 H |
| 56.0 | CH3 | 3.89, 3.87 | 3H, 3H |
| 52.7 | CH | 2.41 | 1H |
| 42.5 | CH | 2.72 | 1H |
| 33.4 | CH2 | 2.55, 2.91 | 1H, 1H |

Having obtained these results, COSY and HMBC signals were then analyzed to identify adjacent carbon atoms. HSQC data had allowed the identification of corresponding hydrogen – carbon peaks so COSY data could then be translated from hydrogen signals to carbon signals. Table 2 summarizes this data with $^{1}$H-$^{1}$H coupling signals listed on the left and the corresponding $^{13}$C signals on the right. The major coupling signals were given by $^{1}$H peaks at δ = 2.41 ppm ($^{13}$C δ = 52.7 ppm) and δ = 2.72 ppm ($^{13}$C δ = 42.5 ppm) so these two signals are given as heading with the peaks they couple to listed below. All signals are given in ppm.

**Table 2. Lariciresinol COSY signals (ppm) ($CDCl_3$)**

| $^{1}H$ δ = 2.41 linked to: | $^{13}C$ δ = 52.7 linked to: |
|---|---|
| 2.55 | 33.4 |
| 2.91 | 33.4 |
| 2.72 | 42.5 |
| 3.75 | 61.0 |
| 3.89 | 61.0 |
| 4.78 | 82.9 |
| **$^{1}H$ δ = 2.73 linked to** | **$^{13}C$ δ = 42.5 linked to:** |
| 3.75 | 61.0 |
| 4.05 | 73.0 |
| 2.55 | 33.4 |
| 2.91 | 33.4 |

HMBC data provided information regarding the location of the methoxy groups and assisted in determining the presence of the tetrahydrofuran ring. The carbon atoms making up the tetrahydrofuran ring (δ = 42.5, 52.7, 73.0, and 82.9 ppm) produced coupling signals to one another as well as to carbon atoms on their adjacent aromatic rings. This data is summarized in Table 3. As in Table 2, the most commonly paired signal is listed at the top. All signals are given in ppm.

**Table 3. HMBC Signals for Lariciresinol (ppm) ($CDCl_3$)**

| $^{1}H$ δ = 3.89, 3.87 ($^{13}C$ δ = 56.0) linked to: | $^{1}H$ δ = 4.78 ($^{13}C$ δ = 82.9) linked to: | $^{1}H$ δ = 2.55, 2.91 ($^{13}C$ δ = 33.4) linked to: |
|---|---|---|
| 145.1 | 61.0 | 42.5 |
| 146.6 | 73.0 | 52.7 |
| 146.7 | 108.3 | 73 |
| | 118.8 | 111.2 |
| | | 121.3 |
| | | 134.8 |

Based on mass spectroscopy data, the structure of pure compound **1** was positively identified as lariciresinol. Based on several optical rotation values of 0.0˚ the compound was determined to be a racemic mixture. The structure of lariciresinol is given in Figure 31 with the $^{1}$H-NMR peaks labeled according to the numbered carbon atoms on the sub-structure.

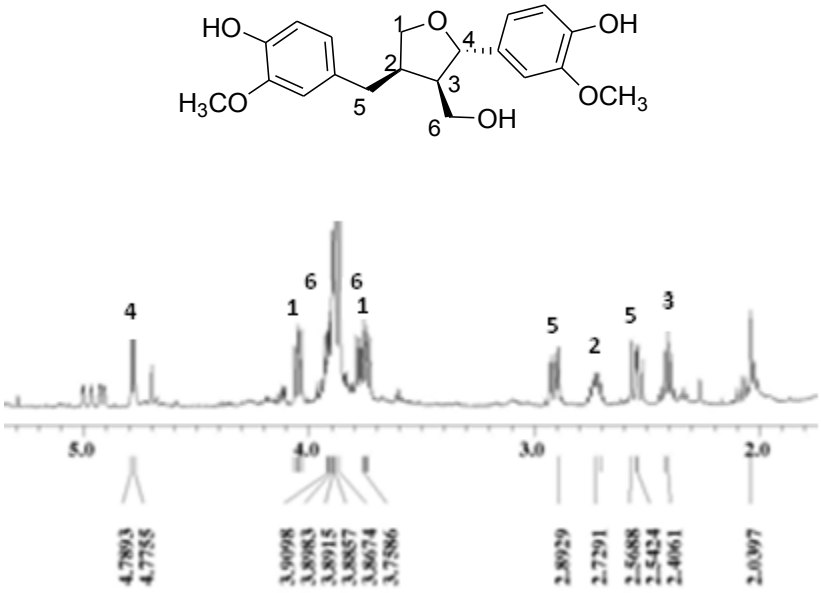



**Figure 31. Lariciresinol peak assignment**

***Identification of Compound 2***

The identification of compound **2** thus far has been based on the similarities between the experimental spectroscopy data for compound **2** and the spectroscopy values reported in the literature for pinoresinol. Despite compound **2** being relatively pure, there are extra signals present in the experimental NMR spectra that cannot be ignored. This

suggests that while compound **2** is most likely very similar to pinoresinol, the two structures are not exactly identical. Analysis of the spectroscopy data, mass spectrometry, and optical rotation suggests that compound **2** shares everything in common with the structure of pinoresinol except the location of one methoxy group and one hydroxy group. The structure of pinoresinol is given in Figure 32 next to a proposed structure for compound **2**. The difference between the two structures is highlighted in red.

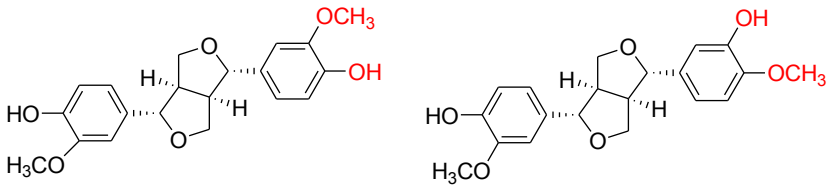



**Figure 32. Structure of pinoresinol (left) and proposed compound 2 (right)**

As illustrated in Figure 32, pinoresinol is a symmetrical compound with both hydroxy groups para to their corresponding furan rings and both methoxy groups meta to the furan. The proposed structure of compound **2** switches this orientation on one of the aromatic rings, making the methoxy para to the furan and the hydroxy meta to the furan.

The structure of pinoresinol is such that the $^{13}$C-NMR spectra gives only ten signals for 20 carbon atoms. For every one carbon atom in the molecule, there is another one that is equivalent. This is not the case with the proposed structure of compound **2**. This structure is not symmetric and therefore gives 20 carbon signals on the $^{13}$C-NMR, one for each carbon atom in the structure. Table 4 compares the $^{13}$C-NMR and $^{1}$H-NMR

literature data for pinoresinol with the experimental data for compound **2**.[54] The far right column lists the extra experimental $^{13}$C-NMR signals observed for compound **2**. All chemical shits are reported in ppm.

**Table 4. Comparison of Literature Data for Pinoresinol (ppm) ($CDCl_3$)[54]**

| Pinoresinol Literature δ $^1$H | Compound 2 δ $^1$H | Pinoresinol Literature δ $^{13}$C | Compound 2 δ $^{13}$C | Compound 2 Extra δ $^{13}$C |
|---|---|---|---|---|
| 6.78-6.90 | 6.81-6.93 | 146.9 | 146.8 | 147.5 |
| 5.64 | 5.63 | 145.2 | 145.3 | 142.4 |
| 4.73 | 4.72-4.79 | 132.9 | 132.9 | 133.0 |
| 4.25 | 4.25 | 118.9 | 119.1 | 124.1 |
| 3.89 | 3.95 | 114.3 | 114.4 | 121.0 |
| 3.86 | 3.81-3.87 | 108.6 | 108.6 | 108.2 |
| 3.09 | 3.11 | 85.8 | 85.9 | 86.0 |
| | | 71.7 | 71.8 | 71.9 |
| | | 55.9 | 56.1 | 56.3 |
| | | 54.2 | 54.3 | 54.2 |

As shown in Table 4 above, the ten additional $^{13}$C-NMR signals observed for compound **2** are not far offset from the signals matching pinoresinol. This suggests that compound **2** is most likely a non-symmetrical isomer of pinoresinol. Figure 33 shows the $^1$H-NMR (δ 3.0 – 4.8) and $^{13}$C-NMR (δ 54.2 – 86.0) spectra for compound **2**, showing the peaks being slightly offset from one another. This can even be seen in the $^1$H-NMR spectra, such as in the peak at δ 3.11 – 3.20, which integrates to 2H, and the two doublets at δ = 4.72 ppm and δ = 4.79 ppm, which both integrate to 1H. In the $^{13}$C-NMR, the slight shift is evident around δ = 56.3, 71.8, and 85.9 ppm.

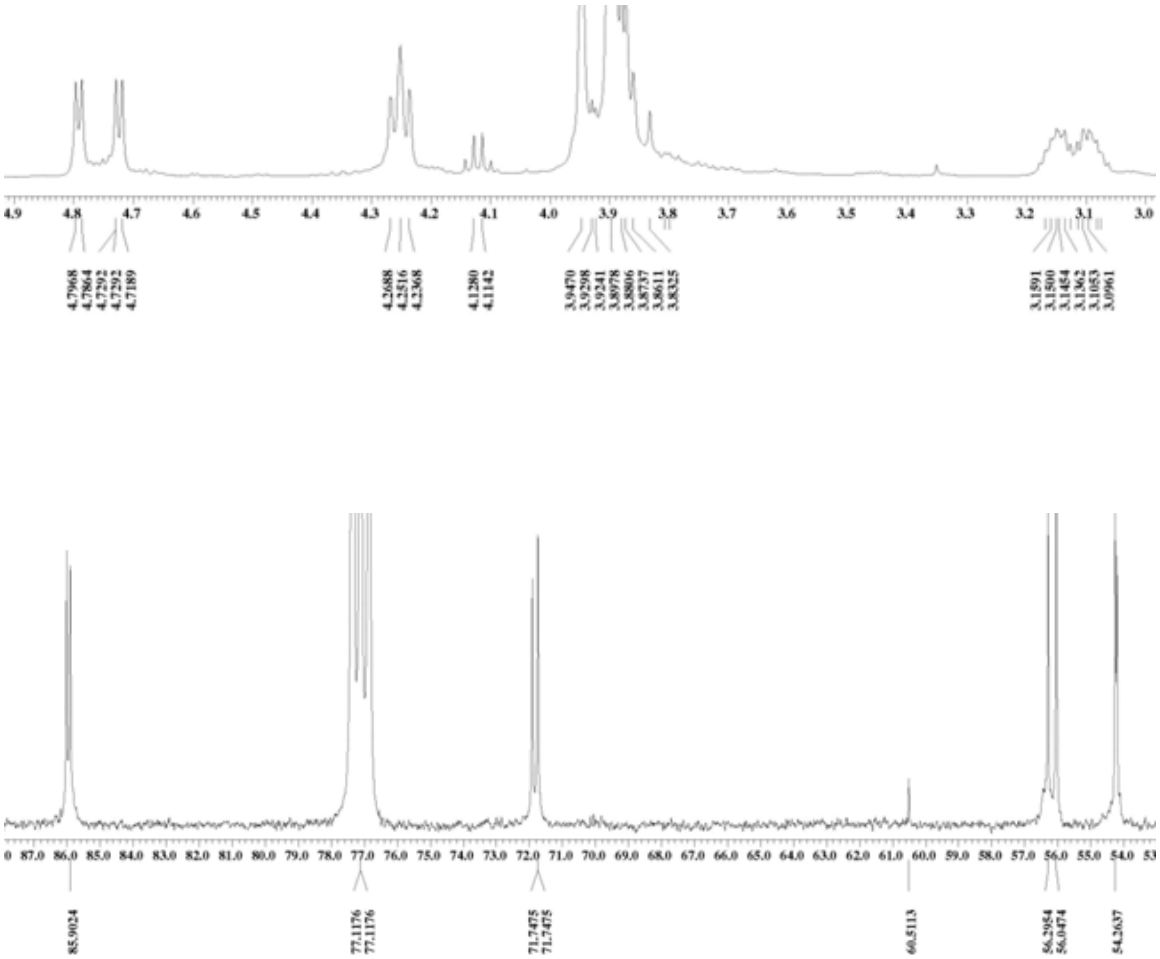



**Figure 33. $^{1}$H-NMR (top) and $^{13}$C-NMR (bottom) spectra for compound 2**

Table 5 below provides a summary of the non-aryl $^{13}$C-NMR, $^{1}$H-NMR, DEPT 135, DEPT 90, and HMQC data for compound **2**. The $^{13}$C and $^{1}$H signals are paired based on coupling signals from the HMQC. Some of the $^{13}$C signals (such as δ 71.8 and 71.9) are too close together to determine which $^{13}$C signal is coupling to which $^{1}$H-NMR signal, so both $^{1}$H shifts have been listed.

**Table 5. Summary of Spectroscopy Data for Compound 2 (ppm) ($CDCl_3$)**

| δ $^{13}C$ | DEPT | δ $^{1}H$ | δ $^{1}H$ Integration |
|---|---|---|---|
| 86.0 | CH | 4.79 | 1H |
| 85.9 | CH | 4.72 | 1H |
| 71.9 | CH2 | 3.81, 4.25 | 2H, 2H |
| 71.8 | CH2 | 3.81, 4.25 | 2H, 2H |
| 56.3 | CH3 | 3.87, 3.95 | 5H, 3H |
| 56.1 | CH3 | 3.87, 3.95 | 5H, 3H |
| 54.3 | CH | 3.11-3.20 | 2H |
| 54.2 | CH | 3.11-3.20 | 2H |

As shown in Table 5, the $^{1}H$-NMR signal at δ 3.11 – 3.20 integrates to 2H, but gives coupling signals to two $^{13}C$-NMR signals at δ 54.2 and 54.3. This is shown in the HMQC spectrum provided in Figure 34.

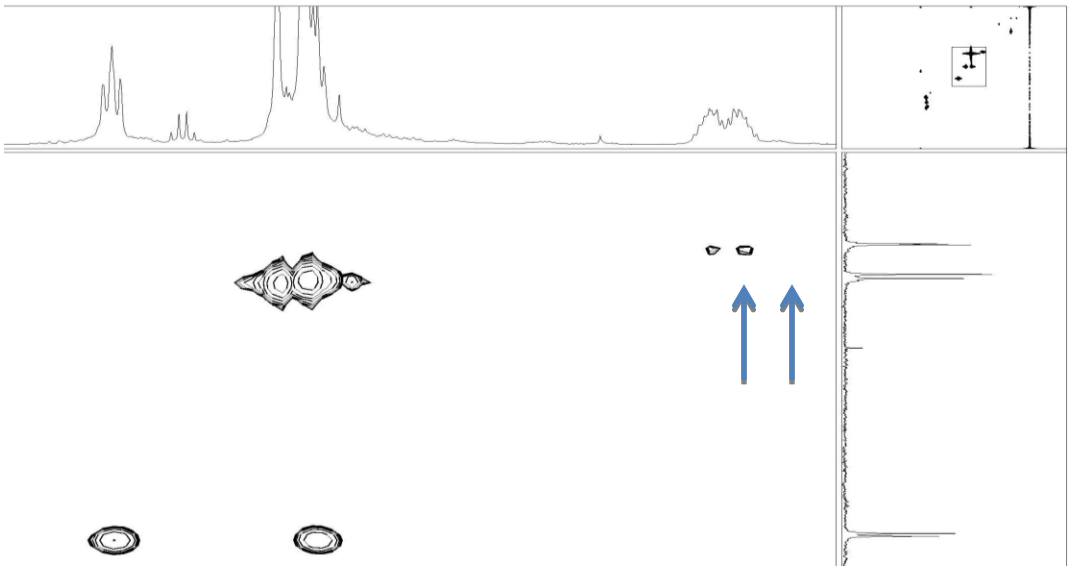


**Figure 34. HMQC of compound 2**

HRMS confirmed that compound **2** had *m/z* ($C_{20}H_{22}O_6$) 358.14, the same as pinoresinol. Additionally, compound **2** showed an optical rotation of 29.2° in methanol which is very close to the 28.0° rotation of pinoresinol reported in the literature. It is important to note that the NMR data reported in the literature data for pandanusphenol A does not match the experimental data for compound **2**. Furthermore, pandanusphenol A has *m/z* ($C_{21}H_{22}O_6$) 372.41.

After conducting a thorough literature review, it appears the proposed structure of compound **2** has not been reported in the literature as a naturally occurring compound. Furthermore, there does not appear to be a reported compound in the literature that matches the spectroscopy data for compound **2** more closely than pinoresinol. As such, a structure has been proposed for compound **2** that would correspond to the data obtained and justify the appearance of extra spectroscopic signals. Additional research, however, will be required in order to confirm this proposed structure.

**Conclusion**

Two plants of Chinese origin were fractionated and purified to afford four isolated compounds. One of these compounds was identified positively as lariciresinol while the structure of the second compound has been proposed with a reasonable amount of certainty as **2** (Figure 32). Additional procedures should be performed, such as recrystallization and x-ray crystallography in order to confirm the proposed structure. The remaining two compounds were not identified due to low yields and an insufficient amount of crude starting material. Unfortunately, lariciresinol exhibited <5% cancer inhibition and <29% anti-inflammatory activity.[51, 52] Anti-viral and anti-protozoan tests

have not yet been performed. The second compound isolated has also not been tested for bioactivity yet.

The results of this research provide opportunities for future work in this area. More research is required to adequately identify the sources of bioactivity in the plants *Euphorbia hirta* and *Pandanus tectorius* as many compounds have yet to be isolated and identified. The hexane extract of *Euphorbia hirta* could yield unexpected bioactivity upon testing and the possibility of isolating additional bioactive compounds. Futhermore, other fractions of interest from *Pandanus tectorius* remain unexamined. Many demonstrated high cancer inhibition and should be examined more closely.

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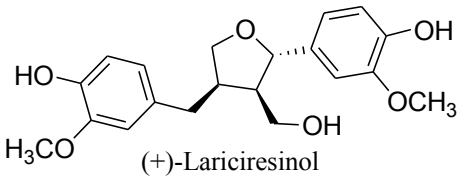
# APPENDICES

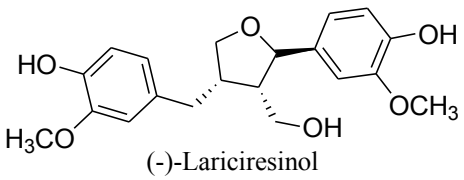
# APPENDIX A

## Spectroscopy Data for Lariciresinol

**(±) 4-[5-(4-Hydroxy-3-methoxyphenyl)-4-(hydroxymethyl)oxolan-3-yl]methyl]-2-methoxyphenol ((±) lariciresinol, PtC2b*1*).**



(+)-Lariciresinol

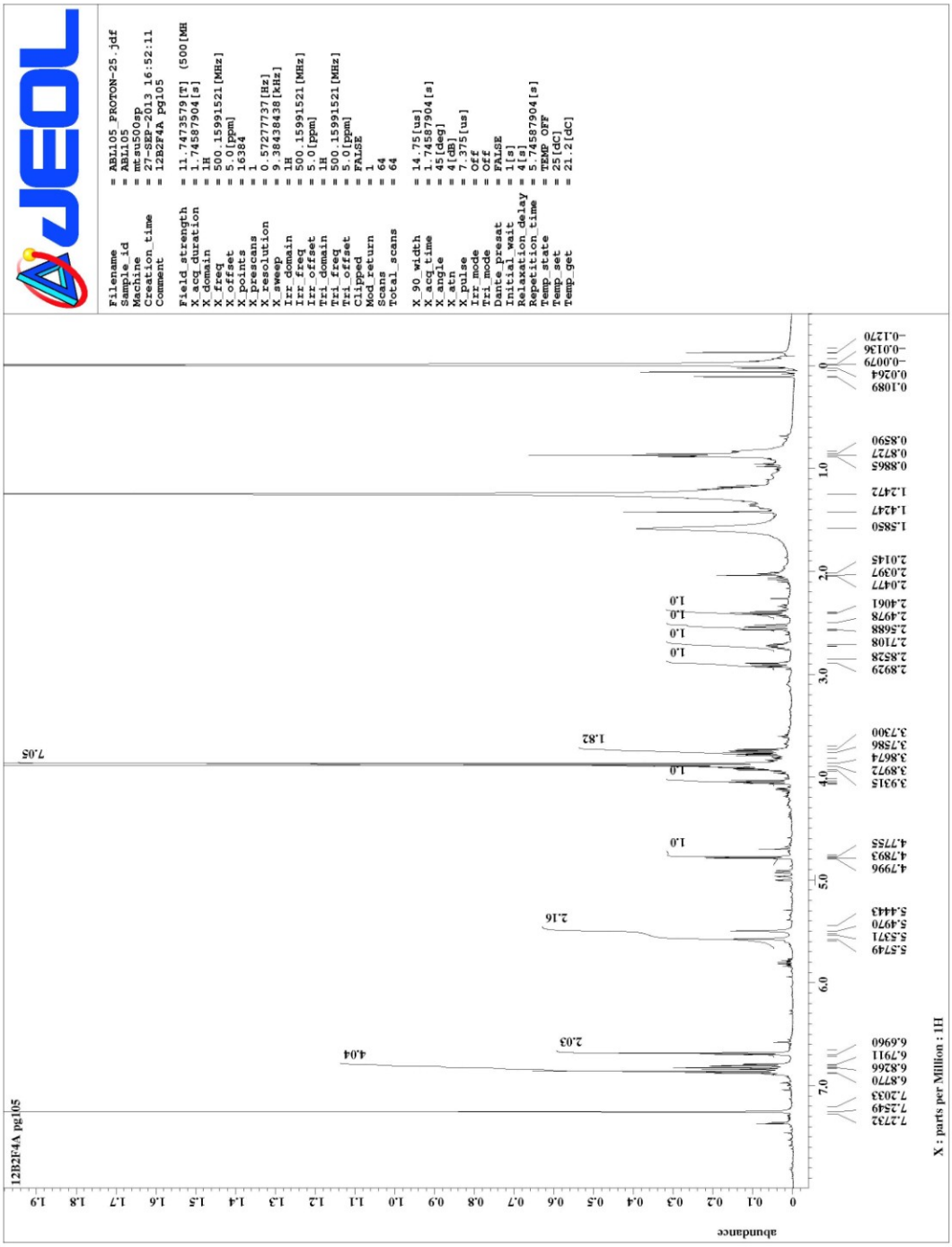


(-)-Lariciresinol

-NMR

- $^{1}H$ ($CDCl_3$)
- $^{1}H$ ($CD_3OD$)
- $^{13}C$ ($CDCl_3$)
- $^{13}C$ ($CD_3OD$)
- DEPT 90 ($CDCl_3$)
- DEPT 135 ($CDCl_3$)
- COSY ($CDCl_3$)
- HSQC ($CDCl_3$)
- HMBC ($CDCl_3$)

$^{1}$ H-NMR ($CDCl_3$)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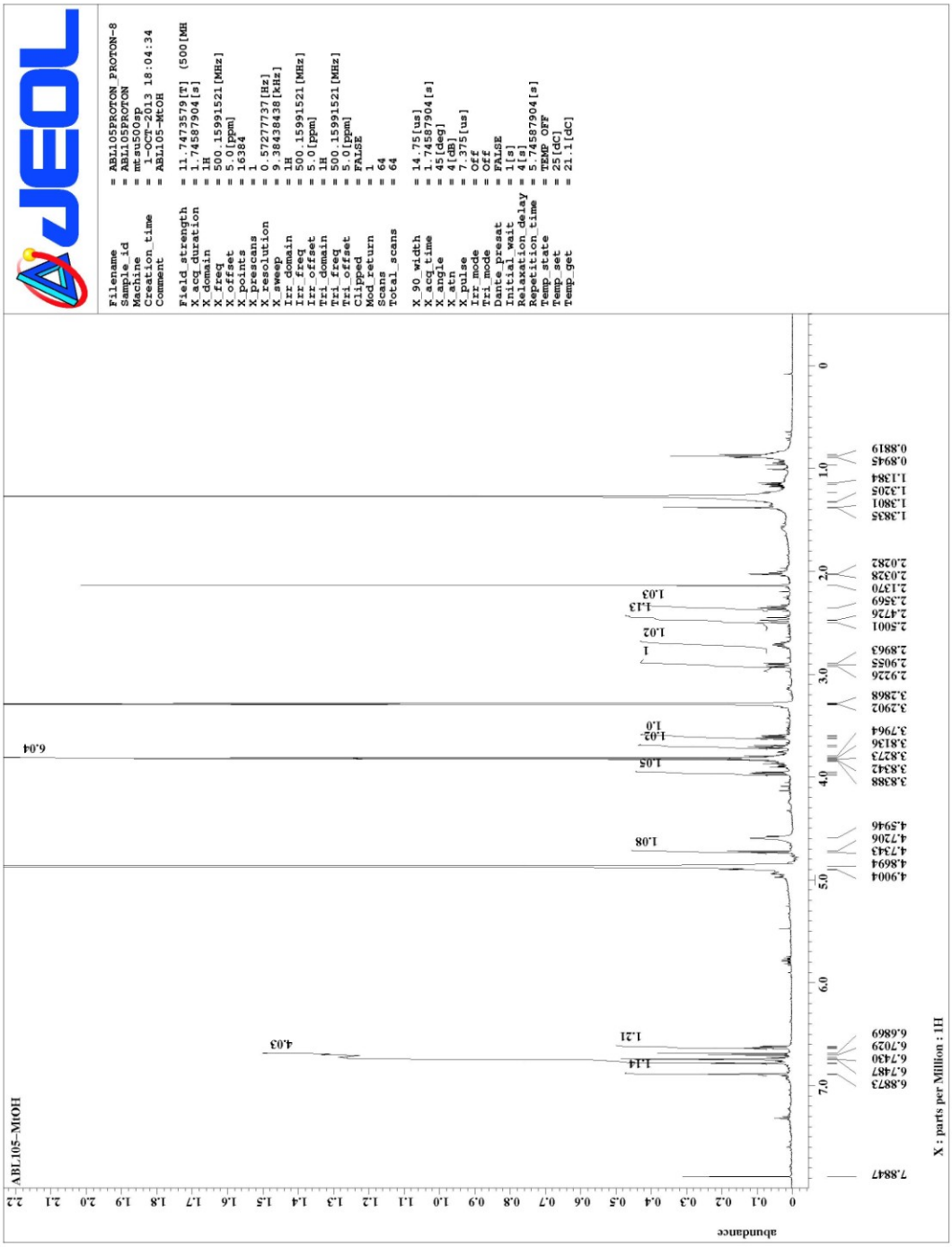
1.0

$^{1}$H-NMR ($CD_3OD$)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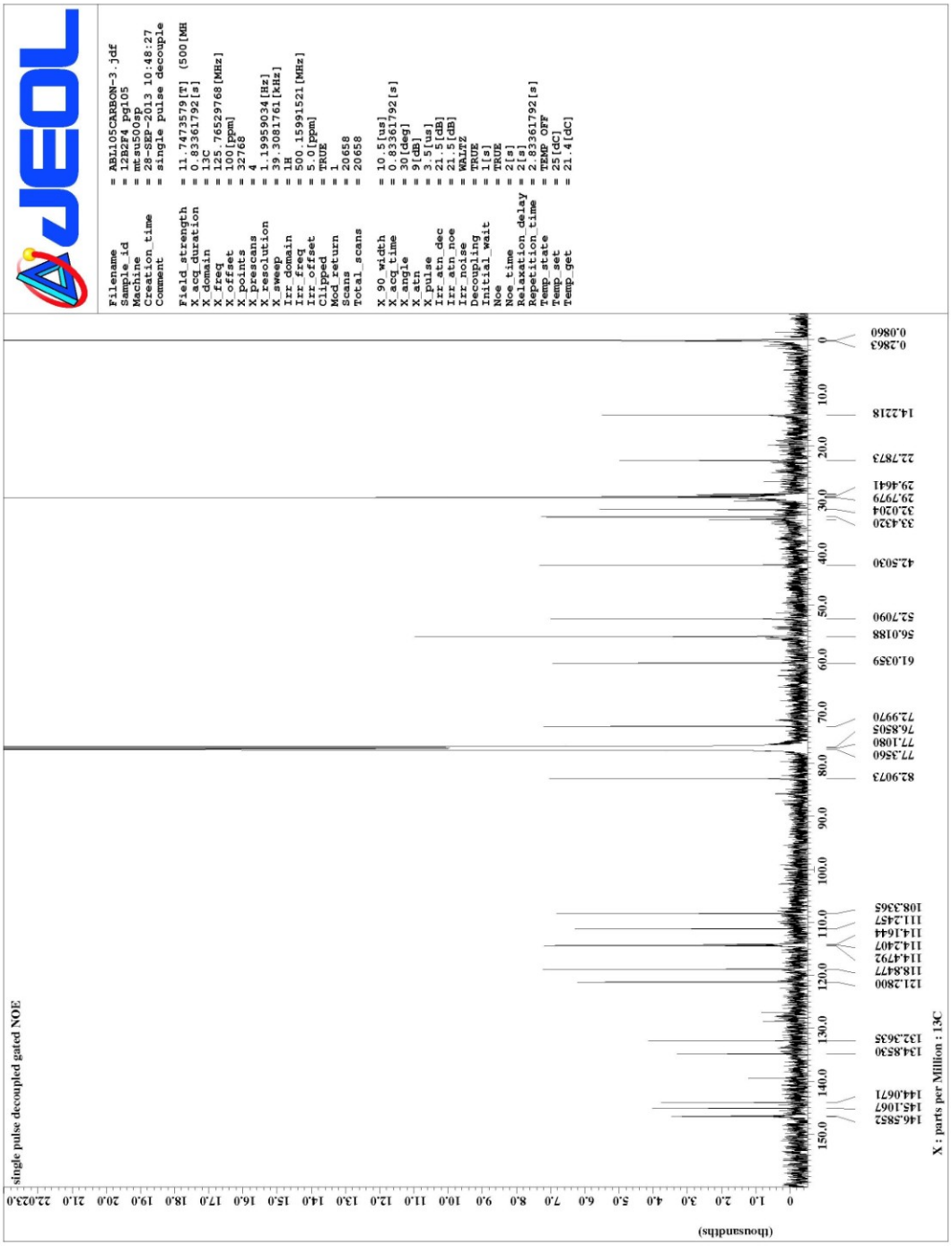
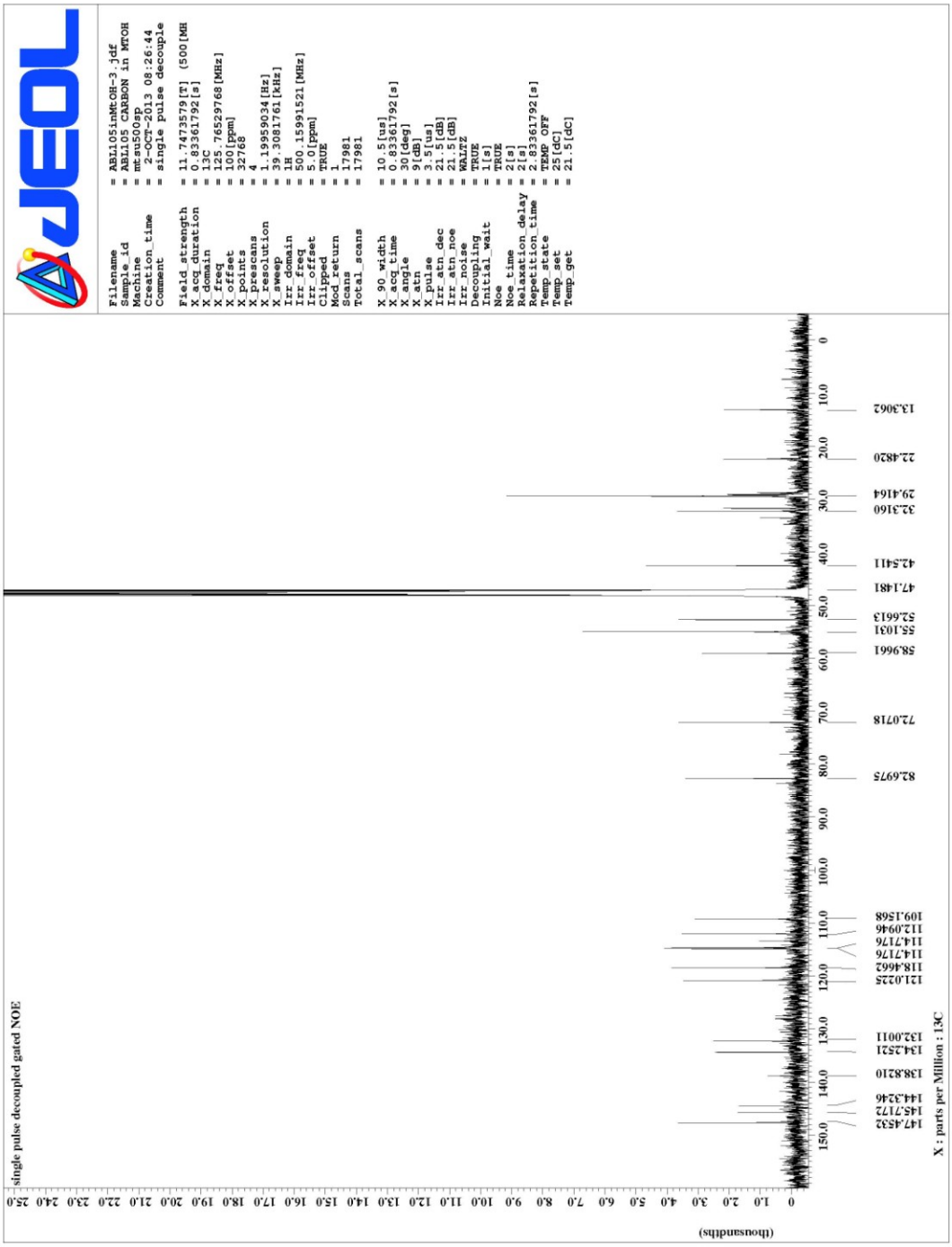
X_points = 16384
X_prescans = 1
X_resolution = 0.572777737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 64
Total_scans = 64
X_90_width = 14.75[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 4[dB]
X_pulse = 7.375[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 21.1[dC]
ABL105-MtOH
6.04
4.03
1.21
1.14
1.08
1.05
1.02
1.0
1.03
1.13
1
1.02
7.8847
6.8873
6.7487
6.7430
6.7029
6.6869
4.9004
4.8694
4.7343
4.7206
4.5946
3.8388
3.8342
3.8273
3.8136
3.7964
3.2902
3.2868
2.9226
2.9055
2.8963
2.5001
2.4726
2.3569
2.1370
2.0328
2.0282
1.3835
1.3801
1.3205
1.1384
0.8945
0.8819
abundance
X : parts per Million : 1H

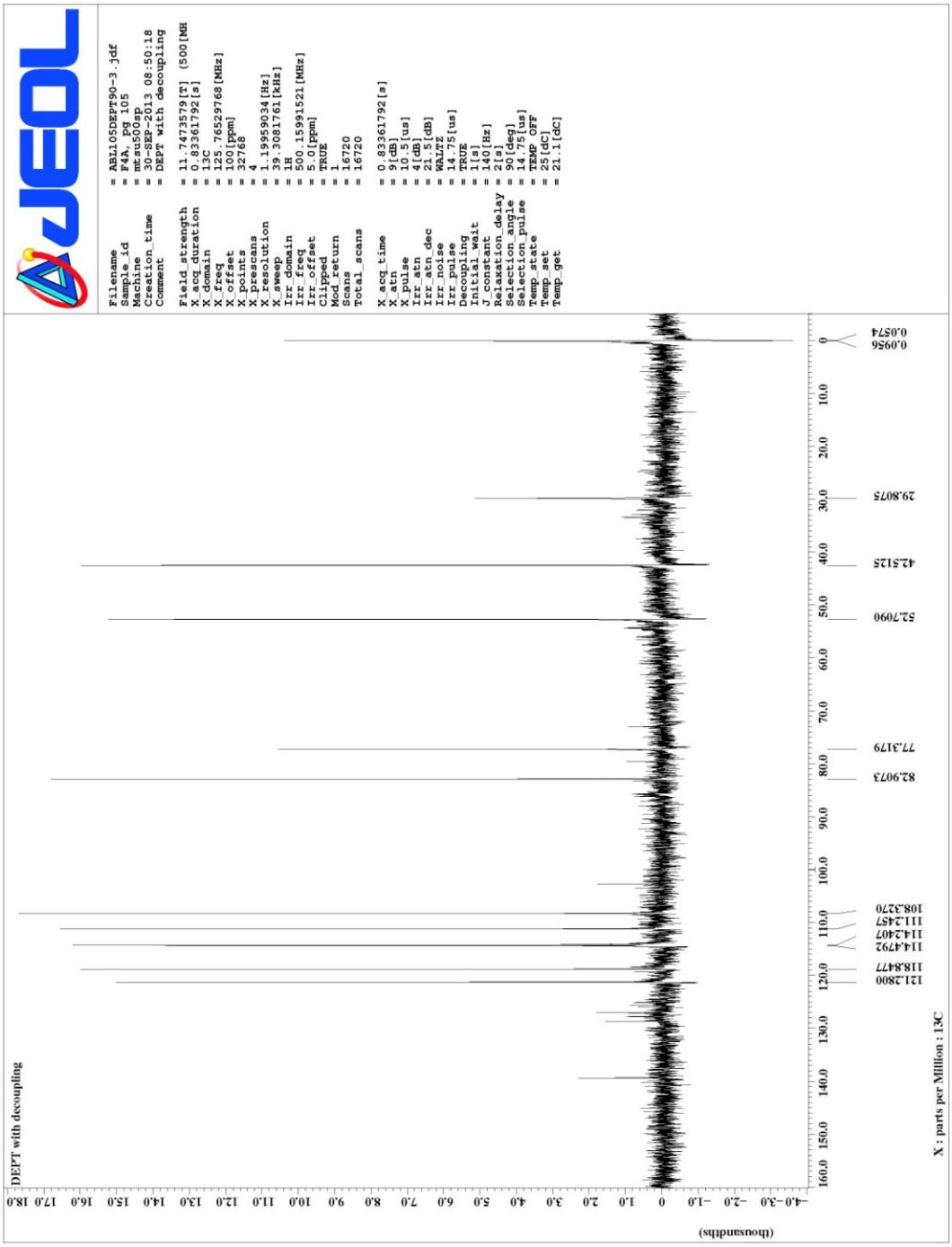
$^{13}$C-NMR ($CDCl_3$)



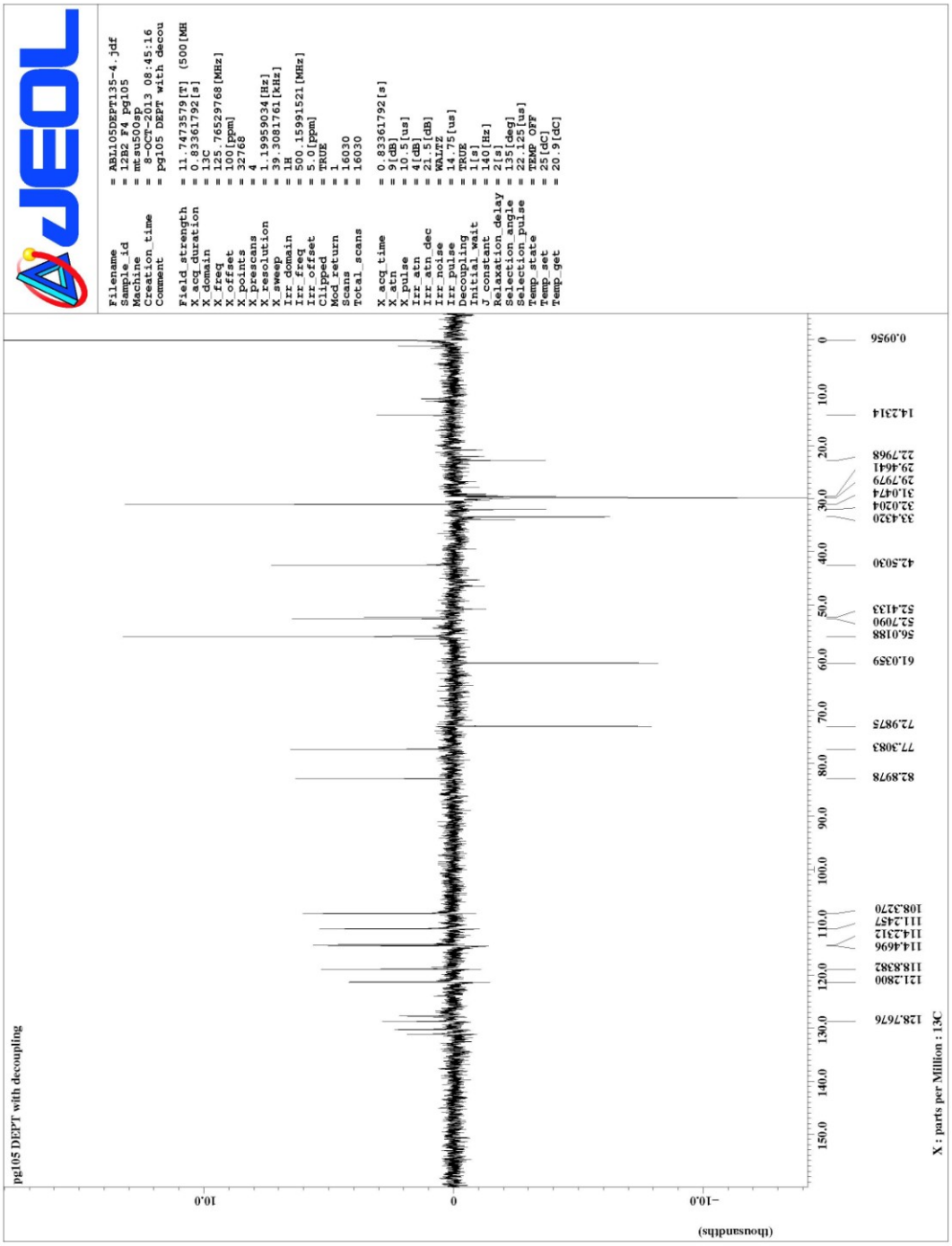
# $^{13}$C-NMR ($CD_3OD$)



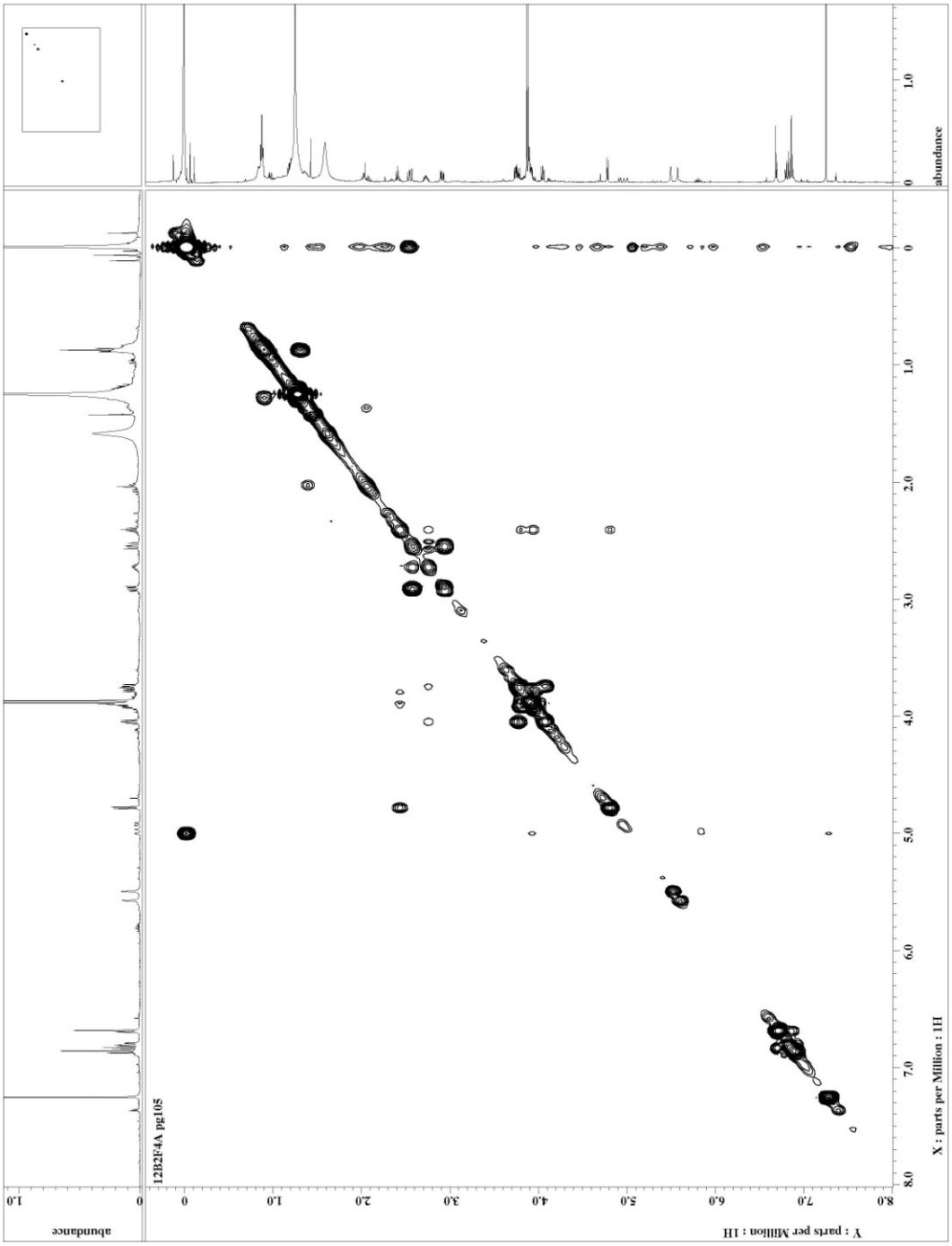
DEPT 90 ($CDCl_3$)



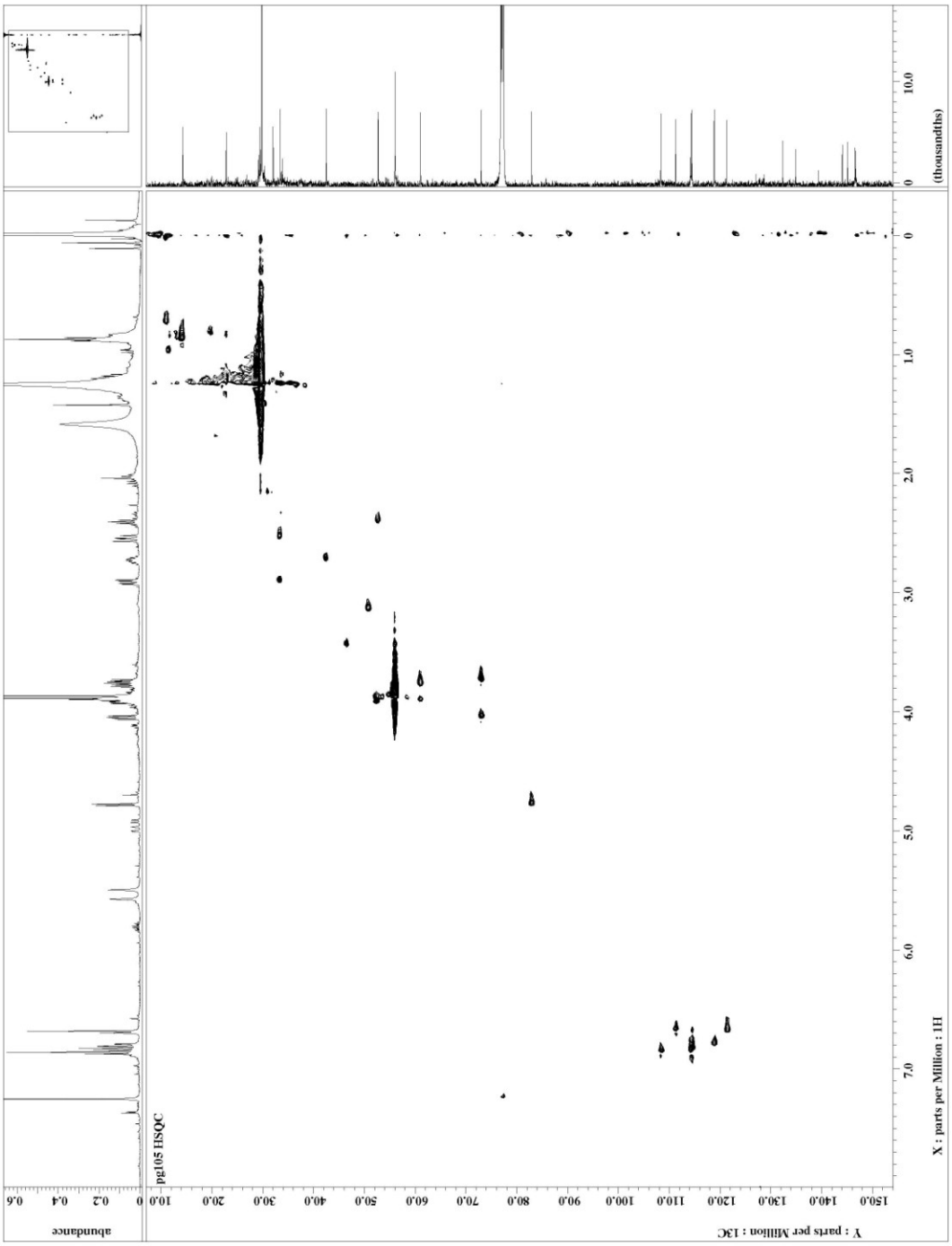
DEPT 135 ($CDCl_3$)



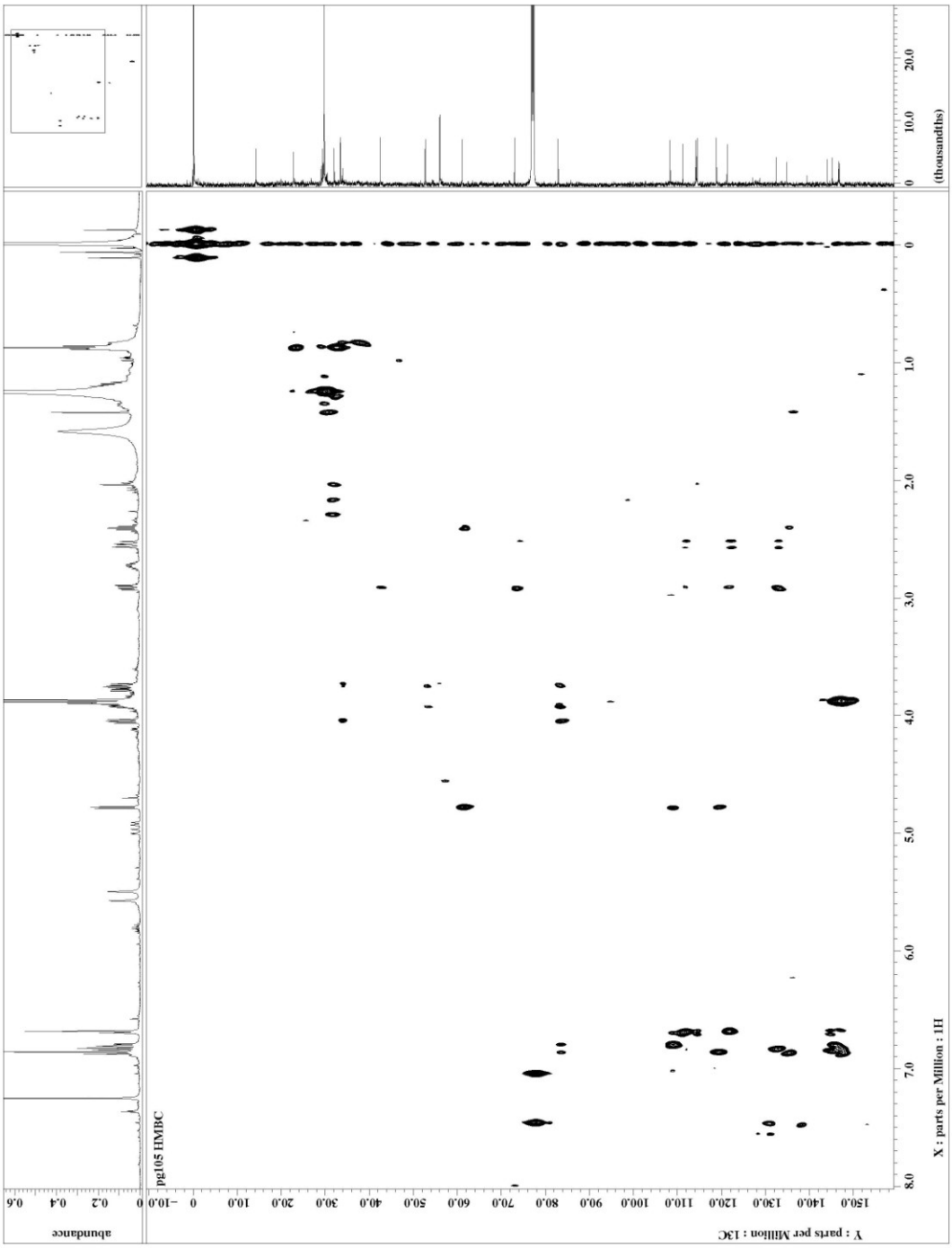
COSY ($CDCl_3$)


abundance
12B2F4A pg105
Y : parts per Million : 1H
X : parts per Million : 1H

HSQC ($CDCl_3$)


pg105 HSQC
X : parts per Million : 1H
Y : parts per Million : 13C
abundance
(thousandths)

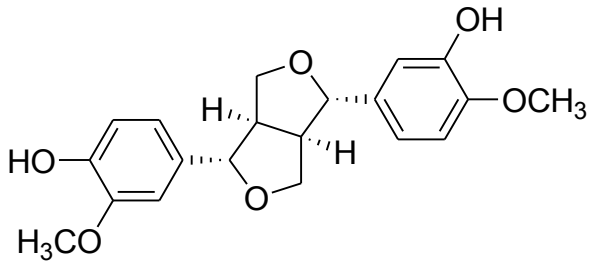
HMBC ($CDCl_3$)


pg105 HMBC
X : parts per Million : 1H
Y : parts per Million : 13C
abundance
(thousandths)

# APPENDIX B

## Spectroscopy Data for Compound 2

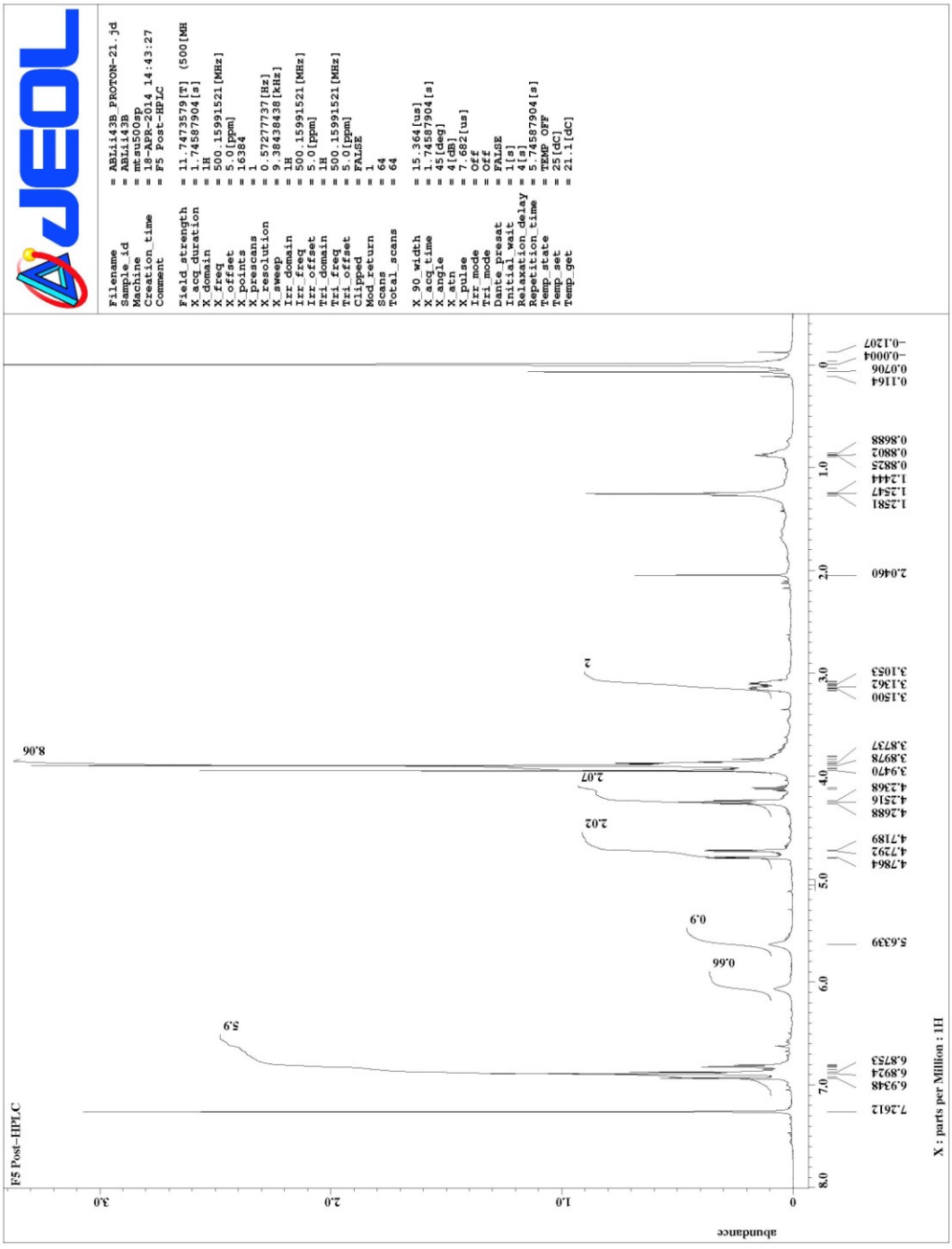
**(+)-4,5'-(1S,3aR,4S,6aR)-Hexahydrofuro[3,4-c]furan-1,4-diyl)bis(2-methoxyphenol), ((+)-compound 2, PtC3c).**



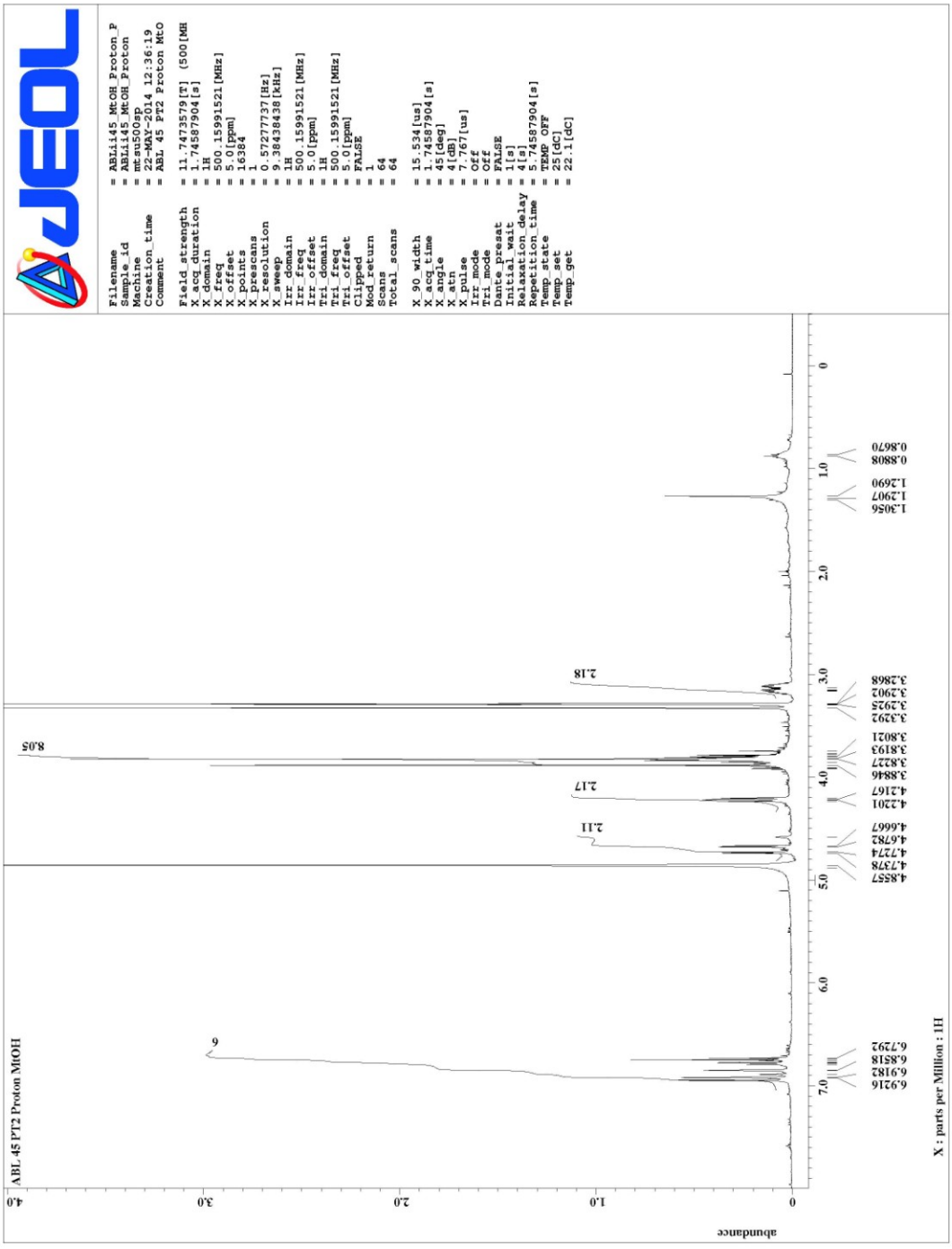

-NMR

- $^{1}H$ ($CDCl_3$)
- $^{1}H$ ($CD_3OD$)
- $^{13}C$ ($CDCl_3$)
- DEPT 90 ($CDCl_3$)
- DEPT 135 ($CDCl_3$)
- COSY ($CDCl_3$)
- HMQC ($CDCl_3$)
- HMBC ($CDCl_3$)

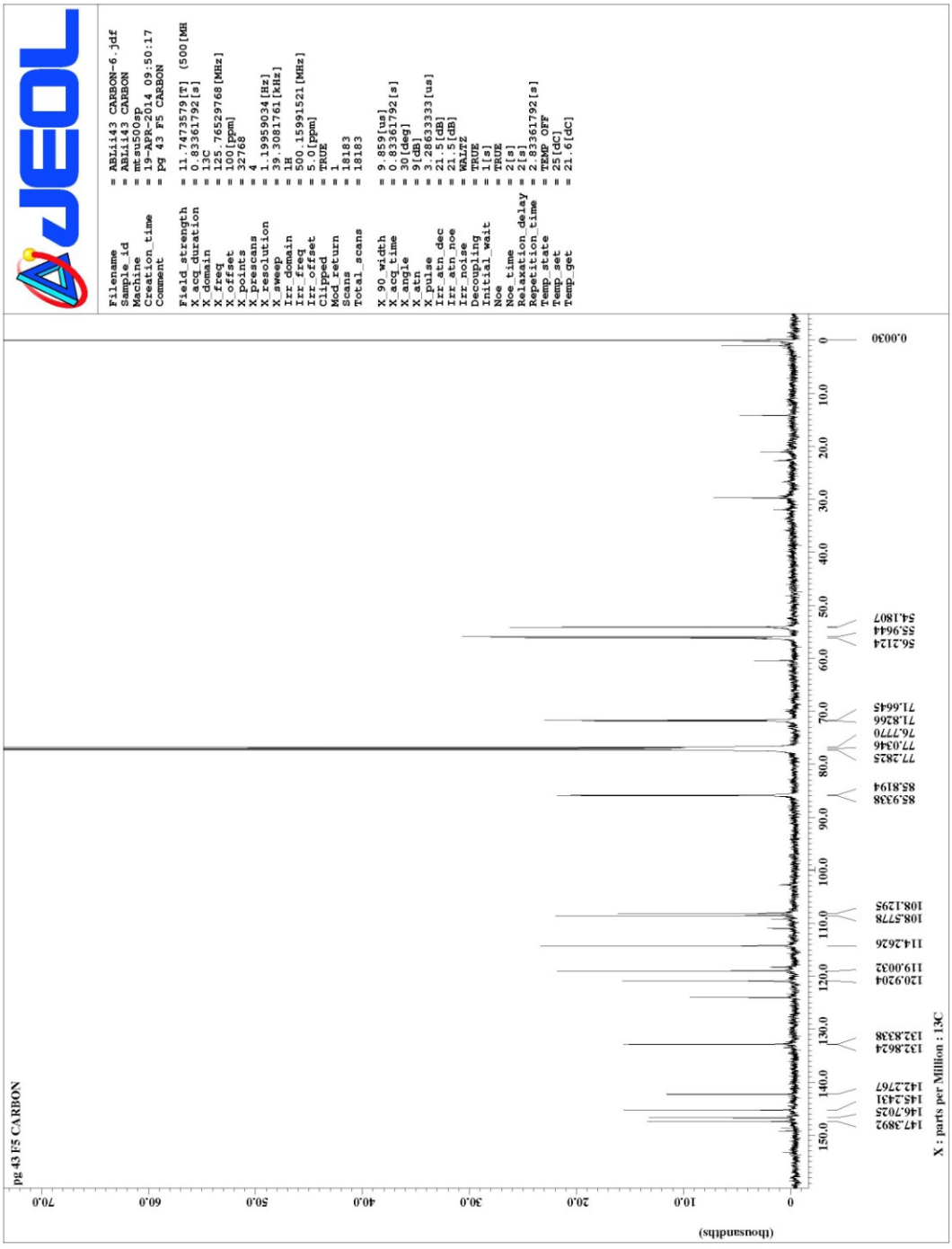
$^{1}$H-NMR ($CDCl_3$)


JEOL
Filename = ABLi143B_PROTON-21.jd
Sample_id = ABLi143B
Machine = mcsu500sp
Creation_time = 18-APR-2014 14:43:27
Comment = F5 Post-HPLC
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 64
Total_scans = 64
X_90_width = 15.364[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 4[dB]
X_pulse = 7.682[us]
Irr_mode = Off
Tri_mode = Off
Date_presat = FALSE
Initial_wait = 1[s]
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 21.1[dC]
F5 Post-HPLC
abundance
X : parts per Million : 1H
8.06
5.9
2.07
2.02
0.9
0.66
2
7.2612
6.9348
6.8924
6.8753
5.6339
4.7864
4.7792
4.7189
4.2688
4.2516
4.2368
3.9470
3.8978
3.8737
3.1500
3.1362
3.1053
2.0460
1.2581
1.2547
1.2444
0.8825
0.8802
0.8688
0.1164
0.0706
-0.0004
-0.1207

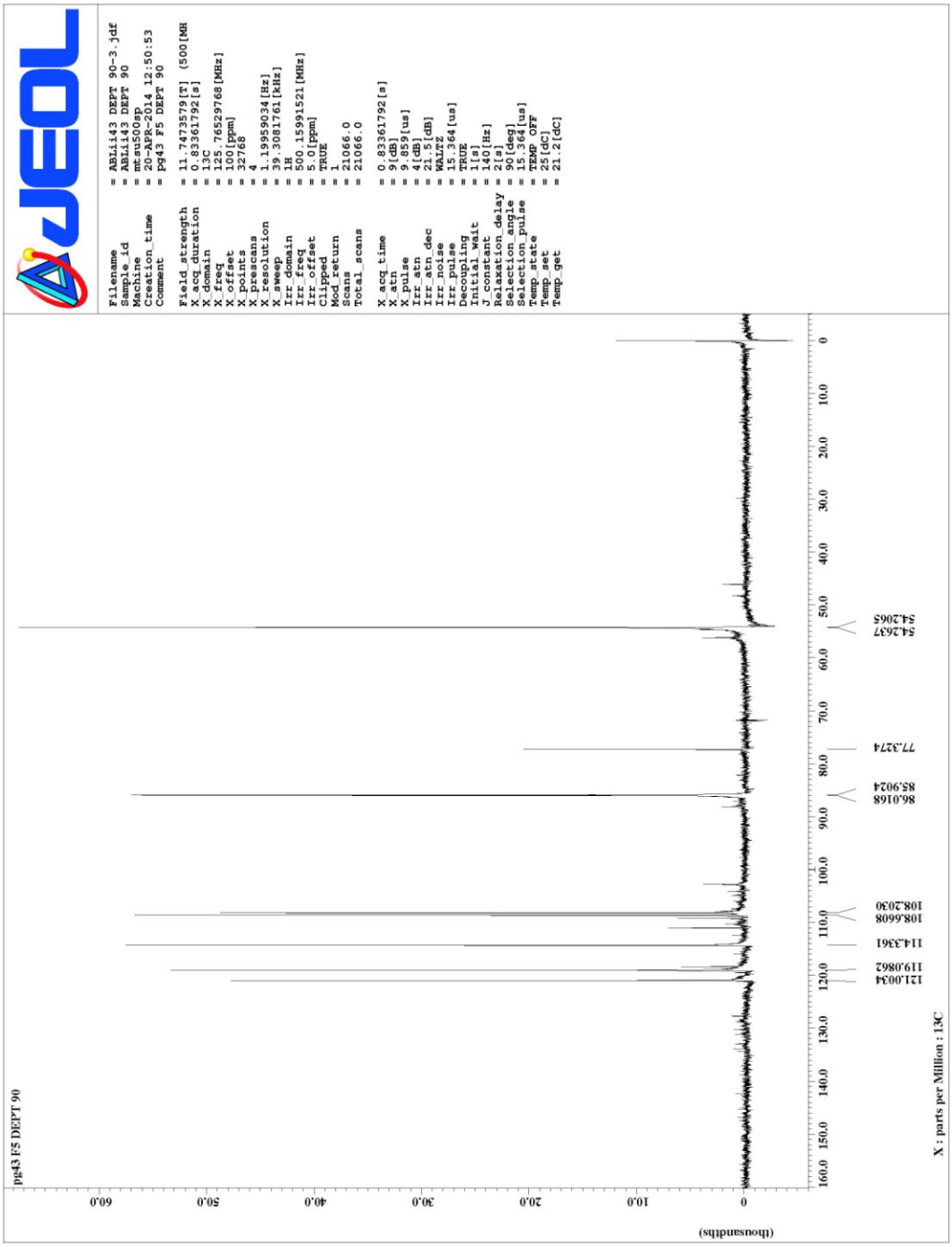
$^{1}$H-NMR ($CD_3OD$)


JEOL
Filename = ABLi145_MtOH_Proton_P
Sample_id = ABLi145_MtOH_Proton
Machine = mtesu500sp
Creation_time = 22-MAY-2014 12:36:19
Comment = ABL 45 P72 Proton MtO
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 64
Total_scans = 64
X_90_width = 15.534[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 4[dB]
X_pulse = 7.767[us]
Irr_mode = Off
Tri_mode = Off
Date_presat = FALSE
Initial_wait = 1[s]
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 22.1[dC]
ABL 45 PT2 Proton MtOH
abundance
X : parts per Million : 1H
8.05
6
2.18
2.17
2.11
6.9216
6.9182
6.8518
6.7292
4.8557
4.7378
4.7274
4.6782
4.6667
4.2201
4.2167
3.8846
3.8227
3.8193
3.8021
3.3292
3.2925
3.2902
3.2868
1.3056
1.2907
1.2690
0.8808
0.8670

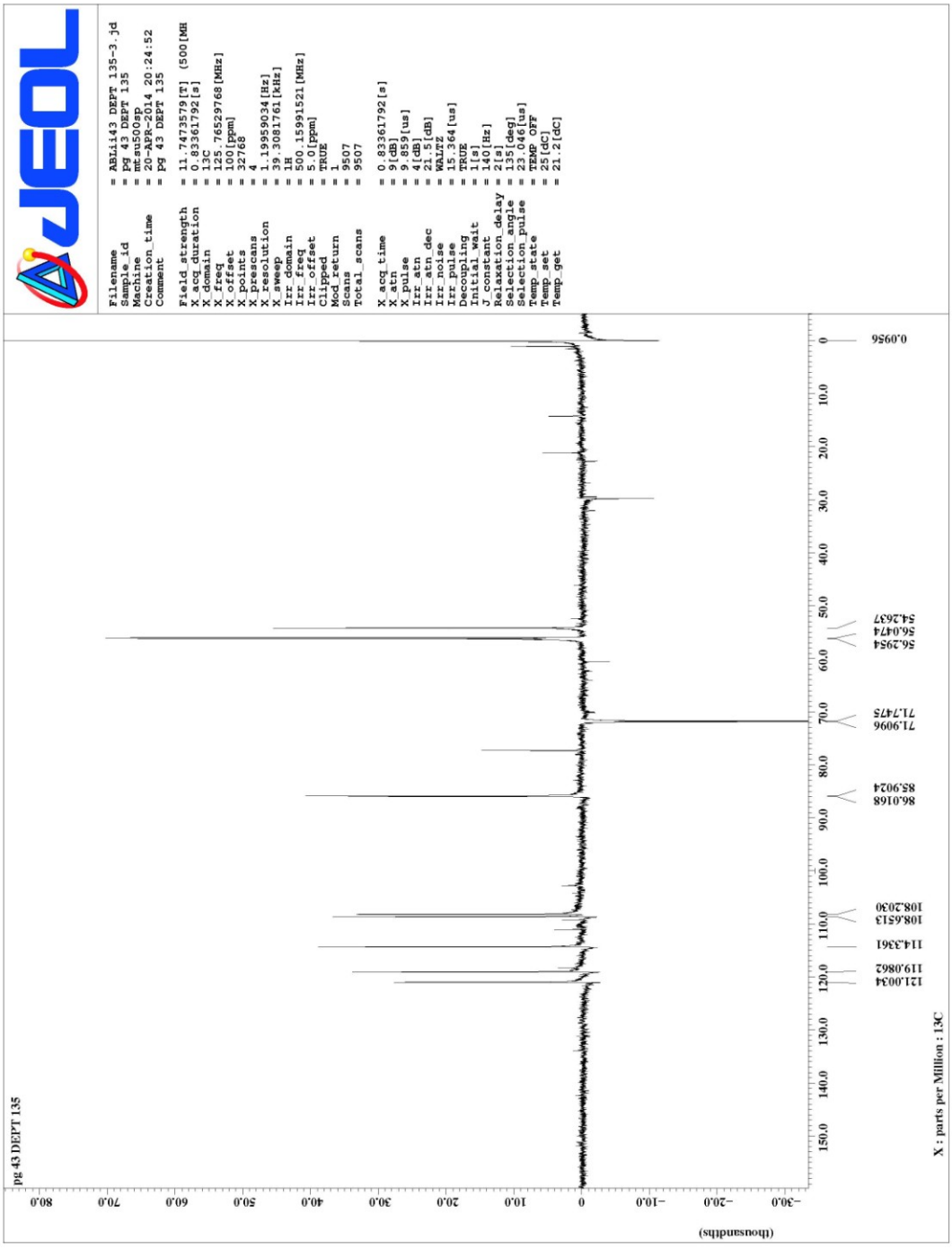
$^{13}$C-NMR ($CDCl_3$)



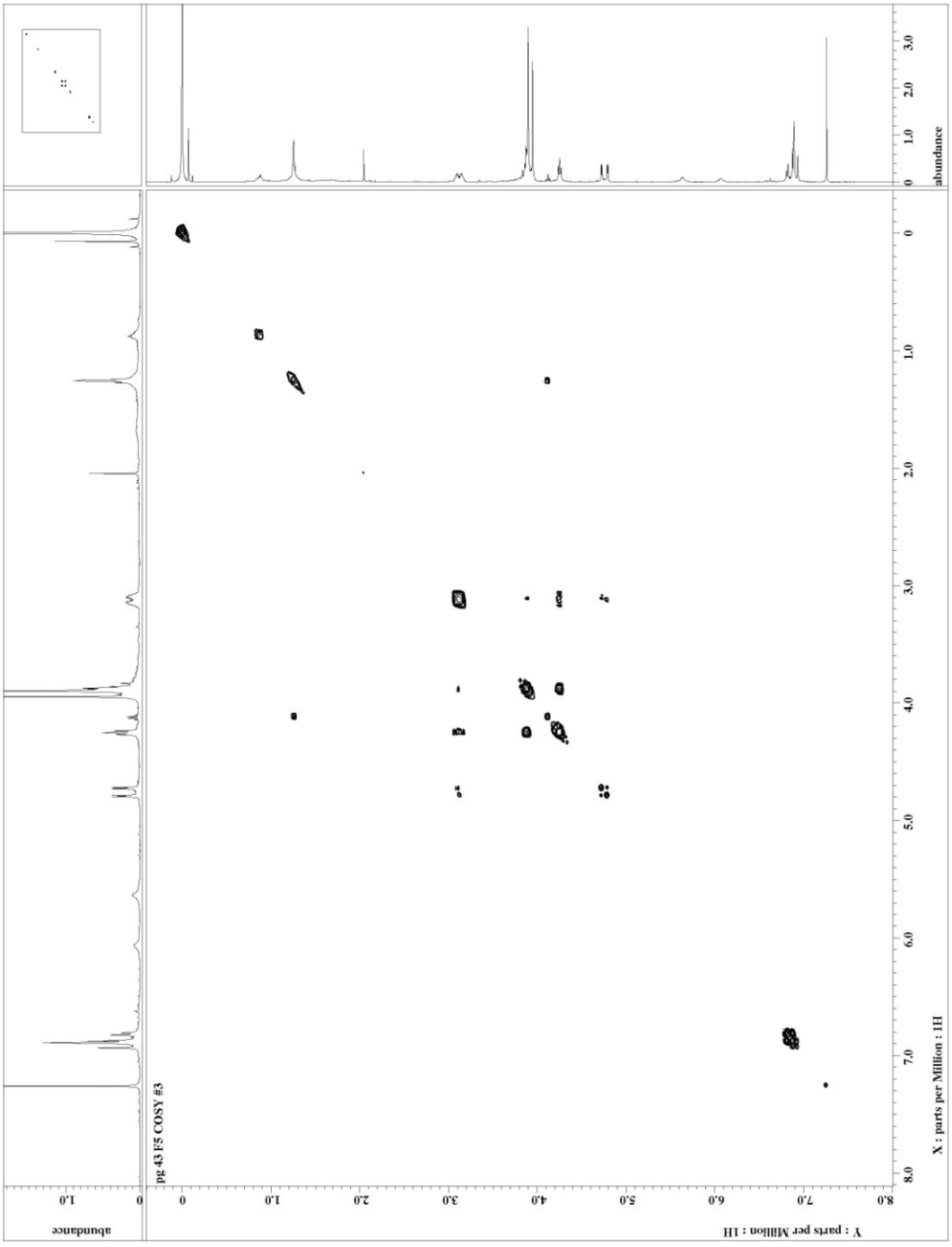
DEPT 90 ($CDCl_3$)



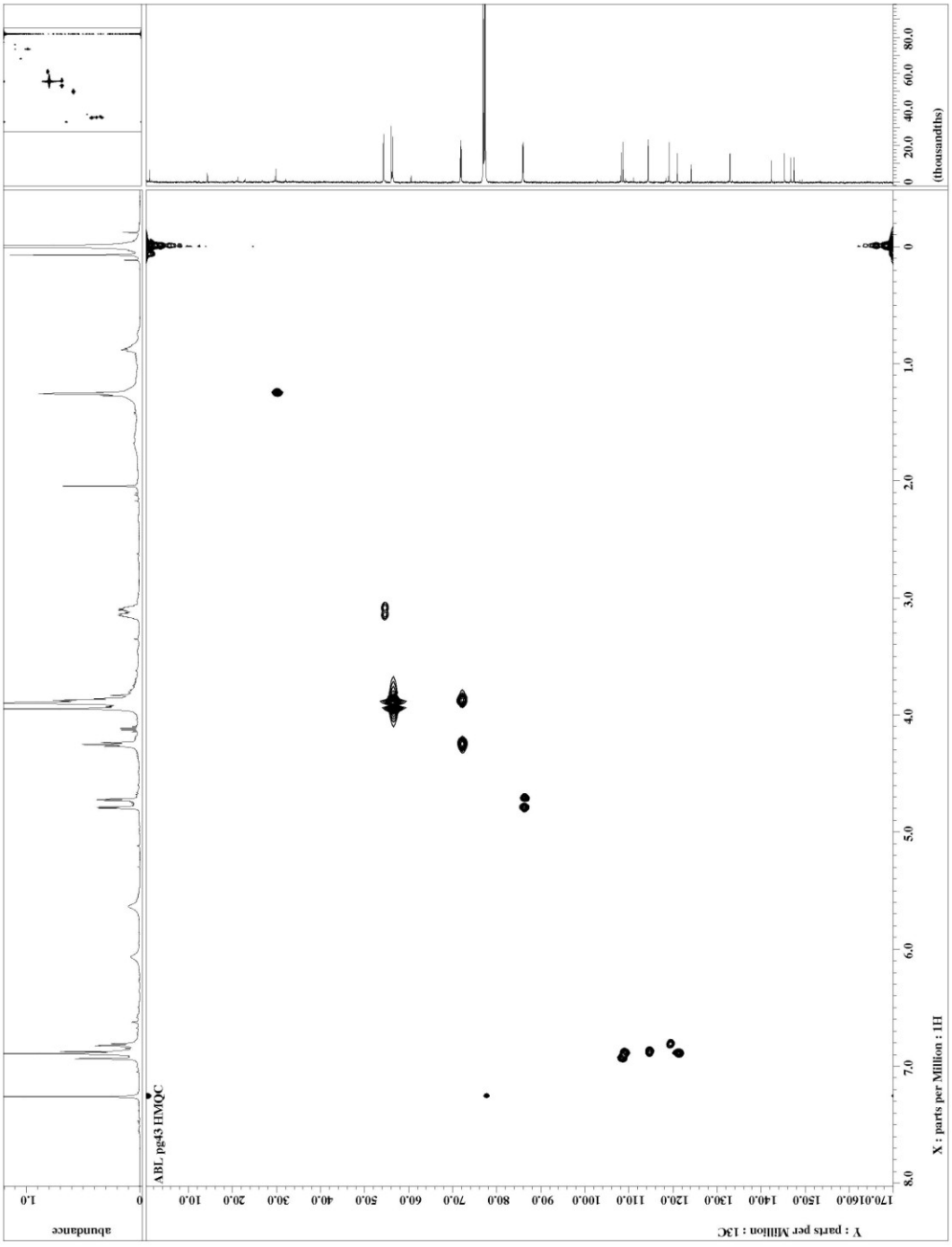
DEPT 135 ($CDCl_3$)



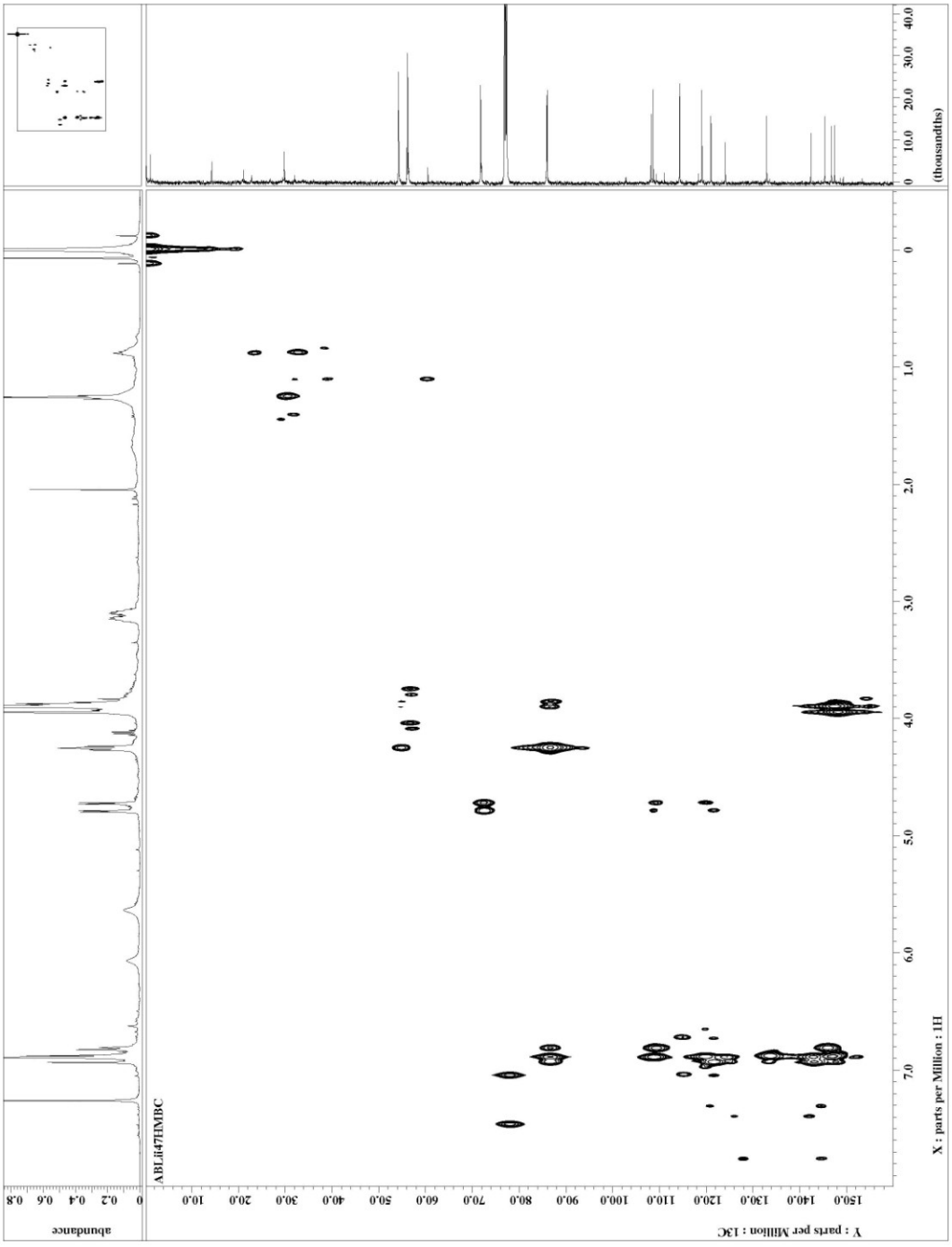
COSY ($CDCl_3$)


abundance
pg 43 F5 COSY #3
Y : parts per Million : 1H
X : parts per Million : 1H

HMQC ($CDCl_3$)


ABL pg43 HMQC
X : parts per Million : 1H
Y : parts per Million : 13C
abundance
(thousandths)

HMBC ($CDCl_3$)


ABLii47HMBC
X : parts per Million : 1H
Y : parts per Million : 13C
abundance
(thousandths)