Antimicrobial and Chemical Studies on a Medicinally important Plant: *Curcuma longa*

BY

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A thesis submitted to the Faculty of Graduate Studies of

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A thesis submitted to the Faculty of Graduate Studies of The University of Winnipeg in partial fulfillment of the requirement of the degree

MASTER OF BIOSCIENCE TECHNOLOGY AND PUBLIC POLICY

Abstract

This thesis describes phytochemical studies on the methanolic extract of *Curcuma longa*; a medicinally important plant used as indigenous medicine for the treatment of wounds in South Asian and African countries. The crude extract displayed antibacterial activity against the Gram-positive bacterium, *Staphylococcus aureus,* but was inactive against Gram-positive bacterium *Streptococcus agalactiae*. This extract also did not show antibacterial activity against Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. Based on the observed antibacterial activity, it was decided to carry out phytochemical investigation of crude methanolic extract of *C. longa* to isolate and characterize natural products and evaluate them for antimicrobial activity. These efforts resulted in the identification of three known natural products: curcumin (**39)** sclareolide (**40**) and atalantoflavone (**41**). Structures of these compounds were established with the aid of NMR spectroscopic studies. Compounds (**40)** and **(41)** have been isolated for the first time from this plant. Compounds (**39-41**) were found to be inactive against the Gram-positive bacterium, *Staphylococcus aureus,* and Gram-negative bacterium, *Escherichia coli*.

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GLOSSARY

^{1}H-NMR spectrum	Proton Nuclear Magnetic Resonance: Shows the electronic environment of proton atom in a molecule.
^{13}C-NMR spectrum	Carbon Nuclear Magnetic Resonance: Shows the electronic environment of carbon atoms in a molecule.
COSY spectrum	^{1}H-^{1}H Correlation Spectroscopy (COSY): Shows the correlation between hydrogens which are coupled to each other in the ^{1}H NMR spectrum
DEPT spectrum	Distortionless Enhancement by Polarization Transfer: Helps to differentiate between, methyl, methylene, and methine and quaternary carbons.
APT spectrum	Attached Proton Test: Used to differentiate between methyl, methylene, and quaternary carbons.
HSQC spectrum	Heteronuclear Single Quantum Coherence: Shows which hydrogens are directly attached to which carbon atoms
HMBC spectrum	Heteronuclear Multiple Bond Correlation: shows the correlations between protons and carbons that are separated by multiple bonds.

CHAPTER 1

Natural products chemistry

1.1 Introduction

Natural products (NPs) are organic chemical substances that are produced from living organisms [1]. They can be produced by partial or full chemical synthesis. The source of NPs are animal and plant cells, and the secretions of microorganisms. Since ancient times, natural products have been widely used for the medical treatment of human and domestic animal diseases. Many researchers all over the world are investigating different samples to analyze and examine effectivness in drug discovery by bioassays [2,4]. Researchers are currently conducting multiple experiments with plants and organisms to explore what biological activity they present [5, 6]. NPs and medicine have been closely associated using typical medicines and toxins for thousands of years [7,8]. For example, many modern drugs for cardiovascular, cancer, malaria and mental health diseases are made from medicinal plants. These drugs come from different parts of medicinal plants such as leaves, seeds, fruits and sometimes the whole plant [1].

Metabolism is divided into two types: primary and secondary. Primary metabolites are considered to be the building blocks of life, so they are necessary for survival. However, secondary metabolites are not always important for growth, instead, they have important roles in protecting themselves against herbivores and microorganisms. Secondary metabolites are signal compounds that attract pollinating and seed dispersing animals or the can function as agents of symbiosis between germs, plants, insects, and animals [1]. Natural products are known as secondary metabolites and are derived from organisms such as plants, animals, insects, marine organisms and microorganisms [1].

A primary metabolite is directly involved in the growth, development, photosynthesis, respiration or reproduction of a plant. Plants produce primary metabolites to drive their primary life function involving development and growth, respiration, cell differentiation, and internal storage of the plant. Primary metabolites vitamins, amino acids, nucleosides, and organic acids that are essential for microbial growth during the logarithmic process. Products like alkaloids, steroids, antibiotics, gibberellins, and toxins are secondary metabolites. These are produced in the stationary phase of microorganism growth and play a vital role in ecological functions [76]. The biosynthesis of secondary metabolites comes from the essential processes of photosynthesis, glycolysis, and the Krebs cycle to deliver intermediate biosynthesis, which results in the formation of secondary metabolites, also called natural products [10].

The most important building blocks associated with biosynthesis of secondary metabolites are derived from the intermediates, acetyl-CoA, shikimic acid, amino acids, and phenylalanine. The main classes of secondary metabolites are alkaloids, polyketides, fatty acids, and phenylpropanoids. Modern organic chemistry started with the extraction and the characterization of definite substances from plants and animal tissue. In the second half of the 19th century, discoveries of new metabolites were rapidly increasing. Organic chemists called these unknown metabolites “natural products”. These compounds have many challenges in structure determination and overall synthesis [77]. A few examples of primary and secondary metabolites are given in **Table 1-1** [11].

Table 1-1: Examples of primary and secondary metabolites [11].

Primary Metabolites	**Example**	**Secondary Metabolites**	**Example**
Alcohol	Ethanol	Pigments	Carotenoids, Anthocyanins
Amino acids	Glutamic acid, Aspartic acid	Alkaloids	Morphine, Codeine, etc
Organic acids	Acetic acid, Lactic acid	Toxins	Abrin, Ricin
Polyols	Glycerol	Drugs	Vinblastin, Curcumin
Vitamins	B_2	Polymeric substances	Rubber, cellulose

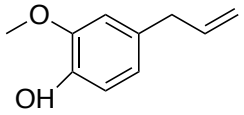
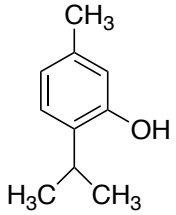
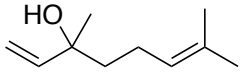
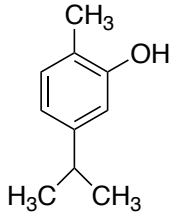
1-2 Classification of natural product:

Metabolites are naturally abundant with various types of biological activity and present great value as bioactive natural products. NPs can contribute to meditation of many diseases [20]. In living organisms, primary metabolites are produced in larger quantities than secondary metabolites. Although there are no clear functions of every type of secondary metabolites, they are potential sources of new antibiotics with fewer side effects. Natural products can be used as dyes, fibers, glues, oils, waxes, flavoring agents, and perfumes. Recently, secondary metabolites have become widely used in human nutrition research since using natural food has long benefited prevention of cancer and several diseases [21]. NPs provide sugar, flavour, color, and promising new drugs [1].

1.2.1 Phenols

Phenols are cyclic compounds and are the largest group of secondary plant metabolites. Phenols contain an aromatic ring with an –OH (hydroxyl) functional group. Many plant phenols contain the phenyl (C_3) - propane radical (C_6) [12]. From a chemical standpoint, phenols are acidic and more robustly reactive than alcohols. There are a couple of pathways of phenol synthesis: the first one is condensation of acetic acid molecules; another is the metabolism of phosphorylated sugar. Shown below are a few simple phenol structures: eugenol (**1**), thymol (**2**), linalool (**3**), and carvacrol (**4**), listed in **Table 1-2** [13]. Eugenol is found in essential oils of *clove*, *cinnamon* leaf, *pimento* leaf, and rose. It is a little spicy and has a pungent odor. Thymol and carvacrol are also found in essential oils of thyme, sage, and oregano. Thymol has an herbaceous odor but carvacrol has a phenolic spicy odor [12, 13].

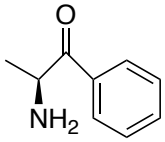
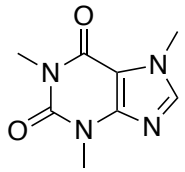
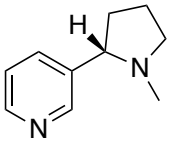
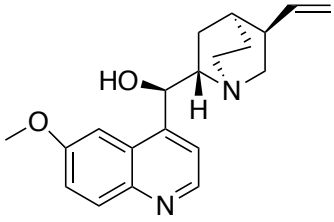
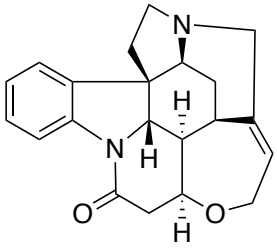
Table 1-2: Examples of phenol structures (**1-4**)

The Name of Compounds	The Structures
Eugenol (**1**) [13]	
Thymol (**2**) [13]	
Linalool (**3**)[13]	
Carvacrol (**4**) [13]	

1.2.2 Alkaloids

The structures of all alkaloids contain nitrogen and possess the ability to interact with other complex structures via the nitrogen. For many years, alkaloids have been used in medicine as common drugs. There are many active compounds extracted from plants that contain oxygen with nitrogen in the tertiary position, though some secondary and quaternary types are known. Some alkaloids activate the central nervous system as cathinone (**5**), caffeine (**6**), nicotine (**7**), quinine (**8**), listed in **Table 1-3** and some inole alkalodis act as psychoactive agents, which cause hallucinations. Morphine isolated from Opium became the first pure naturally extracted medicine. It is a strong painkiller that is still used in the clinic [23]. Strychnine (**9**), the crystalline alkaloid, is used as a pesticide [24].

Table 1-3: Examples of some alkaloid structures (**5-9**)

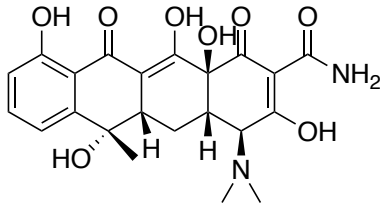
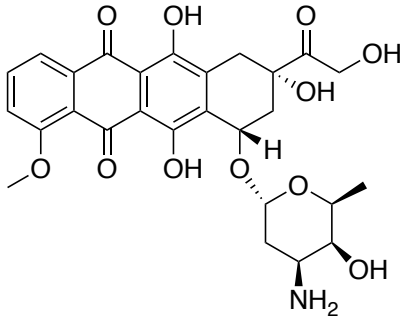
The Name of Compounds	**The Structures**
Cathinone (**5**) [23]	
Caffeine (**6**) [23]	
Nicotine (**7**)[23]	
Quinine (**8**)[23]	
Strychnine (**9**)[24]	

1.2.3 Polyketides

Polyketides are one class of natural products that have alternating carbonyl and methylene groups (-CO-CH2-). Some Polyketides have antimicrobial activity (e.g., tetracycline) (**10**) and can reduce the activation of the immune system (e.g., rapamycin). They also have many different activities such as antifungal (e.g., amphotericin B), antiviral (e.g., balticolid), cholesterol-reducing (e.g., lovastatin), anti-inflammatory activity (e.g., flavonoids), antifungal (e.g., amphotericin B), anticancer (e.g., doxorubicin) (**11**), and immune suppressing (e.g., rapamycin) **Table 1-4** [28].

Toxic substances produced by fungi are often polyketides. The biosynthesis of polyketides starts with condensing of the starting unit (acetyl-CoA or propionyl-CoA) with an extender unit (malonyl-CoA or methylmalonyl-CoA), then processed by decarboxylation of the extender unit. Polyketides have many different structures and functions that allow them to have significant pharmaceutical and agrochemical potential. Most polyketides are produced by microbes such as bacteria and fungi and are found everywhere as they are produced by a host of organisms including plants, insects, mollusks, algae, protists, insects, and sponges. Examples of this are: bacteria (e.g., tetracycline from *Streptomyces aureofaciens*), fungi (e.g., lovastatin from *Phomopsis vexans*), plants (e.g., emodin from *Rheum palmatum*), protists (e.g., maitotoxin-1 from *Gambierdiscus australes*), insects (e.g., stegobinone from *Stegobium paniceum*), and mollusks (e.g., elysione from *Elysia viridis*) [28, 29].

Table 1- 4: Examples of some polyketides structures (**10-11**)

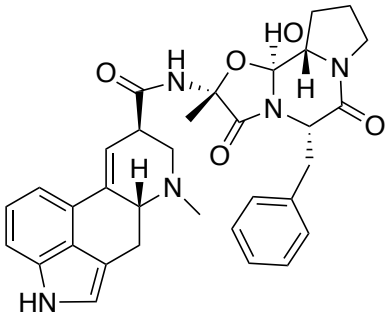
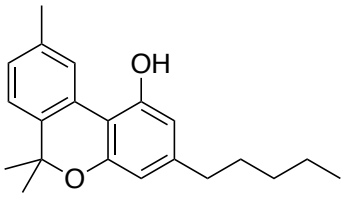
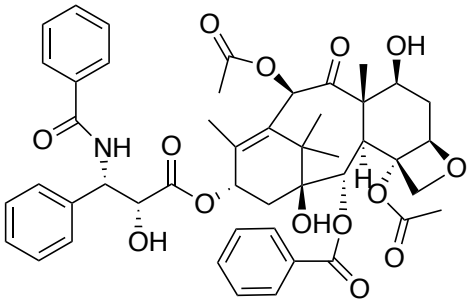
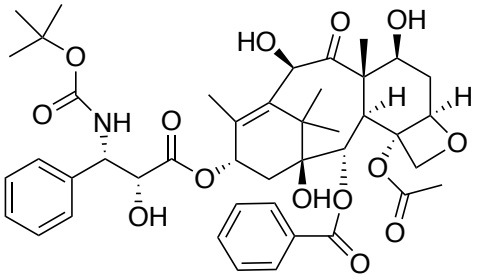
The Name of Compounds	**The Structures**
Tetracycline (**10**) [28]	
Doxorubicin (**11**) [28]	

1.2.4 Terpenoids

Terpenoids (isoprenoids), which contain five carbon units, are the biosynthetic precursors of different natural compounds. Terpenoids are considered to be the largest class of secondary metabolites, which have diverse functionalized hydrocarbons or chiral, carbocyclic skeletons and have different substituents or moieties within the molecules such as hydroxyl, carbonyl, ketone, aldehydes, and peroxide groups. Terpenoids are responsible for aroma and flavour since they are volatile substances in plants and are located in leaves and fruits in some plants such as conifers, citrus, and eucalyptus [30]. Terpenoids make up nearly 60 % of all natural products. They have several cyclic structures that have oxygen functional groups. Most terpenoids are used for chemical reactions and protection in the abiotic and biotic environment. In the past, terpenoids were used in food, pharmaceutical, and chemical industries, and most recently in the biofuel industry [31]. Even though there is huge chemical diversity, natural products are synthesized from a few building blocks such as acetate that has five carbon atoms; mevalonate has five carbon atoms, and shikimate that has nine carbon atoms [32]. Terpenoids or isoprenoids be modified from ergotamine (**12**), cannabinol (**13**), quinine, and vitamin-E. Terpenoids are important for every living organism. Cyanobacteria synthesize terpenoids from the methylerythritol-phosphate (MEP) pathway and process them by utilizing glyceraldehyde 3-phosphate and pyruvate produced via the substrates from photosynthesis. The products of MEP pathway are isomeric the five-carbon compounds isopentenyl diphosphate and dimethylallyl diphosphate that became the formation of building blocks to make terpenoids [34, 78].

Terpenes have different numbers of carbons; hemiterpenes contain 5 carbon atoms, monoterpenes contain 10 carbon atoms, sesquiterpenes contain 15 carbon atoms, diterpenes contain 20 carbon atoms, sesterterpenes contain 25 carbon atoms, triterpenes contain 30 carbon atoms, and polyterpenes contain more than 30 carbon atoms [33]. Terpenoids are synthesized from the mevalonic acid (MVA) pathway and from the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway. Terpenoids have a wide range of roles that have been studied and they have been applied as food additives, in perfume making, and as alternative medicines. Terpenes have many properties, the most important application of them is the prevention and treatment of cancer; paclitaxel (**14**) and docetaxel (**15**) are examples of drugs that are used to treat cancer and are listed in **Table 1-5** [34]. Other important properties of terpenoids are antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory, antioxidant, and antiparasitic activities. Since the natural production of terpenoids is limited, synthetic biology and metabolic engineering give other options for higher level of production [33].

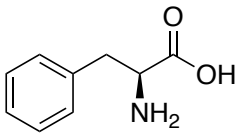
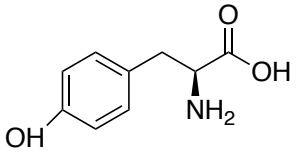
Table 1-5: Examples of some terpenoid structures (**12-15**)

The Name of Compounds	The Structures
Ergotamine (**12**) [34]	
Cannabinol (**13**) [34]	
Paclitaxel (**14**) [34]	
Docetaxel (**15**) [34]	

1.2.5 Phenylpropanoid

Phenylpropanoids come from the plant kingdom *Apiaceae* or *Umbelliferae* family and they are synthesized by plants from the amino acids phenylalanine (**16**) or tyrosine (**17**) (**Table 1-6)**. Phenylpropanoids are good sources of metabolites in plants that have a cyclic structure that has many physiological functions; the enzyme phenylalanine-ammonia lyase (PAL) removes the ammonia from l-phenylalanine to produce theses compounds [34]. Phenylpropanoids are considered good starting points for making other useful compounds such as coumarins and lignans and are used to synthesize ligning, which gives structural support and pathogen resistance to plants. Phenylpropanoids play important roles in the making of hydroxycinnamyl alcohols and its monolignols are building blocks of lignin. Phenylpropanoids are promising in human health with many useful applications. Carrots are important in providing a good source of Phenylpropanoids compounds and are reported to have positive affects on our health such as epilaserine oxide. Phenylpropanoid compounds such as 2-epilaserine was isolated from carrots and reported to be toxic to living cells such as HL-60 cells [35, 36]. Phenylpropanoids contribute to biosynthetic reactions to create a large number of compounds such as lignins, flavanols, isoflavanoids, anthocyanins, and stilbenes. These compounds have many different functions other than plant defense and structural strength. They also have pharmaceutical properties that give them value in nutrition, antioxidants, insecticides, dyes, and medicine [36].

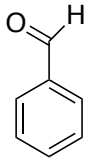
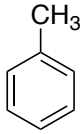
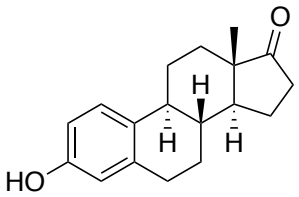
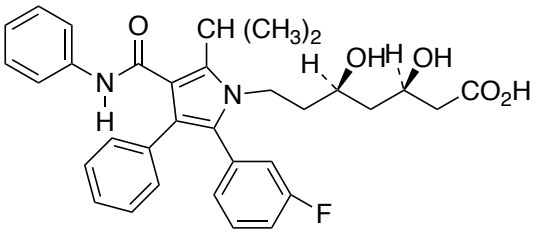
Table 1-6: The structures of phenylalanine and phenylpropanoids (**16-17**)

The Name of Compounds	**The Structures**
Phenylalanine (**16**) [34]	
Tyrosine (**17**) [34]	

1.2.6 Aromaticity

Aromaticity is the possession of (ring-shaped), planar (flat) structures and a ring of resonance bonds which grants increased stability compared with other geometric or conjunctive grouping with similar sets of atoms. Aromatic rings are very stable and do not break apart easily. Compounds that are derived from benzene (**18**) are known as aromatic compounds although there are some aromatic compounds not derived from benzene found in living organisms such as the double-ringed bases in RNA and DNA. The word aromatic was used to describe fragrant substance such as benzaldehyde (**19**) (from cherries, peaches, and almonds), toluene (**20**) (from Tolu balsam), and benzene (from coal distillate). It was found that compounds that have an aromatic group behave differently than other organic compounds. Nowadays, the word aromatic is used to describe the category of compounds that contain six-membered benzene-like rings that have three double bonds. There are numerous natural compounds that are aromatic steroids such as estrone (**21**) and compounds that are used as a medicine such as the cholesterol-lowering treatment Lipitor (**22**) and are listed in **Table 1-7**. Benzene affects depressed white blood cell count (leukopenia) and depression [34, 35].

Table 1-7: The aromatic structures of some compounds (**18-22**)

The Name of Compounds	**The Structures**
Benzene (**18**) [34,35]	
Benzaldehyde (**19**) [34,35]	
Toluene (**20**) [34,35]	
Estrone (**21**) [34,35]	
Atorvastatin Lipitor (**22**) [34,35]	

1.2 Importance of Natural Products:

1.3.1 Modern Drug Discovery Process From Terrestrial Plants:

Natural products are considered a significant source for the discovery of new drugs. Throughout history, natural products have played a fundamental role in eliminating harmful bacteria and fungi that have threatened human life [43]. Most modern drugs currently used to treat medical conditions come from nature since they offer structure diversity and clinical specificity. More attention and development should be given to all steps in the development process of natural products including collecting the samples, choosing strains, metabolic expression, genetic engineering, and isolation of compounds in plants by solvents. Natural products could bind by particular interspecific interactions to proteins with similar drug-like properties [43]. Cyclosporin A and FK-506 are drugs that come from natural products that work as immunosuppressants and are antiviral, antifungal, and antiparasitic agents [43].

New technology such as high-throughput screening, combinatorial chemistry, computational chemistry, gene chip research, and gene-drug cannot achieve what NPs have provided, **Figure (1):** Sources of compounds for drug discovery [1]. Lead compounds are chemical substances which display demanded biological or pharmacological activity. They can lead to the development of a new compound for clinical use as lead substances are considered starting points in drug design. Drug design methods are used to develop a specific compound's properties. The resulting compounds undergo many studies and become clinical candidates. Natural products, synthetics, and combinatorial chemistry are possible sources of lead compounds and novel drugs [60].

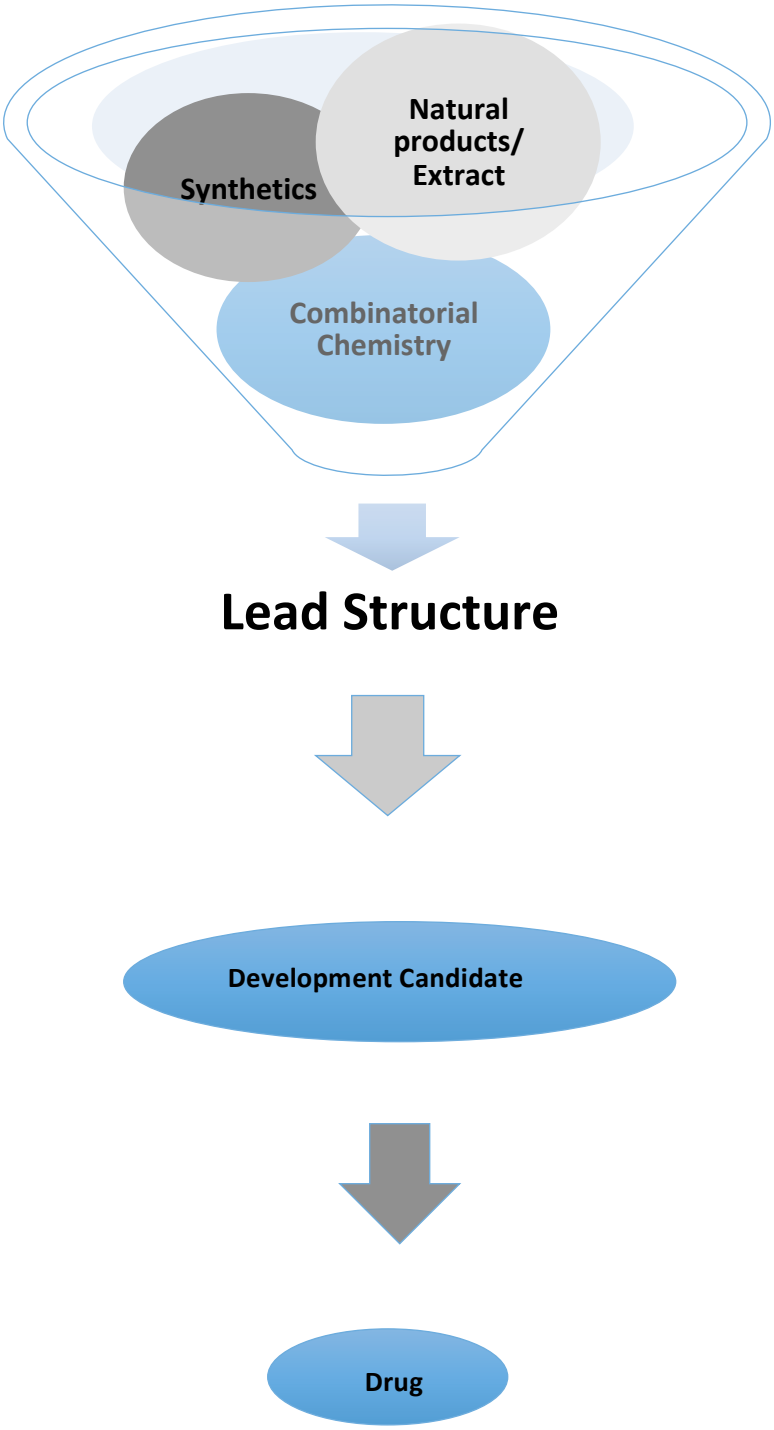


Figure (1): Sources of compounds for drug discovery [60]

Plants play a role in medical treatment to improve our health and well–being, to reduce drug costs, and in the bio prospecting for new naturally sourced compounds. Medical plants of interest are overused by the pharmaceutical industry and traditional experimenters, which lead to some challenges in the collection of desired plants. There is not enough knowledge about the amount of harvest, limited legislative and policy guidance, and with some lands having restricted use of rights [79]. In fact, it is difficult to meet the needs of the pharmaceutical industry using plants. Earth have plants are reaching the point of extinction for the following reasons: 1- desired plants are in remote, hard to reach areas; 2- some plant species have disappeared already; 3- by the 17th century, humans started to replace the large forest with farms and cities and most plants species are found in large forests; 4- plants that have not reached maturity are harvested [80].

It is important to start systematic cultivation of plants to provide biodiversity and protect the species that are rare or disappearing and find other solutions in order to preserve biodiversity. Hundreds of millions of people are still affected by the benefits of collecting medicinal plant and animal products, especially in developing countries [81]. Trial and error have provided people with the knowledge that they need to develop alternative medicine, which is transferred from generation to generation, and sometimes from country to country. In fact, plants, minerals, animals, and fungi were the sources that people relied on throughout history of the study of material medica, pharmacy, and pharmacognosy. Around 80 % of the inhabitants of developing countries rely on alternative medicines; especially plant sourced natural products for their primary health care according to the World Health Organization [50, 81]. Around 25 % of new pharmaceutical drugs come from plants or are synthesized analogues. Researchers have discovered drugs from medical plants for the treatment of diseases. Plants have many biological activities such as antioxidant and antimicrobial properties [50].

There is no reliable estimated number of medical plants in the world; however, there are estimates of the number of species that are used as medicine in the range of 35,000 – 70,000 worldwide [19]. Choosing a candidate species for examination and investigations would be done through long-term use by humans; modern isolation of active compound from plants is safer than the investigations of plant species without any previous history. In fact, one pharmaceutical drug can be effective for more than one disease [83]. There are many patients that have been cured and received the benefits of drugs that were extracted from plants although the medicinal species is at risk of extinction. Currently, increasingly large amounts of medicinal plants are grown and marketed by France, U.K., Canada, Turkey, and U.S.A. Indians consume around 200 tons yearly of these herbs while locally 60 tones are produced. China dominates most of the global markets for medical plants; other important consumers are France, Germany, Italy, Japan, Spain, UK, and U.S.A [50].

1.3.2 Origin of natural products and drug discovery

Plants described in the earliest clay cuneiform tablets from Mesopotamia are still used for the treatment of coughs, cold, and inflammation [9]. China has great numbers of medical plants; around 12,000 and they grow naturally. Thousands of years ago, Chinese people had to use herbs as medicine for many illnesses. In China, the use of alternative treatments, which involve herbs, is still highly appreciated. Nowadays, well-known drugs used around the world such as ergometrine, santonin, scopolamine, reserpine, vincristine, digoxin, deslanoside, and camptothecine are extracted from plants that are grown in China. In addition, there are many antibiotics isolated from Chinese medicinal plants, some with antimalarial properties [1, 7]. As of now, researchers have only studied a small amount of all the plants in the world, therefore the future of drug discovery from plants is promising [8].

Thousands of years ago, extracts from natural products were used as medicine. In the beginning of civilization, people had to use specific plants and some microbes to treat sickness and injuries [37]. Around 3,000 years ago, the Mayans applied fungi grown on green corn to address intestinal diseases [84]. A paleoanthropological study was carried out at the cave site at Shanidar, Iraq and it was known that more than 60,000 years ago Neanderthals were aware of the value of medicinal plants and their properties. *Achillea-type, Centaurea solstitialis, Senecio-type, Muscari-type, Ephedra altissima,* and *Althea-type* are examples of flowers, which were discovered and had a written description that was found in the cave site at Shanidar [38]. The oldest medical records from ancient Mesopotamia in 2600 BC record nearly 1,000 medical

plants. By 1550 BC, there were 800 complex prescriptions reported in ancient Egypt [61]. The importance of natural products for drugs and health has developed significantly over the past several years. In the past, our ancestors commonly used various medical plants to overcome pain by eating leaves or by using herbs for healing diseases, infections, and other ailments. Natural products play an important role in discovering new drug candidate molecules due to their diverse molecular biology and diversity of structures that contribute to development of novel compounds [61, 62].

Chemists are interested in natural products recently since they are a rich source of compounds that are supporting and developing drug discovery. Discovering new drugs promises new treatment for many different diseases such as dementia and cancer. Since natural products have different biological activities and medicinal potency, different cultures have various approaches to the use of plants as treatments. Around 2600 BC, ancient Mesopotamian medical texts described 1,000 plants and plant-derived substances such as the oils of *Cedrus* species (cedar), *Commiphora myrrha* (myrrh), and the juice of the poppy seed, *Papaver somniferum*. Around 460–377 BC, the Greek practitioner, Hippocrates of Cos, listed around 400 natural agents in his written texts, including the laxative properties of melon juice and the wound healing properties of olive oil. Roman physicians contributed to further development with various approaches and different plants [61, 63]. Around 40–90 AD, Pedanius Dioscorides gathered De Materia Medica, in which he recorded around 600 plants with specific dosages and their effects that led to the establishment of pharmacology in Europe. In (129–200 AD), Galen, the Greek physician and pharmacist, listed 540 plants and mentioned that a plant can have both useful and harmful properties depending on how the plant is used and applied medicinally. Following the Greeks, the Orient succeeded in using natural product as treatments. Charaka Samhita, the first medicine

treatise, contained the insights and experiences of Indian Ayurveda, which was recorded around 900 BC and had 341 plant-derived medicines. The Sushruta Samhita (around 600 BC) was interested in surgeries and described 395 medicinal plants and 57 animal-derived products. It is common for traditional Chinese medicine to use natural products, which is the most primitive Chinese medicine and is still used today [64, 61]. The medicinal book, Wu Shi Er Bing Fang, translated treatments for 52 diseases, was gathered around 350 BC and recorded 247 natural agents and about 150 drug formulae, including some recommendations, properties and the effects of drugs from natural sources. The Orient and the Occident were using natural products extensively and continuously without knowing what compounds they possessed until the eighteenth and nineteenth centuries [61].

1.3.3 Natural products usage

Over the last few decades, the use of herbal medicinal products has been increasing tremendously worldwide with more than 80% of the people depending on them and using them as primary healthcare, especially in developing countries [14,15,16]. The consumption of medicinal plants is increasing because of self-medication by patients or individuals. In addition, herbal medicine has fewer side effects and lower costs than synthetically developed drugs. Moreover, there is a fear about the possibility of misdiagnosis and side effects of varying nature by modern drugs and medicines. Some examples of medicinal plants are Irish moss or carrageen moss, which is the source of a beverage, used to treat colds, sore throat, and tuberculosis [17, 18]. Alga is used to treat kidney diseases and red alga is used to treat cancer medications [10, 17, 18]. In modern era, 35,000-70,000 species plants have been used for the preparation of medicine [19].

1.3.4 Drug discovery process:

In 2008, Newman reported that more than 60 % of drugs came from natural products directly or synthetically such as artemisinin, camptothecin, lovastatin, maytansine, paclitaxel, penicillin, reserpine, and silibinin [39]. By 1805, German pharmacist Friedrich Wilhelm Sertürner extracted morphine (**23**) from opium, the first pure form natural product that is now used commercially [39, 66]. By 1826, Western pharmaceutical companies started to extract drugs as separate compounds instead of using whole plants and chemists began to synthesize compounds with known structures instead of extracting them. **Figure (2)** and **Figure (3)** show some approved drugs and sources of small molecule approved drugs below and the codes used in analyses showed in Table 1-8 [39].

Table 1-8: Codes Used in Analyses

Code	Brief definition/year
B	Biological macromolecule, 1997
N	Unaltered natural product, 1997
NB	Botanical drug (defined mixture), 2012
ND	Natural product derivative, 1997
S	Synthetic drug, 1997
S*	Synthetic drug (NP pharmacophore), 1997
V	Vaccine, 2003
/NM	Mimic of natural product, 2003

Figure (2): All new approved drugs; n = 1355 (58)

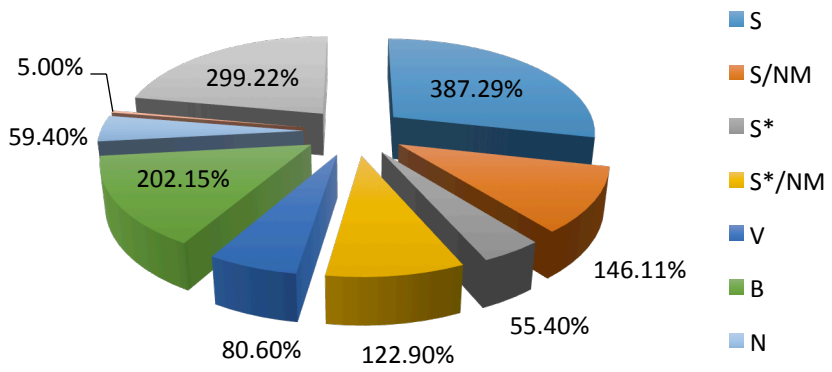
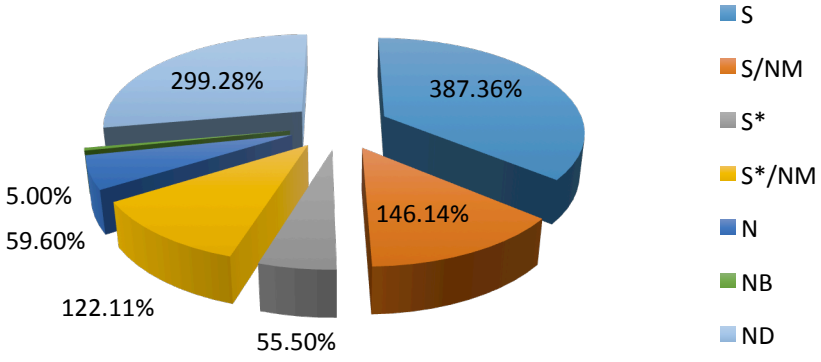
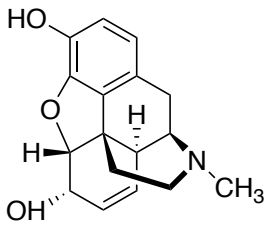


Figure (3): Source of small molecule approved drugs; n = 1073 (58)

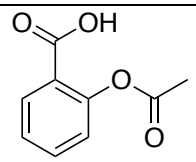
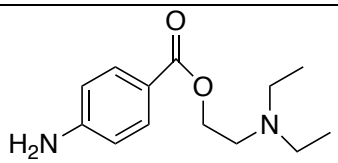
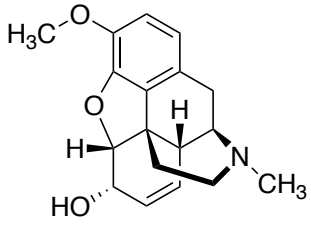
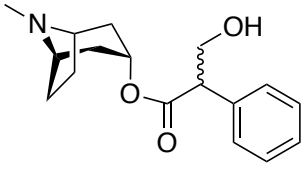


There are examples of drugs derived from plants that are still used in the clinics [2]. One of the oldest drugs currently used is aspirin (**24**). It is synthesized from salicylic acid, extracted from plants by acetylation, and used to reduce fever, relieve pain, and also as an anti-inflammatory drug [3]. Another example is procaine (**25**), an anesthetic that is derived from the active plant compound, cocaine. In the 17th century, the Chinese found aconitine crystals from *Aconitum carmichaeli*, which was later, studied in 19th century by German scientists [4]. Some common drugs isolated from plants are the pain killer morphine, the antitussive codeine (**26**) [5], and the parasympathetic inhibitor atropine (**27**) [6] listed in **Table 1-8**.



Morphine (**23**)

Table 1-9 Examples of drugs derived from plants **(24-27**)

The Name of Compounds	The Structures
Aspirin (**24**) [3]	
Procaine (**25**) [4]	
Codeine (**26**) [5]	
Atropine (**27**) [6]	

Some examples of known natural compounds that have been named, extracted, and synthesized include; salicin from *Salix alba* (white willow), emetine from *Cephaelis ipecacuanha* (ipecacuanha), strychnine and brucine from *Strychnos nux-vomica* (strychnos), quinine from *Cinchona ledgeriana* (cinchona bark), colchicine from *Colchicum autumale* (colchicum), caffeine from *Coffea arabica*, nicotine from *Nicotiana tabacum*, atropine from *Atropa belladonna*, and cocaine from *Erythroxylum coca*. These compounds are still used as treatments [67, 39].

Discovered in the twentieth century, penicillin is the most famous discovery in antibacterial drugs and is extracted from the mold, *Penicillium notatum.* This compound opened the doors for other antibacterial drugs that fight many infections. Synthesis of natural compounds and having the exact structures of them leads to modification of these compounds by chemists and determination of properties such as solubility, efficiency, or stability in the human body [68].

1.4 Bioassay

1. 4.1 Anti-microbial assays

Antibiotics are commonly used as bactericidal or bacteriostatic treatment or prevention of infection. Antimicrobial resistance is rising, leading to more challenges in the treatment against bacteria to overcome the infections. Antimicrobial resistance has resulted incorrect empirical treatment that delays an effective cure, resulting in the use of less effective, more toxic, and further costly drugs. [47]. Antibiotics halt the growth of the cell by inhibiting chemical reactions inside the cells, some of which affect metabolic process. Other antibiotics affect the polymerization reaction leading to the inhibition of the growth of cells, the result of antibiotics [46].

The “golden age of Antibiotics" started in the 1940s and lasted until the 1970s, following the discovery of penicillin, accidentally, by Alexander Fleming in 1928 and development by Chain and Florey in the 1940s [43]. There are different kinds of microbial natural products such as antidiuretic drugs, hormone, antagonists, anticancer drugs, and agricultural and pharmaceutical agents [43]. Natural products continuously produce diverse structures that have effective antimicrobial activity. Nowadays, there are other approaches to cultivate bacteria to enhance the production of new natural products. Natural products are considered as starting points to produce new antibacterial compounds. Polymyxins, including polymyxin B and polymyxin E (colistin) are considered as antibiotics, which have started to be used globally to treat people who are suffering from multidrug-resistant (MDR) Gram-negative Infections of bacteria. [47].

1.4.2 Antimicrobial compounds:

Antibiotics play an important role in fighting bacterial infections, however, after discovery they faced resistance and its still the efficiency of antibiotics. As a result, there is urgent need to resolve infections of bacteria by discovering antibiotics from natural products. Natural products play a significant role in the discovery of antibiotics since 1941, when penicillin was discovered and entered the market. Nowadays, natural products are a major source for discovering new effective drugs. It is supposed that new drugs from non-natural sources could be more active and stronger, yet they have provided disappointing results. The lack of 'privileged structures' of synthetic chemistry leads to lower rate of production of discovering drugs. Therefore, microbials would have resistance to antibiotics easily. Compounds from natural sources with 'privileged structures' provide excellent antimicrobial agents [48]. Natural products have been isolated and purified for many reasons including treating human infections and diseases. Research tends to study activities of secondary metabolites; theses include antimicrobial, antifungal, anticancer, and anti-inflammatory activities. Vinca alkaloid is an example of secondary metabolites used in cancer chemotherapy. Digitoxin is a second example of a secondary metabolite that is extracted from digitalis plant, which cures heart failure. The secondary metabolite, an alkaloid called quinine, is extracted from *Cinchona* tree and is used to heal malaria. Moreover, secondary metabolites have other uses besides human medicine such as food preservation and as antioxidants. In addition, secondary metabolites kill microorganisms or stop their growth. Occasionally antioxidant and antimicrobial properties are found in the same molecule, which gives the drug more efficacy [49].

1.4.3 Recent approaches in natural product research

It has been reported that the FDA approved several antibiotics that are all new antibacterial agents that have been approved after the year 2000 as shown in **Table 1-9A** [59]. Natural products play important roles in the discovery of antibiotics, of which more than half came from natural products. Synthetic routes could not provide leads and natural products proved ability to produce drug-like properties [56]. There are many new antimicrobial agents that have been marketed recently, **Table 1-9B.** Natural products are important for life science applications that could extract target drugs. The actual sales of marketed drugs are the reflection of the success and significance of natural products and the impact of biomechanical tools. Natural products contribute to chemical and biological products that are useful products. Products that are in the market are either from natural products directly or derived from them, **Table 1-10**. However, the success and approval of the drugs faces many challenges starting from the identification of new bioactive compounds from natural sources [59].

Table 1-10A: Newer antibacterial agents [59].

Name of Drug	Class of Drug	Year of FDA Approved/Phase of Trial	Spectrum of Activity
Daptomycin	Lipopeptide	2003	Gram+ve bacteria
Telithromycin	Ketolide	2004	Gram+ve and-ve
Tigecycline	Glycylcycline	2005	Gram+ve and-ve
Doripenem	Carbapenems	2007	Gram+ve and+ve
Retapamulin	Pleuromutilin	2007	Gram+ve
Telavancin	Glycopeptides	2009	Gram+ve
Ceftobiprole	Cephalosporin	2010	Gram+ve
Fidaxomicin	Macrocyclic	2011	Gram+ve
Marketed Agent			

Table 1-10B: Newer antibacterial agents [59].

Name of Drug	Class of Drug	Year of FDA Approved/Phase of Trial	Spectrum of Activity
Ceftobiprole	Cephalosporin	Approval awaited	Gram+ve
Iclaprim	DHFR inhibitor	Approval awaited	Gram+ve
Awaiting FDA Approval			
Torezolid	Oxazolidinones	Phase II	Gram+ve
Radezolid	Oxazolidinones	Phase II	Gram+ve
Cethromycin	Ketolides	Phase III	Gram+ve
Solithromycin	Ketolides	Phase II	Gram+ve
Oritavancin	Glycopepetide	Phase III	Gram+ve
Dalbavancin	Glycopepetide	Phase III	Gram+ve
Agents in Clinical Development			

1.4.4 Development of antibiotics

Antibiotics have been synthesized from either natural products or from synthetic compounds. Natural products were the first source for discovering antibiotics from a mold by Alexander Fleming in 1928. Drugs can be extracted from soil, bacteria, molds, marine organisms, and plants as natural sources for discovering new chemical entities (NCE) and using them in the clinic. Most antibiotics that are used around the world come from natural sources. The main reason for the urgency of discovering antibiotics is that there is rapidly increasing resistance against current drugs [49].

1.4.5 Drugs resistance

Antibiotics were shown to effectively fight infections when used. Nevertheless, in his 1945 Nobel Prize acceptance speech, Fleming mentioned that the misuse of antibiotics would cause resistance. He said ‘there may be danger, though, in under dosage. It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them’ and the same thing has occasionally happened in the body and by 1947, there was a huge global antimicrobial resistance. It was reported that 38 patients of 100 people were infected with staphylococcal after the staphylococcal became resisted against penicillin. [54]. Since resistance to many antibiotics appeared this encouraged the researchers to discover new antimicrobial activities. By 1975, there was a new approach to making penicillin chemically; a semisynthetic way starting from a lactam ring, 6-aminopenicillanic acids. In 1960 and 1961, methicillin and ampicillin were synthesized [55]. Discovering new antibiotics is an

attempt of resistance. By 1961 resistance to methicillin by a strain of *S. aureus* was shown. By 1984, the susceptibility of *S.aureus* against penicillin decreased from 85% before 1946 to between 20% and 30%. Moreover, the report from the Italian Epidemiology Observatory reported a total resistance of 14.3% to penicillin [57]. The relationship between using antibiotics and increasing the resistance was less by 3% in children than adults. Additionally, in Taiwan by 1998-2001, it was reported that resistance was 76% in isolates from people who got infected with invasive pneumococci. A study was conducted in Iceland to prove the relationship between the amount of resistance and the use of antibiotics [26]. It was reported that the resistance to penicillin was reduced when the use of it was reduced, however, the rate of resistance to macrolides got higher after an increased use of it. By 2003, resistance to penicillin of methicillin resistant *Staphylococcus aureus* (MRSA) from nasal swabs in Korea was 91% and 82.1% in Germany. In addition, in Canada, MRSA resistance to fluoroquinolones was 92% and 90% to clarithromycin. By 2004, in the US, Jacobs and co-workers published that the susceptibility of *Streptocuccus pneumoniae* to penicillins and clindamycin was 49% [71].

The reduction or decrease in effectiveness of medication (e.g., antimicrobial or antineoplastic) for the curing of disease is called drug resistance. When an organism is resistant to more than one drug it is called multi-drug resistance. Drug resistance generally happens because of misuse or overuse of antimicrobials, inappropriate use of antimicrobials, and patient not completing the recommended course of treatment. From the last half-century, the number of drug resistant people increased worldwide. Globally, every year, 700,000 die because of drug resistant illness [71, 72].

Antimicrobial resistance: It is the ability of a microbe to impede the proper function of medication that once could perfectly treat the microbes. Antimicrobial resistance arises due to

genetic changes in microorganism (viruses, bacteria, fungi, etc.) that arise during treatment. Microorganisms that are resistant to many antibiotics are called "superbugs" [72, 73]. Resistant microbes are much more difficult to treat and need alternate or higher doses of antimicrobials.

Antineoplastic resistance*:* This type of resistance is similar to chemotherapy resistance. Antineoplastic resistance, usually utilized interchangeably with chemotherapy resistance, is the resistance of neoplasm, or the capacity of cancer cells to continue to live and grow through anti-cancer treatment. Sometimes cancers can develop resistance to many drugs, which is called multiple drug resistance. Antineoplastic therapies can fail by two ways; inherent resistance and acquired resistance. Inherent resistance is the genetic characteristics that give cancer cells resistance from the beginning. Acquired resistance occurs after drug exposure. To reduce drug resistance, it is essential to discover alternate chemotherapy agents that have less resistance and side effects, such as natural products [74].

The most important attribute of antibacterial drugs is their selectivity of toxicity in only killing or inhibiting the growth microbial targets. Each antibacterial drug has an individual mode of function. The first discovered antibiotic drug is penicillin. The group of drug compounds including penicillins, monobactams, cephalosporins, and carbapenemes are characterized by the presence of a β-lactam ring that exists in the central structure of the drug molecules. During the biosynthesis of peptidoglycan around the bacterial cell, β-lactams prevent the crosslinking of the peptide chains [75, 76].

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CHAPTER 2

Phytochemical investigation of *Curcuma longa*

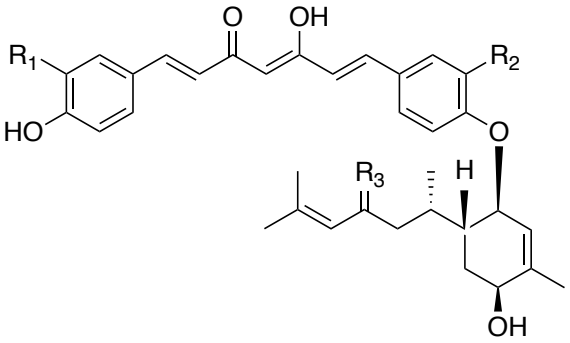
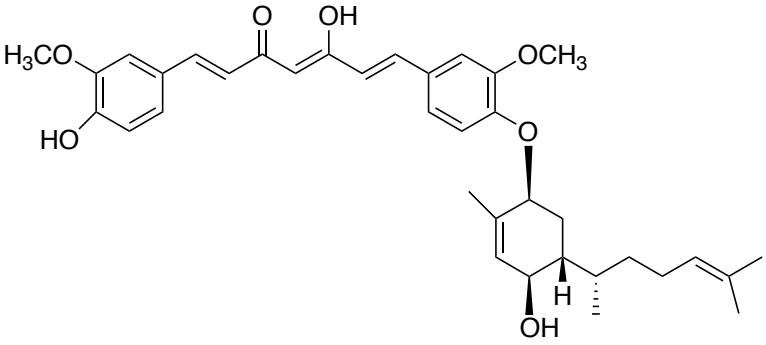
2.1 Introduction

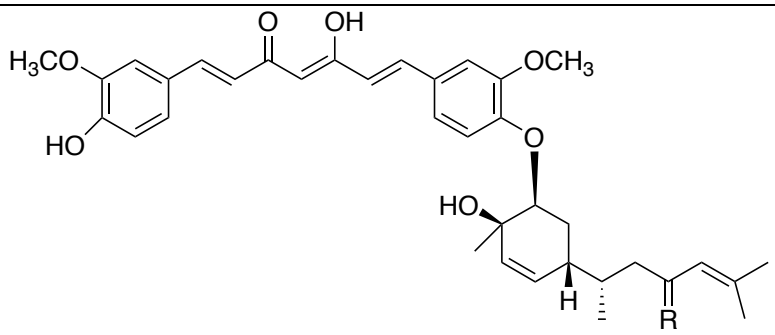
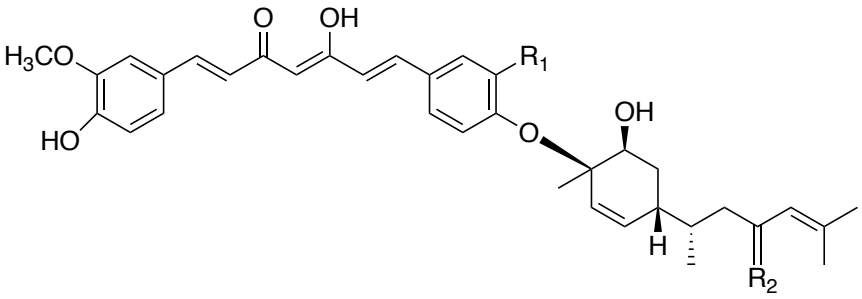
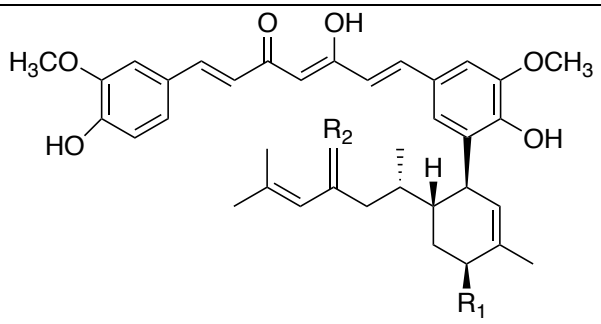
Curcuma longa, commonly known as turmeric, is a perennial herb. This plant belongs to the Zingiberaceae (ginger) family and grows to a height of 3 to 5 feet [4]. This plant is abundant in countries of tropical climate including Bangladesh, India, Pakistan, Sri Lanka, and other Asian countries [5]. It has oblong, pointed leaves and funnel shaped yellow flowers. Its rhizome, in boiled, dried, and powdered form, is used as folk medicines to treat various ailments such as stomach disorders, inflammation, wounds, joint pain, coughing, diabetes, hepatitis, and anorexia [1, 3]**.** This plant is also used to flavor food in Bangladesh, India, Pakistan, Sri Lanka, and other Asian countries. Phytochemical studies on various plants of genus Curcuma have resulted in the identification of over 235 different classes of natural products. These phytochemicals include diarylheptanoids and diarylpentanoids, phenylpropene, flavanoids, terpenoids, steroids, and alkaloids. Previous phytochemical reports on *C. longa* have shown the presence of terpenoids, flavonoids, and curcuminoids and these compounds are assumed to be responsible for the folk medicinal applications of this plant [1, 2]. These compounds have been reported to exhibit different biological activities. Names and structures of curcuminoids isolated from *C. longoa* (**1-22**) have been listed in **Table 2-1**

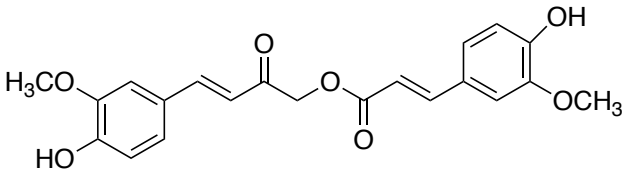
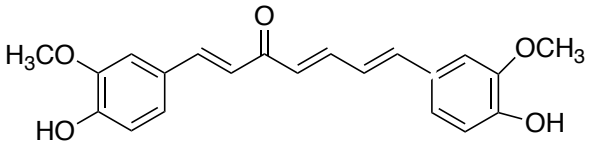
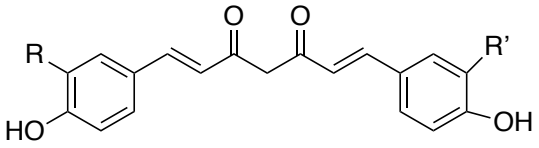
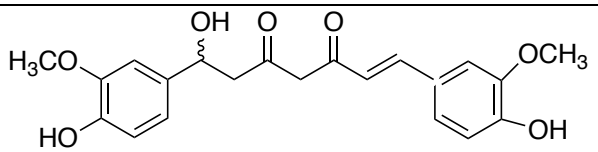
Compounds **4, 6, 7, 10** and **11** showed the cytotoxicity activities against three human cancer cell lines, namely, human alveolar adenocarcinoma (A549), human breast cancer (MDA-MB-231), and human hepatocellular liver carcinoma (HepG2). The bioactivity of these compounds is listed in **Table 2-2.** The bioactivity of these compounds is moderate compared to taxol (**18**), listed in **Table 2-2**, and are currently used in clinics as an anticancer agent [6].

Phytochemical studies on *C. longa* have afforded compounds namely, curcumin (**15**), demethoxycurcumin (**16**), bisdemethoxycurcumin (**17**), alpha- turmerone (**24**), and ar-turmerone (**27**). The compounds have been reported to exhibit concentrations producing 50 % growth inhibition (IC50) of curcuminoids and turmerones isolated from *Curcuma longa* on cancer cell lines and normal skin fibroblasts (**15- 16-17, 24** and **27**) **Table 2.3.** Some of theses compounds showed bioactivities and are listed in **Table 2-4.** Structures of compounds (**13−22)** from *C. longa* protect cells from Beta-Amyloid Insult [10].

Table 2-1: Names and structures of curcuminoids isolated from *C. longoa* (**1-22**)

Name of Compounds	Structures
Terpecurcumin A (**1**) [6] Bisabolocurcumin ether (**10**)[6] Demethoxybisabolocurcumin ether (**11**) [6] Didemethoxybisabolocurcumin ether (**12**)[6]	 **1** R1=OCH3 R2=OCH3 R3=H2 **10** R1=OCH3 R2=OCH3 R3=O **11** R1=H R2=OCH3 R3=O **12** R1=H R2=H R3=O
Terpecurcumin B (**2**)[6]	

Terpecurcumin C (**3**) [6] Terpecurcumin D (**4**) [6]	 **3** R=H_2 **4** R=O
Terpecurcumin E (**5**) [6] Terpecurcumin F (6) [6] Terpecurcumin G (**7**) [6]	 **5** R_1=OCH_3 R_2=H_2 **6** R_1=OCH_3 R_2=O **7** R_1=H R_2=O
Terpecurcumin H (**8**)[6] Terpecurcumin I (**9**)[6]	 **8** R_1=OH R_2=H_2 **9** R_1=H R_2=O

4″- (3‴-methoxy-4‴-hydroxyphenyl)-2″-oxo-3″-enebutanyl 3-(3′-methoxy-4′hydroxyphenyl) propenoate (calebin-A) (**13**) [10]	
1,7-bis (4-hydroxy-3-methoxyphenyl)-1,4,6- heptatrien-3-one (**14**) [10]	
- 1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene- 3,5-dione (Curcumin) (**15**) [6,9] - 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3, 5-dione (demethoxycurcumin) (**16**) [6,9] - 1,7-bis (4-hydroxyphenyl)-1,6-heptadiene-3, 5-dione (bisdemethoxycurcumin) (**17**) [6,9]	 **15** R, R' = OCH_3 **16** R= H, R=OCH_3 **17** R,R'= H
1-hydroxy-1, 7-bis (4-hydroxy-3-methoxyphenyl)-6-heptene-3, 5- dione (**19**) [6]	

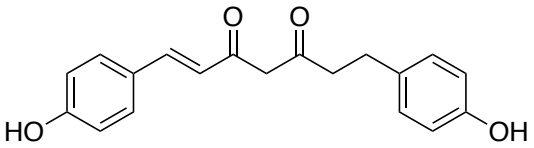
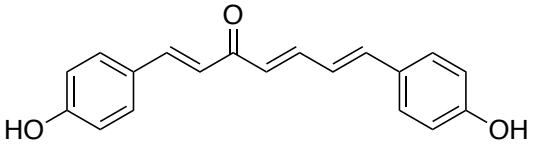
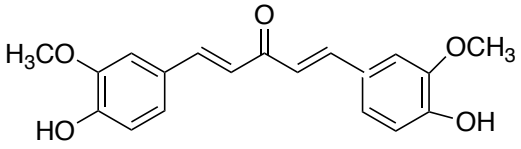
1,7-bis (4-hydroxyphenyl)-1-hep- tene-3, 5-dione (**20**) [6]	
1,7-bis (4-hydroxyphenyl)-1,4,6- heptatrien-3-one (**21**) [6]	
1,5-bis (4-hydroxy-3-methoxy- phenyl)- 1,4-pentadien-3-one (**22**) [6]	

Table 2-2: Cytotoxic activity of compounds (**1-11** and **15-18**) [6]

Compound	**Cell lines**		
	A549	**HepG2**	**MDA-MB-231**
1	30.6	25.0	27.3
2	61.9	64.1	74.9
3	35.0	29.5	24.6
4	14.3	18.3	15.9
5	41.4	35.9	84.1
6	15.2	15.7	19.4
7	14.5	17.1	15.9
8	84.3	54.6	43.7
9	41.1	50.3	69.5
9	16.1	15.4	10.6
10	16.6	13.0	10.3
11	27.4	22.7	30.8
Curcumin (**15**)	49.2	31.3	37.8
Demethoxycurcumin (**16**)	38.1	58.1	44.3
Bisdemethoxycurcumin (**17**)	38.1	62.9	38.5
Taxol (**18**)	0.056	0.054	0.052

Table 2-3 Concentrations producing 50% growth inhibition (IC50) of curcuminoids and turmerones isolated from *Curcuma longa* on cancer cell lines and normal skin fibroblasts (**15,16**, **17,24** and **27**) [9].

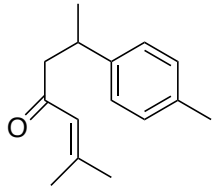
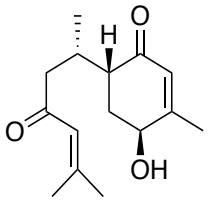
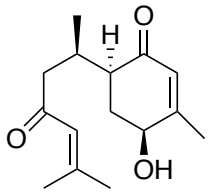
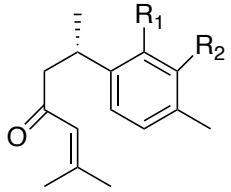
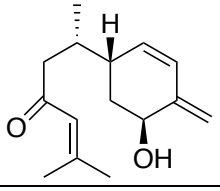
	IC_{50} (µg /ml)			
	HepG2	**MCF-7**	**MDA-MB-231**	**Hs-68**
Curcumin (**15**)	15.8	24.8	11.0	>50
Demethoxycurcumin (**16**)	18.1	22.1	11.4	>50
Bisdemethoxycurcumin (**17**)	19.5	28.2	12.1	>100
Alpha- turmerone (**24**)	32.9	41.8	30.2	>100
Ar-turmerone (**27**)	>100	>100	>100	>100

Table 2-4 Protection of PC12 cells from ßA Insult by natural products (**13-22**) isolated from *C. longa* [10].

Compounds	**PC12 cells** **Anti-*ß*A (25-35)** **ED50*b* (*µ*g/mL)**	**PC12 cells** **Anti-*ß*A (1-42)** **ED50*b* (*µ*g/mL)**
13	1.0 +- 0.3	2.0+-0.4
14	>50	>50
15	7.0 +- 1.1	10.0+-0.9
16	4.0 +- 0.5	5.0+-0.5
17	2.0+-0.6	3.5+-0.7
19	30.7+-3.3	44.3+-3.1
20	0.5+-0.2	1.0+-0.3
21	>50	>50
22	>50	>50
(Congo red) (**23**)	37.5+-5.4	39.2+-5.2

Phytochemical investigation of *C. longa* also resulted in the identification of terpenoids (**24**-**38**), and their names and structures are listed in **Table 2-5.** Some of these terpenoids have shown IC50 values in compounds (**25-38**) inhibiting NO production in BV-2 cells [8], listed in **Tables 2-6.** Some of these terpenoids such as alpha- turmerone (**24**) and ar-turmerone (**27**) have also shown bioactivities that are listed in **Table 2-3** [9].

Table 2-5 Structures of terpenoids (**24-38**) reported from *C. longa*.

The Name of Compounds	**The Structures**
Alpha-turmerone (**24**) [8,9]	
Longpene C (**25**) [8]	
Longpene D (**26**)[8]	
Ar-tumerone (**27**) [8,9] Turmeronol B (**28**)[8] Turmeronol A (**29**)[8]	 **27** $R_1 = R_2 = H$ **28** $R_1 = OH$, $R_2 = H$ **29** $R_1 = H$, $R_2 = OH$
Intermedin B (**30**)[8]	

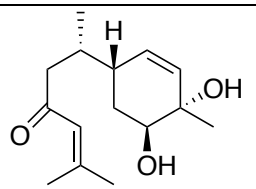
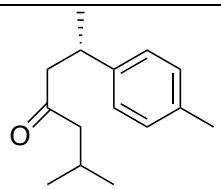
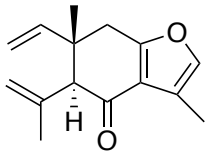
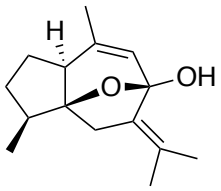
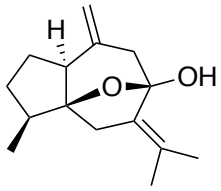
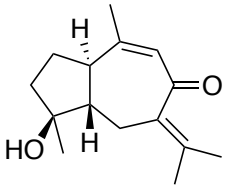
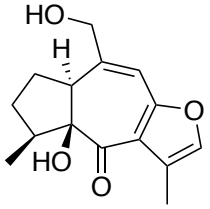
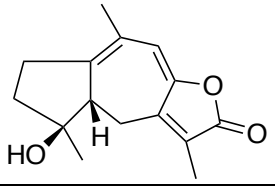
Bisacurone (**31**)[8]	
Ar-dihydroturmer- one (**32**)[8]	
Curzerenone (**33**)[8]	
Curcumenol (**34**)[8]	
Isocurcumenol (**35**)[8]	
Procurcumenol (**36**)[8]	
Zedoardiol (**37**)[8]	
Zedoalactone F (**38**)[8]	

Table 2-6 IC50 values of compounds (**25-38**) inhibiting NO production in BV-2 cells [8]

Compound	**IC_{50} (μM)**	**Compound**	**IC_{50} (μM)**
25	87.3	**32**	>100
26	98.1	**33**	>100
27	92.1	**34**	>100
28	49.0	**35**	23.6
29	>100	**36**	97.0
30	30.0	**37**	37.1
31	29.8	**38**	95.4

This plant has shown antimicrobial activity and this activity is assumed to be associated with the presence of a major metabolite, curcuminoids terpenoids, and other compounds. It was decided to carry out antimicrobial activity directed phytochemical studies on the crude methanolic extract of this plant in order to identify the antimicrobial natural products to answer two questions: (i) to confirm the aforementioned claims or (ii) bioactivity is associated with other natural products present in this plant.

2.2. Experimental Section

2.2.1 General Experimental Conditions

Solvents including methanol, ethyl acetate, chloroform, dichloromethane, and hexane used in this research for extraction and chromatographic work were of ACS grade and purchased from VWR-USA. NMR one-dimensional experiments (^{1}H-, ^{13}C- NMR spectra), and two-dimensional NMR experiments (COSY, HSQC, and HMBC spectra) were carried out on a Bruker Avance at 400MHz NMR spectrometer. These experiments were performed in deuterated chloroform ($CDCl_3$) and acetone (C_3D_3O) which were purchased from Cambridge Isotope Laboratories, Inc. USA. Thin layer chromatography (TLC) was performed on silica gel GF254 pre-coated plates purchased from SiliCycle, Canada while silica gel (230-400 mesh) was used as the stationary phase in the column chromatography and was purchased from Caledon Laboratory Chemicals, Canada. TLC plates were also developed using 10% H_2SO_4.

2.2.2 Extraction and Isolation:

The dried root of *C. longa* (9 kg) was extracted with methanol (8L; 3 × times) at room temperature. The crude extract was filtrated using filter paper and solvent was removed under vacuum to afford yellow colored gum (320 g). This extract was then dissolved in a mixture of 80% methanol (MeOH) and 20% water (H_2O) (2 L-80: 20) to make an aqueous methanolic extract. The antimicrobial activity was performed on the crude extract against Gram- positive and Gram-negative bacteria and the detailed procedure is described on page (29-32).

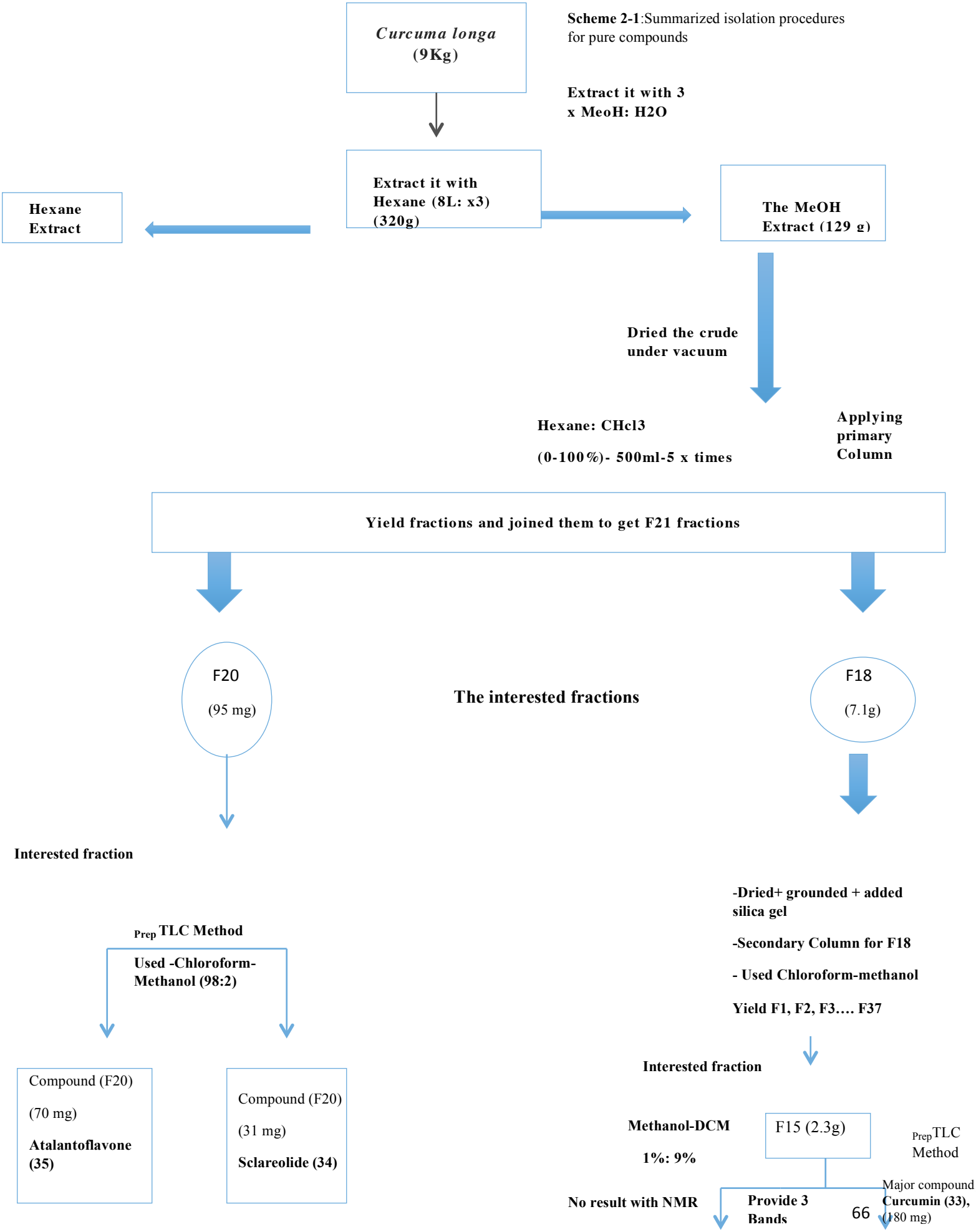
The crude methanolic extract was active against Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*) but was inactive against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and this aqueous-alcoholic extract was defatted

with hexane. Both hexane and defatted extracts were dried using rotatory evaporator. The defatted extract (129 g) was found to be bioactive and was loaded onto a column. This column was eluted with hexane-chloroform (0-100%) to afford several fractions, which were pooled based on the results from analytical TLC data to afford 21 fractions (**F1-F21**). Antimicrobial assay on these fraction revealed that fractions F18 and F20 were bioactive. Fraction **F18** (7.1g), was subjected to column chromatography using chloroform-methanol (0-100%) as eluent to yield several fractions. All of these fractions were pooled on the basis of analytical TLC. A fraction obtained on elution of silica gel column with chloroform-methanol (85%-15%) was subjected to analytical TLC using 9% dichloromethane (DCM) and 1% Methanol (MeOH) and this showed one major spot along with some minor compounds. The prep-TLC of this fraction using the same mobile phase as that of analytical TLC afforded one major compound (180 mg) along with minor compounds. The ^{1}H- and ^{13}C-NMR spectra of the major compound were acquired and were found to be identical as those of curcumin (**F18**). The minor compounds were not obtained in sufficient quantities to record their ^{1}H and ^{13}C-NMR spectra. Analytical TLC of (**F20**) (95 mg) was performed using chloroform-methanol (98:2) and it revealed the presence of two spots. One of these spots was UV active while other was UV inactive. The latter spot along with the former was visualized by developing TLC with 10% sulfuric acid. The prep-TLC of this fraction using the same mobile phase as that of analytical TLC resulted in the isolation of a white coloured compound (**F20**) (31 mg) and yellow coloured compound (**F20**) (70 mg). The ^{1}H- and ^{13}C-NMR spectra of these compounds were found to be identical to those of sclareolide (**F20**) and atalantoflavone (**F20**) reported in the literature. The isolation work has been show in **Scheme 2-1**.

Curcumin (39): brown coloured amorphous solid, (180 mg); ^{1}H-NMR (acetone-d_6, 400 MHz): see **Table 2-7**; ^{13}C-NMR (acetone-d_6, 100 MHz): see **Table 2-7**.

Sclareolide (40): white coloured amorphous solid, (31mg). ^{1}H-NMR (acetone-d_6, 400 MHz:) see **Table 2-8;** ^{13C}-NMR (acetone-d_6, 100 MHz): see **Table 2-8**.

Atalantoflavone (**41**): yellow coloured gum, (70 mg); ^{1}H-NMR (acetone-d_6, 400 MHz): see **Table 2-9**; ^{13}C-NMR (acetone-d_6, 100 MHz): see **Table 2-9**.



Scheme 2-1:Summarized isolation procedures for pure compounds

2.2.3 Antimicrobial assay

Antibacterial activity was determined against cultures of Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The antimicrobial activity was performed using Kirby-Bauer disc method. The stock solution was prepared with different concentrations, which were applied onto 6 mm diameter filter-paper disks. A final disc loading concentration of 300 mg/10 ml for the crude extract and 1 mg/3 ml for the pure compounds was used. The impregnated discs were placed on prepared plates with the selected test organisms, along with discs containing a disc of negative control that was prepared by loading methanol and ciprofloxacin was used as a positive control standard. The plates were incubated at 37 °C for 24 hours and antimicrobial activity was recorded as the clear zones of inhibition surrounding the discs. The diameter was measured in mm and the experiment was carried out one time. The aseptic condition was applied with all throughout the assay.

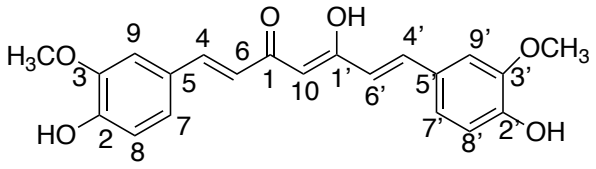
2.2.4 Results and discussion

Phytochemical studies on the methanolic extract of C. longa resulted in the isolation of three natural products, Curcumin (**39**), sclareolide (**40**), and atalantoflavone (**41**). The detailed isolation procedure has been described in **section 2.2.1** of this chapter (page **63**).

Structure of these compounds were elucidated with the aid of NMR spectroscopic studies, summarized as follows.

2.2.5 Structure Elucidation Compounds (39, 40 and 41)

2.2.5.1 Curcumin (39)



(39)

Curcumin (**39**) was isolated as a brown amorphous solid. The ^{1}H-NMR spectrum (acetone-d_6, 400 MHz) of this compound displayed a three-proton singlet at δ 3.79 due to the O-methyl protons substituted at C-3/C-3'. A broad singlet centred at δ 8.20 was ascribed to C-2/C-2' phenolic proton. Resonances at δ 7.64, δ 6.75, δ 7.15, δ 6.94 were due to H-4/H-4', H-6/H-6', H-7/H-7', H-8/H-8' methine protons, respectively. The H-9/H-9' and H-10 methine protons

resonated at δ 7.37, and δ 6.07, respectively. C4/C-4', C-6/C-6', C-7/C-7'and C-8/C-8' methine carbons resonated at δ 140.5, δ 122.9, δ 121.4, and δ 115.3, respectively.

It showed two one-proton of cis isomerism doublets at δ 7.64 and δ 7.64 (J = 15.0 Hz) and δ 6.75 and δ 6.75 (J = 15.0 Hz) due to H-4/H-4' and H-6/H-6' respectively. Two one-proton doublets at δ 7.17 and δ 7.17 (J = 8.0 Hz) and δ 6.94 and δ 6.94 (J = 8.0 Hz) showed sp^3 hybridization of neighbouring protons due to H-7/H-7' and H-8/H-8' respectively.

The COSY spectrum of Curcumin (**39**) showed vicinal coupling of H-4/H-4' (δ 7.64) with H-6/H-6' (δ 6.75). Similarly, H-7/H-7' (δ 7.15) exhibited cross peaks with H-8/H-8' (δ 6.94).

The ^{13}C-APT-NMR spectrum (acetone-d_6, 100 MHz) of **(39)** showed the presence of eleven methine, two methyl, and eight quaternary carbons. It also showed twenty-one carbons and the complete ^{13}C-NMR chemical shift assignments of (**39**) are shown in **Table 2-7.**

The HSQC spectrum of (**39**) was acquired in order to establish ^{1}H/^{13}C one-bond shift correlations of all the hydrogen bearing carbons. H-4/H-4' (δ 7.64) showed cross peaks with C-4/C-4' (δ 140.5). H-6/H-6' (δ 6.74) exhibited ^{1}H/^{13}C one-bond shift correlations with C-1 (δ 185.0). In addition, the H-8/H-8' (δ 6.94) showed correlation with C-8/C-8' (δ 115.3). Complete ^{13}C-NMR chemical shift values and ^{1}H/^{13}C one-bond shift correlations of all hydrogen bearing carbons, as determined from HSQC spectrum, are shown in **Table 2-7.**

The HMBC spectrum of (**39**) showed long-range couplings of H-7/H-7' (δ 7.15) with C-9/C-9' (δ 110.6). H–4/H-4' (δ 7.64) showed other coupling with C-7/C-7' (δ 121.4). H-9/H-9' (δ 7.37) was coupled to C-4/C-4' (δ 140.5). In addition, H-8/H-8' (δ 6.94) exhibited coupling with C-3/C-3' (δ 146.8).

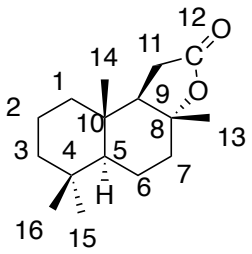
Table 2-7 ^{1}H and ^{13}C NMR chemical shift assignments of **(39)** and $^{1}H/^{13}C$ one-bond shift correlations, as determined by HSQC.

Position	δH (*J* in Hz)	δC (multiplicity†)
1	-	185.0 (C=O)
2	8.20, bs	149.2 (-C-OH-)
3	3.79, s	146.8 (-C-OH_3-)
3	3.79, s	55.4 (-OCH_3)
4	7.64, d (15.0)	140.5 (-CH-)
5	-	127.5 (-C-)
6	6.75, d (15.0)	122.9 (-CH-)
7	7.15, d (8.0)	121.4 (-CH-)
8	6.94,d (8.0)	115.3 (-CH-)
9	7.37,s	110.6 (-CH-)
10	6,07,s	100.7 (-CH-)
1’	-	185.0 (-C-OH-)
2’	8.20,bs	149.2 (-COH-)
3’	3.79 s	146.8 (-C-OH_3-)
3’	3.79, s	55.4 (-OCH_3)
4’	7.64,d (15.0)	140.5 (-CH-)
5’	-	127.5 (-C-)
6’	6.75,d (15.0)	122.9 (-CH-)
7’	7.15,d (8.0)	121.45 (-CH-)
8’	6.94,d (8.0)	115.3 (-CH-)
9’	7.37,s	110.6 (-CH-)

†Multiplicity was determined with the help of ^{13}C-APT and DEPT spectra

The ^{1}H- and ^{13}C-NMR spectroscopic data of compound **(39)** was identical to those of curcumin found in literature [11]. These spectroscopic studies led us to identify compound (**39**) as curcumin.

2.2.5.2 Sclareolide (40)



(**40**)

The second compound, sclareolide (**40**), was isolated as white amorphous solid. Its ^{1}H-NMR spectrum (acetone-$d_{6,}$ 400 MHz) showed three three-proton resonances at δ 1.35, δ 0.98, δ 0.88 and δ 0.91 due to C-13, C-14, C-15, and C-16 methyl protons, respectively. A set of two one-proton double doublets, resonated at δ 2.56 and δ 2.53, was due to C-11 methylene protons. The downfield chemical shift values of these protons were indicative of the presence of germinal carbonyl moiety. A one-proton double doublet centered δ 2.10 was ascribed to C-9 methine proton. Complete ^{1}H-NMR chemical shift assignments of compound (**40**) are shown in **Table 2-8.**

The COSY- spectrum of compound (**40**) showed the vicinal couplings of H_2-11 (δ 2.56) with H-9 (δ 2.10). Similarly, ^{1}H-^{1}H vincinal and germinal couplings of other protons in this compound were traced from the COSY spectrum that helped in assigning ^{1}H-NMR chemical shift assignments of compound (**40**). Complete chemical shift assignments of (**40**) are listed in **Table 2-8**.

The ^{13}C-APT-NMR spectrum (acetone-d_6, 100 MHz) of (**40**) showed the signals for all sixteen carbons and revealed the presence of four methyl, six methylene, two methine, and four quaternary carbons in (**40**). Complete ^{1}H and ^{13}C-NMR chemical shift assignments of (**40**) are shown in **Table 2-8**.

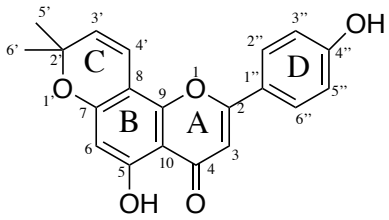
The ^{1}H and ^{13}C-NMR spectroscopic data of compound (**40**) was similar to those of sclareolide, reported in literature [12]. This compound was previously reported from *Arnica angustifolia* [13]. These spectroscopic data led us to identify compound (**40**) as sclareolide. Compound (**40**) has been isolated for the first time from *C. longa.*

Table 2-8 ^{1}H and ^{13}C NMR spectroscopic data for **(40)** and $^{1}H/^{13}C$ one-bond shift correlations.

Position	δH (*J* in Hz)	δC (multiplicity†)
1	1.53,1.79,m	39.5 ($-CH_2-$)
2	1.28,1.29,m	18.8 ($-CH_2-$)
3	1.53,1.53,m	42.0 ($-CH_2-$)
4	-	33.3 (-C-)
5	1.48,dd (11.2,13.6)	56.6 (-CH-)
6	1.43,1.72 m	20.5 ($-CH_2-$)
7	1.68,1.69, m	36.2 ($-CH_2-$)
8	-	85.0 (-C-)
9	2.10,dd (4.8,7.2)	59.0 (-CH-)
10	-	37.5 (-C-)
11	2.56, 2.53,m	32.6 ($-CH_2-$)
12	-	175.6 (-C=O-)
13	1.35,s	21.1($-CH_3-$)
14	0.98,s	14.4 ($-CH_3-$)
15	0.88,s	27.8 ($-CH_3-$)
16	0.91,s	29.3 ($-CH_3-$)

†Multiplicity was determined with the help of ^{13}C-APT and ^{13}C Broadband spectra.

2.2.5.3 Atalantoflavone (41)



(41)

The third compound, atalantoflavone (**41**), was isolated as yellow amorphous solid. The ^{1}H-NMR spectrum (acetone-$d_{6,}$ 400 MHz) of **(41) s**howed the resonance of two six proton singlets at δ 1.24 due to the C-5'/C-6' methyl protons. A set of two doublets, integrating for two protons each, appeared at δ 7.45 and were assigned to H-2''/H-6''and H-3''/H-5'', respectively. The double set of two one-proton doublets at δ 6.65 and δ 5.70 were due to H-3' and H-4' protons, respectively. The C-3 methine proton resonated at δ 6.29.

It showed two one-proton doublets at δ 6.65 (J = 10.1 Hz) and δ 5.7 (J = 10.1 Hz) due to H-3' and H-4' respectively. The multiplicity of theses signals suggested that para position of two one-proton doublets at δ 7.45 (J = 8.5 Hz) and δ 7.45 (J = 8.6 Hz) due to H-2''/H-6'' respectively.

The broadband ^{13}C NMR spectrum (acetone-d_6, 100 MHz) of **(41)** showed the resonance of all carbons present in this compound. The ^{13}C-APT-NMR was also recorded and it indicated the presence of two methyl, eight methine, and ten quaternary carbons in it. Complete ^{1}H and ^{13}C-NMR chemical shift assignments of are shown in **Table 2.9.**

Table 2-9 ^{1}H and ^{13}C NMR spectroscopic data for **(41)** and $^{1}H/^{13}C$ one-bond shift Correlations.

Position	δH (*J* in Hz)	δC (multiplicity†)
1	-	-
2	-	159.5 (-C-)
3	6.29,s	104.3 (-CH-)
4	-	180.9 (-C=O-)
5	-	157.3 (-OH-)
6	8.05,s	94.8 (-CH-)
7	-	157.4 (-CH-)
8	-	106.0 (-CH-)
9	-	153.3 (-C-)
10	-	105.0 (-C-)
1’	-	-
2’	-	77.9 (-C-)
3’	6.65,d (10.1)	128.3 (-CH-)
4’	5.70,d (10.1)	115.6 (-CH-)
5’	1.24,s	27.6 (-CH_3-)
6’	1.24,s	27.6 (-CH_3-)
1’’	-	122.3 (-C-)
2’’	7.45,d (8.5)	130.7(-CH-)
3’’	6.44,d (8.6)	115.6 (-CH-)
4’’	-	157 (-COH-)
5’’	6.44,d (8.6)	115.6 (-CH-)
6’’	7.45,d (8.5)	130.7 (-CH-)

†Multiplicity was determined with the help of ^{13}C-APT and ^{13}C Broad-Band spectra

The ^{1}H and ^{13}C-NMR spectroscopic data of compound (**41**) was similar to those of atalantoflavone, reported in literature [15]. This compound was previously reported from *Erythrina sigmoidea* [14]. These spectroscopic data led us to identify compound (**41**) as atalantoflavone. Compound (**41**) has been identified for the first time from *C. longa.*

2.3 Antimicrobial activity of crude extract and pure compounds (39-40 and 41)

The antimicrobial activity of the methanolic crude extract was examined by using Kirby-Bauer disc methods. The methanolic crude extract was screened against four pathogenic bacteria namely Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*) and Gram-negative bacteria *(Escherichia coli* and *Pseudomonas aeruginosa*), all of which were organisms provided by Dr. Paul Holloway from the Department of Biology at the University of Winnipeg. A negative control was prepared by loading a disc of methanol and ciprofloxacin was used as positive control standard. The sample was prepared with moderate concentration 300-mg/10 ml methanol (30 mg/ml) at 50 µl, 100 µl, 150 µl and 500 µl with 1.500 µg, 3.000 µg, 4.500 µg, and 15.000 µg Concentrations respectively based on available literature that used high concentrations [16] showed in **Table 2.10**. The disc was left over night for the solvent to dry completely in order to rule out the possibility of biological activity due to the solvent. These plates were incubated for 24 hours at 37 °C and the crude extract exhibited significant antimicrobial activity against *Staphylococcus aureus.* The zone of inhibition around the disc was 8 mm that was measured by subtracting the diameter of the disc (6 mm) from the total diameter. When the crude was investigated against *Staphylococcus aureus,* a zone of inhibition of 2 mm was found with the growth medium TSA at a volume of 500 µl.

The purified known compound curcumin **(39)** was also tested against *Escherichia coli* and *Staphylococcus aureus* by following the above stated methodology. The sample was prepared with 1 mg/3 ml methanol with the growth medium TSA at a volume of 25 µL at 0.0083 **µg** concentrations respectively, shown in **Table 2.11**. This compound was inactive while the purified compounds sclareolide (**40**) and atalantoflavone (**41**), at similar concentration and were also tested against *Escherichia coli* and *Staphylococcus aureus* and showed no inhibition (**figure 4**). All these compounds had the methanol used as a negative control while the positive control was Ciprofloxacin (5 micrograms) discs from BD Diagnostics.

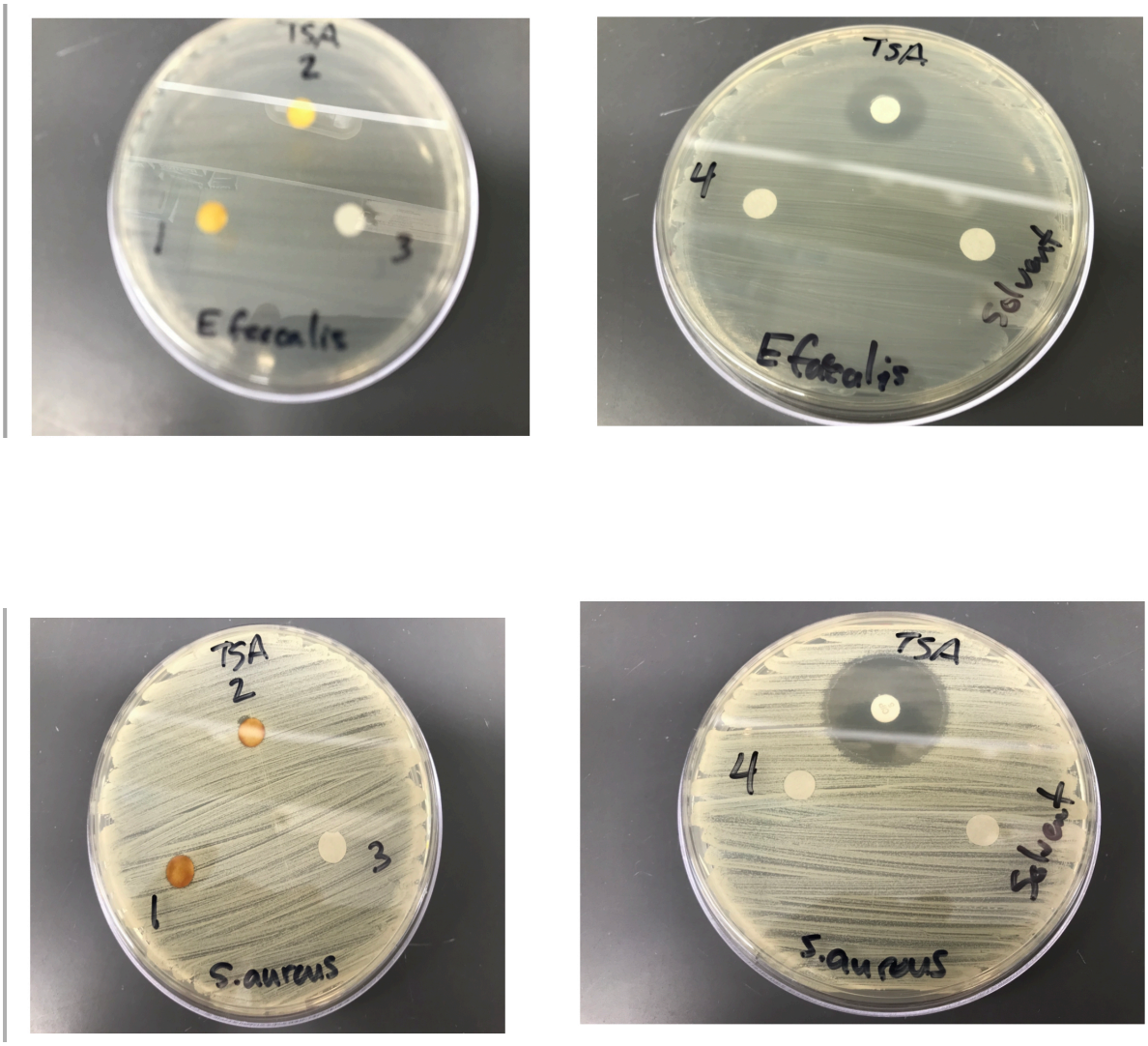


Figure 4: The Figures shows the discs deposited on agar plate inoculated with test organisms *Escherichia coli* and *Staphylococcus aureus.*

Table 2-10: Zone of inhibition (mm) of the crude extract

Bacteria	**Amount** 50 µl	**Amount** 100 µl	**Amount** 150 µl	**Amount** 500 µl
Staphylococcus aureus	Negative	Negative	Negative	2mm
Streptococcus agalactiae	Negative	Negative	Negative	Negative
Escherichia coli	Negative	Negative	Negative	Negative
Pseudomonas aeruginosa	Negative	Negative	Negative	Negative

Table 2.11: Zone of inhibition (mm) of curcumin **(39),** sclareolide (**40**) and atalantoflavone (**41**).

Bacteria	**Amount**	Curcumin (**39)**	Sclareolide (**40**)	Atalantoflavone, (**41**)
Escherichia coli	25 µl	Negative	Negative	Negative
Staphylococcus aureus	25 µl	Negative	Negative	Negative

2.4 References:

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CHAPTER 3

Conclusion

The overall goal of this project was to perform phytochemical studies *Curcuma longa* in order to isolate natural products and to evaluate them for antimicrobial activities. This study afforded three known compounds: curcumin (**39),** sclareolide (**40**), and atalantoflavone (**41**). These three pure compounds did not show antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*.

The antimicrobial data suggested that these compounds need to be re-evaluated for antimicrobial activity at higher concentrations. During these studies, a number of minor metabolites were isolated in trace quantities and we were unable to perform NMR spectroscopic studies on them. There is also a possibility that minor metabolites might be responsible for antimicrobial activity of this plant. This problem can be overcome by re-isolation of minor metabolites from *C. longa* using large-scale plant material. Due to COVID-19 pandemic and time limitation, we were unable to perform these experiments.

Though the proposed objective of this research for the identification of antimicrobial natural products was not achieved, these studies resulted in the identification of sclareolide (**40**) and atalantoflavone (**41**) as major metabolites for the first time from this plant. There is no report available in the literature describing the isolation of compounds (**40)** and (**41)** from *C. longa*.

Curcumin (**39**) and other curcuminoids possess the main phytochemicals of *Curcuma longa L*. Curcuma phytochemicals have received much attention due to their diverse biological effects [21]. Many publications have recorded broad-spectrum antimicrobial activity and it's safety for use at high doses (12 g/day) in the clinic. The best potential of curcumin is restricted due to the low oral bioavailability and lack of solubility in aqueous solvents causing low absorption, quick metabolism, and fast systemic elimination. There is a study to improve the stability and solubility of curcumin, which was a microencapsulation method that was examined. The microcapsule of curcumin with improved solubility is useful as a preservative and colorant in food manufactur and it showed strong antimicrobial activity against foodborne pathogens including *E. coli* and *S. aureus [*17].

Further research on curcumin (39), sclareolide (40), and atalantoflavone (41) could investigate its antibiotic potential against rapidly developing bacterial resistance. Other methods to evaluate the antimicrobial activity of curcumin include screening with bioassays in the lab such as disk-diffusion, well diffusion, and broth or agar microdilution. [22].

Other various laboratory procedures can be applied to evaluate or screen the in vitro antimicrobial activity of these three known compounds such as applying two differentially permeable fluorescent probes, namely, propidium iodide and calcein. They provide information on the integrity of the bacterial membrane and cellular viability. Propidium iodide interacts with deoxyribonucleic acid bases (DNA), fluorescence is produced as a result of this. Only after the bacterial cell membrane has been permeabilized by an agent will propidium iodide enter and bind to DNA. When the membrane is damaged, calcein can leak out of the cells [18]. Both the

membrane permeabilization assays can confirm membrane leakage in Gram-negative and Gram-positive bacteria on exposure to the pure compounds. Scanning electron microscopy and fluorescence microscopy can also be applied to determine membrane disturbances in bacterial cells on exposure to the pure compounds [17].

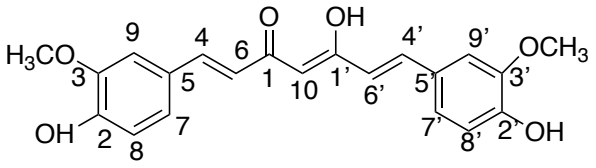
A survival curve can be used to quantify the antimicrobial effect of curcumin. Test bacterial cells are grown in a broth until mid logarithmic phase. Different concentrations of the pure compound can be added to the mid logarithmic bacterial cells. After a time, the cells are plated out on media plates and colonies counted to determine bacterial survival [18].

Another suggestion for further research into the antimicrobial activity of curcumin is the use of the broth microdilution method to determine the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of the compounds. Atalantoflavone (41) was reported in the study and it was tested Gram-negative multi-drug resistant phenotypes using the broth microdilution method [19].

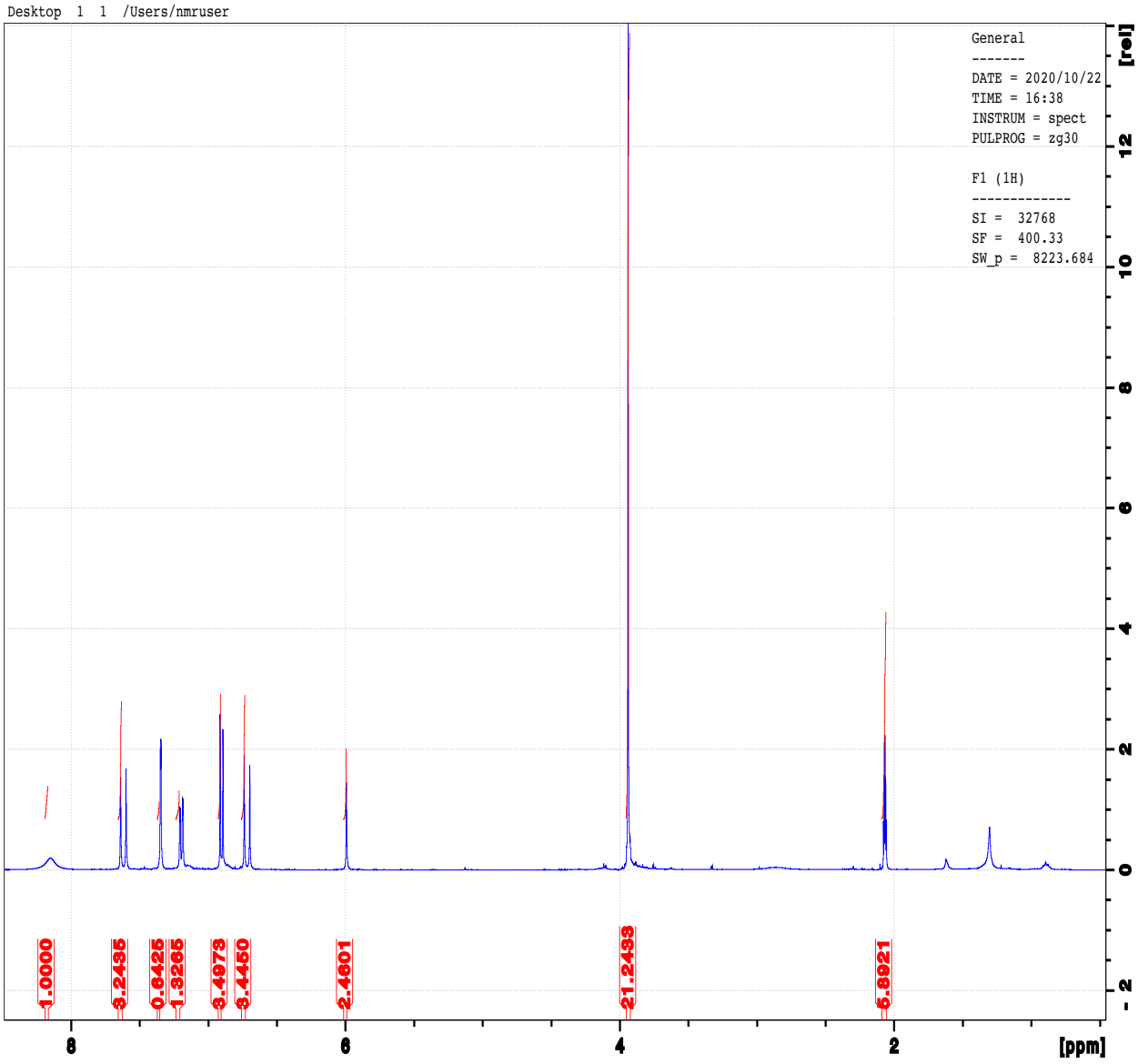
Another suggestion for future research is to determine a relationship between the chemical structure of the compound and its biological activity in order to determine the chemical group responsible for evoking a biological influence in the organism. For example, natural product hedychilactone A was synthesized from sclareolide by an efficient route. Two of the synthetic intermediates, α, β-unsaturated ketone and α, β-unsaturated ketone showed strong growth inhibition effects against five cancer cell lines. [20]

APPENDIX

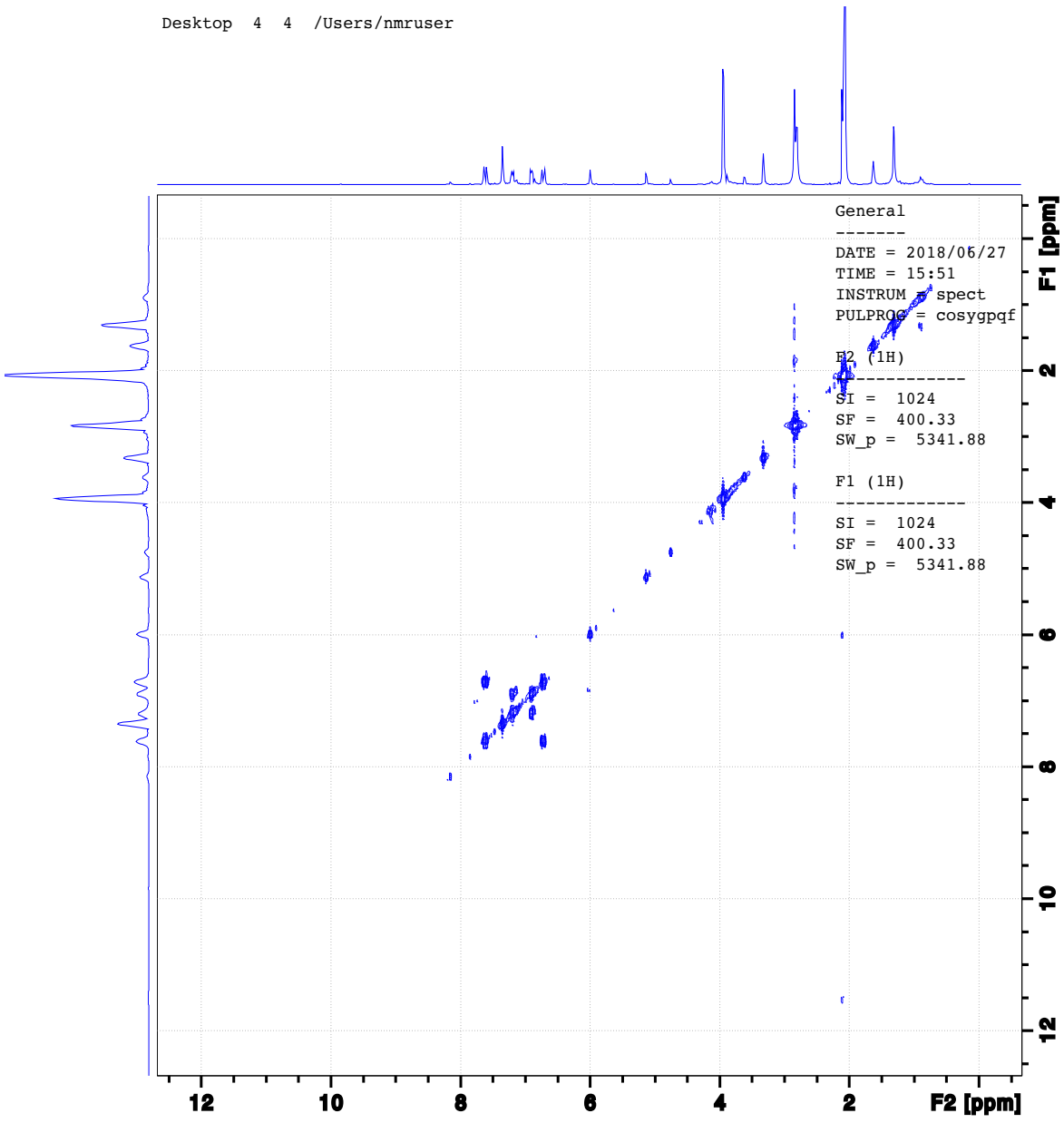
Curcumin (39)



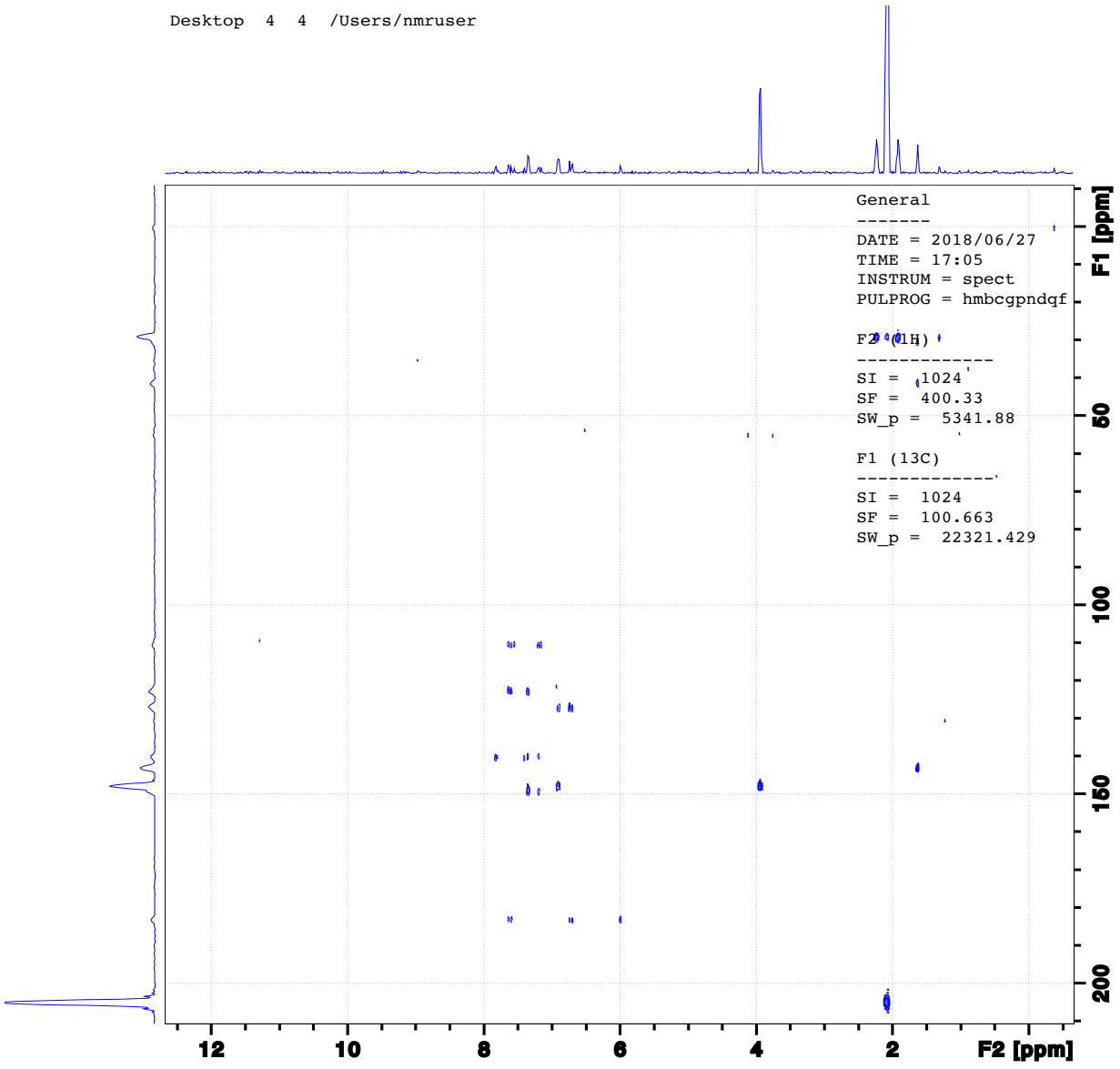
(**39**)



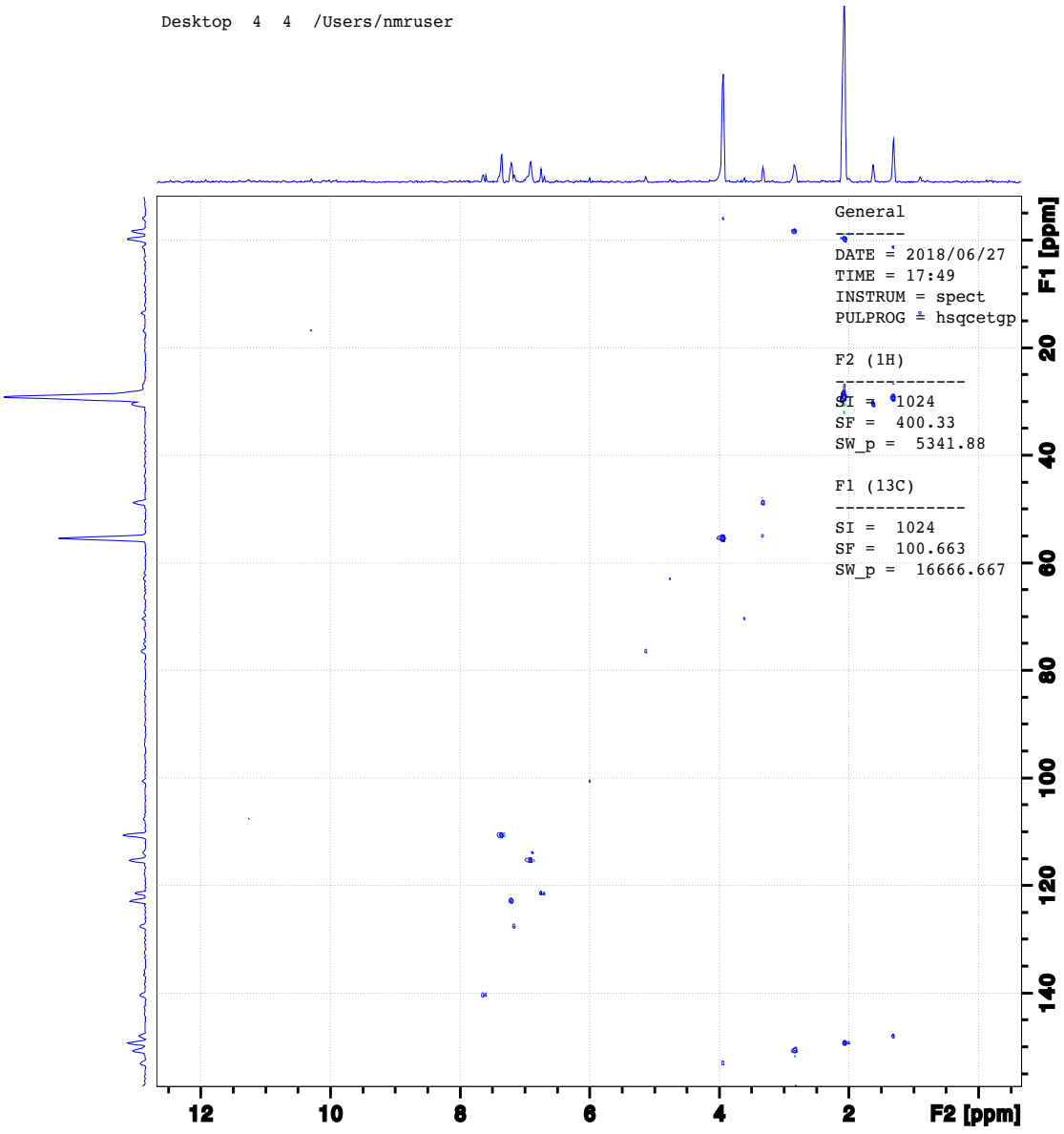
A1: ^{1}H-NMR spectrum of compound (**39**) in acetone-d_6



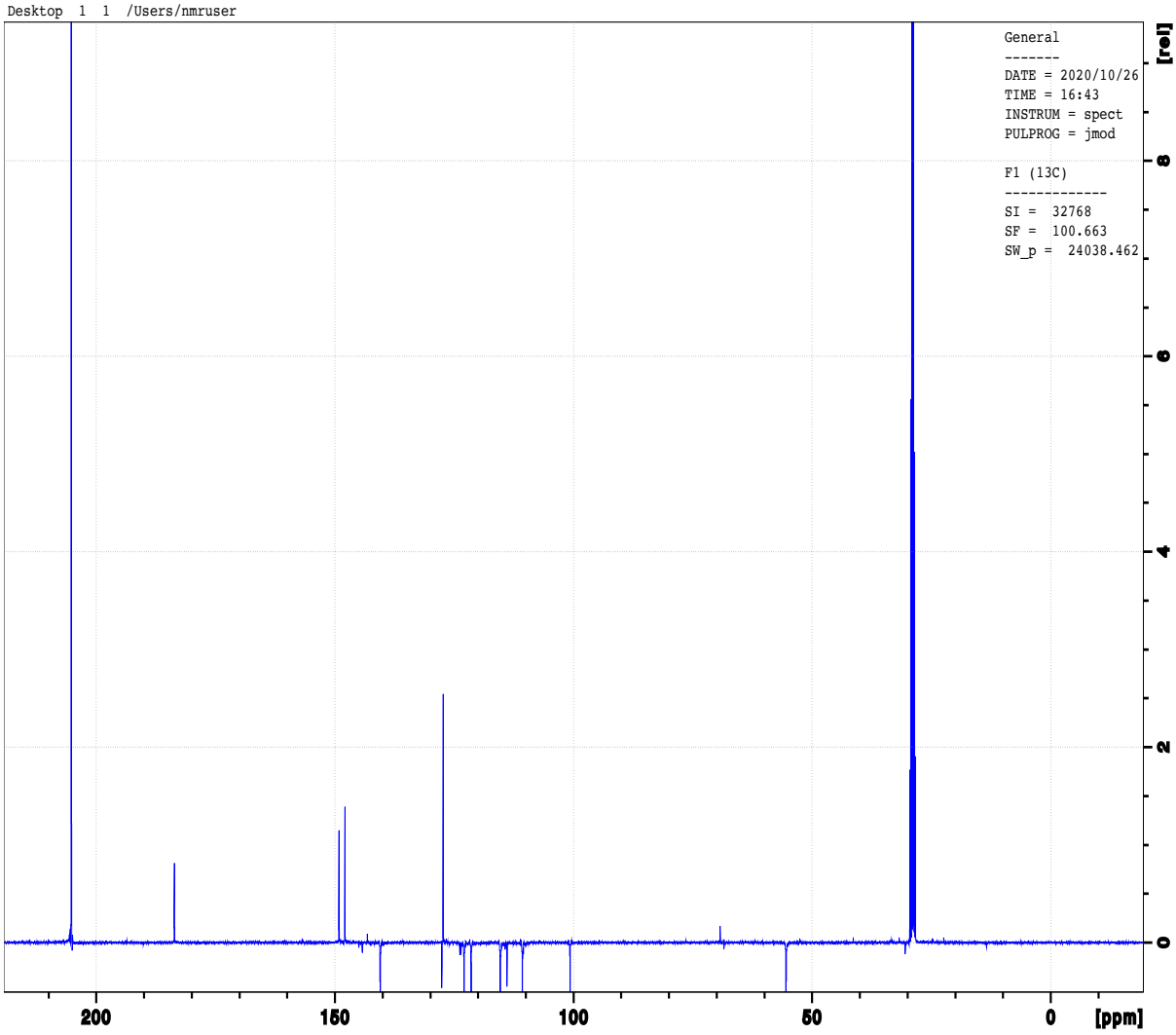
A2: COSY spectrum of compound (**39**) in acetone-d_6



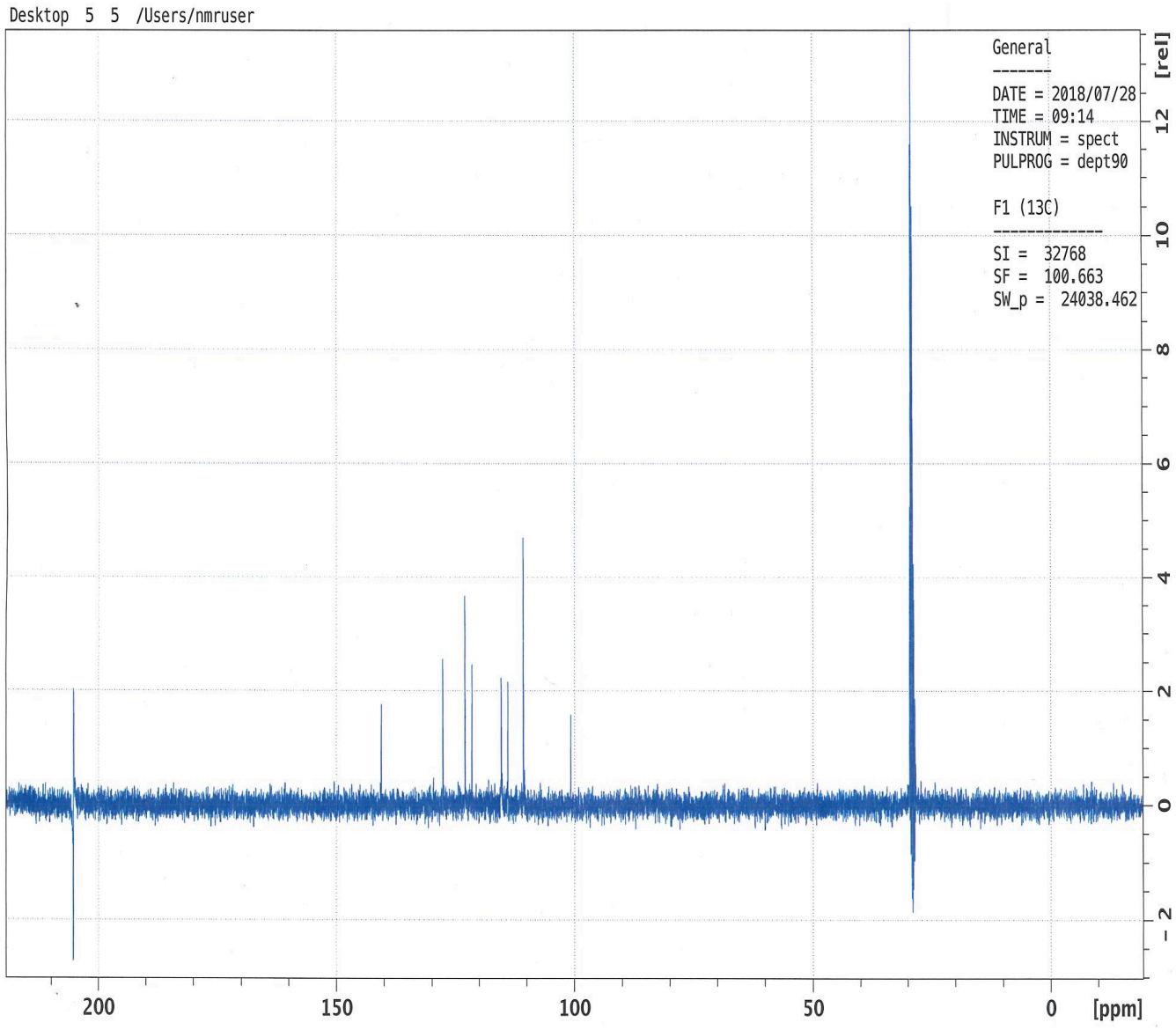
A3: HMBC spectrum of compound (**39**) in acetone-d_6



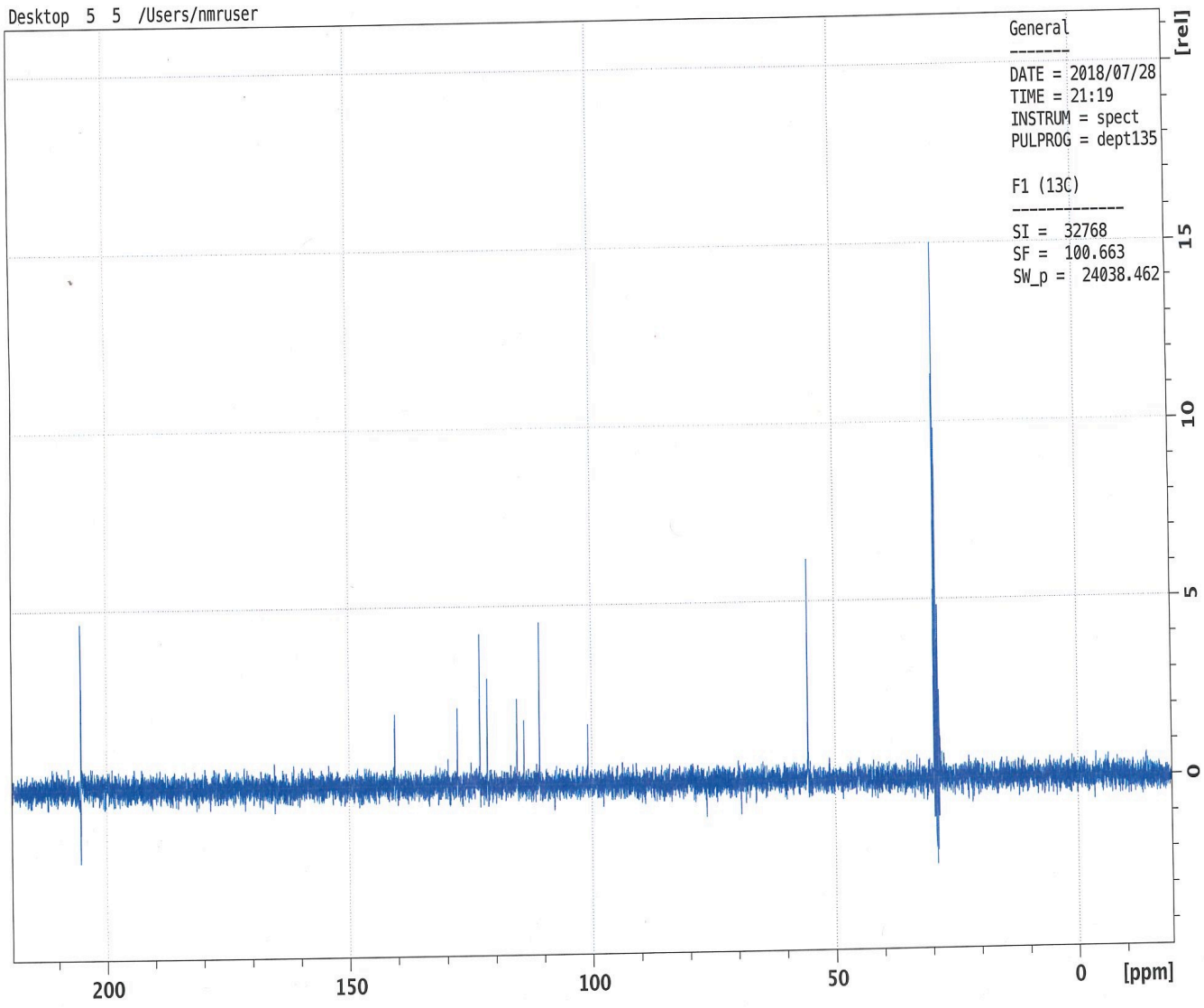
A4: HSQC spectrum of compound (**39**) in acetone-*d*



A5: ^{13}C-NMR spectrum of compound (**39**) in acetone-d_6.

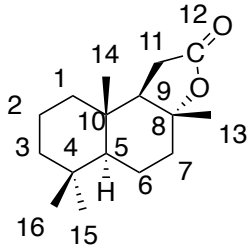


A-6: DEPT-90^0 spectrum of compound (**39**) in acetone-d_6

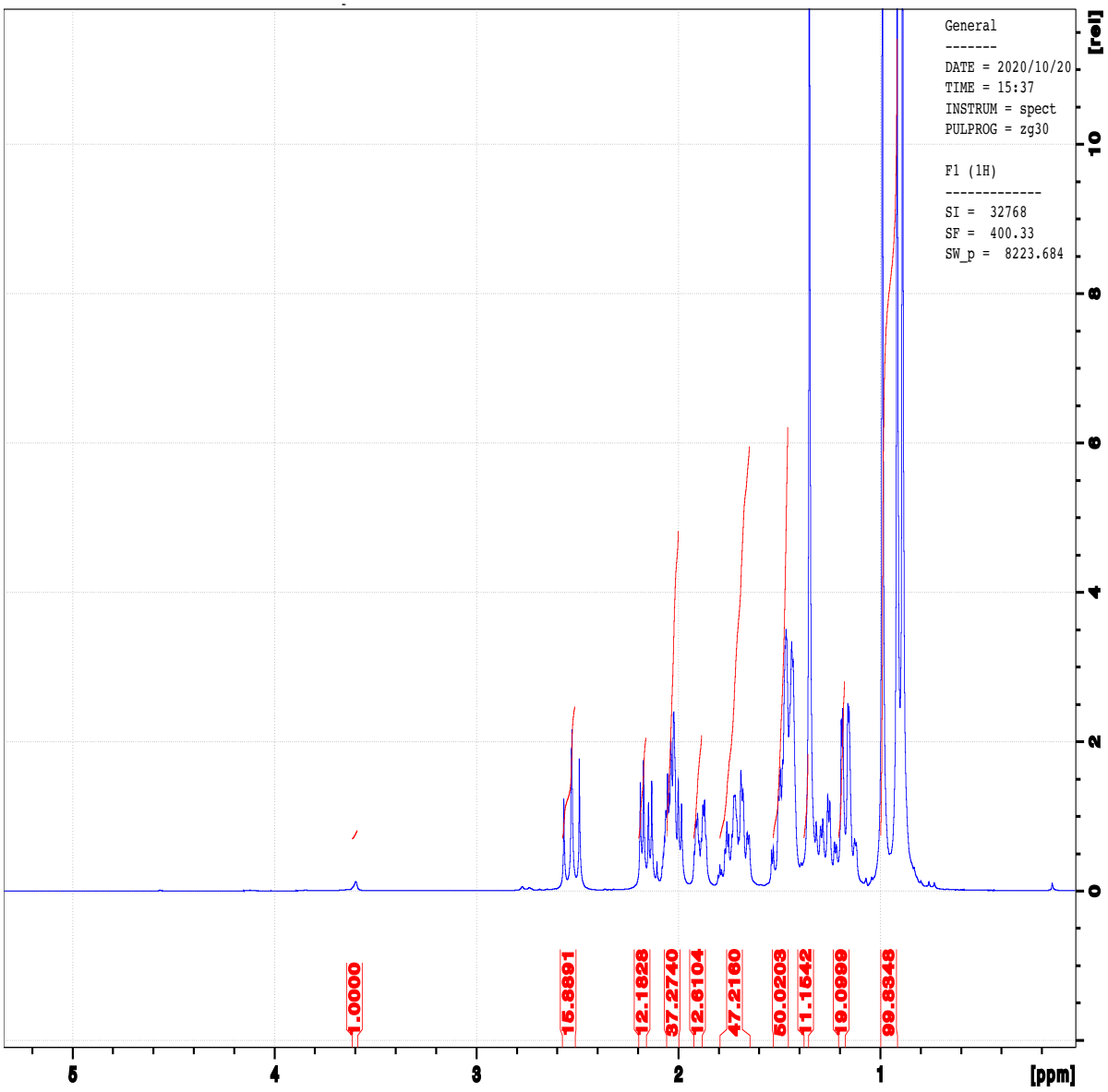


A-7: DEPT-135 spectrum of compound (**39**) in acetone-d_6

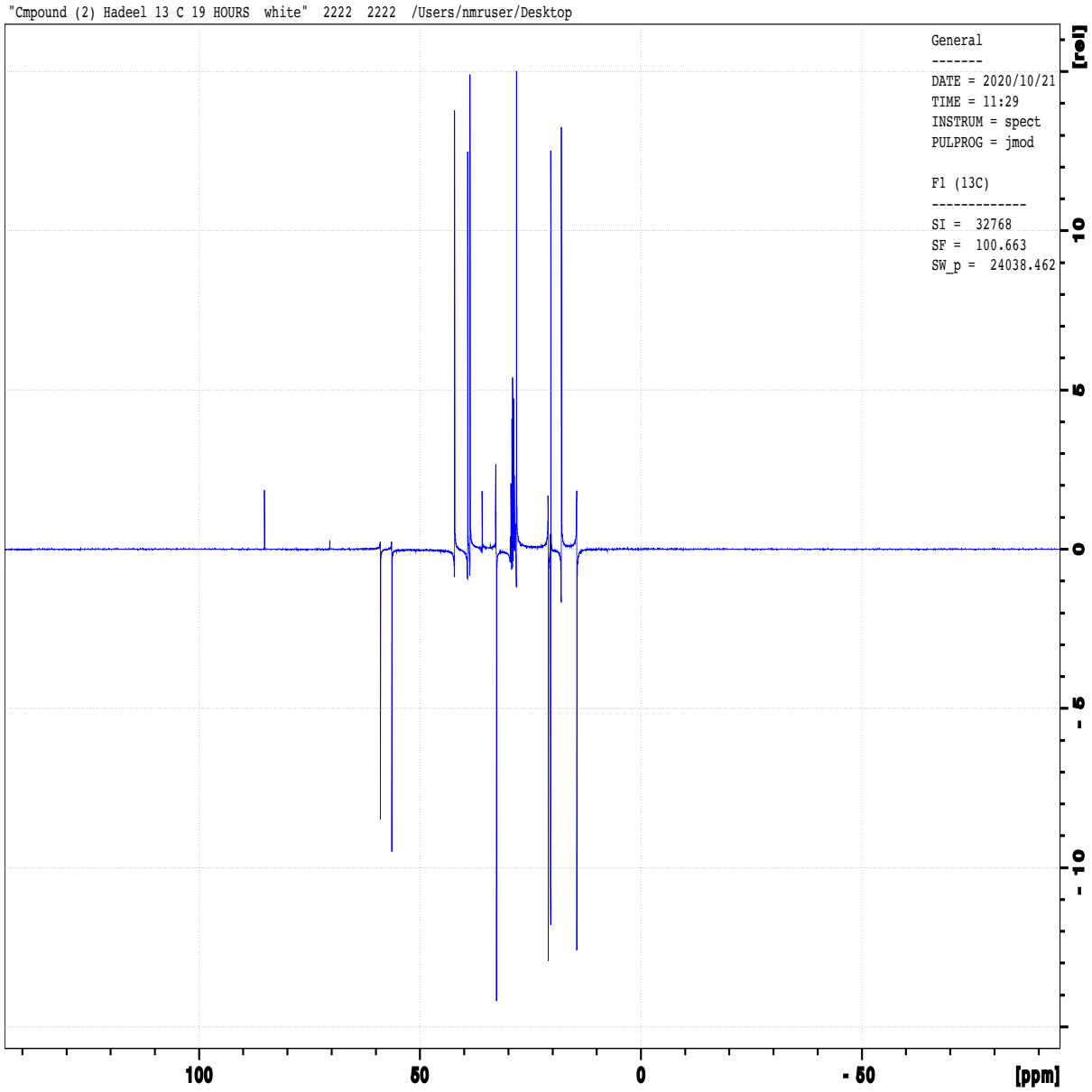
Sclareolide (**40**)



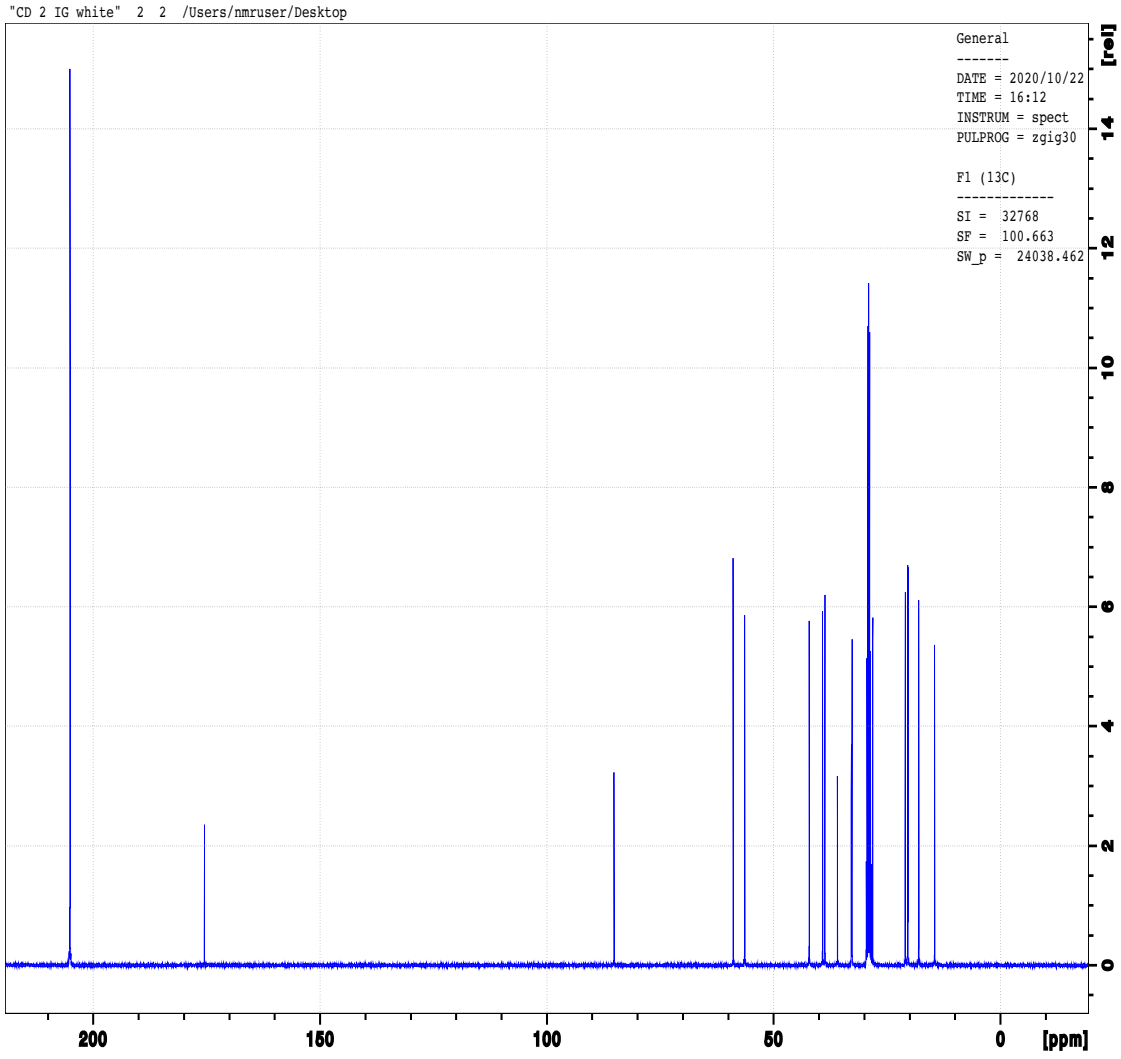
(**40**)



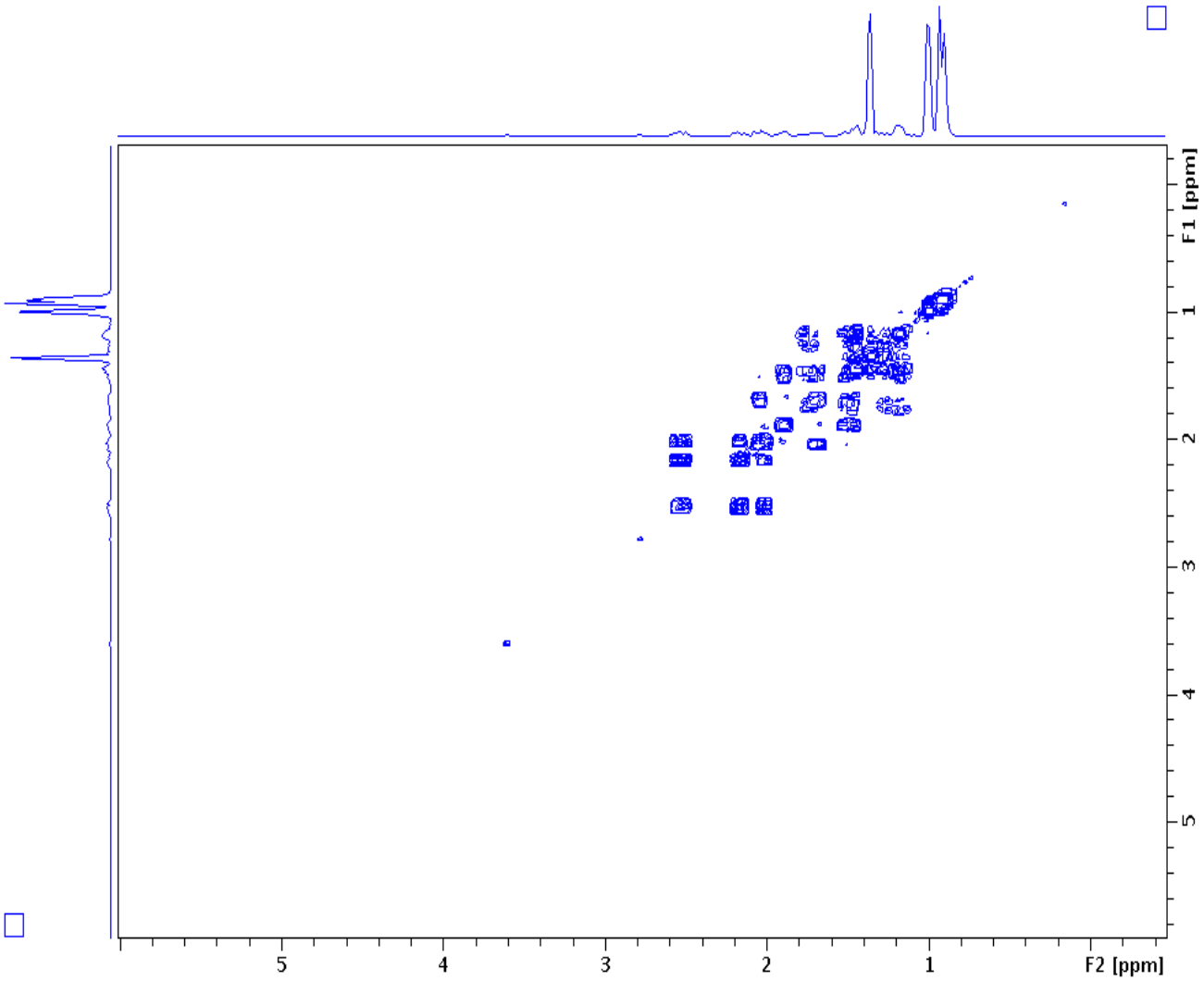
A-8: ^{1}H-NMR spectrum of compound (**40**) in acetone-d_6



A-9: $^{1\,3}$C-APT spectrum of compound (**40**) in acetone-d_6

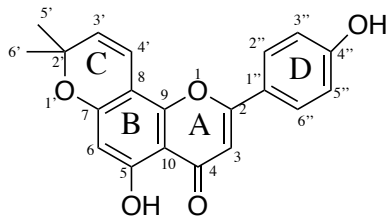


A-10: IG spectrum of compound (**40**) in acetone-d_6

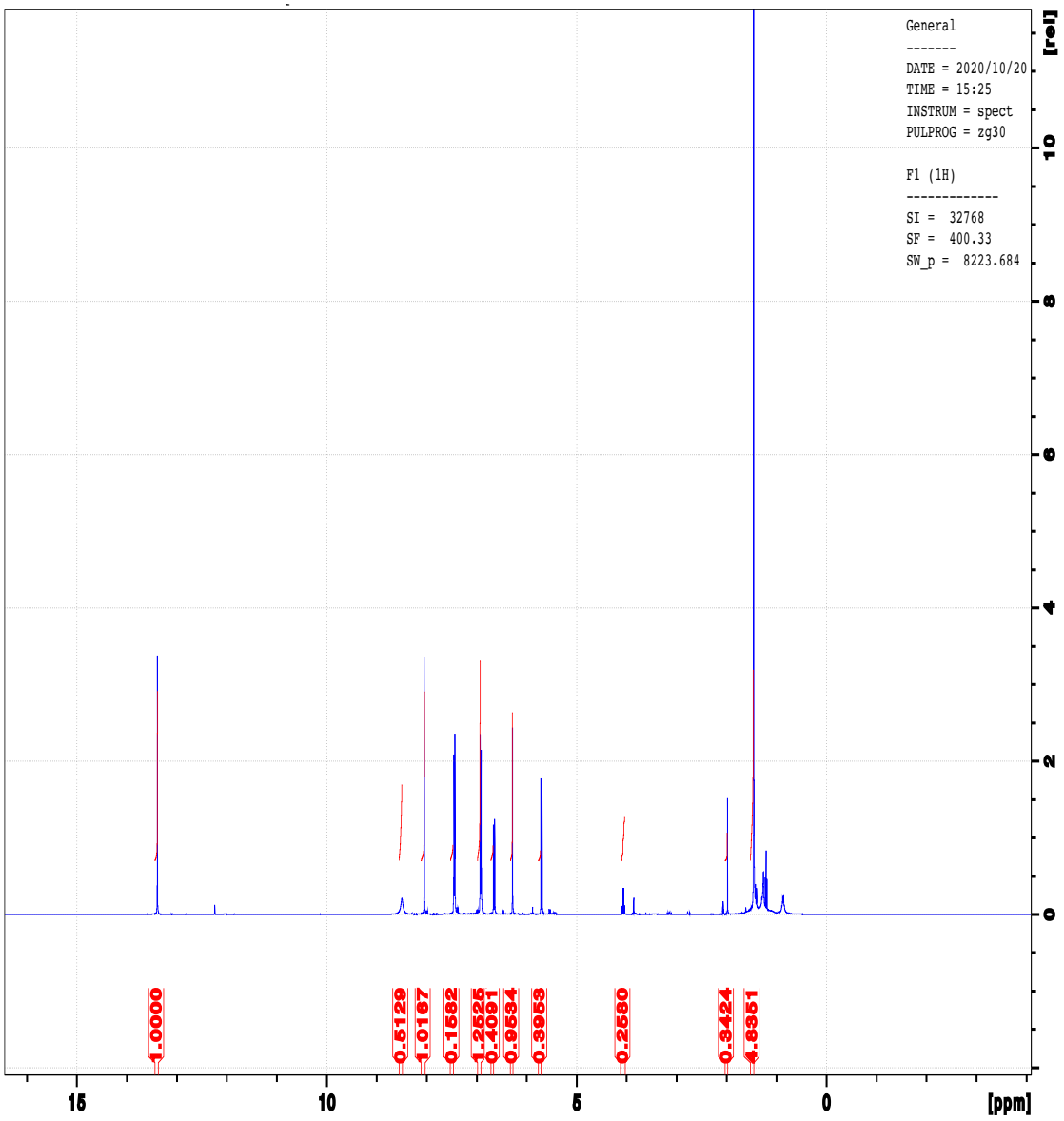


A-11: COSY spectrum of compound (**40**) in acetone-d_6

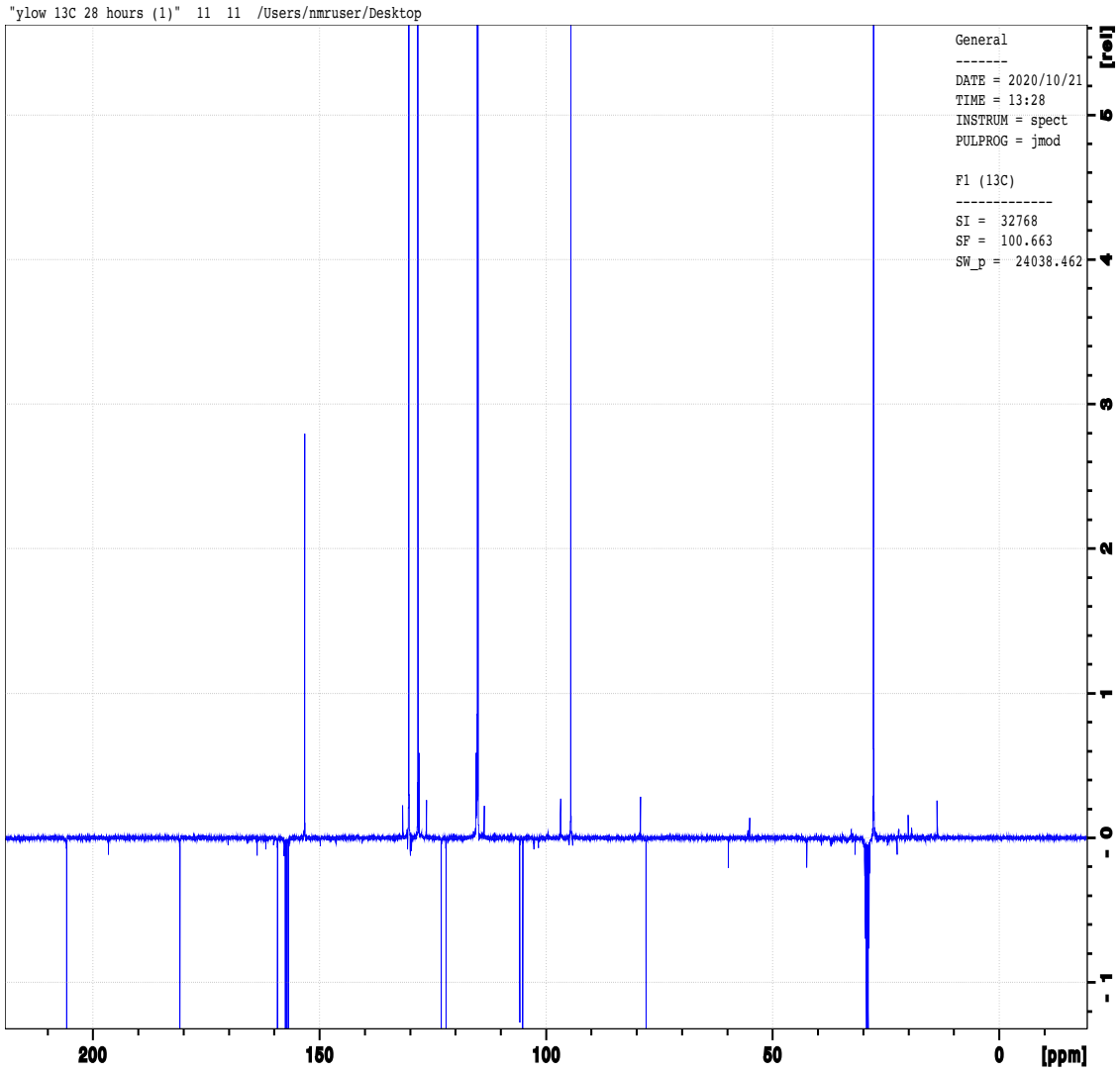
Atalantoflavone (**41**)



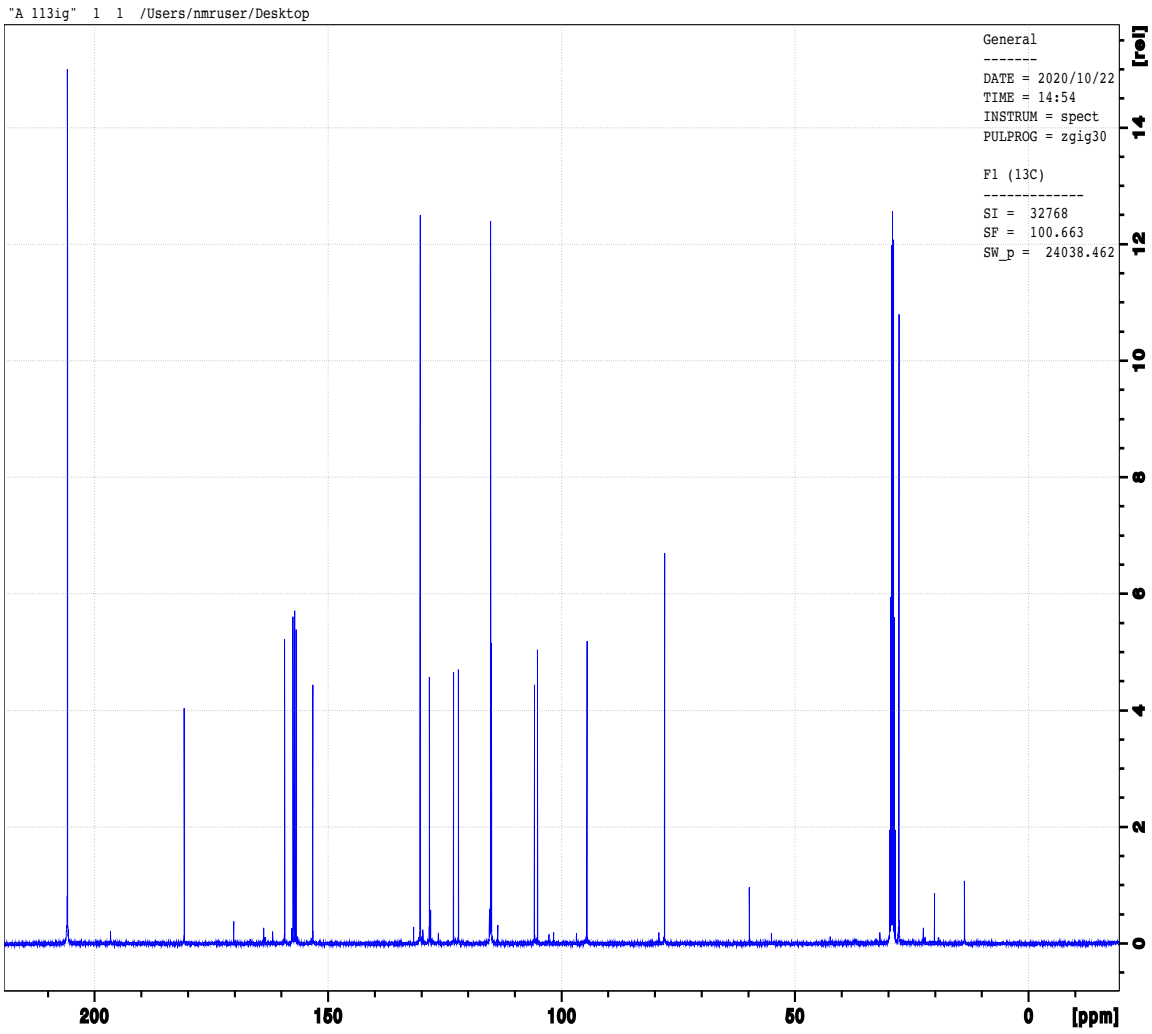
(41)



A-12: ^{1}H-NMR spectrum of compound (**41**) in acetone-d_6



A-13: ^{13}C-APT spectrum of compound (**41**) in acetone-d_6



A-14: IG spectrum of compound (**41**) in acetone-d_6