ISOLATION, CHARACTERISATION AND *IN VITRO* BIOLOGICAL ACTIVITY OF BIOACTIVE PRINCIPLES IN *Hermannia geniculata* Eckl. & Zeyh. LEAF EXTRACTS

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my original work and that I have not previously in its entirety or in part submitted at any university for a degree. I furthermore cede the copyright of the thesis in favour of the University of the Free State.

Signature:…….. …….

Date …….

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DEDICATION

This thesis is dedicated to my late father, Tumo Solomon Mojau, my mother, Tshokolo Gloria Mojau, My wife, Matshidiso and my children Refilwe, Junior and Quinton for their unconditional love.

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LIST OF ABBREVIATIONS

^{13}C	Carbon 13
^{1}H	Proton
%	Percentage
µg/l	Microgram per litre
ABTS	2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid
α	Alpha
°C	Degrees Celsius
CD_3OD	Deuterated methanol
$CHCl_3$	Chloroform
cm	Centimetre
COSY	Correlation spectroscopy
DMSO	Dimethyl sulphoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
ESI^+	Electrospray positive mode
FTIR	Fourier Transform Infrared Spectroscopy
g	Grams
H	*Hermannia*
h	Hour
HSQC	Heteronuclear Single Quantum Coherence
kg	Kilograms

L	Litre
MIC	Minimum inhibitory concentration
MFC	Minimum fungicidal concentration
MHz	Mega hertz
MS	Mass spectroscopy
mL	Millilitre
min	Minute (s)
m	Meter
mg	Milligram
m/z	Mass to charge ratio
NMR	Nuclear Magnetic Resonance
p-TLC	Preparative- Thin Layer Chromatography
PPARy	Peroxisome proliferator-activated receptors
TLC	Thin Layer Chromatography
rpm	Revolutions per minute
UV	Ultraviolet
WHO	World Health Organisation

ABSTRACT

Hermannia geniculata has been used widely as traditional medicine for treatment against infectious human pathogens. The aim of the present study was to determine the phytochemical constituents, antioxidant, antidiabetic and antimycotic activities of *Hermannia geniculata* leaf extracts; and to isolate and test the activity of bioactive compounds from the extract with better activity. The antidiabetic potential of the acetone, hexane, ethanol and ethyl acetate leaf extracts of *H. geniculata* was investigated against activities of α-amylase, and α-glucosidase enzymes; while the antioxidant activity of the extracts was determined using metal chelation, 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical scavenging and 2,2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) assays.

Fresh leaves of *Hermannia geniculata* were collected from vegetation along Wetsi café at Monontsha village, Qwaqwa, Eastern Free State Province, South Africa. The roots were thereafter authenticated and a Voucher Specimen (Mojamed/1/2016/Qhb) was prepared and deposited at the Herbarium of Plant Sciences Department, University of the Free State, Qwaqwa Campus, South Africa.

The fresh leaves were cut into smaller pieces and washed under running water to remove all debris, afterwards dried in an Ecotherm oven at 40°C. Dried plant

materials were then powdered using Waring laboratory blender (Labcon, Durban, South Africa).

Powdered plant materials (150 g each) were extracted separately in ethanol (1500 ml), ethyl acetate (1500 ml), acetone (1500 ml), hexane (1500 ml), the plant in different solvents were put on a Labcon platform shaker for 24 h at the speed of 100 rpm Extracts were filtered using Whatman no. 1 filter paper and each filtrate was concentrated to dryness under reduced pressure at 40°C using rotary evaporator (Cole-Parmer) as depicted in. Finally, extracts were dried to yield ethanol extract (33 g), acetone (10 g), ethyl acetate (44.2 g), hexane (4.5 g). Each extract was re-suspended in its respective solvent to make a 50 mg/ml stock solution.

Phytochemical constituents of *Hermannia geniculata* leaf was determined in the all the extracts adopting standard methods.

Antioxidant activity of *Hermannia geniculata* extracts as per ABTS•+ decolorization assay was measured. The α-glucosidase and α-amylase inhibitory activities were assayed.

For the fractions and the isolated compound, only α-amylase and α- glucosidase assays were used. Fractionation of the ethyl extract was done by thin layer chromatography (TLC) profiles, and further purification of semi-pure compounds was achieved using preparative thin layer chromatography (pTLC) to obtain pure compounds. Isolated compound was characterised using nuclear magnetic

resonance (NMR), mass spectroscopy (MS) and Fourier Transform Infrared (FTIR).

Phytochemical analysis of the extracts revealed the presence of alkaloids, saponins, flavonoids phenols and triterpenes for all the extracts. The isolated compound exhibited significant α-amylase activity with the lowest IC_{50} value at 0.172 compared to other extracts and acarbose (standard) with 2.344 mg/mL. α-glucosidase activity of the isolated compound gave a lower IC_{50} of 4.760 compared to acarbose at 10.450 value. For DPPH activity, the isolated compound had lower value of IC_{50} of 0.474 compared to silymarin (standard) at 16.647. For hydroxyl radical, the isolated compound was more active than the ascorbic acid (standard). However, for metal chelating assay, the compound showed a significantly lower IC_{50} value of 5.242 compared to silymarin at 2.734. For antimycotic activity, the isolated compound showed activity against *candida albicans* with MIC values at high concentrations of 6.5 mg/ml which was higher than that of a positive control (Fluconazole). The ethyl acetate extract and the isolated 1,3-dibutyl1-2,8-dihydroxy-9H-xanthen-9-one exhibited best inhibitory activity on the assays studied. Overall the presence of phytochemicals in the leaves of *H. geniculata* may be suggested to have contributed greatly to the biological activities of the plant.

CHAPTER 1

INTRODUCTION

Medicinal plants have been found to perform an important role in the health of many people around the world for centuries (Hosseinzadeh *et.al*., 2015). In South Africa, large number of people consult traditional healers for primary health care in addition to orthodox medicine (Leistner, 2000). Medicinal plants continue to play a critical role in the healthcare system of enormous proportions of the world's population (Akerele, 1988). Both developing and industrialised nations have seen an increase in the recognition and development of the medicinal and economic use of these plants (WHO, 1998).

Medicinal plants are used worldwide with a rapidly growing economic significance. In developing countries, traditional medicines are often the only accessible and affordable treatment available. For most rural parts in Africa, 80% of the population make use of traditional medicine as the primary health care system (Fisher and Ward, 1994). Traditional medicine usage is rapidly gaining more patronage by national governments and health care providers. Due to poor communication network, poverty, ignorance and unavailability of modern health facilities, most of the rural people are forced to engage in the practice traditional medicine for their common ailments (Khan, 2002).

The efficacy of higher plants as sources of drugs has not been fully studied. Only a small percentage has been investigated phytochemically and the fraction

subjected to both biological and pharmacological screening is even narrower. Based on history, pharmacological screening of compounds of natural or synthetic origin has been the source of many therapeutic agents (Mahesh and Satish, 2008). Plants and plant based medicaments are the basis of many of the modern pharmaceuticals used today for a variety of ailments. This is evidence as plant kingdom harbours an inexhaustible source of active ingredients that are invaluable in the management of many intractable diseases (Shariff, 2001).

Bioactive compounds are normally accumulated as secondary metabolites in all plant cells but their concentrations differ according to the plant parts, season, climate and particular growth phase. Leaf is one of the highest accumulated plant part of such compounds and people generally prefer it for therapeutic purposes. However, some of the active compounds inhibit the growth of disease causing microbes either singly or in combination (Hassawi and Kharma, 2006). Several medicinal plants have been tested for biological, antimicrobial and hypoglycaemic activities. In addition, others have been tested for antiulcerogenic, anthelminthic, hepatoprotective, analgesic, antipyretic, antileishmania and insecticidal activities (Doughari and Obidah, 2008).

CHAPTER 2

LITERATURE REVIEW

2.1 Use of Traditional Medicine in Southern Africa

Southern Africa has one of the richest plant diversity in the world. Most of these plant species have been implicated in traditional medicine in this region for centuries (Lewu and Afolayan, 2009). Nearly 27 million South Africans depend on traditional medicine for their basic health care needs (Street *et al*., 2009). A number of factors can be attributed to the heavy reliance of such a large portion of the population including accessibility to the plants, affordability and the level of extensive knowledge and expertise amongst the local communities (Grundy and Wynberg, 2001). In the past, the field of medicine was dominated by traditional knowledge and most indigenous healers across ethnic and racial populations of the world are not keen to accurately share their experience with outsiders. As a consequence, there is a great bridge in knowledge between modern medicine and traditional healing. The development of Phytomedicine within the last few decades with specific reference to South Africa has rapidly bridged that gap (Van Wyk and Gericke, 2000)

Approximately 72% of Black population of South Africa (about 26.6 million) use traditional medicine. These are people from a variety of age categories, education levels, religions and occupations. The diverse number of people is an indication that traditional medicine is a common practice across most sectors of

black African population, and that use of traditional medicine is not restricted to the needy, rural and illiterate users (Mander *et al*., 2007). Indigenous plants constitute the pre-dominant source of medicine for traditional healers, with at least 771 plant species recorded in the trade in South Africa. It is projected that 20 000 tonnes of indigenous plants are harvested from grasslands, forests woodlands and 4 thickets in Eastern South Africa every year, with a small portion being cultivated (Mander *et al*., 2007).

One of the main reasons for the increasing use of traditional medicine is the growing trend for patients to take a more proactive approach to their own health and to seek out different forms of self-care. In the process, many consumers have turned to natural traditional medicinal products and practices, under the assumption that "natural means safe". However, this is not necessarily the case. A number of reports have revealed examples of incorrect use of traditional medicines by consumers, including incidents of overdose, unrecognised use of suspected or counterfeit herbal medicines, and unintentional injuries caused by unqualified practitioners (WHO, 1998).

Another factor that validates the relevance of herbal medicine is that herbs remain the foundation for a large amount of commercial medications used today for treatment of heart disease, blood pressure regulation, pain remedies, asthma and other health problems (Okigbo and Mmeka, 2006; Calixto, 2000). For instance, Artemisinin which is extracted from the Chinese herbal wormwood

plant *Artemisia annua* is the basis for more effective antimalarial drugs (Okigbo and Mmeka, 2006). Herbal medicines are also being used increasingly as dietary supplements to fight or prevent common maladies like cancer, heart disease and depression. The public and herbal medicine community is extolling the miraculous medical benefits of the Ginkgo biloba, St. John‟s wort, Moringa, sunflower seed, black cohosh and many other herbs (Okigbo and Mmeka, 2006; Cohen *et al*., 2000).

2.2 Phytochemical attributes of medicinal plants

Medicinal plants have less toxicity with minute side effects and are believed to be safer than many synthetic drugs. Plants have been reported to produce vast and distinct organic compounds. These naturally occurring chemical compounds have many health benefits to humans in treating and preventing diseases. The non-nutrient plant chemical compounds or the bioactive components are normally referred to as phytochemicals (*Phyto* from Greek meaning plant) (Saxena *et al*., 2013). Phytochemicals protect plants from disease and damage either caused by other plants, herbivores or insects or contributes to the plant fragrance, flavour and colour (Saxena *et al*., 2013).

Phytochemicals can be classified into two groups according to their functions and properties, namely, primary and secondary metabolites. Primary metabolites play an important role in metabolism and reproduction of cells and are vital for the survival of the plant. These metabolites include carbohydrates, lipids,

proteins, nucleotides, fatty acids and steroids (Croteau *et al*., 2000; Hanson, 2003). Secondary metabolites are unevenly distributed and their main functions among other things involve defence against herbivorous animals, chemical communication, and assistance during interactions and reproduction (Salisbury and Ross, 1992).

2.3 Role and classification of secondary metabolites

Secondary metabolites are organic compounds that do not play any role in the normal growth and development of plants. The production of secondary metabolites normally emanates from the maximum level during the transition from active growth to stationary phase. The organism that produces this secondary metabolite normally grows without their production, thus, the secondary metabolism in the organism may not be significant, yet, is pivotal for short survival of the organism (Agostini-costa *et al*., 2012). There are numerous protective roles that these secondary metabolites provide. For example, they act as free radical scavenging, antioxidants, UV-light absorbing, and anti-proliferation agents. They also protect plants and defend them from harmful pathogens, microorganisms such as bacteria, fungi and viruses (Kennedy and Wightman, 2011). This was supported by the fact that some plants manufacture these chemicals as part of their defence system. For instance, phytoalexins are produced by plants when they are attacked by bacteria and fungi, hence, their antibacterial and antifungal properties (Gurib-Fakim, 2006).

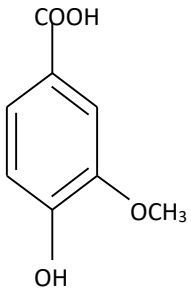
There are various classes of secondary metabolites present in plants; they include phenolics, flavonoids, steroids, terpenoids and alkaloids. The different categories in which the secondary metabolites belong to share a distinct structure, their derivatives are made up of structural units or some composed of complex molecules that are compiled by large numbers of simple molecules (Hopkins and Hüner, 2009). The following sections reflect a brief account on some of the classes known.

2.3.1 Phenolics

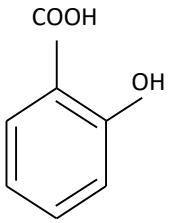
Phenolics are known to be the largest category of phytochemicals and are cosmopolitan across the plant kingdom with about 10 000 structures being identified (Agostini-costa *et al*., 2012). Some of the phenolics are soluble in organic solvents and water which are known as carboxylic acid and glycosides; while others are large, insoluble polymers (Castillo *et al*., 2012). Phenolics structures differ greatly from simple low molecular weight compounds such as the simple phenols, phenylpropanoids, coumarins, catechols and benzoic derivatives (Fig 2.1) to the most complex higher molecular weight structures such as catechol, melanins, lignins, tannins, flavonoids, stilbenes and vitamins (Kennedy and Wightman, 2011; Agostini-costa *et al*., 2012). The phenolic phytochemicals play a significant role as defence compounds; manifest several properties that are beneficial to humans. Their antioxidant properties are one of

the most important properties that determine their roles as protecting agents against free radical mediated disease processes (Saxena *et al*., 2013).

Fig. 2.1 Examples of important naturally occurring phenolics, (a) phenolic acid and (b) caffeic acid chemical structures (PubChem, 2016)



(a)



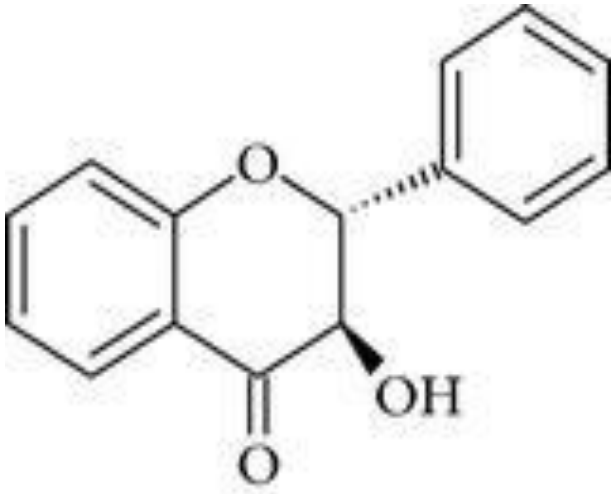
(b)

2.3.2 Flavonoids

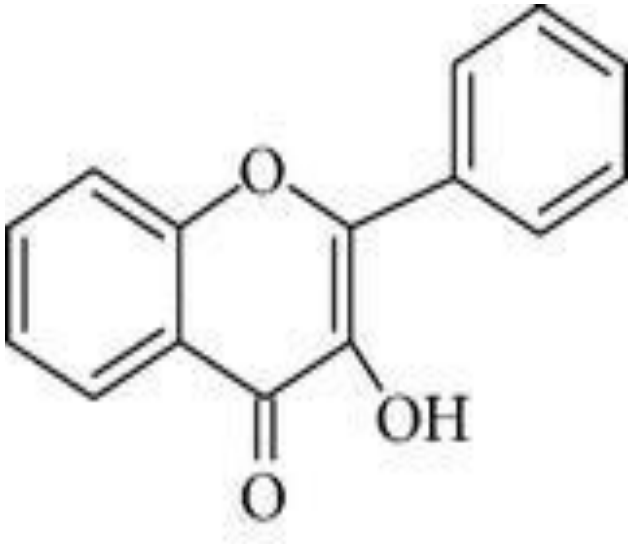
Flavonoids are polyphenolic compounds that are present among vascular plants. The adequate availability of this group has led to more than 4 000 which have been described so far within the part of the plants normally consumed by humans and approximately 650 flavones and 1030 flavonols are known (Harbone and Baxter, 1999). They occur as aglycones, glycosides and methylated derivatives

(Veitch and Grayer, 2008). The six-membered ring condensed with the benzene ring is either -pyrone (flavones and flavonols) or its dihydroderivative (flavonones and flavan-3-ols) (Fig. 2.2) (Saxena *et al*., 2013). Flavonoids are involved in the formation of colour of flowers, fruits and sometimes the leaves. They also play a significant role in protecting plants against damaging effects of UV light damaging effects as well as pollination and seed dispersal by attracting pollinators. This class of secondary metabolite has been subject of considerable scientific and therapeutic interest since they play important role in physiological and dietary antioxidants, thereby increasing the body's natural resistance to oxidative damage (Shahidi, 2000).

Fig. 2.2 Examples of classes of flavonoids.



(a) Flavanonol



(b) Flavonol

2.3.3 Terpenoids

There are more than 36 000 terpenoids compounds that have been identified, making terpenoids one of the largest groups of plant metabolites. They are a class of natural products which have been derived from five- carbon isoprene units (Bruneton, 1999). Terpenoids that are composed of one unit are classified as a hemiterpene; those incorporating two isoprene units are monoterpenes; sesquiterpenes incorporate three, diterpenes comprise four, sesterpenes include five, triterpene incorporate six and tetraperpenes have eight units (Gurib-Fakim, 2006). The term terpene usually refers to a hydrocarbon molecule while terpenoid refer to a terpene that has been modified such as addition of oxygen. Therefore, the isoprenes are the building structures of other metabolites such as plant hormones, carotenoids, sterols, rubber, the phytol tail of chlorophyll, and turpentine (Zwenger and Basu, 2008). Most of the terpenoids have cyclic structures. The cyclizations of most of the terpenoid take place in the living systems and they are of an acid catalysed type (Hanson, 2003).

According to Chang *et al*. (2011), more than 80 different triterpenoid structures have been isolated and identified from plants. The triterpenoids are important since they are used as preventive medicines and also a good source of food. Terpernoid compounds that have been isolated from plants and are available for pharmaceutical application include artemisinin for malaria and taxol for anti-

cancer (Goto *et al*., 2010). Both are useful in prevention of diseases and plays important role in chemotherapy. Terpenoids have been found to be useful in cancer treatment, have antimicrobial properties, antifungal, antiviral, antiparasitic, antipasmodic, antiallergic, antiinflammatory, antihyperglycemic, and immunomodulatory properties (Wagner and Elmadfa, 2003; Shah *et al*., 2009). They are found in vegetables, fruit, and that dietary terpenoids may contribute to a decrease in risk of metabolic syndrome. Figure 2.2 depict and example of terpernoid, limonene.

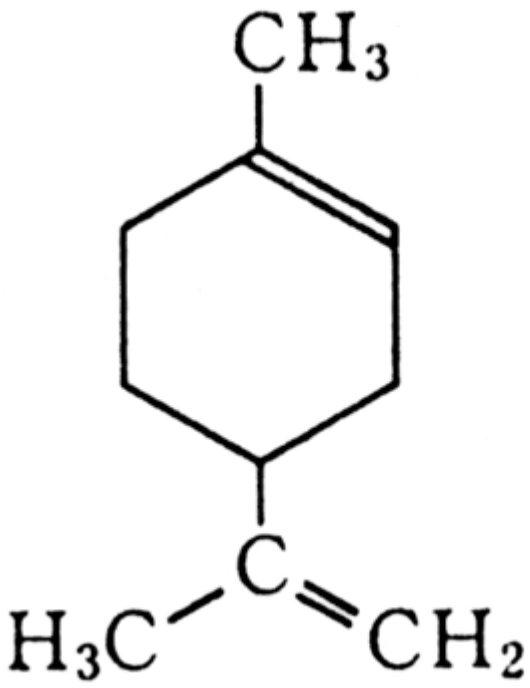


Figure 2.3 Limonene (Yadav *et al.*, 2014)

2.3.4 Alkaloids

Alkaloids are structurally diverse group of over 12 000 cyclic nitrogen-containing compounds that are available in more than 20% of plant species (Aniszewski, 2007). They have bitter taste and appear as white; they form water soluble-salt and most of them have well defined crystalline substances which reacts with acid to form salts (Wisniak, 2013). Nicotine is the only one that has brown liquid (Aniszewski, 2007). They are known as true alkaloids and are highly reactive. Even at low quantities, they still possess biological activity. As stated by Woolley (2001), alkaloids have many pharmacological activities and they often provide lead in the search for new synthetic drugs such as oral hyperglycaemic agents, antibacterial (berberine), antimalarial (quinine, alstonine) antihypertensive (cevadine, veratrine, reserpine, serpentine, rubijervine) and anticancer actions (camptothecine, demecolcine (desacetylmethylcolchicine), ellipticine, indicine N-oxide, maytansine, pacletaxel, vincristine and vinblastine).

2.3.5 Saponins

Saponins are well known for their potential to produce a soapy lather when shaken with water and they also have the ability of precipitating cholesterol by forming insoluble complexes. They are classified chemically into two groups, steroidal saponins and triterpenoid saponins based on the chemical structures of

their aglycones or saponegins (Shibata, 1977). Steroidal saponins are widely distributed in nature and exhibit various biological activities. They are found to possess properties such as haemolytic activity, toxicity to fish and form complex formation with cholesterol. They have been found to possess antidiabetic, antitumor and antitussive properties (Mimaki and Sashida, 1996). Triterpenoid saponins are naturally occurring surface active glycosides of triterpenes. They can be categorized into two major groups, monodesmosides, in which the aglycone has a singly attached linear or branched chain set of sugars and bisdesmosides in which there are two sets of sugars.

Saponins are one of the great molecules that are structurally broad. They occur as complex mixtures and have novel bioactivities of significance to the pharmaceutical industry and agriculture. Their relevance as ingredients of cosmetics, allelochemicals, food and feeding stuff has generated great interest in the study of these molecules (Guclu-ustundag and Mazza, 2007). Saponins possess a variety of biological activities such as antioxidant, immunostimulant, antihepatotoxic, anticarcinogenic, antidiarrheal, antiulcerogenic, antioxytoxic, hypocholesterolemic, anticoagulant, anti-insect, hepatoprotective, hypoglycemic, neuroprotective, antiinflammatory, haemolytic and inhibition of dental caries and platelet aggregation (Guclu-ustundag and Mazza, 2007; Barbosa, 2014).

2.4 Role of extracts in the medicinal properties of Bioactive compounds

The qualitative and quantitative studies of bioactive compounds from plant materials are mostly reliant on the choice of proper method of extraction (Smith, 2003; Sasidharan *et al*., 2011). Extraction is the preliminary step for any medicinal plant study as they play a crucial role on the final result and outcome of compounds obtained. Extraction methods are sometimes referred to as 'sample preparation techniques'. At times, this part of study is neglected and done by non-trained research personnel (Hennion *et al*., 1998), though two-third of effort of an analytical chemist account for sample preparation techniques. A study conducted by Majors (1999), showed that most of researchers believe in the importance of sample preparation during any analytical study. It is true that development of modern chromatographic and spectrometric techniques makes bioactive compound analysis easier than before but, the success still depends on the extraction methods, input parameters and exact nature of plant parts (Poole *et al*., 1990). The most common factors affecting extraction processes are matrix properties of the plant part, solvent, temperature, pressure and time (Hernandez *et al*., 2009). The increased understanding about dynamic chemical nature of the diverse bioactive molecules is the pioneer fuel for the progress of bioactive analysis during the past decade (Torssell, 1997). As a result of these huge technological and technical improvements; pharmaceuticals, food additives even

on natural pesticides sectors have become interested in bioactive molecules from natural sources (Anklam *et al*., 1998; Ambrosini *et al*., 1999). Characteristically, bioactive compounds remain together with other compounds present in plants and they can be identified and characterized from various plant parts such as leaves, stem, flower and fruits.

2.5 Importance of natural products to drug discovery

For many decades, synthetic chemicals as drugs have been effective in the treatment of most diseases (Lahlou, 2013). The pharmaceutical industry has synthesized over 3 million new chemicals in their effort to produce new drugs. Despite their success in developing drugs to treat or cure many diseases, the treatment of certain diseases such as cancer, AIDS, heart disease and diabetes has not been a complete success due to the complexity of these diseases. Over the centuries, people have been living in close association with the environment and relying on its flora and fauna as a source of food and medicine. As a result, many societies have their own rich plant pharmacopoeias. In developing countries, due to economic factors, nearly 80% of the population still depends on the use of plant extracts as a source of medicine. Natural products also play an important role in the health care system of developed countries. The isolation of the analgesic morphine from the opium poppy, *Papaver somniferum*, in 1816 led to the development of many highly effective pain relievers. The discovery of penicillin

from the filamentous fungus *Penicillium notatum* by Fleming in 1929 had a great impact on the investigation of nature as a source of new bioactive agents. Natural products can also be used as starting materials for semisynthetic drugs. The main examples are plant steroids, which led to the manufacture of oral contraceptives and other steroidal hormones. Currently, almost every pharmacological class of drugs contains a natural product or natural product analogy. The investigation of higher plants has led the discovery of many new drugs. So far, only a small portion of higher plants has been investigated. Consequently, there is a big reservoir of useful chemical compounds not only as drugs, but as templates for synthetic analogy (*www.americancancersociety.org*).

Plant preparations have a very special attribute that contrasts them from chemical drugs: a single plant may contain numerous bioactive phytocompounds and a combination of plants even more. This complexity is one of the most pivotal challenges to phytoscientists attempting to identify a single bioactive phytocompound or chemical group in the enormous universe that comprises a single crude extract. The field of biotechnology in the 1970s and 1980s made tremendous strides and brought in a new era for the pharmaceutical industry. Many enzymes and receptor proteins of therapeutic importance were made available in large quantities by recombinant expression, while signal transduction

pathways could be interrogated by reporter gene carrying cellular constructs. Such mechanism-based *in vitro* assays are amenable to large scales of operation, and the concept of high-throughput screening rapidly became the paradigm for lead discovery (Ganesan, 2002).

2.6 Mycology

Mycology is the study of fungi; their genetic and biochemical properties, as well as their taxonomy. Pathogenic fungi have the ability to actively attack and invade tissues (Hawksworth, 1974); Bauman, 2007). The study also focuses on the impact of fungi on human health in some way. Surprisingly, the causative relationship of fungi to human health was known before the pioneering work of Pasteur and Koch with pathogenic bacteria. Fungi are omnipresent in the environment, and infections due to fungal pathogens have become more frequent (Walsh and Groll, 1999; Fleming *et al*., 2002). During the early years, mycology was really the study of dermatophytes (tinea and ringworm fungi), with Raimond Sabouraud (1864-1938) being the most well-known name in the field. Sabouraud's agar to date remains the most famous name in the formulation for growing fungi.

It has been estimated that there are between 250,000 and 1.5 million species of fungi on this planet, and about 70,000 of these species have been described.

Fortunately, only about 300 of these species cause human infections, and of these about 30 species are seen regularly (Davis,1994).

The search for novel antifungal agents relies mainly on ethnobotanical information and ethnopharmacologic exploration. The medicinal knowledge of North American First Nations peoples has been shown to be a valid resource. Studies have revealed a high degree of correlation between traditional medicinal uses and laboratory analysis (McCutcheon *et al*., 1994; Bergeron et al., 1996; Jones *et al*., 2000).

Fungal diseases can also be classified broadly on the basis of causative agents; these diseases differ in nature, causative agents, and distribution (Khan *et al*., 2010).

2.6.1 Antibiotics

After Bayarski: an antibiotic is a drug that kills or inhibits the growth of bacteria. It is a one class of antimicrobials, a larger group that includes anti-viral, anti-fungal, and anti-parasitic drugs. They are chemicals produced by or derived from microorganisms (i.e. bacteria and fungi). The first antibiotic was discovered by Alexander Fleming in 1928.

Antibiotics are among the most frequently prescribed medications in modern medicine. Some antibiotics are “bactericidal”, meaning their role is to kill

bacteria. Other antibiotics are “bacteriostatic”, meaning their role is to stop bacteria from multiplying. Some antibiotics can be used to treat a wide range of infections and are known as “broad-spectrum” antibiotics (*http://EzineArticles.com/?expert=Yury_Bayarski*).

2.7 Choice of solvents

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions include, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, multiplicity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process and potential health hazard of the extractants (Eloff, 1998). The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted (Ncube *et. al*, 2008 and Das *et al*., 2010).

2.8 Antimicrobial Bioactive Phytocompounds from Extraction to Identification: Process Standardization

A variety of approaches to drug discovery using higher plants can be distinguished: random selection followed by chemical screening; random selection followed by one or more biological assays; biological activity reports and ethnomedical use of plants (Eloff, 1998). The latter approach includes plants used in traditional medical systems; herbalism, folklore, and the use of databases. The objective is the targeted isolation of new bioactive phytocompounds. When an active extract has been identified, the first task to be taken is the identification of the bioactive phytocompounds, and this can mean either a full identification of a bioactive phytocompound after purification or partial identification to the level of a family of known compounds (Miles *et al*., 1998).

2.8.1 Extraction

Extraction is the crucial first step in the analysis of medicinal plants, because it is significant to extract the desired chemical components from the plant materials for further separation and identification. The basic operation included steps, such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system (Sasidharan

et al., 2011). Proper actions must be taken to ensure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples. If the plant was selected on the basis of traditional uses (Fabricant and Farnsworth, 2001), then, it is needed to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug. The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate while the extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some instances, extraction with hexane is used to remove chlorophyll (Cos *et al*., 2006).

As the target compounds may be non-polar to polar and thermally labile, the suitability of the methods of extraction must be considered. Various methods, such as sonification, heating under reflux, soxhlet extraction and others are commonly used (United States Pharmacopeia and National Formulary, 2002; Pharmacopoeia of the People's Republic of China, 2000; The Japanese Pharmacopeia, 2001) for the plant samples extraction. In addition, plant extracts are also prepared by maceration or percolation of fresh green plants or dried

powdered plant material in water and/or organic solvent systems. A brief summary of the experimental conditions for the various methods of extraction is presented in Table 2.1

The other modern extraction techniques include solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction, and surfactant-mediated techniques, which possess certain advantages. These are the reduction in organic solvent consumption and in sample degradation, elimination of additional sample clean-up and concentration steps before chromatographic analysis, improvement in extraction efficiency, selectivity, and kinetics of extraction. The ease of automation for these techniques also favours their usage for the extraction of plants materials (Huie, 2002).

Table 2.1: Summary of the experimental conditions for various methods of extraction for plants material.

	Soxhlet extraction	**Sonification**	**Maceration**
Common Solvents used	Methanol, ethanol,	Methanol, ethanol,	Methanol, ethanol,
Temperature	Depending on solvent used	Can be heated	Room temperature
Pressure applied	Not applicable	Not applicable	Not applicable
Time required	3–18 hr	1 hr	3-4 days
Volume of solvent required (ml)	150–200	50–100	Depending on the sample size
Reference	Zygmunt and Namiesnik, 2003; Huie, 2002	Zygmunt and Namiesnik, 2003; Huie, 2002	Phrompittayarat *et al*.,2007; Sasidharan *et al*.,2008; Cunha *et al*., 2004; Woisky *et al*., 1998

2.8.2 Identification and characterization

Due to the fact that plant extracts usually occur as a combination of various type of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC, should be used to obtain pure compounds. The pure compounds are then used for the determination of structure and biological activity. Besides that, non-chromatographic techniques such as immunoassay, which use monoclonal antibodies (MAbs), phytochemical screening assay, Fourier-transform infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds.

2.8.3 Role of bio-assays as antidiabetic, antioxidant and antimicrobial agents

Bioassays are required to select crude materials and isolate potential new drug agents from natural sources. The assay must be reliable, reproducible, sensitive and predictive (Dey and Harbone, 1991).

To determine the true efficacy of potential drug agents, it is important to evaluate their potency in more advanced testing systems followed by preclinical trials. *In*

vitro bioassays in these disease can take the form of antidiabetic and antioxidant activities.

2.8.3.1 Anti-diabetic activity

Diabetes is a defect in the body's ability to convert glucose (sugar) to energy. Carbohydrates, when digested, change to glucose. In order for glucose to be transferred from the blood into the cells, the hormone - insulin is needed which is produced by the beta cells in the pancreas In individuals with diabetes, this process is impaired. Diabetes develops when the pancreas fails to produce sufficient quantities of insulin (Type 1 diabetes) or the insulin produced is defective and cannot move glucose into the cells. In Type 2 diabetes, either insulin is not produced in sufficient quantities or the insulin produced is defective and cannot move the glucose into the cells. *In vitro* screening of anti-diabetic drug is carried out by estimating the levels of Peroxisome proliferator-activated receptor gamma (PPARγ) and the two principle enzymes which involved in the carbohydrate digestion and glucose absorption process. Various methods are reported for antidiabetic activity (Duff, 1965; Conforti *et al.,* 2005). In this study, inhibition of α-Amylase and α-glucosidase assay methods was adopted.

2.8.3.2 Anti-oxidant activity

Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “free radicals.” Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction. Overall, free radicals have been implicated in the pathogenesis of at least 50 diseases (Duh, 1998). Fortunately; free radical formation is controlled naturally by various beneficial compounds known as antioxidants. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation process, so that they can act as primary and secondary antioxidants. In reducing power assay, the antioxidant compounds convert the oxidation form of iron from ferric chloride (Fe^{+3}) to ferrous (Fe^{+2}). The reducing power increased with increasing amount of the extract (Babu *et* a.,l 2001).

DPPH assay is based on the reduction of DPPH in presence of methanol due to the hydrogen–donating antioxidant leading to the formation of the non-radical form of DPPH. This transformation results in a colour change from purple to yellow, which is measured spectrophotometrically. DPPH radicals react with suitable reducing agent, the electrons become paired off and the solution loses

colour stoichiometrically depending on the number of electrons taken up. Decrease in the absorbance of the DPPH solution is an indication of an increase in the DPPH radical-scavenging activity. The result of the different tests carried on the plant materials are compared with standard result of ascorbic acid (Robak and Gryglewski, 1998; Olaive and Rocha, 2007).

2.9 Chromatographic techniques

For the separation of compounds within the extract, chromatographic techniques are employed. Chromatographic techniques have been instrumental in the separation of natural products. Chromatography is a process whereby a mixture of solutes may be resolved into components by exploiting differences in affinity of the solutes for particles of an insoluble matrix over which a solution of the components is passing. The insoluble matrix is called the stationary phase, while the solution which passes through it is called the mobile phase (Wei *et. al*., 2008). There are different types of chromatographic techniques which can be utilised to separate compounds, in the present study, three chromatographic techniques will be discussed

2.9.1 Thin layer chromatography

Thin layer chromatography (TLC) is one of the fastest and most widely used chromatographic techniques in the separation of natural products. TLC is mostly used for phytochemical analysis of plant extracts and to check purity of isolated compounds. TLC method employs glass or aluminium plates pre-coated with the sorbent (e.g., silica gel) to varying thickness depending on the amount of the sample to be loaded. The compound mixture is loaded on plates at around 1-2 cm from the bottom of the plate and lowered in a tank containing the solvent. The latter migrates up the plates and separates the compound mixture according to the polarity of the components. Several reagents are available for visualization of the separated materials. TLC has the advantage of being a highly cost-effective qualitative technique since a large number of samples can be analysed simultaneously.

2.9.2 Column chromatography

Column chromatography (CC) is a popular technique which is used for fractionation and isolation of bioactive natural compounds. This technique is usually employed after solvent/solvent partition. To fractionate or isolate bioactive compounds, the stationary phase normally used is silica gel with the mobile being the solvent(s) of choice. There are eluting techniques which can be

used which are either isocratic elution and or gradient elution. Isocratic elution employs only one mobile phase while gradient elution employs a sequence of mobile phases usually in order of polarity. For example, increasing polarity for normal phase chromatography and decreasing polarity for reverse phase chromatography. Gradient elution is normally employed when isolating and/or fractionating natural bioactive compounds from crude samples. After elution, fractions collected are analysed using chemical tests, TLC, bioassays etc. to identify fractions of interest, similar fractions are grouped together for future work (Gurib-Fakim, 2006).

2.9.3 Nuclear Magnetic Resonance (NMR)

In the process of structure elucidation, obtaining of 1H- NMR and 13C- NMR is crucial. The sample in the NMR spectrometer is exposed to radiofrequency radiation in the presence of a strong external magnetic field. In 1H- NMR, the spectrometer measures the energy levels of the nucleus of hydrogen, which is possible because the radiation in the presence of the magnetic field can change the orientations of protons in the nucleus (Johnson, 1999). The NMR spectra are generated by the magnetic properties of the atomic nuclei of the analysed elements. These magnetic properties are generated by the spinning charge of electrons (Crews and Jaspars, 1998). This gives information about the hydrogens in the molecule. The properties of the proton of hydrogen appear also in 13C. The

13C- NMR spectroscopy is based on the presence of 13C in a mixture with 12C, since only 13C is detected in the analysis. These spectra are often simplified by decoupling the effects of the hydrogens, and reveal the different kinds of carbon atoms in the compound (Johnson, 1999). Other NMR methods used in this work are HSQC (Heteronuclear Single- Quantum Correlation) and HMBC (Heteronuclear Multiple-Bond Correlation). These spectra are often coupled together giving information about the correlation between two different nuclei separated by one bond (HSQC) where each unique proton coupled to a carbon gives a 13 peak, and correlations over longer ranges approximately 2-4 bonds (HMBC). With COSY (Correlation Spectroscopy) one can detect which atoms are connected to each other. NMR is a non- destructive method of analysis.

2.9.4 High resolution Mass spectrometry

High resolution mass spectrometry is used to accurately determine the mass of the molecular ion in structure elucidation to identify or confirm the molecular formula for a compound. The spectrometers have evolved over time to overcome limitations of this technique like peak broadening and interfering ions. This evolution led to the more recent techniques like electrospray ionization (ESI), matrix- assisted laser desorption/ ionization (MALDI) and time- of- flight (TOF). In this work ESI and TOF were used (Russel, 1997).

2.10 The choice of *Hermannia geniculata* for this study

Hermannia geniculata belongs to the Sterculiaceae family and commonly known as ‘Seletjane’ among the Basotho tribe of the Eastern Free State Province of South Africa (Moffett, 2010). There are six to eight species of genus *Hermannia* globally but only *Hermannia geniculata* is often used in the traditional Basotho medicine after *H. depressa* (Kazeem and Ashafa, 2015). In Basotho traditional medicine; the dry root material is chopped, boiled in water and taken three times daily to ameliorate blood sugar disorders. Moreover, it is also used in the management of diarrhoea, heartburn, stomach disorder and flatulency called “leletha” in pregnant Sotho women (Moffett, 2010).

2.11 Southern African distribution of *Hermannia* genus

Southern Africa has nearly 150 species, including some of those found further north in Africa. The greatest diversity is within Cape Province and Namibia, but, there are relatively few species within the Southern coastal areas of Cape Province (Cape Floristic Province). The other South African provinces have between 18 (Gauteng) and 34 (Free State) species (Fig 2.4). There are 8 species in Lesotho, 20 in Botswana and 13 in Swaziland. There are perhaps 20 species in southern tropical Africa, of which 12 occur in Zimbabwe and 3 in Zambia. Mozambique has 6 species, 4 of these are also shared with Zimbabwe. At least 6

species occur in Angola. The majority of the remaining species are presumably to be found in Natal, Transvaal, the Orange Free State, Namibia and Angola. Madagascar has a single species (*Hermannia exappendiculata*) which is shared with East and North East Africa (Leistner, 2000).

2.12 Medicinal Properties of *Hermannia* genus

The genus *Hermannia* has been used traditionally by people of diverse cultures for the treatment of fever, cough, and respiratory diseases such as asthma, wounds, burns, eczema, and stomach-ache. This plant is also used as purgative, diaphoretic, heartburn, flatulence in pregnant women, colic and haemorrhoids (Essopa *et al*., 2008).

In addition, the Xhosa use a decoction of the root of *H. incana* for dysuria; while a decoction of the root of *H. salviifolia* is utilized as an old-fashioned European household remedy for convulsions.

Hermannia incana is used as an emetic and the leaf sap extracted in cold water, is used to treat stomach-ache and diarrhoea, having purgative and diaphoretic effects. Decoctions of the whole plant are taken to soothe coughs. However, no other studies relating to the chemical composition of this species have earlier been reported (Van Wyk *et al*., 1997).

Hermannia geniculata is a species under the genus *Hermannia* of the subfamily *Byttnerioideae* and tribe *Hermanieae* of the family *Malvaceae* (previously called

Sterculiaceae). The wide diversity of species in a restricted geographical region is suggestive of a recent origin and diversification of the species. The lack of reported variation in chromosome counts may be further evidence in favour of this interpretation, or may reflect a limited sampling of the species of the genus. On the other hand, the genus seems less derived than the other genera of the tribe (for example in the presence of 5-locular ovaries with pluri-ovulate locules, which is a widespread condition in *Byttneroideae*, whereas the other genera show reduction in both the number of locules and ovules).

2.13 Morphology of *Hermannia geniculata*

Hermannia is a genus of small shrubs, ranging from upright to sprawling prostrate shrublets. They are characterized by the presence of minute glandular or star-like hairs on the leaves and stems. The stems often have a dark grey bark. Leaves are alternate and entire, lobed or incised. Flowers consist of 5 petals which are slightly or very strongly spirally twisted into an upended rose (Fig 2.5). Most *Hermannia* species have a thick woody stem and root, forming an underground stem, which enables the plants to survive dry periods and fires. In the veld, the plants appear woody, some species being very palatable to stock and browsed down to the main branches (*www.plantzafrica.com*). Figure 2.4 shows the distribution map of *H. geniculata* in South Africa.

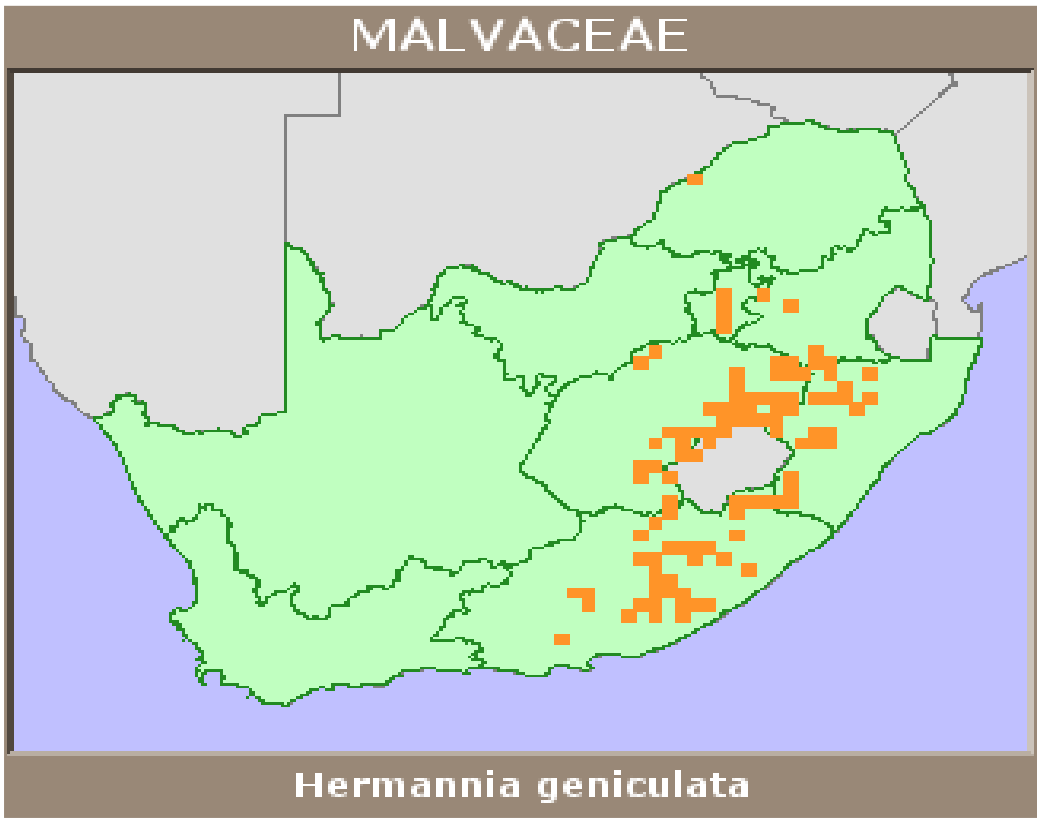


Figure 2.4 Distribution map of *H. geniculata* in South Africa (*www.redlist.sanbi.org*)

Hermannia geniculata is a decumbent, leaves petiolate, elliptic-oblong, obtuse, sub-cordate at base, corrugated and first pubescent, but grows glabrous on the upper side, stipules membranous, broadly ovate.

Fig. 2.5. *H. geniculata* growing on a rocky hill (*www.colinpatersonjones.co.za*)

2.14 Motivation of the study

Plants are chemical store houses of many chemical compounds which offer protection to the plants harbouring them from free radicals and pathogenic microorganisms. This makes plants a good source of natural antioxidants and

antibacterial compounds. These compounds are in most cases produced as secondary metabolites. Due to an increase attributed to antibiotic resistance of many microorganisms and numerous incidences in diseases that are associated with the presence of free radicals, plants are therefore studied to discover novel compound(s) which can be used as antibiotics and/ or antioxidants. The selected medicinal plant in this study is often used by practitioners of traditional medicines to treat a variety of bacterial infections and other ailments and for blood purifications.

2.15 General aim

The aim of the present study was to evaluate the antidiabetic and antimicrobial activities as well as isolation of the bioactive compounds of *Hermannia geniculata* leaves.

2.15.1 The specific objectives are to:

- Extract the leaves of *H. geniculata* using solvents of different polarity and screen the resultant crude extracts for inhibitory activity against carbohydrate metabolizing enzymes (α-amylase and α-glucosidase) using standardized *in vitro* enzyme inhibition bioassays.
- Evaluate antioxidants activity of the crude leaf extracts of *H. geniculata* using standard protocols
- Isolation and identification of bioactive compounds from active *H. geniculata* extract using preparative Thin Layer Chromatography fractionation to isolate the active chemical ingredient from the active fraction.
- Test the isolated compound (s) for antidiabetic, antioxidant and antimycotic activities
- Elucidate the structure of the isolated compound (s).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Introduction

The current chapter discusses the materials and methods used in the sampling of *Hermannia geniculata* plant materials, as well as performance of phytochemical activity screening of the respective extracts of the plants, isolation of bioactive compounds responsible for such activity.

3.2 Plant collection and identification

Fresh leaves of *Hermannia geniculata* were collected from vegetation along Wetsi café at Monontsha village, Qwaqwa, Eastern Free State Province, South Africa. The plant was thereafter authenticated and a Voucher Specimen (Mojamed/1/2016/Qhb) was issued and deposited at the Herbarium of Plant Sciences Department, University of the Free State, Qwaqwa Campus, South Africa.

3.3 Extract preparation

The fresh leaves were cut into smaller pieces and washed under running water to remove all debris, and dried in an Ecotherm oven at 40°C. The dried plant materials were then pulverised with the help of Waring laboratory blender (Labcon, Durban, South Africa).

Powdered plant (leaves) material (150 g) each were extracted separately in 1500 ml each of ethanol, ethyl acetate), acetone and hexane, for 24 h on a Labcon platform shaker at the speed of 100 rpm (Fig.3.1)

Extracts were filtered using Whatman no. 1 filter paper (Fig.3.2) and each filtrate was concentrated to dryness under reduced pressure at 40°C using rotary evaporator (Cole-Parmer) as depicted in Fig.3.3.

Finally, the extracts were dried to yield ethanol extract (33 g), acetone (10 g), ethyl acetate (44.2 g), hexane (4.5 g). Each extract was re-suspended in its respective solvent to make a 50 mg/ml stock solution.

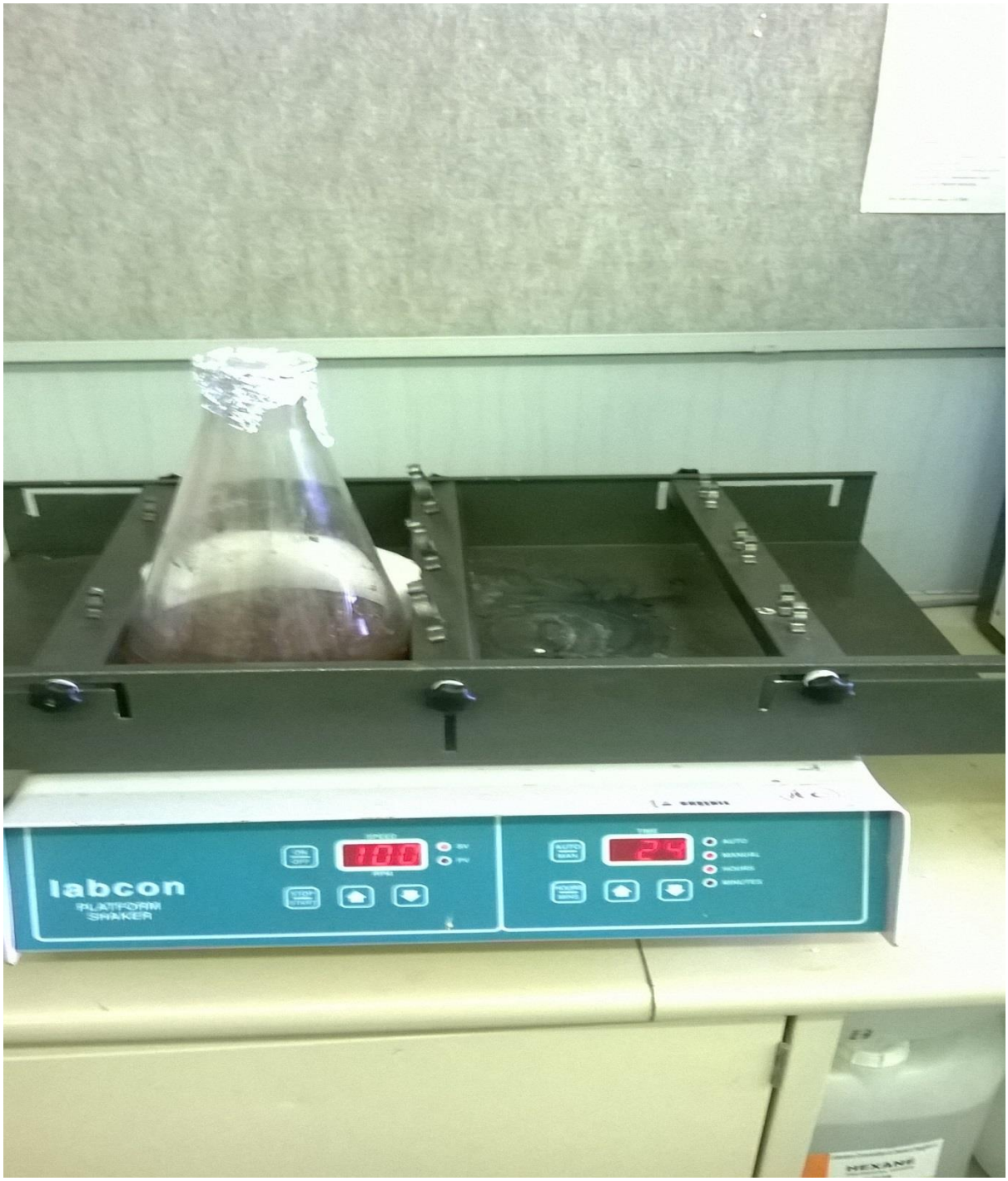


Fig. 3.1 *H. geniculata* leaf extraction process

Fig. 3.2 Filtration process of *H. geniculata* leaf

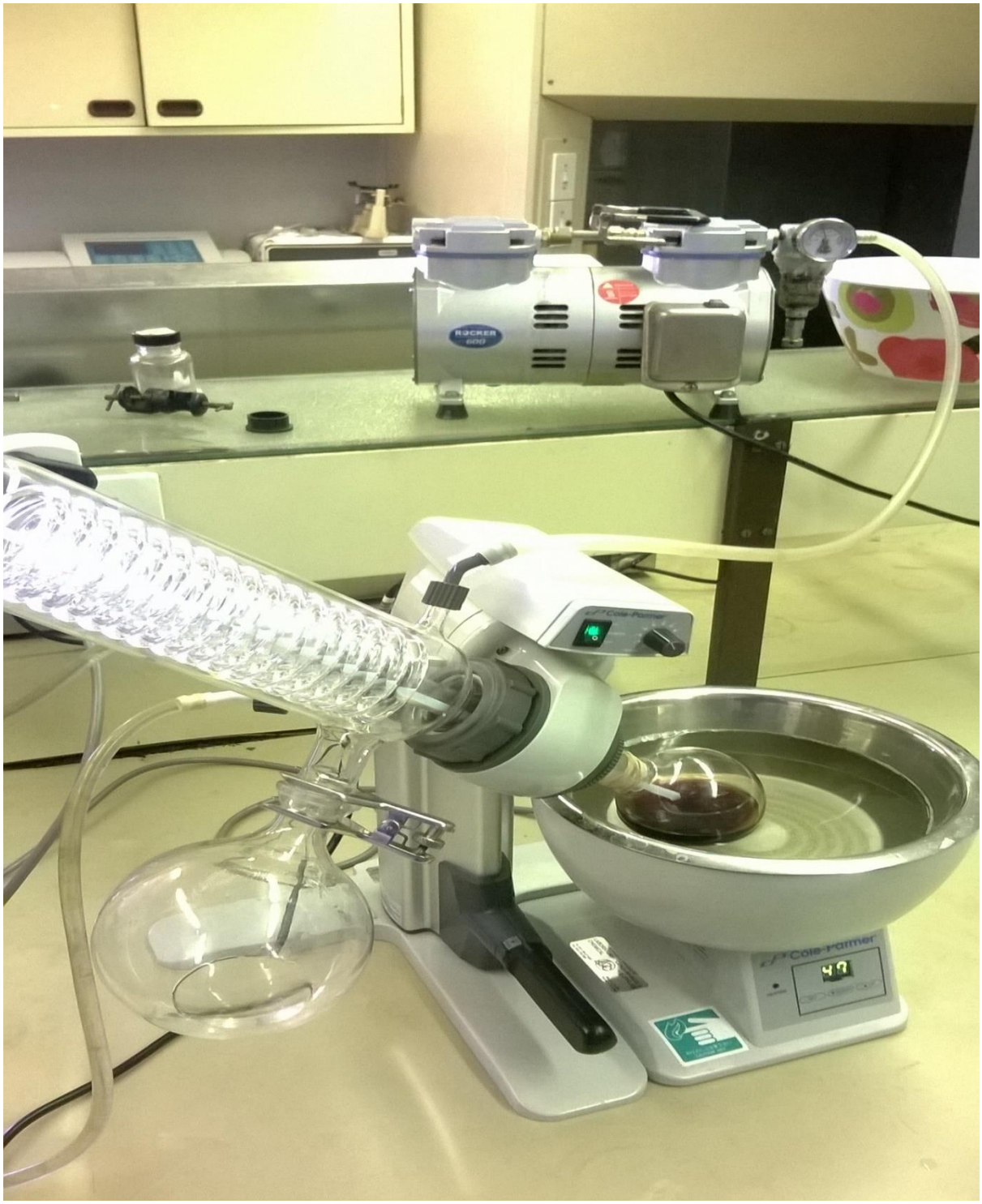


Fig. 3.3 The process of concentrating the extract

3.4 Phytochemical Analysis

Phytochemical constituents of *Hermannia geniculata* leaf were determined in the ethanol, acetone, hexane and ethyl acetate extracts adopting standard methods described by various authors (Harbone, 1973; Trease and Evans, 1989; Edeoga, 2005; Sofowora, 2006)

Preliminary phytochemical screening was carried out to determine the presence of various bioactive constituents in the crude extracts of ethanol, ethyl acetate, hexane and acetone.

3.4.1 Detection of Alkaloids

Half a gram of the powdered root material was dissolved in 5 ml of 1% aqueous hydrochloric acid on a water bath and filtered. 1 ml of the filtrate was subjected to Wagner's reagent (2 g of iodine and 6 g of potassium iodide in 100 ml of water) treatment and the presence of alkaloids was confirmed by brown/reddish precipitate.

3.4.2 Detection of anthraquinones

Exactly 2 ml of chloroform was added to 0.2 g of the extract and the resulting mixture was vigorously shaken for 5 minutes before filtration. Equal amounts of filtrate obtained and 10 % ammonia solution was thoroughly mixed and the formation of a bright pink colouration layer of the mixture was observed which confirmed the presence of anthraquinones.

3.4.3 Detection of flavonoids

Amount of 0.5 g of the extract was treated with few drops of 10% sodium hydroxide solution. Formation of intense yellow colour, which became colourless upon addition of aqueous hydrochloric acid, suggested the presence of flavonoids.

3.4.4 Detection of phenols

Amount of 0.5 g of the extract was treated with 3-4 drops of 10% ferric chloride solution. Formation of bluish black colour indicated the presence of phenols

3.4.5 Detection of saponins

Amount of 2 g of powdered material was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water, shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously again and then observed for the formation of emulsion as indication of saponin.

3.4.6 Detection of triterpenes

Amount of 2 g of the extract was treated with chloroform and filtered. The filtrate was subjected to few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour was an indication of triterpenes.

3.4.7 Detection of phytosterols

Amount of 0.5 g of the extract was treated with chloroform and filtered. The resulting filtrate was thereafter subjected to few drops of anhydride treatment, boiled and allowed to cool down. Following addition of concentrated sulphuric acid, the formation of brown ring at the layer junction indicated the presence of phytosterols.

3.5 Determination of antioxidant activity

3.5.1 Determination of DPPH radical scavenging activity

The free radical scavenging activity of ethanol crude extract and active band (S1A) was measured by using DPPH radical (Williams *et al*., 1995; Miliauskas *et al*., 2004). DPPH radicals have strong absorption maximum at 515 nm which decreases due to the reduction by antioxidants. The DPPH solution in ethanol (6 $\times 10^{-5}$M) was prepared freshly, and 3 mL of this solution was mixed with 100 µL of various concentration (0-0.5 mg/ml) of crude extracts. The samples were incubated for 20 min at 37 °C in a water bath, and then the decrease in absorbance at 515 nm was measured (Ab). A blank sample containing 100 µL of methanol in the DPPH solution was prepared and its absorbance was measured (Ab). All tests and analyses were run in triplicates and the results obtained were averaged.

Radical scavenging activity was calculated using the following formula:

% inhibition = [(Ab-Ae)/Ab] × 100

3.5.1.1 Hydroxyl Radical Scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell et al. (1987). Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), Ascorbic Acid (1 mM), H_2O_2 (10 mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml H_2O_2, 0.36 ml of deoxyribose, 1.0 ml of the extract of different concentration (125, 250, 500 & 1000 μg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.9), 0.1ml of ascorbic acid in sequence. The mixture was then incubated at 37ºC for 1 hour. 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10 % TCA and 1.0 ml of 0.5 % TBA (in 0.025 M NaOH containing 0.025 % BHA) to develop the pink chromogen measured at 532 nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation is calculated by using the following equation

Hydroxyl radical scavenging activity= {(A0 –A1)/A0)*100}

Where, A0 is the absorbance of the control reaction, and A1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

3.5.2 Total antioxidant activity

Spectrophotometric method was used for the determination of total antioxidant activity (Shirwaikar et al., 2003). Various concentrations (0.05–0.75 μg/ml) of the crude extracts and active band (S1A) were prepared separately in methanol. The reaction mixture contained 1 ml of the extract and 2 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated in water bath at 95 °C for 90 min and after cooling at room temperature, the absorbance of reaction mixture was measured at 695 nm against blank using microplate reader (Biorad, model 680. South Africa). Ascorbic acid was used as the standard and total antioxidant capacity was expressed in terms of ascorbic acid equivalents. All tests were run in triplicates and averaged.

Fig. 3.4 Preparation of DPPH assay**.**

3.5.3 Metal chelating activity

Metal chelating activity was evaluated using standard colorimetric method (Benzie and Szeto, 1999). The reaction mixture contained 0.5 ml phenanthroline (0.05 %) in methanol, 1 ml ferric chloride (200 μM) and 1 ml different concentrations (0.05-0.075 μg/ml) of the extracts. The reaction mixture was further incubated at room temperature for 10 min and the absorbance of the sample was read at 510 nm. Ascorbic acid was used as the standard and ion chelating activity was expressed as ascorbic acid equivalents. All tests and analyses were run in triplicates and the results obtained were averaged.

3.5.4 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) activity

The concept of antioxidant capacity first originated from chemistry and was later adapted to biology, medicine, epidemiology and nutrition (Cao and Prior, 1998; Pellegrini *et al*., 2003 Floegel *et al*., 2010;). It describes the ability of redox molecules in foods and biological systems to scavenge free radicals. This concept provides a broader perception of the antioxidants present in a biological sample as it takes into cognisance the additive and synergistic effects of all antioxidants rather than the effect of single compounds, and may, therefore, be useful to study

the potential health benefits of antioxidants on oxidative stress mediated diseases (Brighenti *et al*., 2005; Puchau *et al*., 2009).

Antioxidant activity of *Hermannia geniculata* extracts as per ABTS•+ decolorization assay was measured using the method reported by Re *et al*. (1998). The working solution of ABTS•+ radical was made by reacting ABTS (9.5 mL, 7 mM) with potassium persulfate (245 μL, 100 mM), and raising the volume to 10 mL with distilled water. The solution was kept in the dark at room temperature for 18 h, and then diluted with potassium phosphate buffer (0.1 M, pH 7.4) to an absorbance of 0.70 (±0.02) at 734 nm. Plant samples were prepared in methanol with dilutions 50–1250 μg/mL. A sample (10 μL) was placed in a test tube and mixed thoroughly with 2.99 mL ABTS radical working solution. Absorbance of the resulting clear mixture was recorded at 734 nm. The percent antioxidant activity of the sample was determined using the following formula:

$$\%\text{Antioxidant activity} = [(Ac - As)/Ac] \times 100$$

where Ac and As are the absorbances of the control and sample, respectively. The control was prepared by adding 10 μL of methanol in place of the sample.

3.6 *In vitro* antidiabetic Assays

3.6.1 α-glucosidase activity

The α-glucosidase inhibitory activity was assayed following the method of Elsnoussi *et al*. (2012). In brief, different concentrations (0.25–8.0 mg/mL) of *Hermannia geniculata* leaf extracts were prepared in distilled water. Then, 50 ml from the stock solution was mixed with100 ml of 0.1m phosphate buffer (pH 6.9) containing1.0 M of α-glucosidase solution. The mixtures were then incubated in 96-well plates at 25 °C for 10 min. Following this, 50 mL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. The absorbance readings were thereafter taken at 405 nm using a micro-plate reader (Thermomax, USA) and the values compared with a control which contained 50 ml of the buffer instead of the extract. Acarbose (Bayer Medicals, Germany) was prepared in distilled water at same concentrations as the extract and used as control. The experiments were conducted in triplicate and the α-glucosidase inhibitory activity was expressed as % inhibition using the expression: % Inhibition = [(ΔAcontrol - ΔAextract)/ΔAcontrol] x 100,

where Δ A control and Δ A extract are the changes in absorbances of the control and extract sample respectively. Using standard calibration curve, the concentration of *Hermannia geniculata* leaf extract causing 50% inhibition (IC_{50})

of α-glucosidase activity was estimated. For the enzyme kinetics on inhibition of α-glucosidase activity by aqueous extract of *Hermannia geniculata* leaf, a modified method of Dnyaneshwar and Archana (2013) was adopted. Briefly, 50 µL of 5 mg/mL extract was pre-incubated with 100 µL of α-glucosidase solution for10 min at 25 °C in one set of tubes. In another set of tubes, α-glucosidase was pre-incubated with 50 µL of phosphate buffer (pH6.9). 50 mL of pNPG at concentrations (0.63–2.0 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then placed in incubator for 10 min at 25 °C, and 50 mL of Na_2CO_3 was added to halt the reaction. The amount of reducing sugars released was determined colourimetrically using a p-nitrophenol standard curve.

3.6.2 α-amylase inhibition

Adopting the methods of Elsnoussi *et al*. (2012) and Kazeem *et al*. (2013), the α-amylase inhibitory activity and mode of inhibition were evaluated. Briefly, varying concentrations (0.25– 10.0 mg/mL) of the extracts were prepared and 500 mL of each was mixed with 50 ml of 0.02 M sodium phosphate buffer (pH 6.9) containing 0.5 mg/ml of α-amylase solution and incubated in test tubes at 25 °C for 10 min. After pre-incubation, 50 ml of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube at timed intervals. The reaction mixtures were incubated at 25 °C for 10 min and halted with 1.0 mL of

dinitrosalycyclic acid colour reagent. The tubes were then heated in a boiling water bath for 5 min and subsequently cooled to room temperature. The reaction mixtures were then diluted with distilled water (15 ml), and the absorbance readings were measured at 504 nm using a spectrophotometer (Biochrom WPA Biowave II, Cambridge, England) and the values compared with a control which contained 50 ml of the buffer instead of the extracts. Acarbose was prepared in distilled water at same concentrations as extracts and used as control. The experiments were conducted in triplicate and the α-amylase inhibitory activity was expressed as % inhibition. The concentration of the extract causing 50 % inhibition (IC_{50}) of α-amylase activity was estimated from its standard calibration curve.

3.7 Antimycotic activity

3.7.1 Minimum Inhibitory Concentration (MIC) Determination

Micro-dilution technique was used to evaluate the MIC using 96-well plates according to the method of Ogundajo *et al*. (2017) with modification. Different concentrations (0.051–26.000) mg/mL of the pure compound were prepared using two fold serial dilutions in sabouraud dextrose broth (Lab M, UK) for fungi strains. Fluconazole (Austell Laboratory, South Africa) was used as positive controls (0.0195–10.000 0) µg/mL and 100% solvent of dilution as negative control. Each strain (50 mL) deposited in the wells was adjusted to be equal to 0.5 McFarland standards and incubated at 37ºC for 24 h. Exactly, 40 mL 0.2 mg/mL of colourless tetrazolium salt (P-iodonitrotetrazolium) solution was pipetted into each well and further incubated for 30 min. The assay was performed in duplicate and concentration at which 50% (IC_{50}) of microbial growth was inhibited was determined.

3.7.2 Minimum Fungicidal Concentration (MFC) Assay

Fresh PDA (Lab M, UK) plates prepared for fungi were inoculated with 100 µL of culture taken from each of the MIC wells after dilution in 900 µL of broth for the determination of minimum fungicidal concentration (MFC). After the incubation periods, the lowest concentration of the extract that did not produce

any or >90% fungal growth on the solid medium was regarded as the minimum fungicidal concentration (MFC) of the extract.

3.8 Isolation of the bioactive molecule

The ethyl acetate fraction thus obtained was dissolved in 1 ml hexane. For the TLC analysis, 25 µl of the extract was loaded on the TLC (Silica gel 60) plates (Merck, Germany) (Fig.3.5) The solvent ratio used for the separation of the compounds was hexane: ethyl acetate (90:10, v: v). UV-trans-illumination of the plates at 365 nm revealed bands. Then the TLC plates were dipped inside a spraying agent of 4 ml P-anisaldehyde containing 4 ml of sulphuric acid, 2 ml glacial acetic acid and 100 ml of 95% ethanol. Plates were then heated and the colour developments were observed.

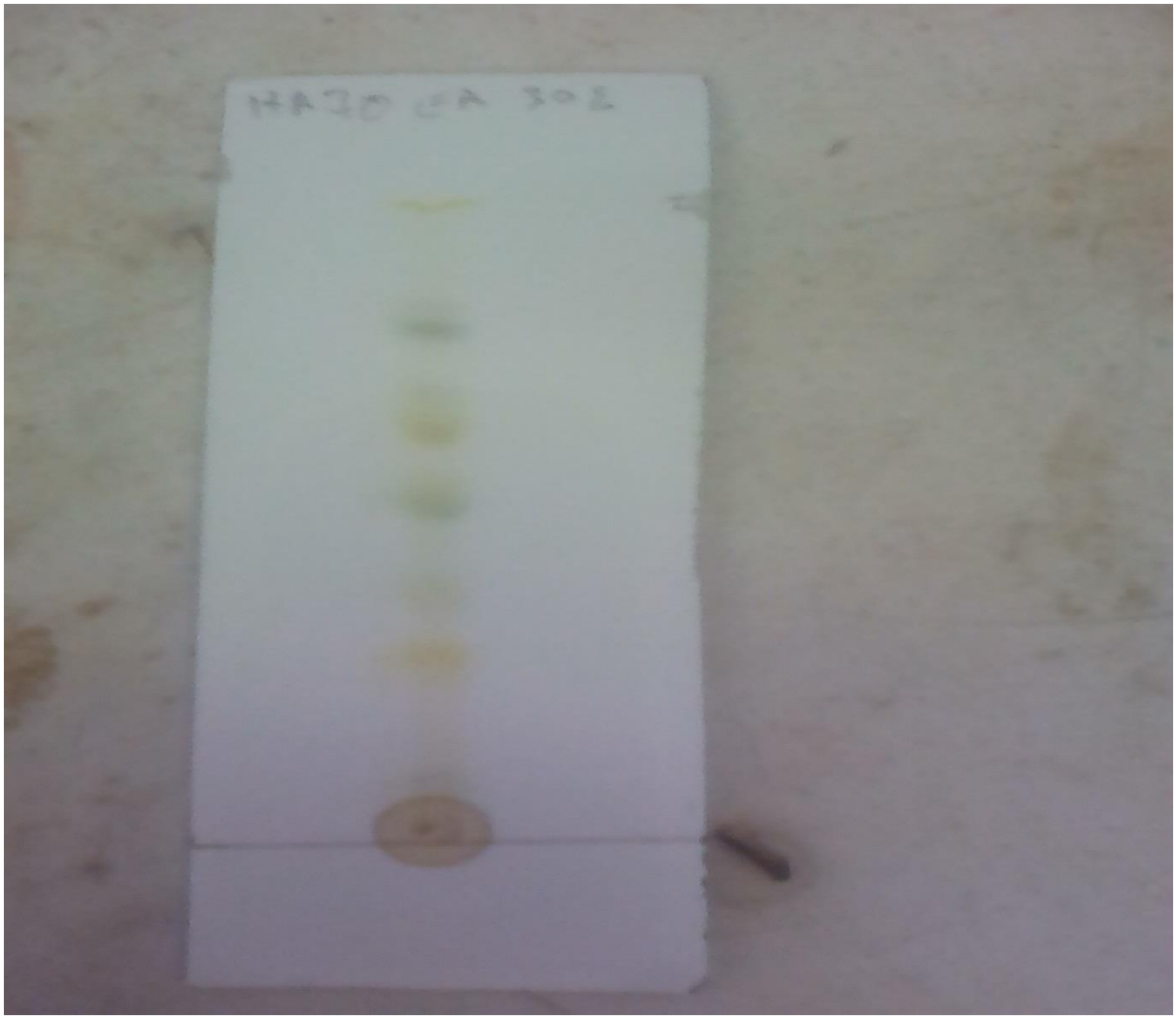


Fig. 3.5. TLC analysis of ethyl acetate extract (Mojau, 2016)

3.8.1 Experimental Techniques for Isolation of Bioactive Principle

Four techniques were used to screen and isolate chemical components of *Hermannia geniculata* leaf extracts. The chromatographic techniques used were Thin Layer Chromatography (TLC) and Column Chromatography (CC). Nuclear Magnetic Resonance (NMR) was used for characterisation of the isolated compounds. Mass spectroscopy (MS) was used to confirm mass-to-charge ratios

(m/z) and the relative abundance of the isolated compound. Fourier Transform Infrared Spectroscopy (FTIR) was used to confirm MS results from NMR.

Activated silica gel (60-120 mesh) was packed on to a glass column (450 mm × 40 mm) using *n*-hexane solvent and 12 g of ethyl acetate extract was loaded on top of the packed silica gel. The column was eluted step-wise with 500 ml of *n*-hexane: ethyl acetate (90:10) to obtain 20 fractions (1-20) collected into 250 ml conical flasks (Fig.3.6). The fractions were concentrated using a Cole Parmer rotary evaporator at 40°C to reduce the volume and then poured into a pre-weighed beaker and evaporated to dryness through air drying system.

The chromatograms were visualised using P-anisaldehyde spray reagent and under UV light 254 nm. Fractions (F1; 2; 3; 4-6; 7; 8; 9-11; 12-15; 16-19; and 20) were selected and combined for further separation (Fig.3.7) The combined fractions were fractionated on a silica gel, eluted with hexane: ethyl acetate (90:10).

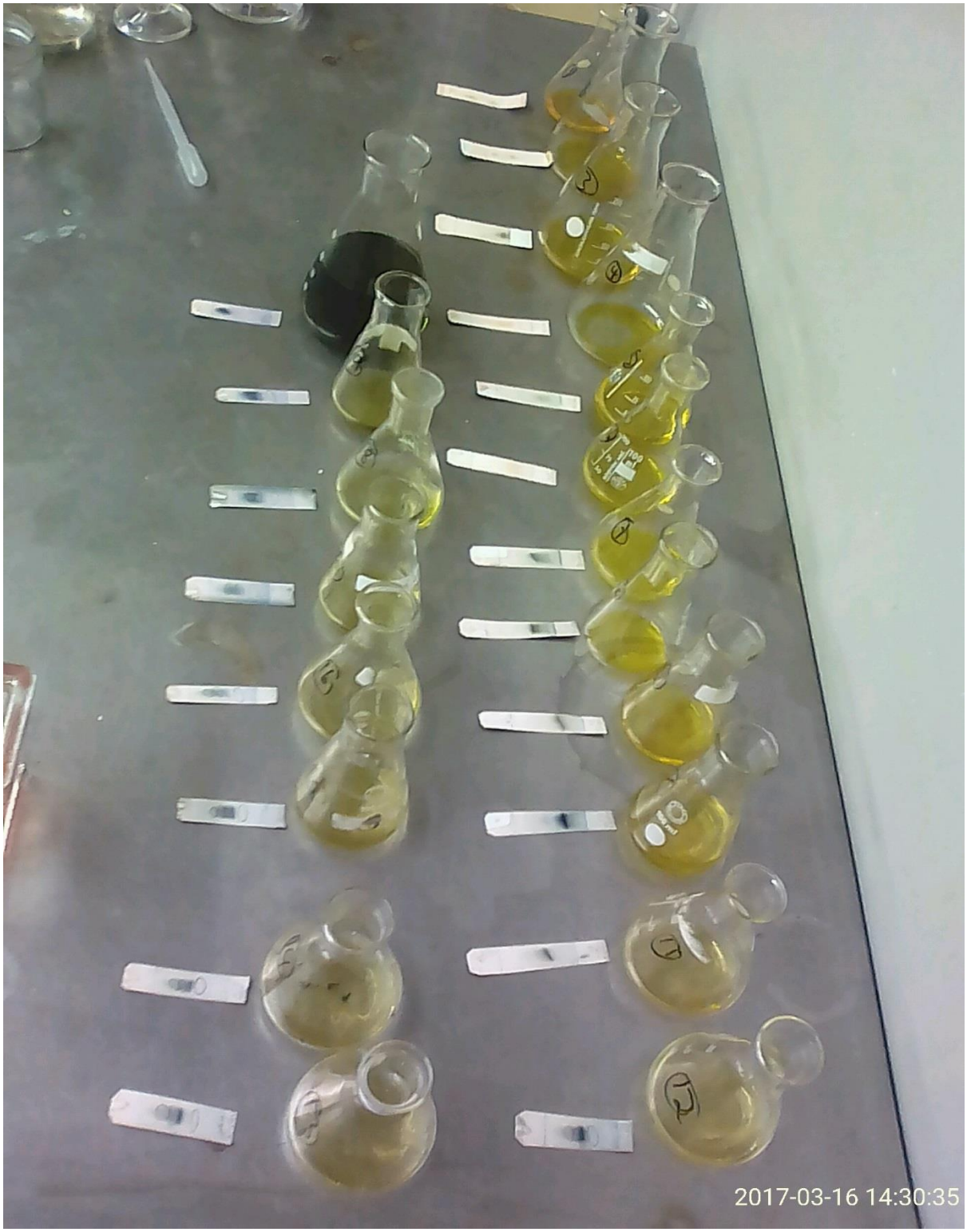


Fig. 3.6 Fractions eluted from the column chromatography (Mojau, 2016)

Fig. 3.7 Chromatogram from selected fractions (Mojau, 2017)

The selected chromatograms were further eluted and 6 fractions were combined (F2-3; F4-5; and F6-7). F4 and 5 were further eluted in a column prepared with sephadex using methanol as a solvent of elution and 0.2 g of ethyl acetate extract was loaded on top of the sephadex. The column was eluted step-wise with 500 ml of methanol: ethyl acetate (90:10) to obtain 9 fractions (1-9) collected into 250 ml conical flasks). The fractions were concentrated using a Cole Parmer rotary

evaporator at 40°C to reduce the volume and then poured into a pre-weighed beaker and evaporated to dryness through air-drying system. F5 and F6 were combined and were prep-TLC was performed for isolation.

3.8.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy gives information on the environment in which the nuclei of the atoms are found in molecules and compounds. It is the study of the interaction of radio frequency (RF) of the electromagnetic radiation (EMR) with unpaired nuclear spins in an external magnetic field to extract structural information about the given sample (James, 1998).

The structure of the isolated compounds was interpreted using various 1D and 2D NMR experiments (such as proton - 1H, carbon - 13C, Heteronuclear Multiple Bond Correlation- HMBC, Heteronuclear Single Quantum Coherence - HSQC, Correlation SpectroscopY - COSY) to determine the proton to carbon relation and the chemical environment they are in for structure elucidation (Silverstein *et al.,* 2005).

Two Varian NMR instruments allowing analysis at 600 MHz and 400 MHz were used. Deuterated Chloroform (CDC_{l3}) and methanol (CD_3OD) were used for dissolving of non-polar and polar compounds respectively. The duration of the experiments depended on the purity and quantity of the samples. For accurate

mass detections of the compounds, analysis by the mass spectroscopy (UPLC-QTOF-MS) was used. All samples were run at a set temperature of 25 °C.

3.8.3 Fourier Transform Infrared (FTIR) Process Analysis

Infrared instrumentation has been used in chemical process control for approximately fifty years, making it one of the first analytical techniques to be put on-line. However, until recently, on-line infrared instruments were generally restricted to one and two wavelengths nondispersive (NDIR) analyzers. Dispersive IR laboratory instruments instruments, the only full spectrum IR spectrometers available prior to 1970, were simply too slow and insensitive to find widespread use in process applications.

The advent of commercial FTIR instruments in 1970 represented a major advance in IR spectroscopy in terms of both raw performance and data manipulation capability. However, the early FTIR's were strictly laboratory instruments, being highly sensitive to ambient temperature variations, vibration, and acoustic disturbances, all of which are typical of the pre-process environment. To understand the reasons for this sensitivity, we need to briefly review the operation of an FTIR spectrometer.

The heart of any FTIR spectrometer is an amplitude division interferometer. The original Michelson design - still employed in the majority of laboratory FTIR's -

consists of a beamsplitter, a compensating plate, and a pair of mirrors. The difference in path length between the two arms is varied by mechanically scanning the position of one of the mirrors. This gives rise to a time dependent variation in transmitted optical intensity, called the interferogram. When the inteferometer is illuminated by a monochromatic source such as a single frequency laser, the interferogram will be a sine wave of intensity versus mirror position. On the other hand, if the source is characterized by a broad infrared spectrum, the interferogram will correspond to the superposition of an infinite number since waves having different periods but a common zero phase point (or central maximum) which occurs when the lengths of the two interferometer arms are equal.

In principle, the intensity of a given spectral point could be determined by simply passing the electrical signal obtained from the IR detector through a narrow band electronic filter. And the complete spectrum could be measured by varying the filter frequency. A much more rapid approach is to use a digital computer to perform a Fourier transformation of the interferogram, thereby directly yielding the composite spectrum of the source, the instrument, and any sample interposed in the optical path. This is the basis of all modern rapid-scan FTIR spectrometers. Over the past several years, FTIR spectrometers have almost completely supplanted the older dispersive instruments for laboratory IR analysis (Doyle,

1992). Due to the sensitivity of the FTIR instrument, the most convenient and satisfactory method involves simple evaporation of a solution of the sample (chloroform, ether, dichloromethane; or even a CDCl3 NMR sample may be used) onto a KBr salt plate and acquisition of the spectrum from the thin film remaining.

CHAPTER 4

RESULTS

4.1 Phytochemical screening analyses

The results obtained in respect of qualitative phytochemical analysis are presented in Table 4.1. Alkaloids, flavonoids and triterpenes were present in all the four extracts of *Hermannia geniculata*. Anthraquinones were only detected in acetone extract. While, saponins were only detected in ethanol and acetone extracts. Phytosterols were detected in ethanol and ethyl acetate extracts only.

Table 4.1. The phytochemical constituents of the leaf extracts of *Hermannia geniculata*

Compound	Ethanol	Acetone	Hexane	Ethyl acetate
Alkaloids	+	+	+	+
Anthraquinones	-	+	-	-
Flavonoids	+	+	+	+
Phenols	+	+	+	+
Saponins	+	+	-	-
Triterpenes	+	+	+	+
Phytosterols	+	-	-	+

(+) detected, (-) not detected

4.2 Alpha amylase inhibition

The ethyl acetate, ethanol and hexane extracts demonstrated the strongest inhibitory activity against the α-amylase at the highest concentrations which was significantly higher ($p < 0.0.5$) than other extracts and standard (acarbose) as well as ascorbic acid. However, acarbose had the highest inhibition at 0.75 mg/ml concentration, but at 0.25 mg/ml, both ascorbic acid and acarbose shown

the similar highest concentration against all the extracts. Acetone extract exhibited the lowest inhibition activity against all concentrations (Fig. 4.1).

Table 4.2 IC_{50} (mg/mL) of alpha amylase capabilities of the isolated compound (S1A) against acarbose

DPPH	Acarbose	Compound (S1A)
IC_{50}	0.070	0.117
R^2	0.735	0.457
Regression equation	Y = -220.41 x + 34.533	Y = 86.387x + 39.858

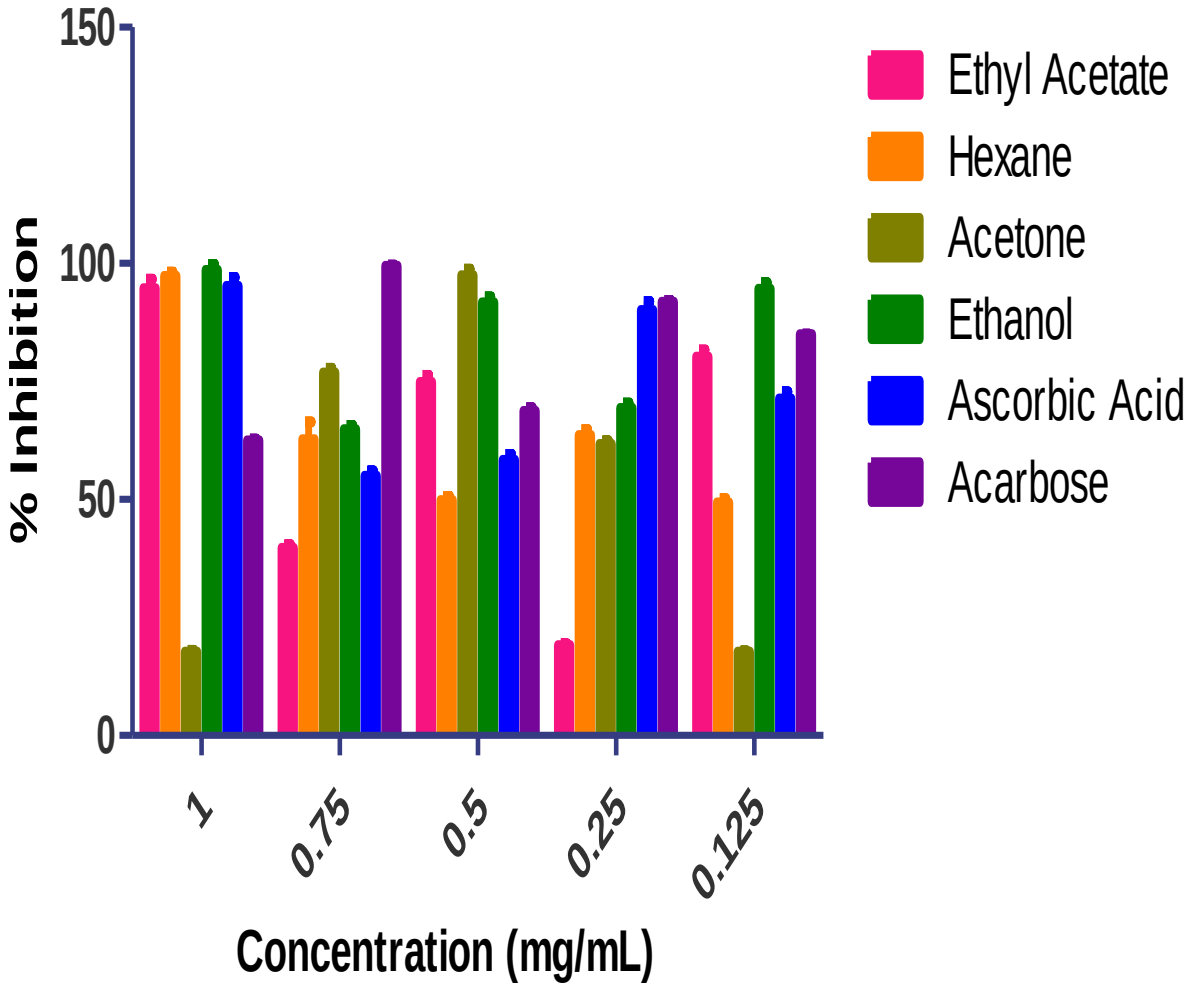


Figure 4.1 Inhibition effects of *Hermannia geniculata* leaf extracts against α-amylase

Table 4.3 IC_{50} values of α-amylase inhibition by acarbose and leaf extracts of *Hermannia geniculata*

IC_{50}	EXTRACTS
0.172	Ethyl acetate
0.240	Hexane
4.201	Acetone
8.101	Ethanol
2.344	Acarbose
0.117	S1A

Ethyl acetate extract had the lowest IC_{50} value at 0.172 compared to other extracts while acarbose (standard) had 2.344. However, the isolated compound S1A showed the highest activity with the value of 0.117 (Table 4.3).

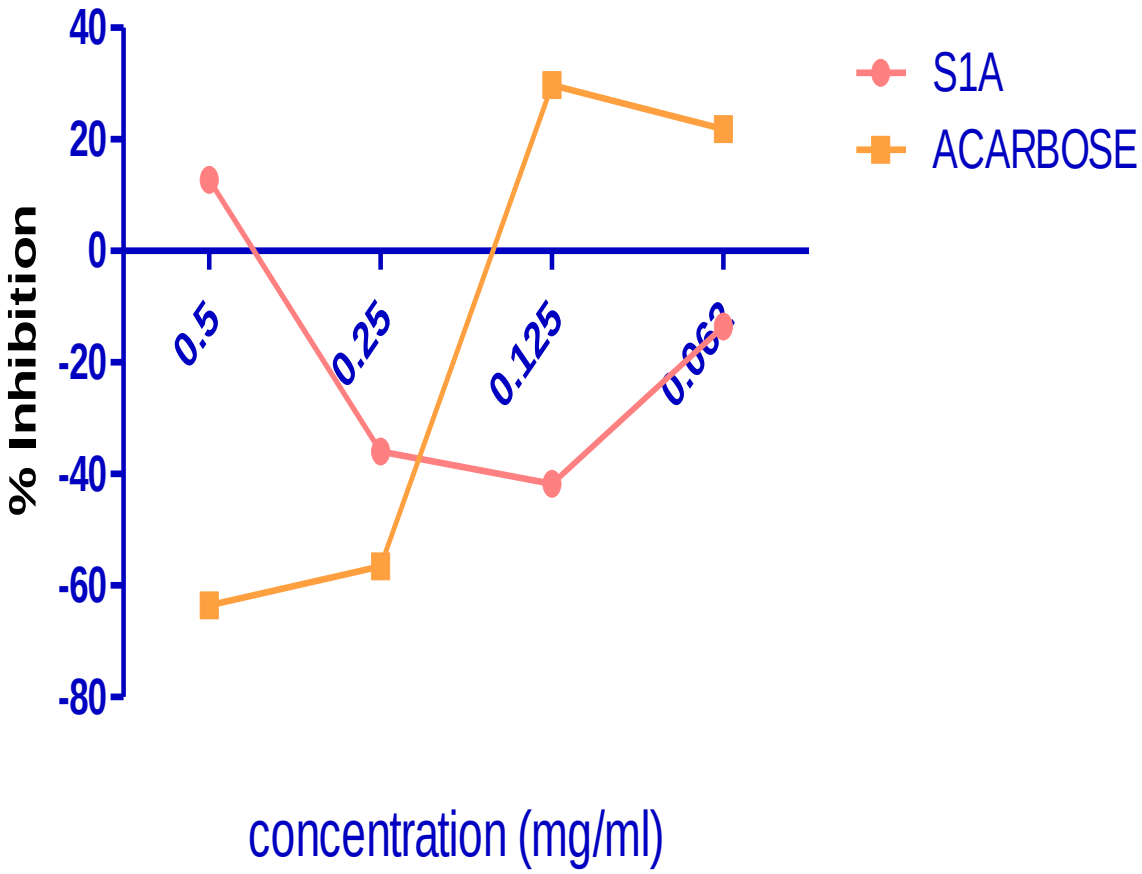


Fig. 4.2 Graph depicting alpha amylase inhibition by the isolated compound (S1A) and the acarbose (standard).

4.3 Alpha glucosidase inhibition

All extracts showed high inhibitory activity against alpha glucosidase at more than 90% inhibition at the highest concentration compared to the standard acarbose. However, at the 0.75 mg/ml concentration; standard acarbose exhibited the highest alpha glucosidase activity followed by hexane. Hexane also exhibited the highest inhibition at 0.125 – 0.5 mg/ml concentrations followed by ethanol, similarly at these concentrations, ethanol extract also showed the least inhibition activity.

Table 4.4 IC_{50} (mg/mL) of alpha glucosidase capabilities of the isolated compound (S1A) against acarbose

DPPH	Acarbose	Compound (S1A)
IC_{50}	10.450	4.760
R^2	0.084	0.626
Regression equation	Y = 4.525 x + 2.705	Y = 9.792x + 3.38

Table 4.4 depicts the behavior of the IC_{50} values of the isolated compound (S1A) compared to acarbose. The S1A showed highest activity with the value of 4.760.

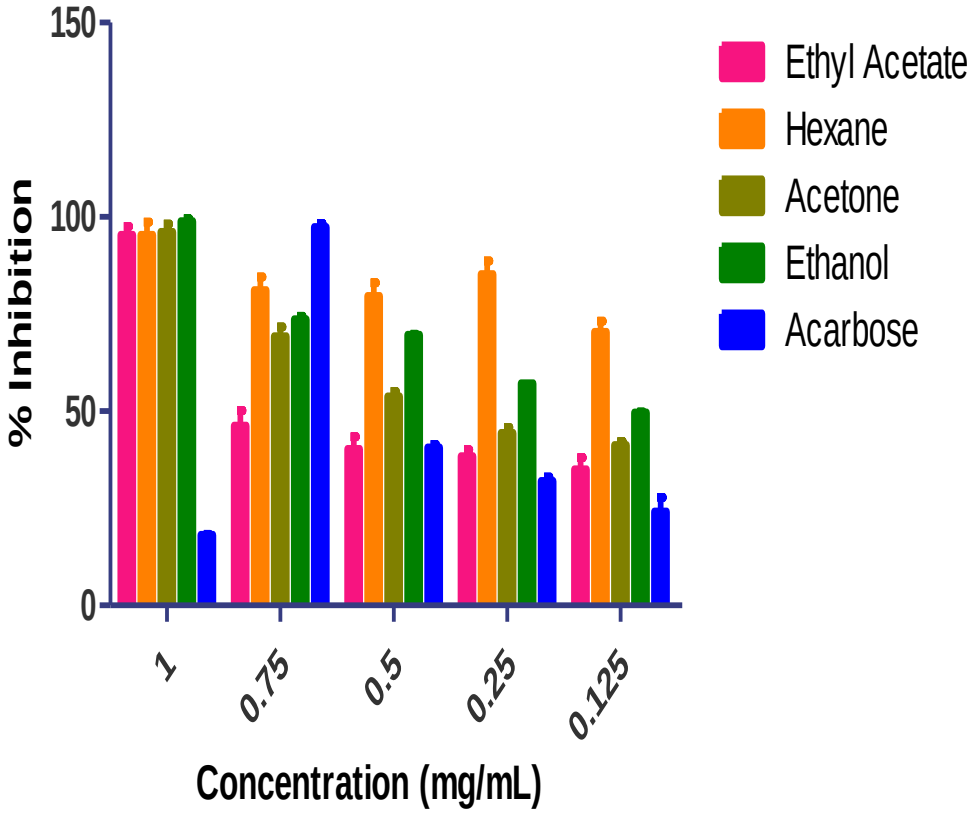


Fig. 4.3 Inhibitory activity of *Hermannia geniculata* leaf extracts and acarbose (standard) against α-glucosidase

Table 4.5 IC_{50} values of α-glucosidase inhibition by acarbose and leaf extracts of *Hermannia geniculata*

IC_{50}	EXTRACT
0.459	Ethyl acetate
0.974	Hexane
0.370	Acetone
0.144	Ethanol
0.88	Acarbose

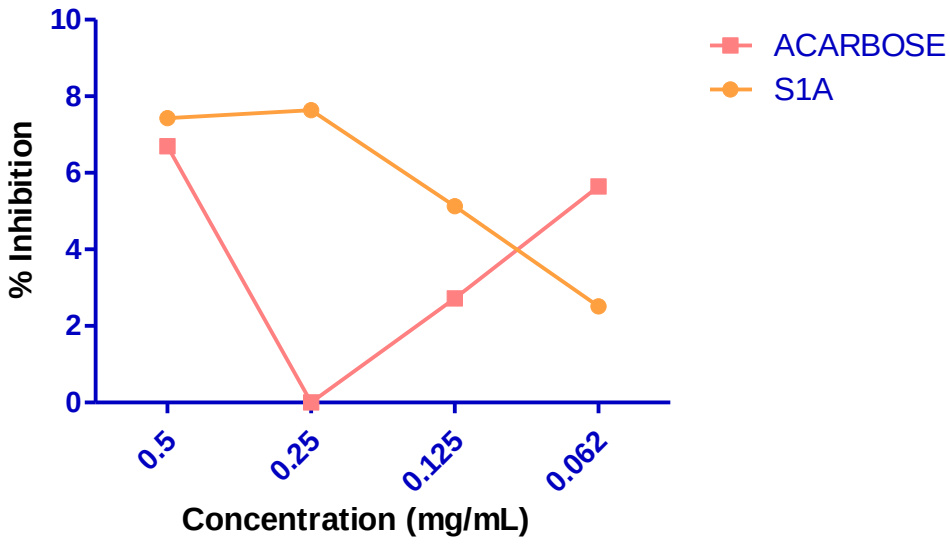


Fig. 4.4. Graph depicting alpha glucosidase inhibition by the isolated compound (S1A) and the acarbose (standard).

4.4. *In vitro* antioxidant effects of leaf extracts of *Hermannia geniculata*

The *in vitro* antioxidant capacities for the extracts under investigation were studied using different models of antioxidant activities. They were determined using 1,1-diphenyl- 2-picrylhydrazyl (DPPH) radical, ABTS, hydroxyl radical and metal ion chelation. In the DPPH assay, the percentage inhibition by Silymarin (standard) was significantly higher ($P < 0.05$) along with the tested extracts at the highest concentration except the isolated compound (S1A). However, for all other concentrations the isolated compound with all other extracts exhibited a significantly higher inhibitory concentration except Silymarin.

At the other concentrations, S1A had significantly higher activity along with all the extracts except silymarin (standard) which showed the lowest activity at the concentrations ranging from 0.05 – 0.036 mg/mL

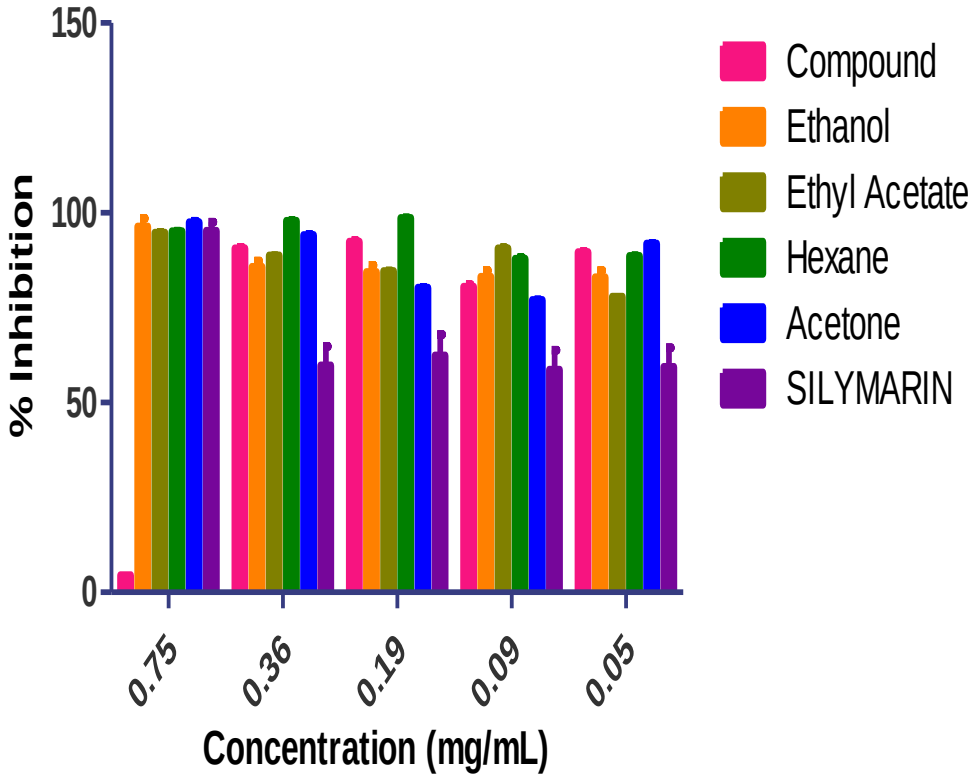


Fig. 4.5. DPPH radical scavenging of *Hermannia geniculata* leaf extracts, isolated compound (S1A) and Silymarin (standard)

Table 4.6 IC_{50} values of DPPH with inhibition by Silymarin and leaf extracts of *Hermannia geniculata*

IC_{50}	EXTRACT
1.263	Ethyl acetate
0.379	Hexane
0.661	Acetone
0.477	Ethanol
0.177	Silymarin

Table 4.7. IC_{50} (mg/mL) of DPPH capabilities of the isolated compound (S1A) against silymarin

DPPH	Silymarin	Compound (S1A)
IC_{50}	0.474	0.474
R^2	0.960	0.771
Regression equation	Y = 2.664x + 5.63	Y = 117.11x + 105.51

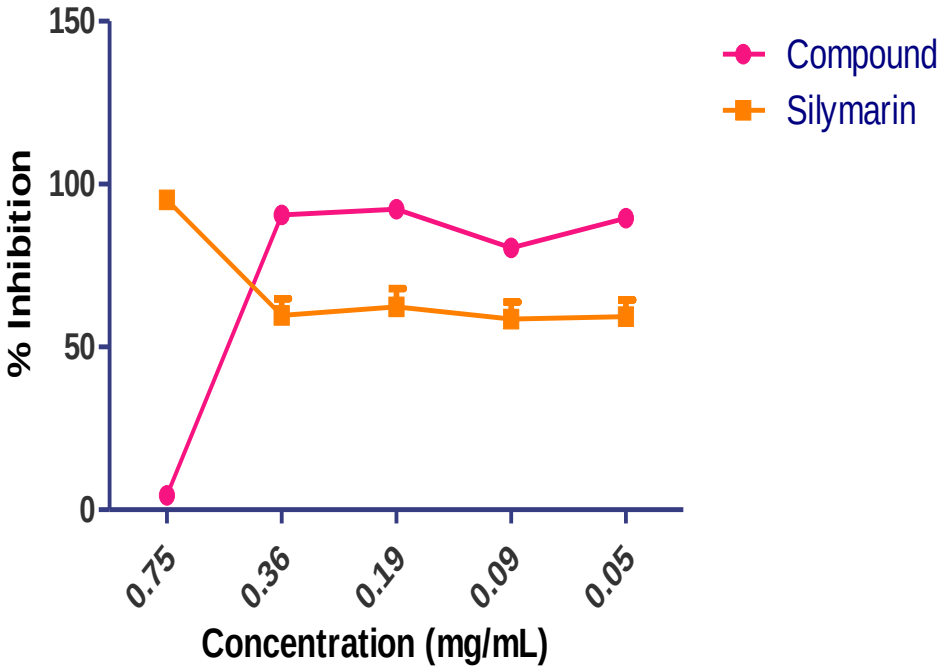


Fig. 4.6 DPPH graph showing activity of isolated compound (S1A) and Silymarin (standard)

The isolated compound (S1A) showed higher activity against silymarin (standard) with the IC_{50} of 0.474 compared to silymarin with 16.647.

4.5 Hydroxyl radical scavenging inhibition activity

At the highest concentration, all extracts including ascorbic acid inhibition were significantly high. Hexane had the highest inhibitory activity at the concentration of 0.75 mg/ml, followed by ethyl acetate, ethanol and ascorbic acid with similar

inhibition concentrations, except for acetone with the lowest activity. At 50 mg/ml concentration, ethanol and ascorbic acid had significantly higher inhibitory concentration compared to all other extracts. In all concentration, acetone had the least inhibitory effect compared to all other extracts and the standard ascorbic acid

Table 4.8 IC_{50} (mg/mL) of hydroxyl radical capabilities of the isolated compound (S1A) against ascorbic acid

DPPH	Ascorbic acid	Compound (S1A)
IC_{50}	8.875	0.474
R^2	0.041	0.771
Regression equation	Y = 3.490x + 80.978	Y = 117.11x + 105.51

The isolated compound showed higher activity against ascorbic acid (standard) with the IC_{50} value of 0.474 compared to ascorbic acid with 8.875.

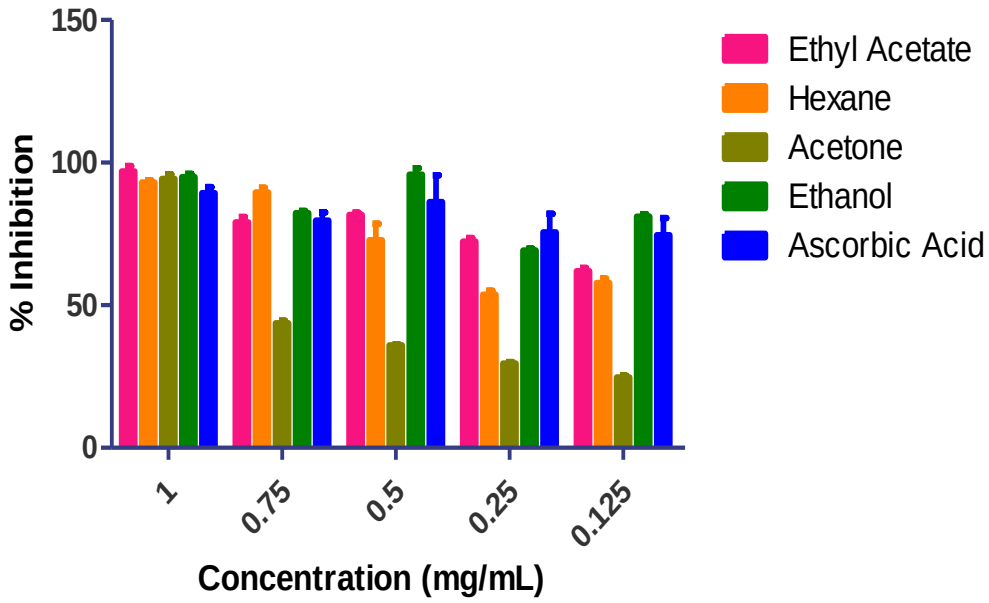


Figure 4.7. hydroxyl radical graph with *Hermannia geniculata* leaf extracts and ascorbic acid (standard)

Table 4.9 IC_{50} values of hydroxyl radical scavenging effects of *Hermannia geniculata* leaf extracts and ascorbic acid

IC_{50}	EXTRACT
0.391	Ethyl acetate
0.132	Hexane
0.600	Acetone
1.379	Ethanol
8.875	Ascorbic acid

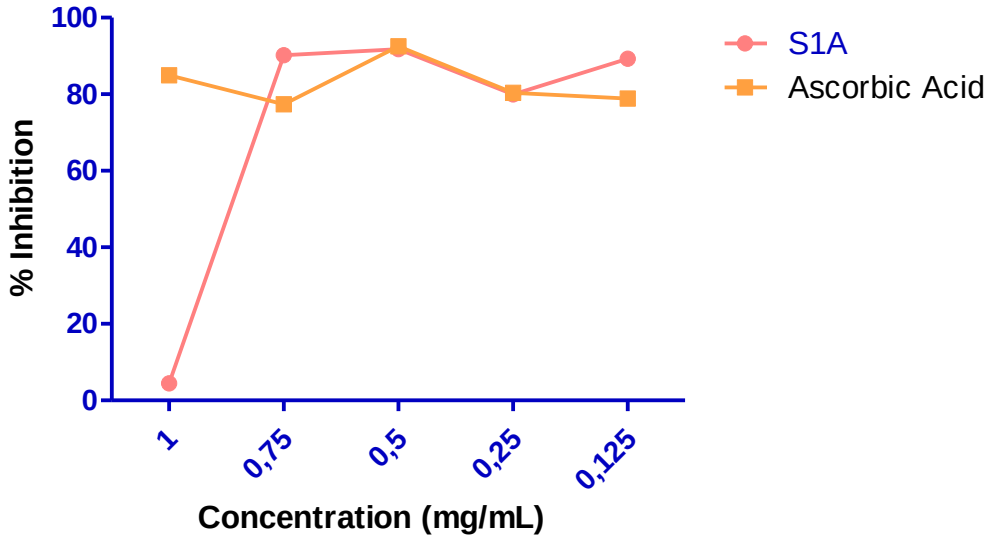


Fig. 4.8. hydroxyl radical graph showing activity of isolated compound (S1A) and ascorbic acid (standard)

4.6 Metal chelating activity

At the highest concentration all extracts exhibited similar inhibitory behaviour except for the ascorbic acid (standard) with a slightly lower activity even though overall activity for all the extracts and ascorbic acid were significantly high ($P < 0.05$). At the concentration of 0.75 mg/ml, ascorbic acid showed the highest activity compared to all extracts, the similar trend was observed at 0.25 mg/ml and 0.125 mg/ml concentrations with ascorbic acid showing the highest activity. Acetone extract showed relatively low activity at the concentrations of 0.75, 0.5,

0.25 and 0.125 mg/ml concentrations (Fig.4.9). The activity of the isolated compound compared with Silymarin (standard) is depicted in Fig. 4.10, with Silymarin showing higher activity at the lowest concentration of 0.625 mg/ml compared to isolated compound. However, Silymarin did not show any activity at the concentration of 0.25 mg/ml, whereas the compound had shown significant activity at that concentration. In general, the isolated compound exhibited significant activity against Silymarin.

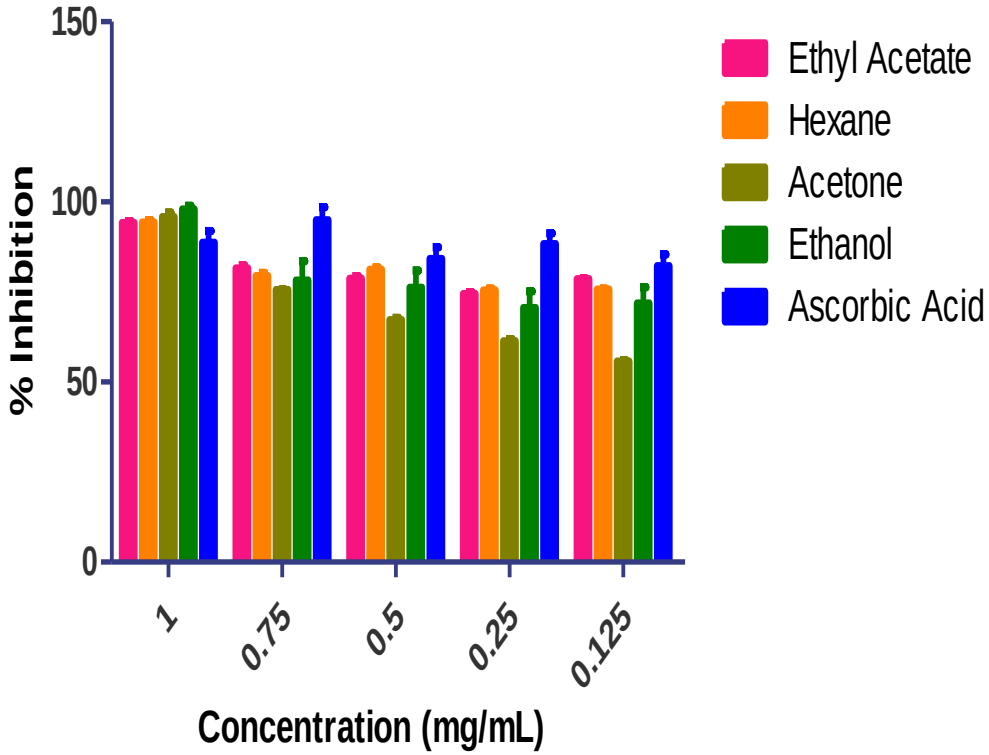


Figure 4.9. metal chelating effects of *Hermannia geniculata* leaf extracts and ascorbic acid (standard).

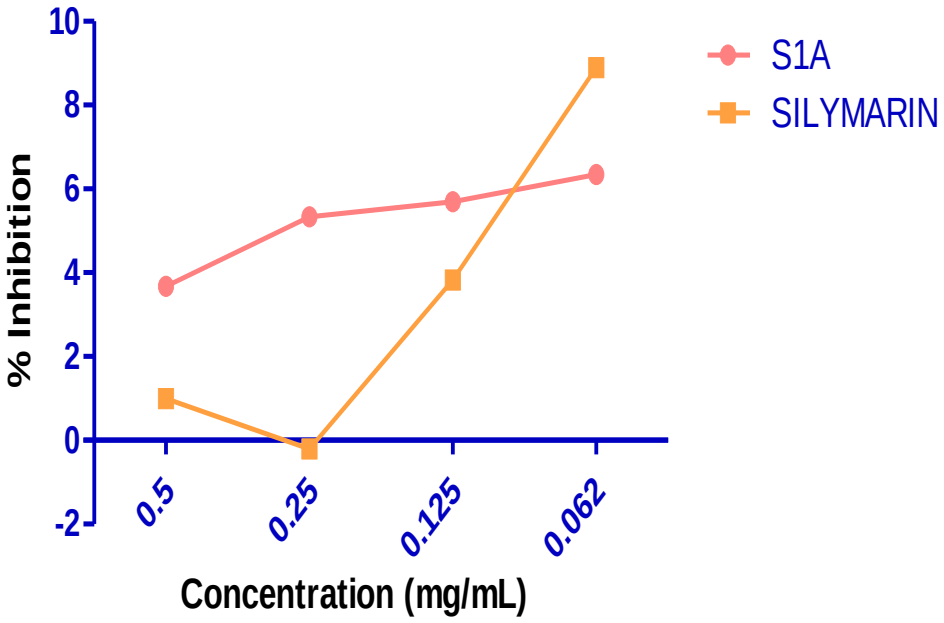


Figure 4.10. Graph showing isolated compound (S1A) and silymarin (standard).

Table 4.10. IC_{50} (mg/mL) of metal chelating capabilities of the isolated compound (S1A)

DPPH	Silymarin	Compound (S1A)
IC_{50}	2.734	5.242
R^2	0.259	0.128
Regression equation	Y = 18.087x – 0.533	Y = 8.0904 + 7.5834

Table 4.11. IC_{50} values of metal chelating with inhibition by ascorbic acid and leaf extracts of *Hermannia geniculata*

IC_{50}	EXTRACT
1.273	Ethyl acetate
1.224	Hexane
0.042	Acetone
0.808	Ethanol
4.862	Ascorbic acid

4.7 ABTS inhibition

At the highest concentration, all extracts exhibited a significantly higher activity except for ascorbic acid (standard) with the least activity. The concentration of 0.75 mg/ml saw ascorbic showing the highest activity compared to all extracts and acetone with the least activity. The concentrations 0.5 mg/ml, 0.25 mg/ml and 0.125 g/ml concentrations saw all extracts and ascorbic acid exhibiting relatively low activity compared to 1.0 mg/ml and 0.75 mg/ml (Fig.4.11).

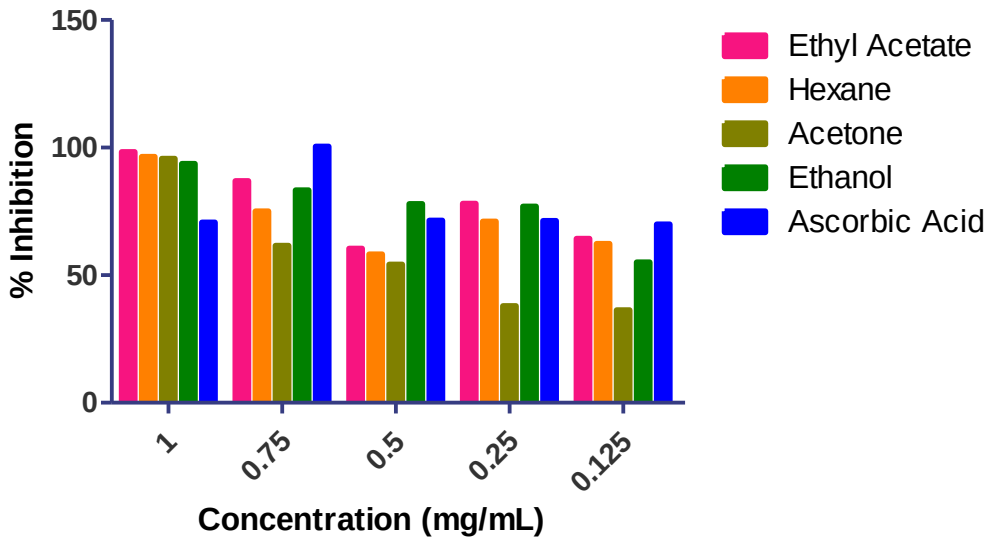


Fig. 4.11. ABTS effects of *Hermannia geniculata* leaf extracts and ascorbic acid (standard).

Table 4.12. IC_{50} values of ABTS with inhibition by ascorbic acid and leaf extracts of *Hermannia geniculata*

IC_{50}	EXTRACT
0.290	Ethyl acetate
0.211	Hexane
0.434	Acetone
0.378	Ethanol
2.521	Ascorbic acid

4.8 Antimycotic activity of the isolated compound (SIA)

The isolated compound (S1A) against strains of fungi showed lower MIC values compared with the fluconazole (positive control). The S1A showed significant activity against two strains, *Candida albicans* HO321 and *Candida albicans* HO325 with the MIC values of 3.25 mg/ml even though this was higher than fluconazole (positive control). All the other fungal strains were inhibited with the MIC value of 6.25 mg/ml. Minimum fungicidal concentration of S1A were significantly lower against *Candida albicans* HO321 and, which HO325 with values of 6.5 mg/ml for both strains. However, all the other strains had MFC values of 13 mg/ml. Fluconazole (positive control) showed highest activity

against *Candida albicans* HO322 with MIC value of 1.25 mg/ml, followed by *Candida albicans* HO325 with the MIC value of 0.313 mg/ml.

4.13 Antimycotic results showing the MIC and MFC of S1A

Strains of fungi	Pure Compound		Flu
	MIC	**MFC**	**MIC**
Candida albicans HO319	6.5	13	0.625
Candida albicans HO320	6.5	13	0.625
Candida albicans HO321	3.25	6.5	0.313
Candida albicans HO322	6.5	13	1.25
Candida albicans HO323	6.5	13	0.625
Candida albicans HO324	6.5	13	0.625
Candida albicans HO325	3.25	6.5	0.313

4.8.1 Bioactive compound isolated from *Hermannia geniculata* (S1A)

The TLC plate with the isolated compound is presented in Figure 4.12. The TLC plate of ethyl acetate fraction, hexane: ethyl acetate (10:90) showed the occurrence of blue spot. This blue spot was deduced to be a compound and this was detected after spraying with P-anisaldehyde reagent and was and viewed under UV-light. At this stage, the compound was not yet pure. As a result, it was further subjected to a ratio of 5:5 (hexane: ethyl acetate) twice for purification.

Thereafter, another TLC plate was prepared, again viewed under UV-light for definite confirmation (Fig.4.12).

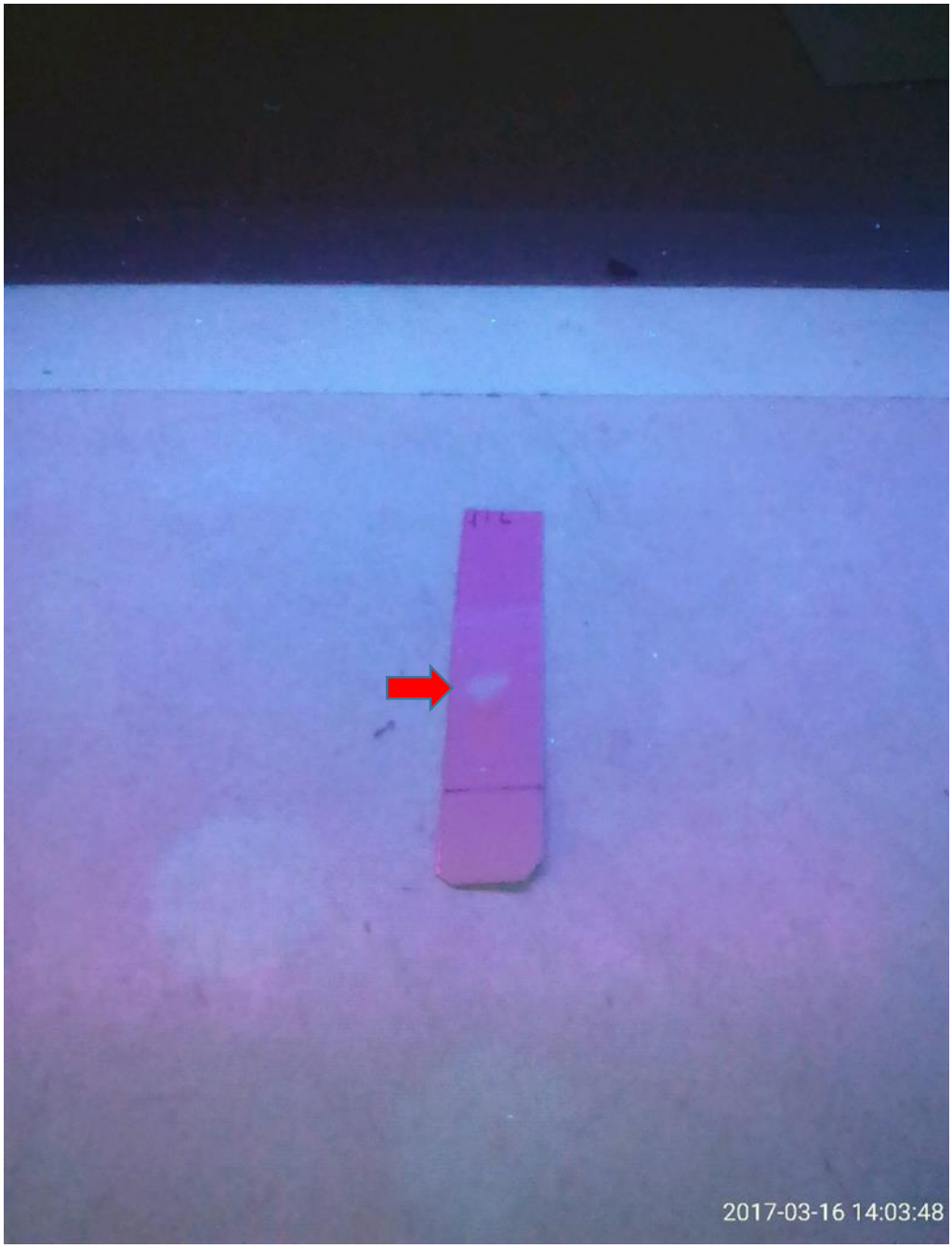
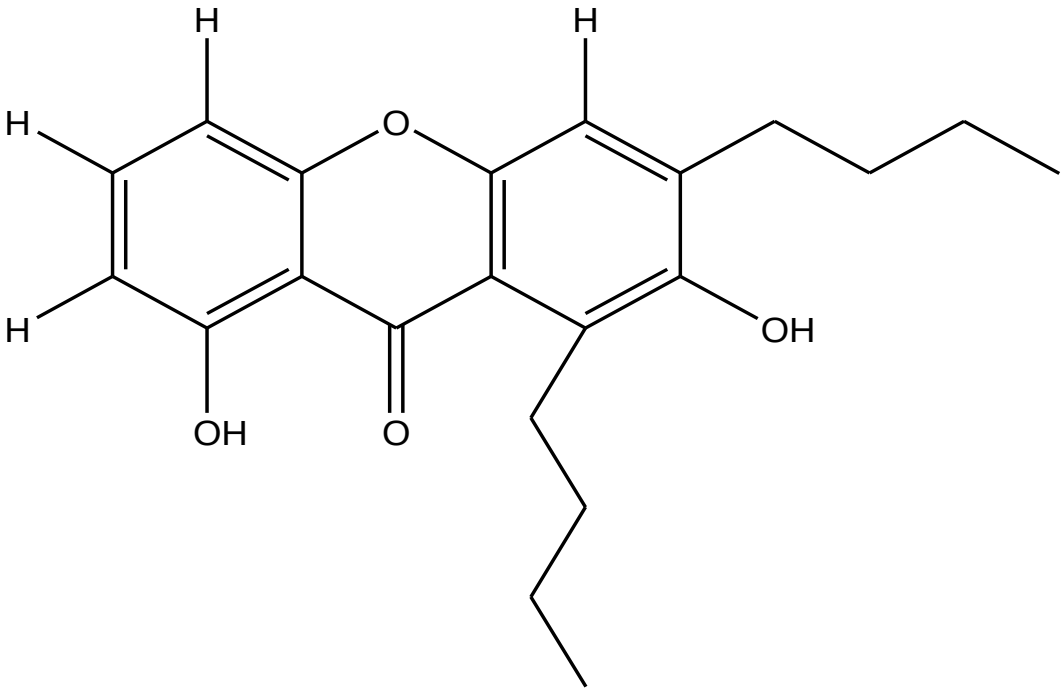


Fig. 4.12 Isolated compound under UV-light.

4.9 Structural elucidation of compound S1A 1,3-dibutyl1-2,8-dihydroxy-9H-xanthen-9-one

1,3-dibutyl1-2,8-dihydroxy-9H-xanthen-9-one (Fig.4.13) was isolated as a single spot which was blue and UV active (visible under UV-light) using ethyl acetate: hexane (90:10) mobile phase. The NMR data obtained was found to confirm this. The accurate mass obtained from the TOFMS spectra confirmed the structure. The mass spectra data obtained showed a pseudo molecular ion at m/z 487.34 and the ESI^+ (positive mode) from the mass spectra showed a pseudo molecular ion at 339 m/z. Supplementary information is given in Appendix 1.



1,3-dibutyl-2,8-dihydroxy-9*H*-xanthen-9-one

Fig. 4.13 The isolated compound structure (S1A)

CHAPTER 5

DICUSSION

5.1 Phytochemical screening

The powdered leaves of *H. geniculata* were extracted with different solvent. The resultant extracts were dried in air until constant weight of the plant extracts were obtained. The plant extracts were then subjected to phytochemical characteristics screening for identification of various phytochemical constituents.

The extracts contained many chemical constituents like alkaloids, glycosides, carbohydrates, proteins, steroids, tannins, saponins, flavonoids etc. These chemical constituents are called as secondary metabolites and are responsible for therapeutic effects (Padmalochana and Rajan, 2014). To confirm the presence or absence of these secondary metabolites in hexane, ethanol, acetone and ethyl acetone extracts were subjected to coloured reactions of chemical tests.

The preliminary phytochemical analysis of hexane extract of *H. geniculata* revealed the presence of alkaloids, flavonoids, phenols and triterpenes and the absence of saponins, anthraquinones and phytosterols. Ethanol extract confirmed the presence of alkaloids, saponins, flavonoids, phenols, phytosterols and triterpenes and absence of anthraquinones. Acetone extract revealed the presence of alkaloids, saponins, triterpenes, phenols, flavonoids and anthraquinones. Only phytosterols were absent. Ethyl acetate presented with the presence of Alkaloids,

flavonoids, phenols, triterpenes and phytosterols. However, anthraquinones and saponins were absent. Diverse tests have been executed to establish the presence of phytochemical constituents in the different solvent derived leaf extracts. Results have shown that each and every phytochemical has the potential to get extracted with different solvents (Pimporn and Srikanjana, 2011). This may differ according to the polarity of the solvent (Arun *et al.*, 2014). From the study, acetone leaf extract has shown that it has extracted most of the compounds.

Saponins provide an array of health benefits such as reducing cholesterol levels in the intestinal tract. Therefore, it helps in lowering the recurrence of obesity and antimutagenicity thus preventing cancer cells from proliferating (Mpofu *et al*., 2014). van Wyk and Wink (2004) reported that saponins possessed antidiabetic, lipid and cholesterol lowering activities. The non-sugar parts of saponins have a direct antioxidant activity; hence, reducing the risk of cardiovascular disorders (Mpofu *et al*., 2014).

Studies have shown that tannins possess multiple biological activities including antioxidant, anticancer and antimicrobial activities (Gin *et al*., 1999; Amarowicz, 2007).

Flavonoids and phenolic acids are the largest classes of plant phenolics and are known to have good antioxidant activity both *in vitro* and *in vivo* (Kasote *et al*., 2015).

Song et al. (2009) reported that flavonoids may preserve beta-cell function by reducing oxidative stress- induced tissue damage. Plants that contain high levels of phenolics are considered to be good sources of antioxidants and therefore it is important to quantify the total phenolics in plants (Gorinstein et al., 2004). All extracts used in this study had high content of phenolic and flavonoid compounds.

According to Li et al. (2010), phenolic compounds possess multiple biological properties such as antitumor, antimutagenic and antibacterial properties. As reported by Mayur et al. (2010), flavonoids are phenolic compounds with antioxidant and antidiabetic potentials due to the presence of hydroxyl groups that confer scavenging ability on them.

The presence of alkaloids in plants do not feature strongly in herbal medicine since they are known to be extremely toxic. However, they have always been important in allopathic systems where the dose is controlled and in homeopathy, where dose-rate is so low as to be harmless (Trease and Evans, 2005). Alkaloids have been reported to show effect on the nervous system, digestive system, blood circulatory system and they can act as anticancer and anti-inflammatory agents (Jacobsen and Salguero, 2003). Various alkaloids such as catharanthine, leurosine and vindoline have been reported to have hypoglycemic effect (Deutschländer, 2009). In this study, alkaloids were found in all leaf extracts of *H. geniculata.*

Similar studies conducted by (Meera, 2009) on the ethyl acetate, butanol and water extract which showed bioconstituents such as amino acids, tannins, phytosterols and cardioglycosides together with have antibacterial, antiviral, antioxidant and anti-inflammatory activity.

5.2 *In vitro* inhibitory effects of *H. geniculata* leaf extracts on α-amylase and α- glucosidase activities

One of the important strategies for the management of diabetes mellitus is to maintain near normal blood glucose levels in fasting and postprandial states (Bailey, 2000; Ortiz- Andrade *et al*., 2007). Management of diabetes without side effects is still posing a challenge in the health sector. Diet rich in carbohydrates causes a sharp increase in the level of blood glucose as the complex carbohydrate in food is rapidly absorbed in the intestine (Kazeem *et al*., 2013). According to Rhabasa-Lhoret and Chiasson (2004), inhibition of the activity of α-amylase and α-glucosidase delay the degradation of carbohydrate, which in turn cause a decrease in the absorption of glucose, and as a result the reduction of postprandial blood glucose level. Acarbose and miglitol are α- glucosidase inhibitors which act competitively and modulate the postprandial digestion and absorption of carbohydrates (Nolte and Karam, 2001; Kim *et al*., 2005). These two compounds have different binding affinities; acarbose and miglitol both target α-

glucosidases: sucrase, maltase, glycoamylase and dextranase. The enzyme (α-amylase) can only be targeted by acarbose while isomaltase and β-glucosidase are targeted by miglitol (Nolte and Karam, 2001). These inhibitors are known to causes gastrointestinal discomfort such as flatulence and diarrhoea (Shai *et al*., 2010).

In this study, the effects of *H. geniculata* on the activities of α-amylase and α-glucosidase were investigated in vitro. The potency of an enzyme inhibitor is usually determined and reported in terms of an IC_{50} value (inhibitor concentration corresponding to 50% inhibition). Low IC_{50} values suggest a higher affinity of the enzyme for the inhibitor. All the extracts (hexane, ethanol, acetone and ethyl acetate investigated in this study demonstrated significant potency in inhibiting the activities of α-amylase and α-glucosidase. The ethyl acetate extract had the strongest inhibitory effect against the α- amylase with IC_{50} of 0.068 µg/mL compared to standard acarbose with 2.282 µg/mL. These results confirm the previous reports which indicated that excessive inhibition of pancreatic *α*-amylase could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon (Apostolidis et al., 2007; Cheplick et al., 2010). Though α-amylase inhibitory activity had positive effects on prevention of hyperglycemia linked to Type II diabetes mellitus, however, mild inhibitory

activity is desirable. Hence, the ethanol extract was found to be the most suitable of the extracts showing mild/moderate activity in inhibiting this enzyme. Ethanol extract had the highest IC_{50} value of 6.071 µg/mL and the reference drug, acarbose was found to be weak inhibitor against α-amylase in this study. This is consistent with earlier reports that either acarbose is described as a very weak inhibitor with an IC_{50} value of about 1 mg/mL or no inhibition of α-amylase (Subramanian *et al*., 2008).

Similarly, the inhibitory activities of the leaf extracts of *H. geniculata* were investigated using α-glucosidase together with maltase and sucrose. Maltase and sucrase are forms of α-glucosidase which catalyse the hydrolysis of maltose and sucrose to their constituent monosaccharides, respectively (Toda *et al*., 2000). In this study, ethanol extract exhibited the strongest activity against α-glucosidase compared to other extracts. The IC_{50} values of this extract on α-glucosidase was lower than those of other extracts and the standard (acarbose). The activity of ethanol extract on α-glucosidase could be attributed to the presence of the phytochemicals present in the extract, thus α-glucosidase can be inhibited by polar compounds. Ethanol extract showed the best activity on α-glucosidase activity but lowest activity on α-amylase.

5.3 *In vitro* antioxidant potential of *H. geniculata* leaf extracts

Antioxidants fight against free radicals and as such protecting the body from different diseases (Khatoon *et al*., 2013). Oxidative damage can be overcomed by many synthetic drugs available but these drugs are associated with adverse side effects (Suwalsky and Avello, 2014). The antioxidant activities of plants may act by preventing the production of free radicals or by scavenging free radicals produced in the body or chelating the transition metal composition (Amic *et al*., 2003). Hence, continuous search and study on the anti-oxidative properties of medicinal plants is imperative and should be afforded dedicated attention. These plant-derived antioxidants include ascorbic acid, carotenoids and phenolic compounds (Ghani, 2003). Ascorbic acid (vitamin C) is a water-soluble micronutrient required for multiple biological functions (Duarte and Lunec, 2005). Gallic acid is an antioxidant which is used as a remote astringent in cases of internal haemorrhage as well as in the treatment of albuminuria and diabetes (Deutschländer, 2009).

The radicals investigated in this study have been implicated in the pathogenesis of Type II diabetes and its associated complications (Maritim et al., 2003). The use of different models for this anti-oxidative studies were supported since a single method cannot give a full evaluation of the anti-oxidative capabilities due

to the involvement of multiple mechanisms in the induction of oxidative stress (Ceriello, 2006).

The antioxidant activity of the extract was determined using the stable DPPH radical. DPPH is a free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule (Auwal and Islam, 2014). The DPPH radical scavenging ability of the extract was based on its ability to decolourize the deep purple colour that was measured from the changes in absorbance (Wettasinghe and Shahidi, 2000). Using this model, the ethyl acetate extract exhibited remarkable activity with the IC_{50} value of 0.199 µg / mL compared to other extracts as well as the standard (ascorbic acid) with IC_{50} value of 2.263 µg / mL. This result was not expected since ascorbic acid (reference drug) is an established anti-oxidative drug for DPPH radicals (Susanti *et al*., 2007).

Muleya *et al*. (2015) reported high activity of acetone extract in DPPH radical scavenging assay. However, results obtained from the current study revealed that acetone extract had the lowest activity in scavenging DPPH radical.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage. They cause reduction of disulfide bonds in proteins specifically fibrinogen resulting in unfolding and scrambled refolding into abnormal spatial configurations (Lipinski, 2011). The hexane extract and the

acetone extracts (with IC_{50} of 0.021 and 0.056 µg/mL respectively) were found to be more effective in hydroxyl radical inhibition than other extracts. As noted by Kazeem and Ashafa (2015), hydroxyl radicals are highly reactive in causing enormous biological damage to any living cell, but this untoward effect may be mitigated by the presence of hexane and acetone extracts of *H. geniculata*.

Humans are unable to eliminate iron released from the breakdown of transfused red blood cells and thus the excess of this iron is deposited in the liver, spleen and endocrine organs. The accumulation of the toxic metal ion causes tissue damage and leads to various complications such as heart failure, endocrine abnormalities like diabetes, liver failure hypothyroidism and ultimately death (Loukopoulos, 2005; Taher *et al*., 2006). In this study, the acetone and ethanol extracts were found to be the most active extracts which effectively interfered with the formation of ferrous and ferrozine complex, thus suggesting that it has chelating activities and captures ferrous before ferrozine formation (Enein et al., 2003). The ability of ethanol extract to successfully interfere with the formation of ferrous and ferrozine complex as noted by Enein et al. (2003) can assist in removal of toxic ions formed that causes damages and other complications which leads to diabetes mellitus (Taher et al., 2006).

The antioxidant activity of the extracts is strongly dependent on the types of solvent used because compounds with different polarity exhibit different degrees of antioxidants potential (Kumaran, 2007; Caunii *et al*., 2012). It is obvious from the results of this study that the extracts with non-polar and polar compounds showed radical scavenging activities. Our assertions are such that it may be logically inferred that *H. geniculata* is endowed with promising antioxidant constituents that protect against a wide range of free radical-induced diseases including diabetes mellitus.

5.4 *In vitro* 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) ABTS potential of *H. geniculata* leaf extracts

This method permits the measurement of antioxidant activity of mixtures of substances and hence helps to distinguish between additive and synergistic effects. The assay was based on interaction between antioxidant and ABTS radical cation (ABTS) which has a characteristic color showing maxima at 645, 734 and 815 nm (Rice-Evans and Miller, 1994).

ABTS assay measures the relative ability of antioxidant to scavenge the ABTS•+ generated in aqueous phase, as compared with a Trolox (water soluble vitamin E analogue) standard. The ABTS•+ is generated by reacting with a strong oxidizing

agent (e.g., potassium permanganate or potassium persulfate) the ABTS salt. The reduction of the blue-green ABTS•+ by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum, during this reaction, the blue ABTS radical cation is converted back to its colorless neutral form (Rice-Evans *et. al*, 1996).

The method is rapid and can be used over a wide range of pH values, which is useful in studying the effect of pH on antioxidant mechanisms. Furthermore, the ABTS•+ radical is stable and soluble in water and organic solvents, enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds/ samples. It also has good repeatability and simple to perform; hence, it is widely reported. However, as the results obtained for samples are related to an antioxidant standard compound that shows different kinetic behavior, the results provided by this assay are dependent of time of analysis.

ABTS assay is frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods (Alam *et. al*, 2013).

The ethyl acetate showed significant activity with the lowest IC_{50} of 0.077 µg/mL Followed by ethanol with 0.111 µg/mL. The standard (Ascorbic acid) was the least active at 0.088 µg/mL. The activity of ethanol may be attributed to the fact that is safer and less toxic as compared to acetone, methanol and other organic solvent. This has been corroborated by Chew *et. al.* (2011) where ethanol

concentration had significant effect ($p<0.05$) on both phenolic contents (TPC, TFC and CTC) and antioxidant capacities (ABTS and DPPH) of crude extract.

5.5 *In vitro* inhibitory effects of the active compound (S1A) isolated from *H. geniculata* on α-amylase and α-glucosidase activities

The activity of the compound was determined by *in vitro* α-amylase and α-glucosidase assays. The isolated compound showed a significant activity with the IC_{50} value lower than that of all the extracts and the acarbose standard. However, this was followed closely by ethyl acetate, which proved to be the best candidate extracted that was consequently used for isolation of S1A compound.

For α-glucosidase, again the isolated compound showed better activity with low values compared to acarbose which is a commercial antidiabetic drug. This activity was much better than that of the extracts

5.6 *In vitro* antioxidant effects of isolated compound of *Hermannia geniculata*

5.6.1 DPPH capabilities of the isolated compound (S1A) against silymarin

The isolated compound showed a significant activity against silymarin (standard antioxidant agent). However, silymarin showed highest activity compared to all extracts followed by ethanol extract.

5.6.2 Hydroxyl radical capabilities of the isolated compound (S1A) against ascorbic acid

The isolated compound exhibited significantly higher activity compared to acarbose (acarbose: a standard antidiabetic drug) with IC_{50}value of 0.474 compared to acarbose at 8.875.

5.6.3 Metal chelating capabilities of the isolated compound (S1A)

The isolated compound was less active compared to silymarin (standard) with high IC_{50} value. However, all the other extracts showed higher activity compared to silymarin.

5.7 Antimycotic activity of the isolated compound (SIA)

The isolated compound (S1A) against strains of fungi showed lower MIC values compared with the fluconazole (positive control). The S1A showed significant activity against two strains, *Candida albicans* HO321 and *Candida albicans* HO325 with the MIC values of 3.25 mg/ml even though this was higher than fluconazole (positive control). All the other fungal strains were inhibited with the MIC value of 6.25 mg/ml. Minimum fungicidal concentration of S1A were significantly lower against *Candida albicans* HO321 and, which HO325 with values of 6.5 mg/ml for both strains. However, all the other strains had MFC

values of 13 mg/ml. Fluconazole (positive control) showed highest activity against *Candida albicans* HO322 with MIC value of 1.25 mg/l, followed by *Candida albicans* HO325 with the MIC value of 0.313 mg/ml. Even though *H. geniculata* plant activity against fungal strains has been explored, the isolated compound holds a promising prospect in the antimycological studies to further ascertain if the S1A compound is an active ingredient or it has to be synergistically used with other compounds to achieve the anticipated goals.

5.8 Structural elucidation of compound S1A 1,3-dibutyl1-2,8-dihydroxy-9H-xanthen-9-one

The isolated compound S1A can be regarded as a novel compound on the basis that it is the first to be isolated form *Hermannia geniculata* plant leaf and no other study on isolation of compound from the leaf of this plant has been elucidated to date. This milestone achievement highlights the mammoth task that lies ahead for researchers to unravel the mystery of the potential this plant holds and also discover if there are other compounds plant that may be employed in curing and managing many diseases in the world.

CHAPTER 6

CONCLUSION

Microorganisms are the concealed enemies to the mankind. They are small but cause a very profound damage in human body as well as other living organisms. These agents, which have capacity to kill microbes or minimize their multiplication, are called the antimicrobial agents or drugs. The last two decades have seen an increase in the investigations of plants as sources of human disease management (Dash *et al*., 2011) and more natural antimicrobials have driven scientists to investigate the effectiveness of inhibitory compounds such as extracts from medicinal plants (Nasar-Abbas and Halkman, 2004)

The fields of ethnopharmacology and ethnobotany, is progressing steadily in South Africa. Ethnopharmacologists, botanists and microbiologists are searching the earth for phytochemicals which could be developed for the treatment of infectious diseases (Tanaka *et al*., 2006) especially in light of the emergence of drug-resistant microorganisms and the need to produce more effective agents against microorganisms.

It has been shown that *in vitro* screening methods could provide the needed fundamental observations necessary to select crude plant extracts with potentially

useful properties for further chemical and pharmacological investigations (Mathekaga and Meyer, 1998).

Modern scientific evaluation of plants and herbs is mainly focused on validating the traditional use of plants and identifying the active components of extracts and preparations. Nevertheless, continuous examination of traditional plant medicines is required to establish the scientific basis for activity, efficacy and safety (Palombo, 2006).

In this study, it was shown that antidiabetic, antioxidant and antifungal properties of *H. geniculata* cannot be overlooked. This was seen with the ability of *H. geniculata* to successfully inhibit the growth of microorganisms of human pathogenic importance with its different leaf extracts and the isolated compound. Since this plant is used in the traditional Basotho medicine for different stomach ailments, the results of this study validates that this plant holds hope.

CHAPTER 7

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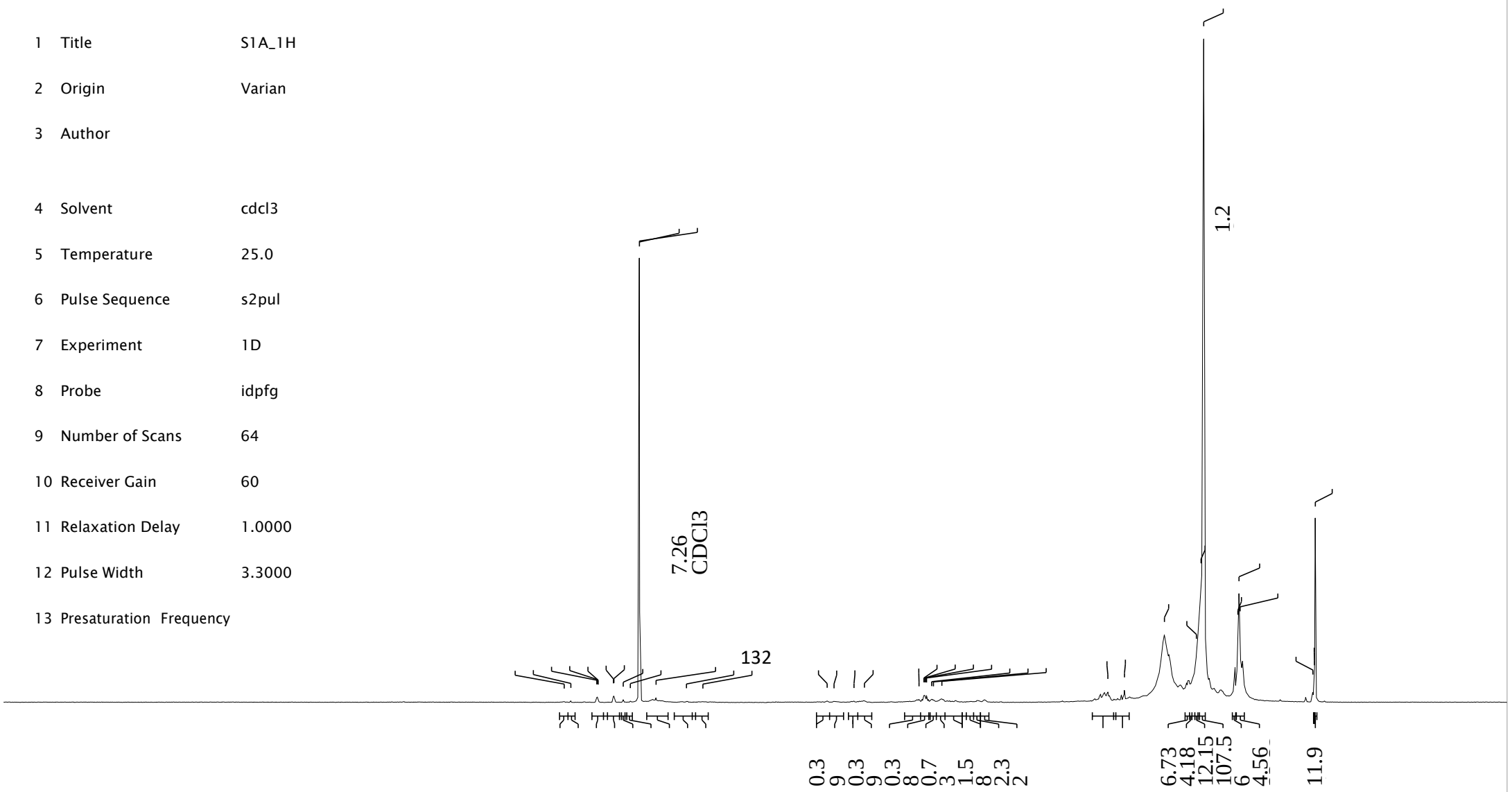
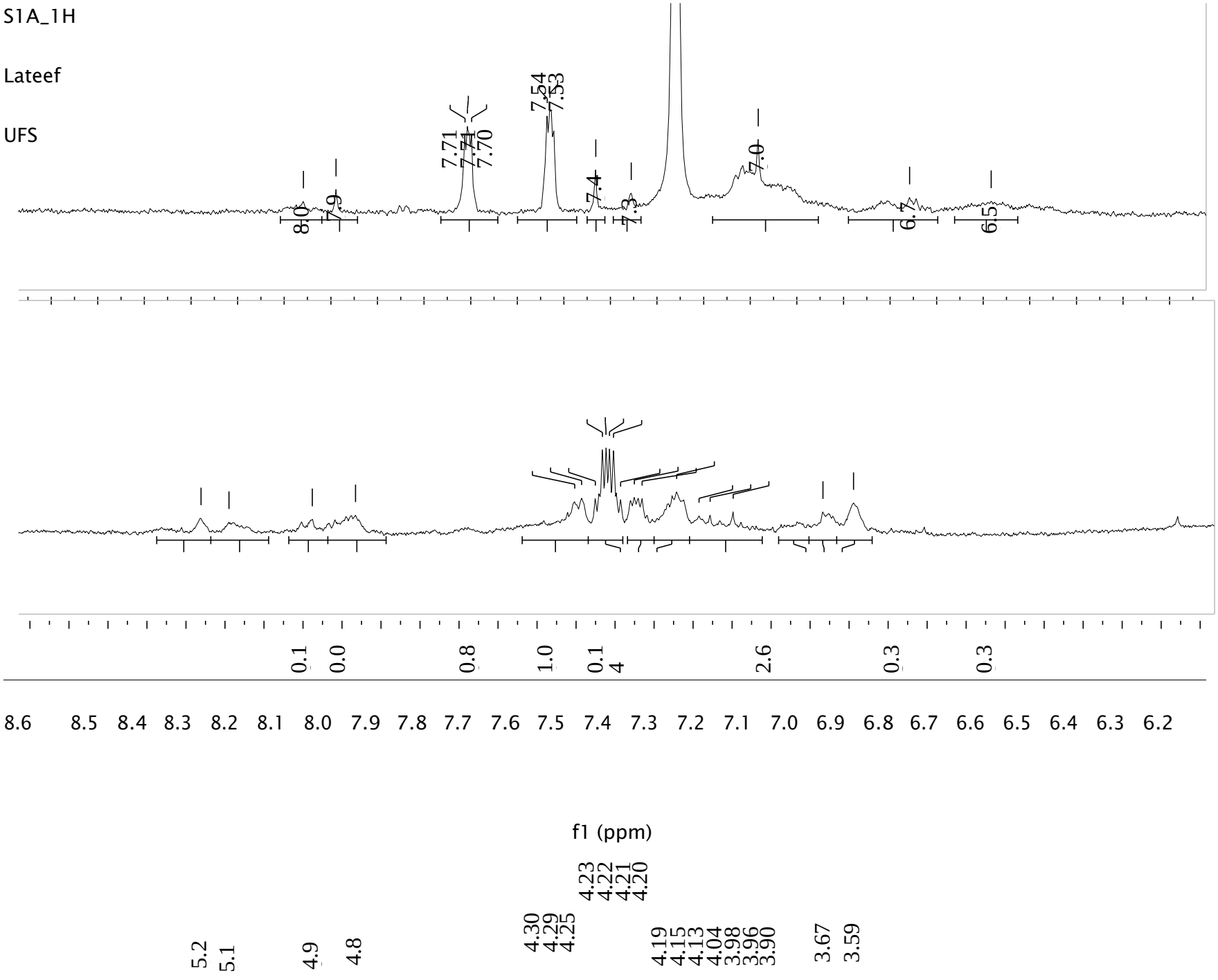
APPENDICES Compound SIA ^{1}H-NMR, C^{13}, COSY, HSQC and HMBC

	Parameter	Value
1	Title	S1A_1H
2	Origin	Varian
3	Author	
4	Solvent	cdcl3
5	Temperature	25.0
6	Pulse Sequence	s2pul
7	Experiment	1D
8	Probe	idpfg
9	Number of Scans	64
10	Receiver Gain	60
11	Relaxation Delay	1.0000
12	Pulse Width	3.3000
13	Presaturation Frequency	



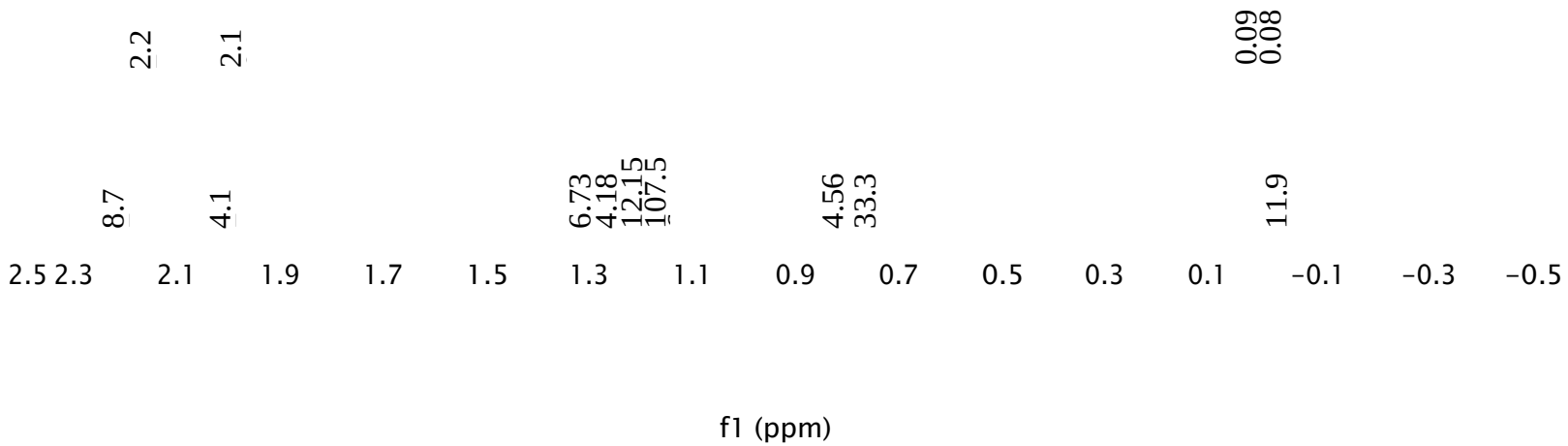
14	Acquisition Time	1.7072
15	Acquisition Date	2017-06-14T11:08:35
16	Modification Date	2017-06-14T11:09:47
17	Spectrometer Frequency	599.99
18	Spectral Width	9596.9
19	Lowest Frequency	-1182.9
20	Nucleus	1H
21	Acquired Size	16384
22	Spectral Size	65536
23	Absolute Reference	

8.06
7.99
7.71
7.71
7.70
7.54
7.53
7.43
7.36
7.08
6.76
6.58
5.2
6
5.1
9
4.29
4.23
4.22
4.21
4.20
4.15
4.13
4.04
2.2
7
1.67
1.33
1.2
0.89
0.88
0.87
0.09
0.08
0.0



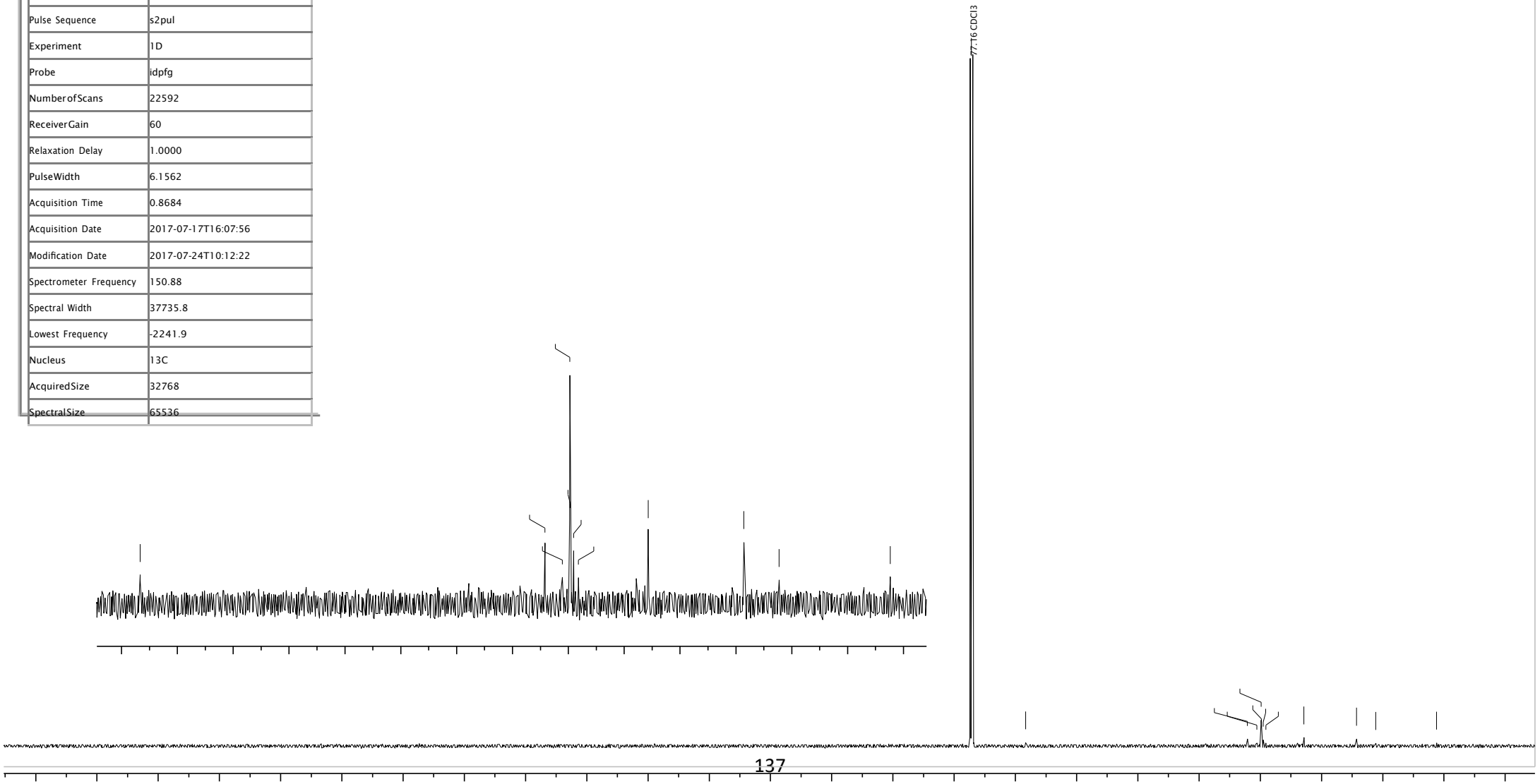
	ppm	Hz
1	8.06	4835.3
2	7.99	4793.2
3	7.71	4627.2
4	7.71	4623.9
5	7.70	4618.5
6	7.54	4521.3
7	7.53	4518.0
8	7.43	4458.8
9	7.36	4413.0
10	7.26	4355.8
11	7.08	4249.7
12	6.76	4054.8
13	6.58	3949.7
14	5.26	3156.6
15	5.19	3113.7
16	4.98	2985.5
17	4.87	2919.1
18	4.30	2582.0
19	4.29	2571.3
20	4.25	2550.4
21	4.23	2539.3
22	4.22	2533.7
23	4.21	2528.4
24	4.20	2522.2
25	4.19	2511.2
26	4.15	2490.2
27	4.13	2478.2
28	4.04	2425.0
29	3.98	2390.6
30	3.96	2373.5
31	3.90	2338.2
32	3.67	2200.1
33	3.59	2152.8
34	2.27	1364.7
35	2.10	1259.4
36	1.67	1004.1
37	1.62	974.8
38	1.50	900.9
39	1.44	861.8
40	1.42	854.1
41	1.41	846.4
42	1.40	838.2
43	1.33	799.4
44	1.28	770.4
45	1.26	753.0
46	1.20	718.1
47	1.19	711.8
48	0.93	559.9
49	0.92	552.5
50	0.91	544.9
51	0.89	534.8
52	0.88	527.9
53	0.87	520.8
54	0.86	513.3
55	0.84	504.4
56	0.09	55.8
57	0.08	46.5
58	0.07	41.2

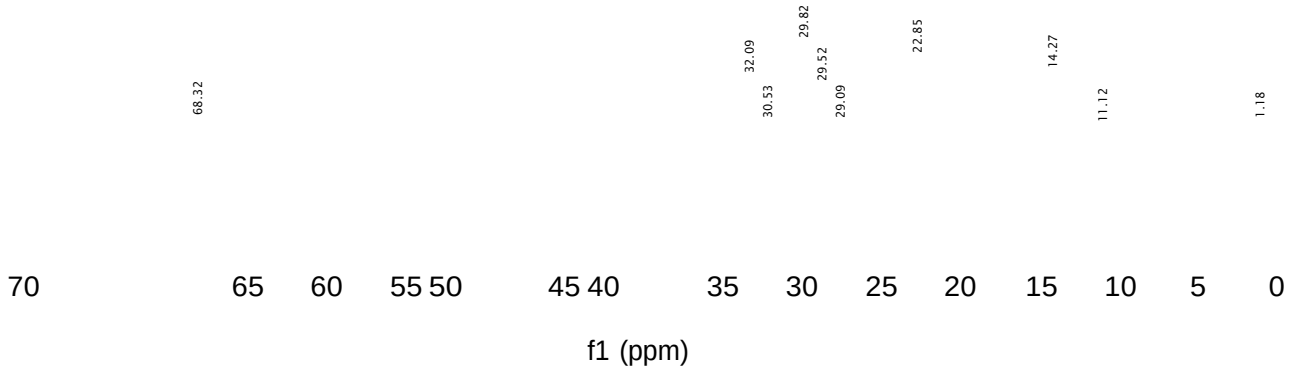
0.3
0.3
0.3
0.7
1.5
2.3
2
1.0
1.0
0.3
7
0.5
5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0 2.9 2.8 2.7
f1 (ppm)
1.67
1.62
1.50
1.44
1.42
1.41
1.40
1.33
1.2
1.20
1.19
0.93
0.92
0.91
0.89
0.88
0.87
0.86
0.84
0.0

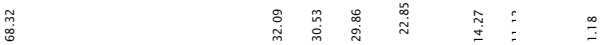

2.2
2.1
0.09
0.08
8.7
4.1
6.73
4.18
12.15
107.5
4.56
33.3
11.9
2.5 2.3 2.1 1.9 1.7 1.5 1.3 1.1 0.9 0.7 0.5 0.3 0.1 −0.1 −0.3 −0.5
f1 (ppm)

^{13}C NMR (151 MHz, cdcl$_3$) δ 68.32, 32.09, 30.53, 29.86, 29.82, 29.52, 29.09, 22.85, 14.27, 11.12, 1.18.

Parameter	
Title	S1A_13C
Comment	STANDARD PROTON PARAMETERS
Origin	Varian
Instrument	inova
Solvent	cdcl3
Temperature	25.0
Pulse Sequence	s2pul
Experiment	1D
Probe	idpfg
NumberofScans	22592
ReceiverGain	60
Relaxation Delay	1.0000
PulseWidth	6.1562
Acquisition Time	0.8684
Acquisition Date	2017-07-17T16:07:56
Modification Date	2017-07-24T10:12:22
Spectrometer Frequency	150.88
Spectral Width	37735.8
Lowest Frequency	-2241.9
Nucleus	13C
AcquiredSize	32768
SpectralSize	65536

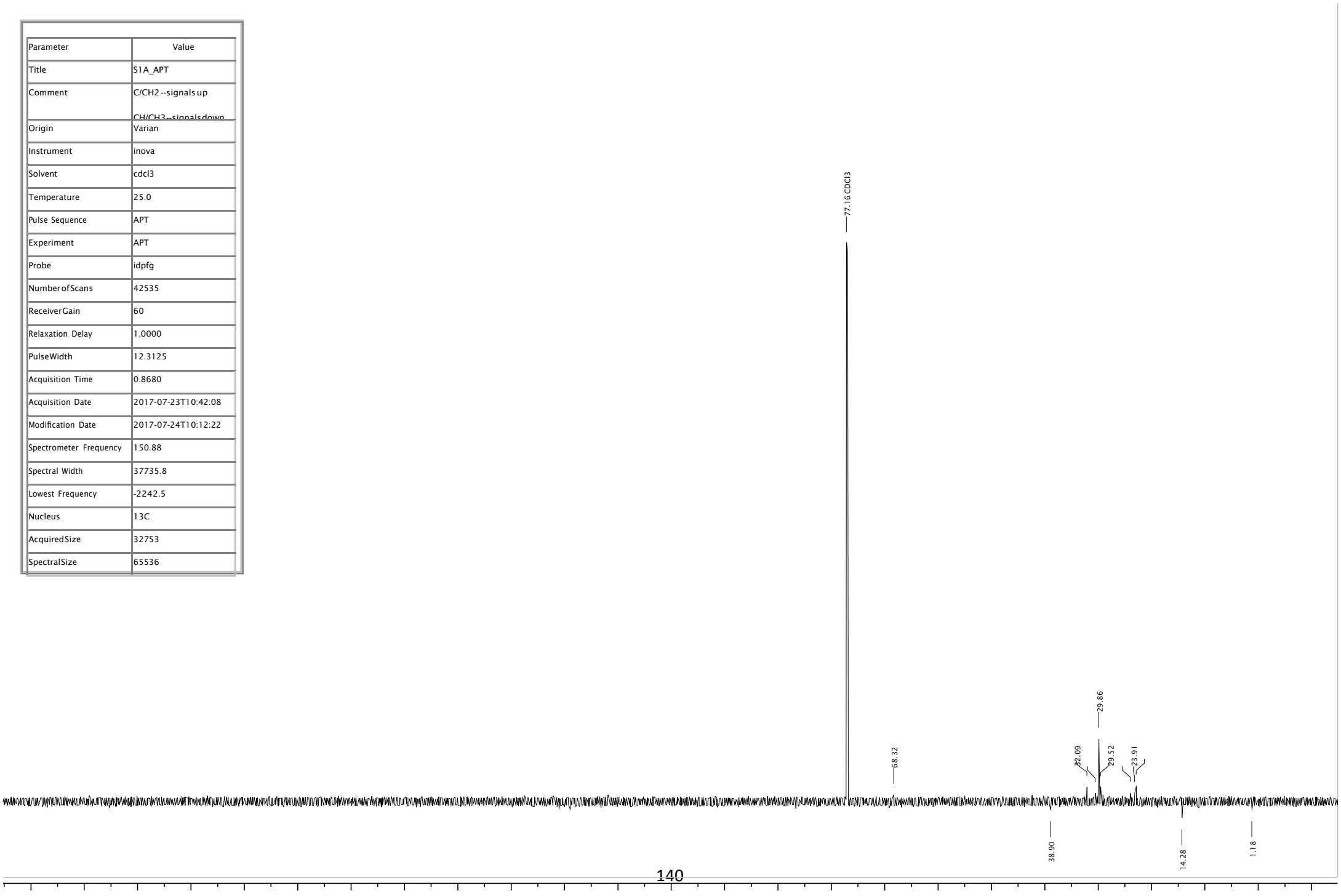



68.32
32.09
30.53
29.82
29.52
29.09
22.85
14.27
11.12
1.18
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
f1 (ppm)


68.32
32.09
30.53
29.86
22.85
14.27
1.18

29.86

Parameter	Value
Title	S1A_APT
Comment	C/CH2 --signals up CH/CH3--signals down
Origin	Varian
Instrument	inova
Solvent	cdcl3
Temperature	25.0
Pulse Sequence	APT
Experiment	APT
Probe	idpfg
Number of Scans	42535
Receiver Gain	60
Relaxation Delay	1.0000
Pulse Width	12.3125
Acquisition Time	0.8680
Acquisition Date	2017-07-23T10:42:08
Modification Date	2017-07-24T10:12:22
Spectrometer Frequency	150.88
Spectral Width	37735.8
Lowest Frequency	-2242.5
Nucleus	13C
Acquired Size	32753
Spectral Size	65536



S1A_COSY

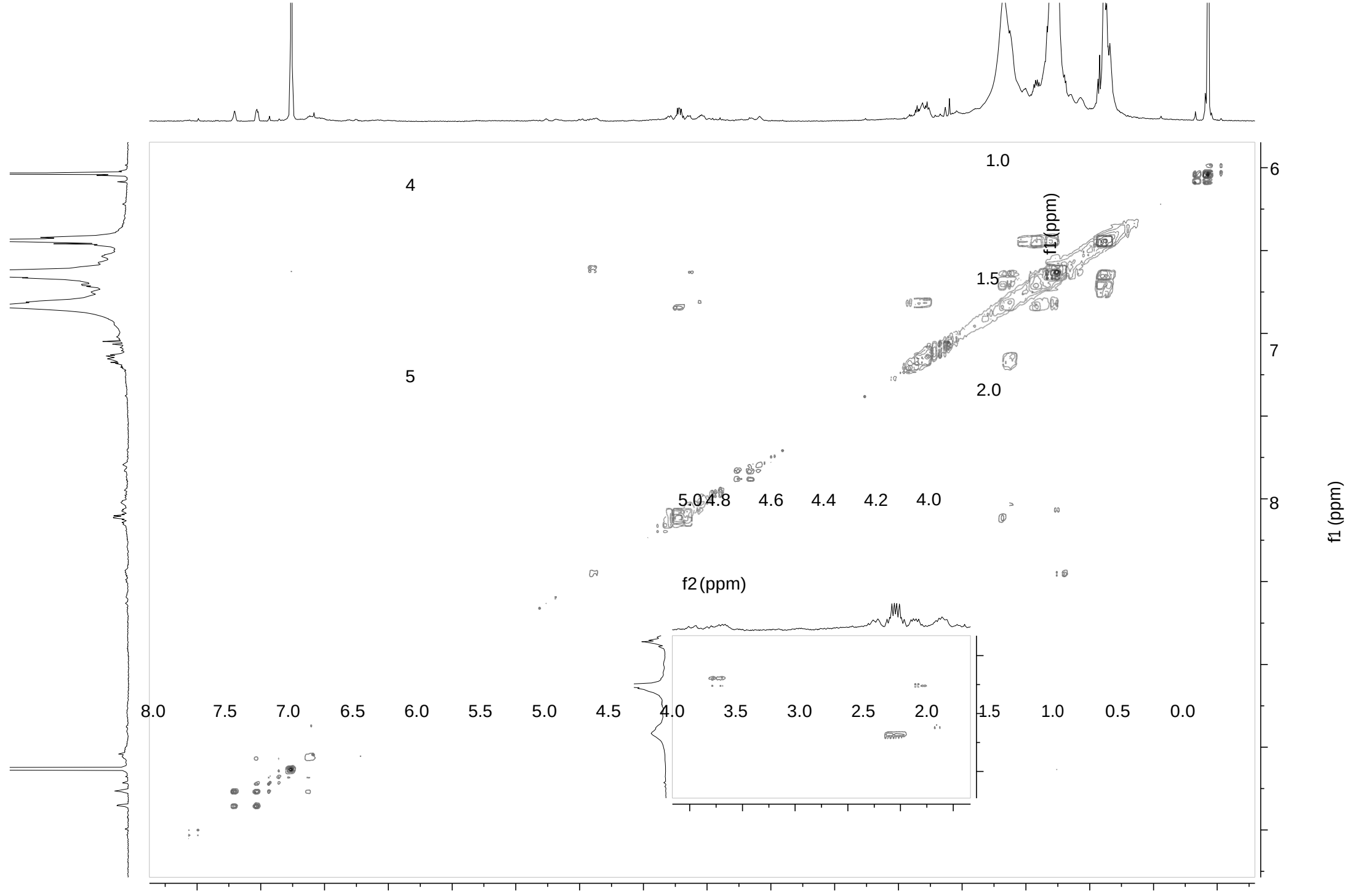
STANDARD PROTON PARAMETERS

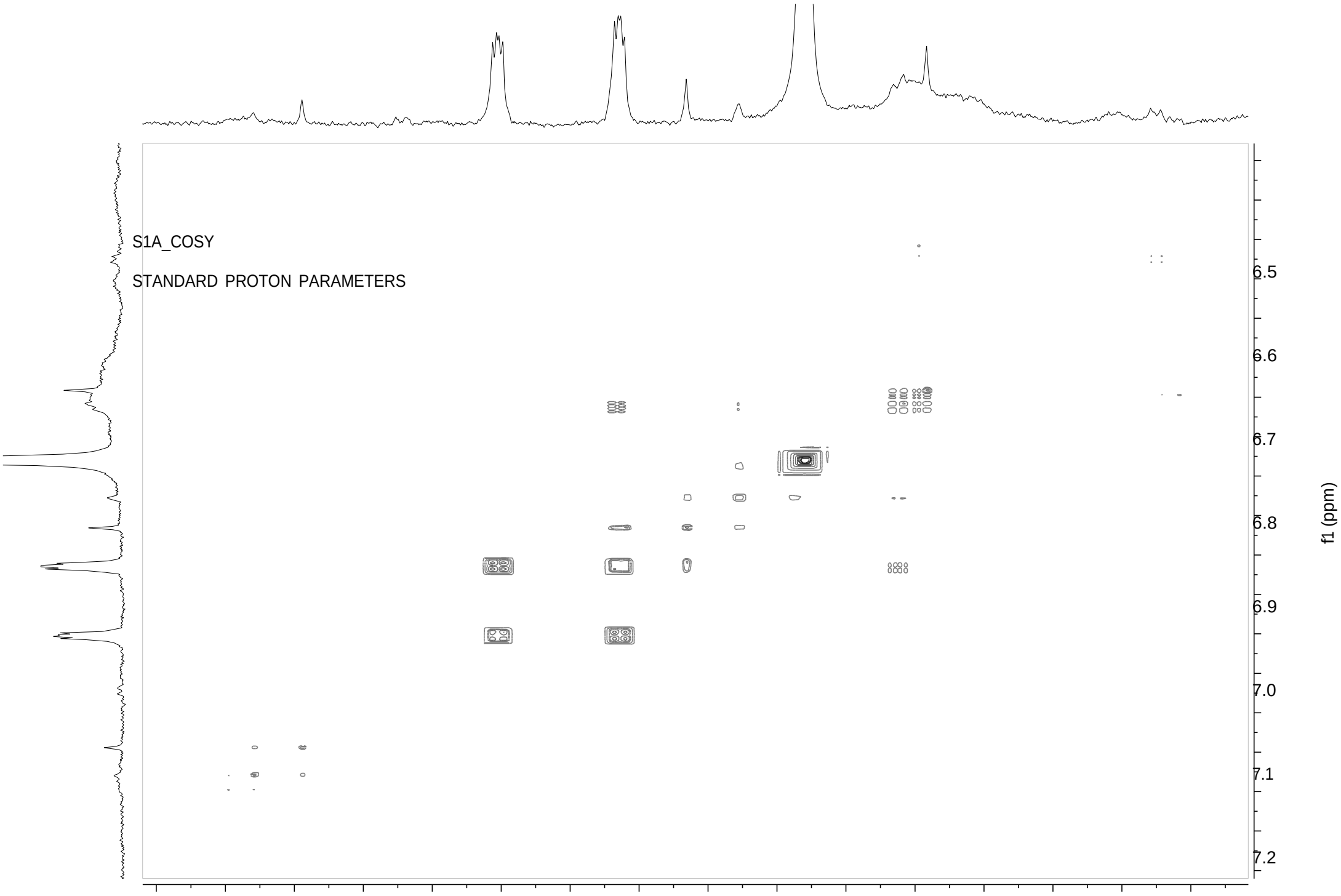
0

1

2

3


f1 (ppm)
f2 (ppm)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4
5
6
7
8
1.0
1.5
2.0
5.0
4.8
4.6
4.4
4.2
4.0


S1A_COSY
STANDARD PROTON PARAMETERS
6.5
6.6
6.7
6.8
6.9
7.0
7.1
7.2
f1 (ppm)

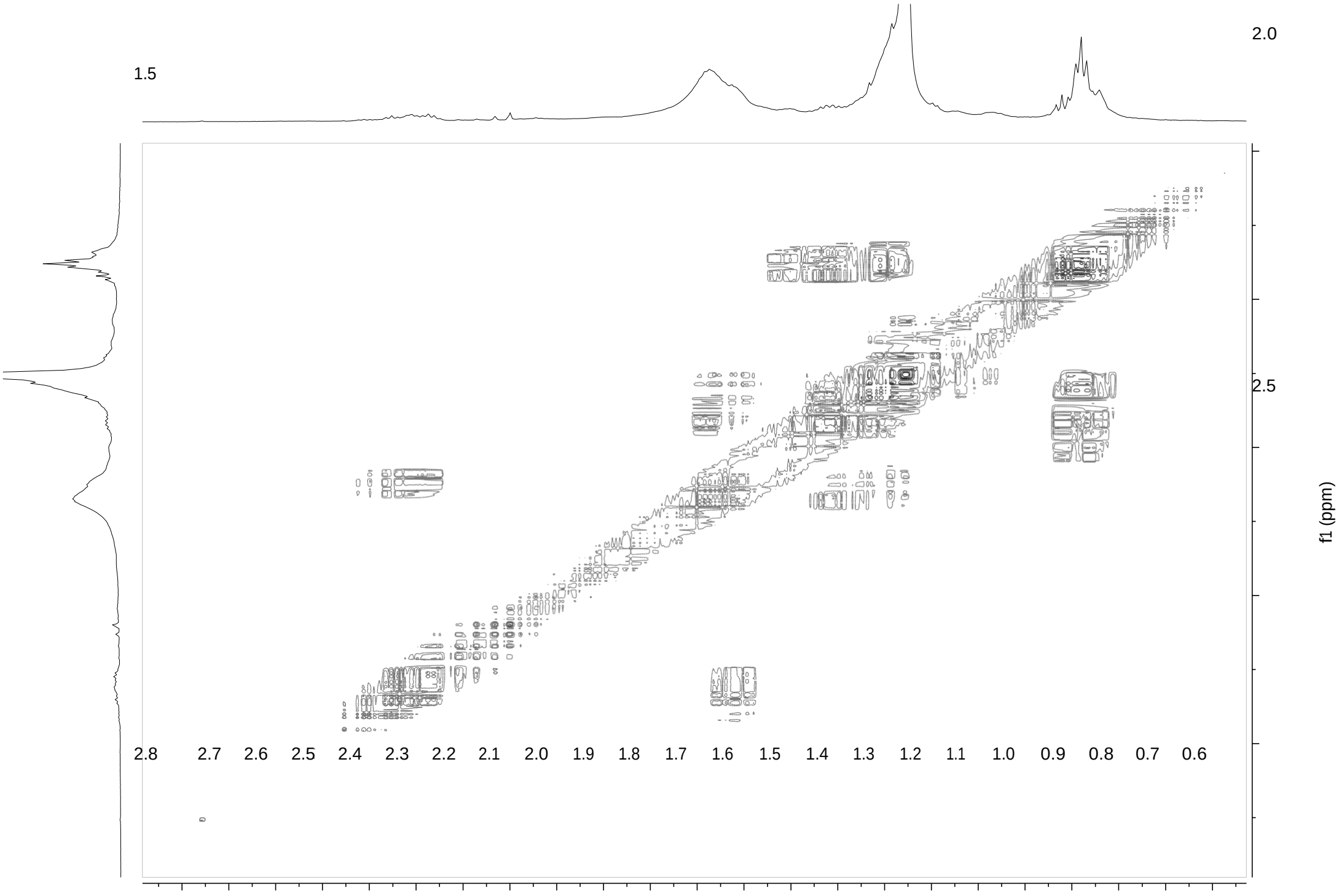

7.3
8.3
7.4
7.5
7.6
7.7
7.8
7.9
8.0
8.1
8.2

0.5

S1A_COSY

STANDARD PROTON PARAMETERS

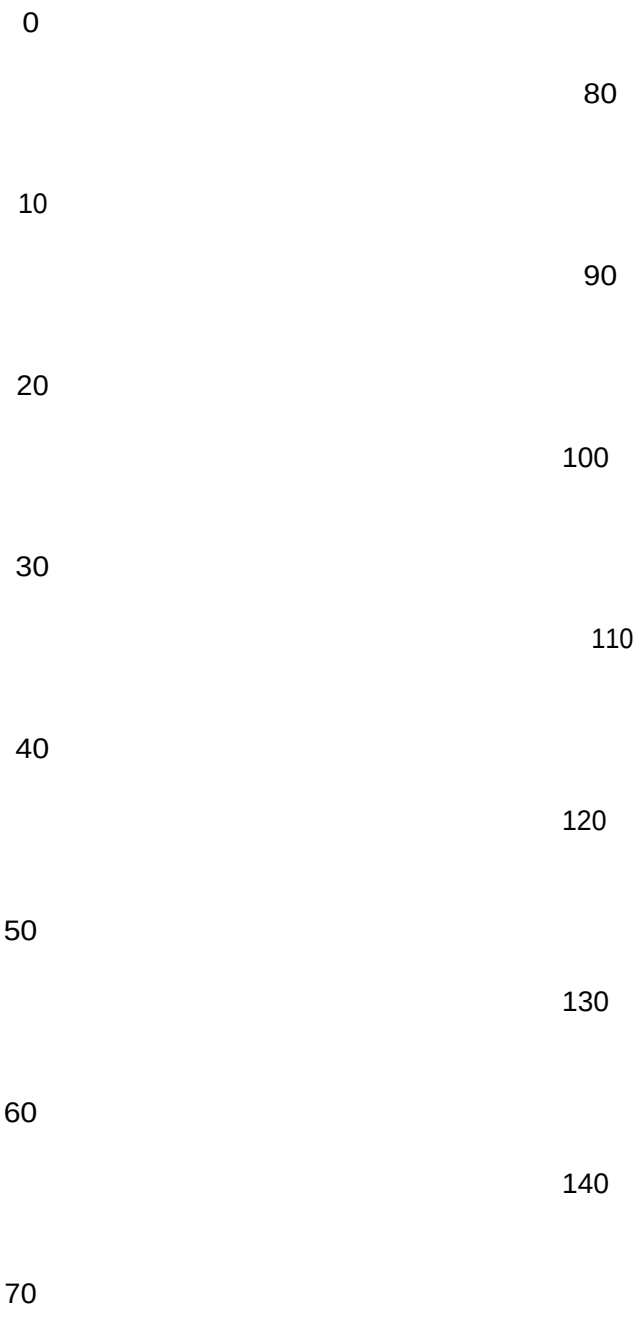
1.0

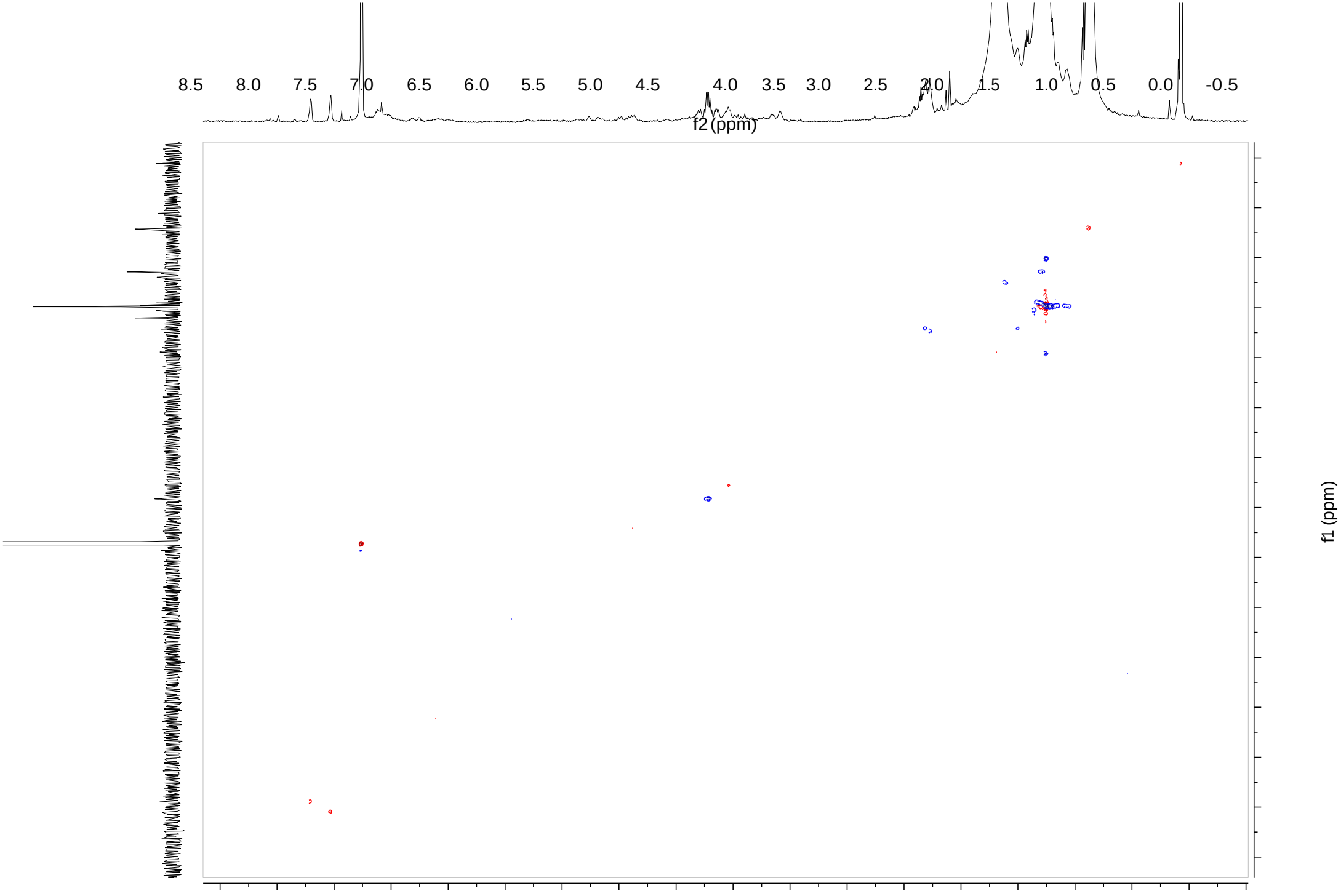

1.5
2.0
2.5
f1 (ppm)
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6

S1A_HSQC

STANDARD PROTON PARAMETERS

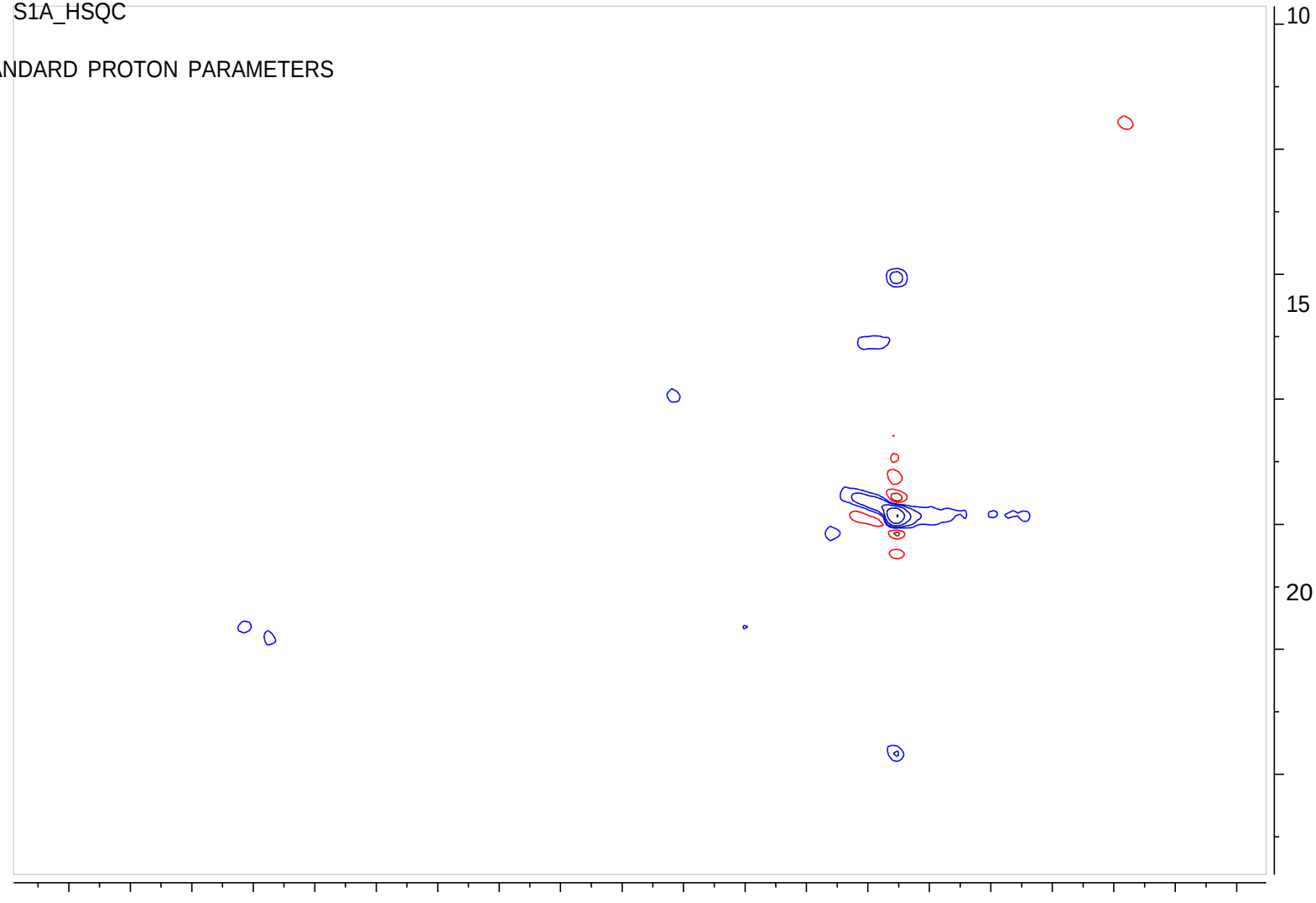
Red = CH/CH3 Blue = C/CH2




8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f2 (ppm)
f1 (ppm)

S1A_HSQC

STANDARD PROTON PARAMETERS

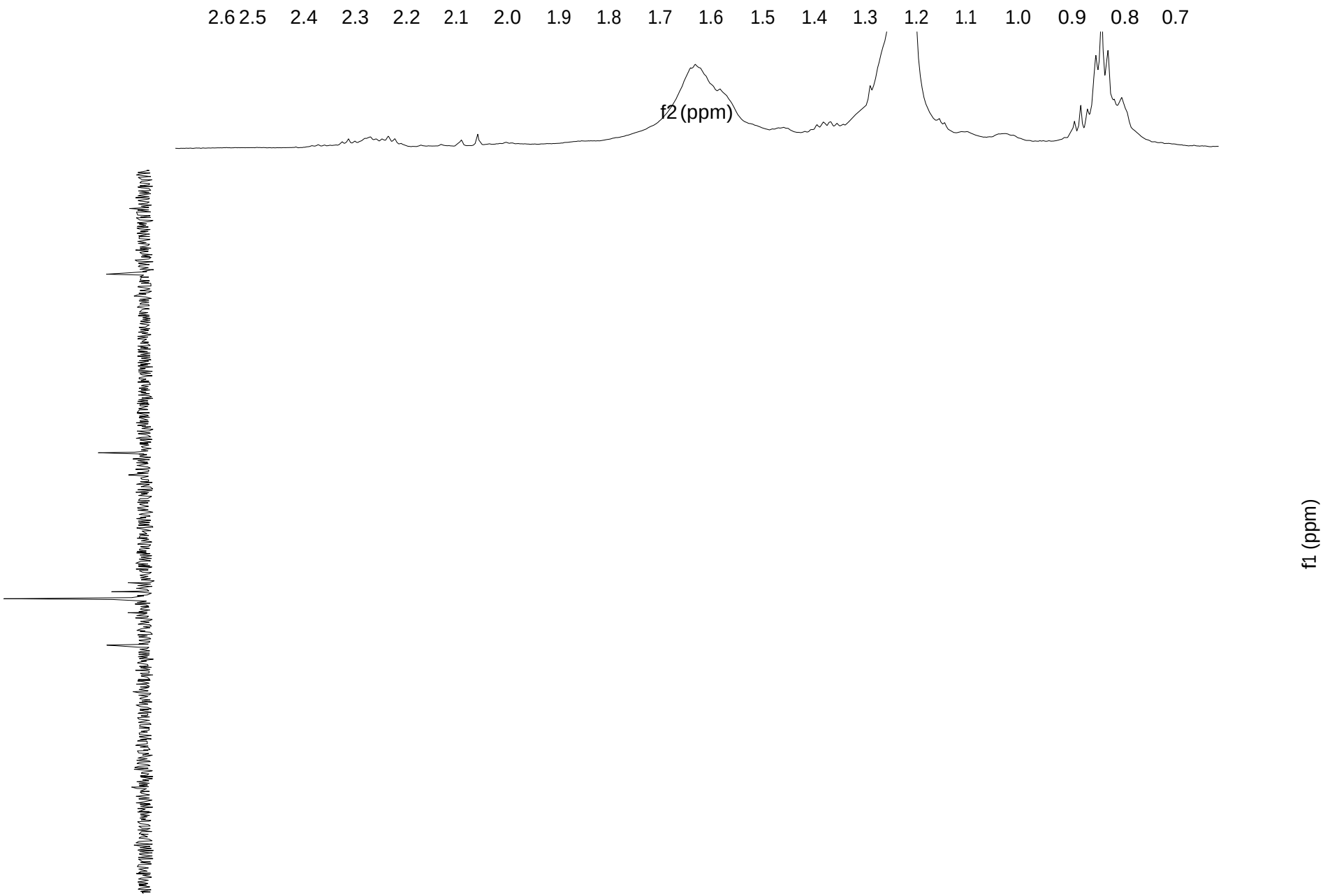


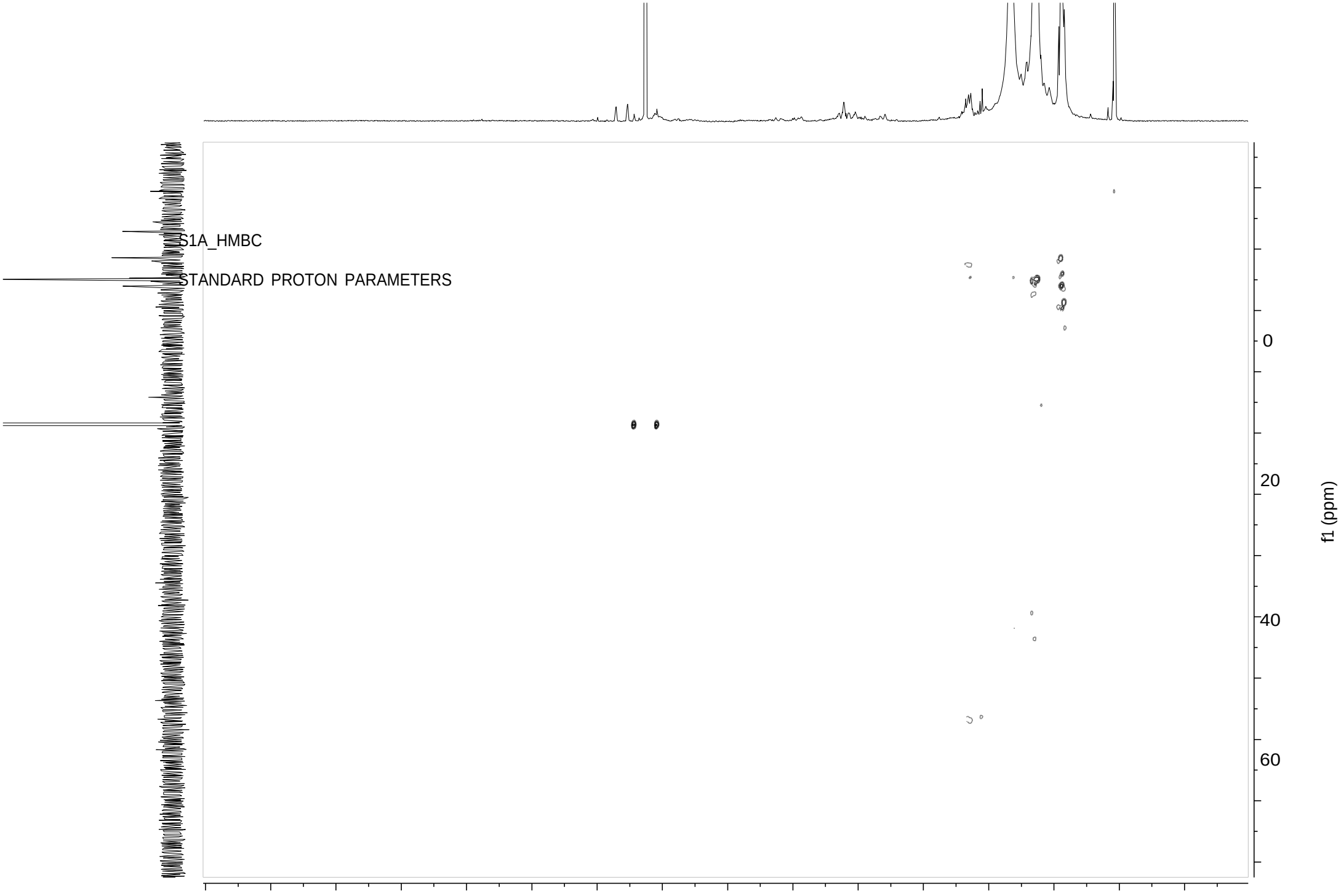
25

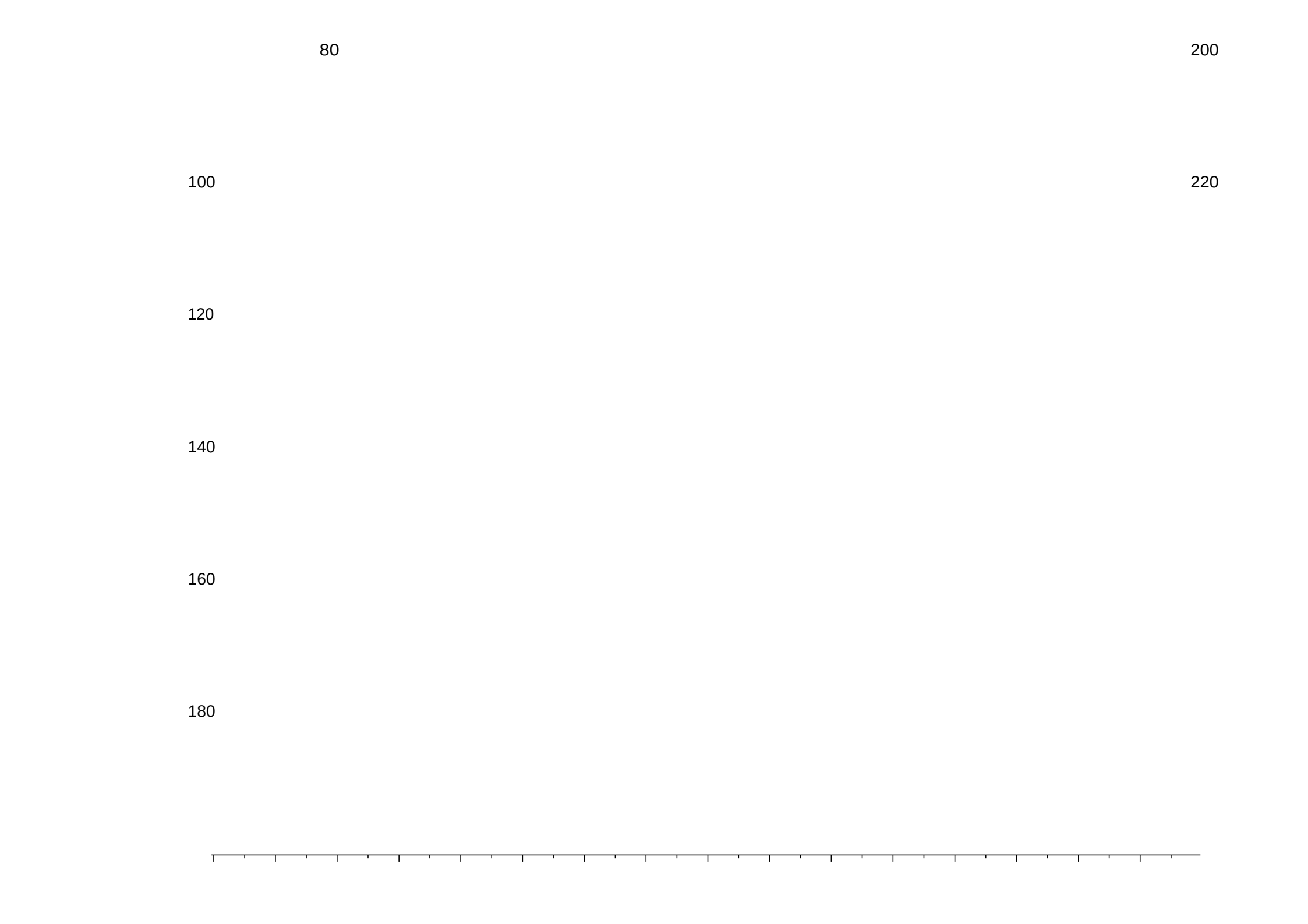
30

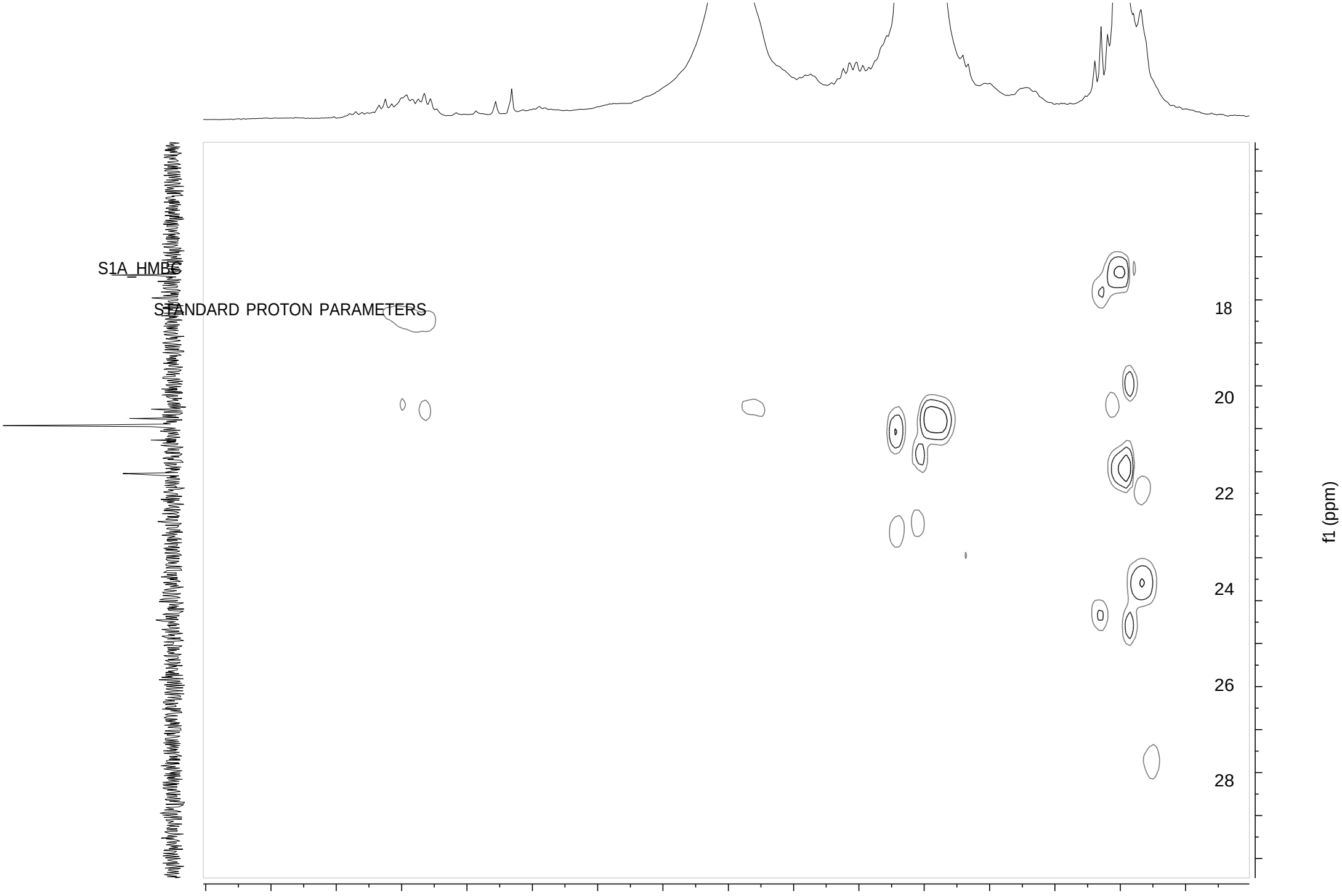
35

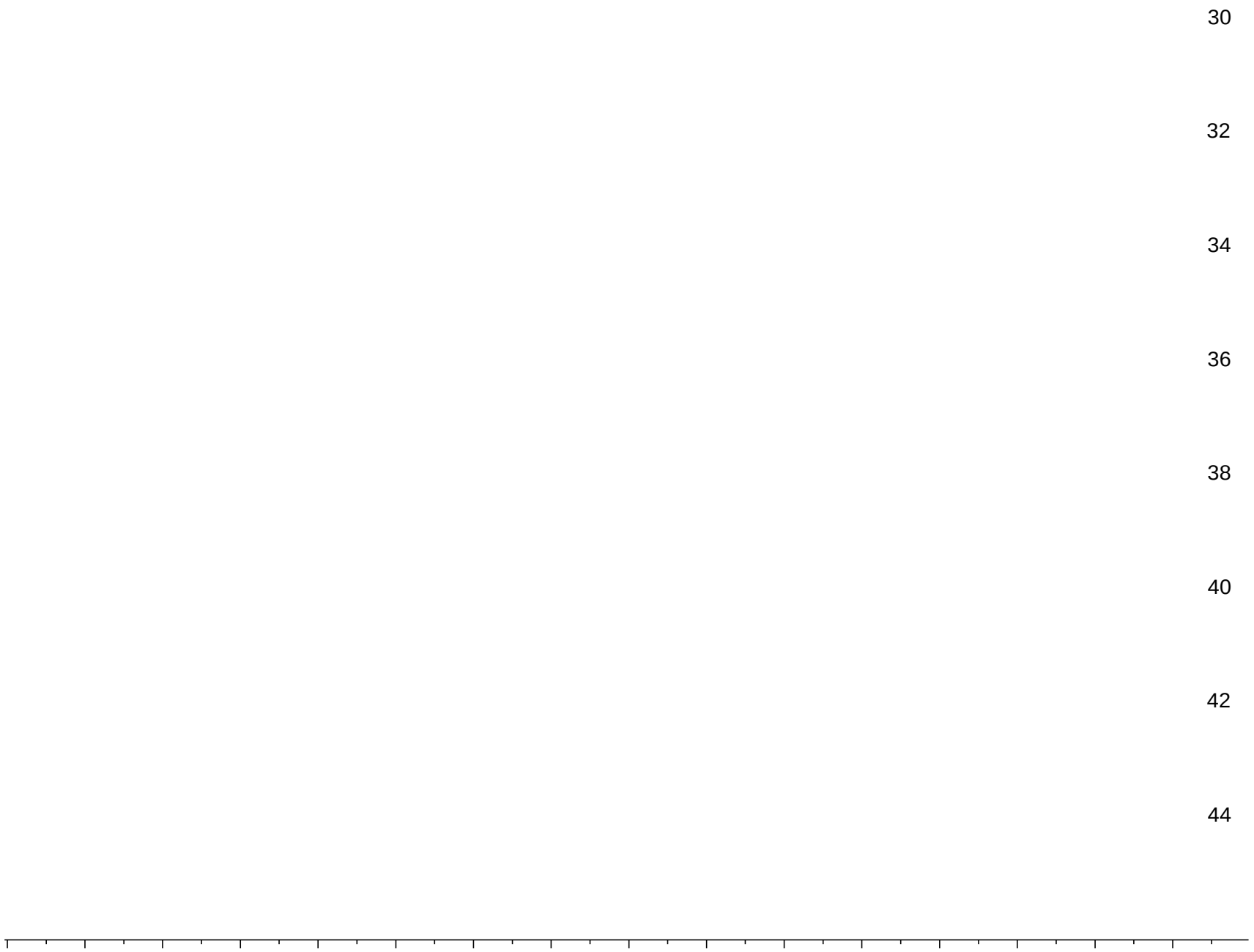
40

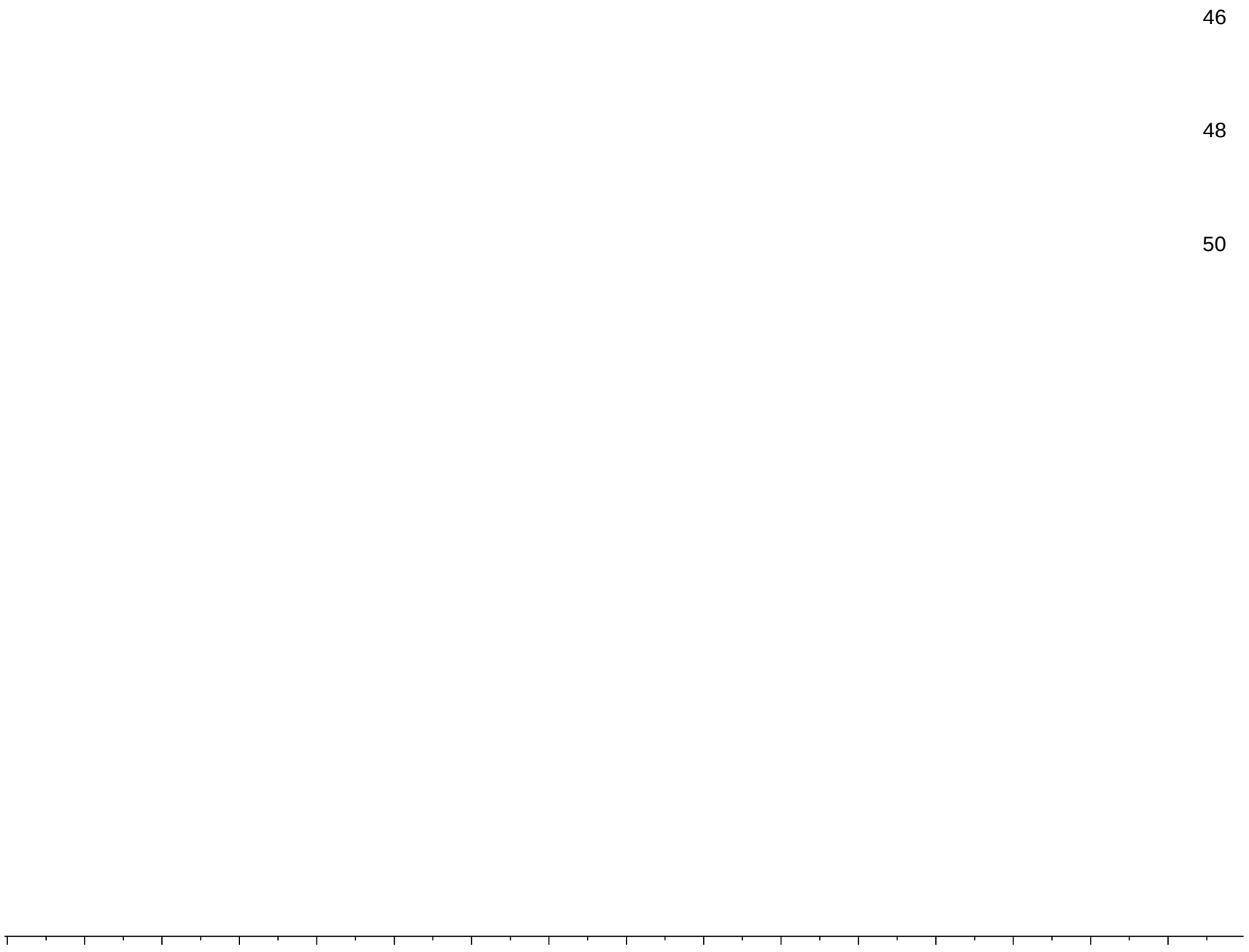

2.6 2.5 2.4 2.3 2.2 2.1 2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 0.9 0.8 0.7
f2 (ppm)
f1 (ppm)

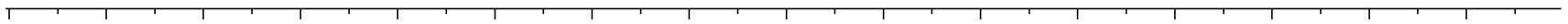

S1A_HMBC
STANDARD PROTON PARAMETERS
0
20
40
60
f1 (ppm)

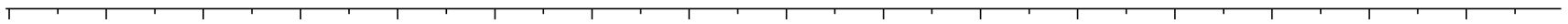

80
200
100
220
120
140
160
180

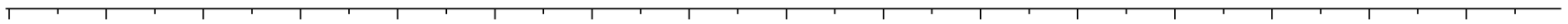

S1A_HMBC
STANDARD PROTON PARAMETERS
18
20
22
24
26
28
f1 (ppm)

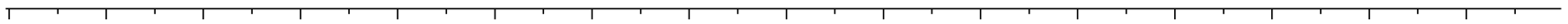

30
32
34
36
38
40
42
44


46
48
50

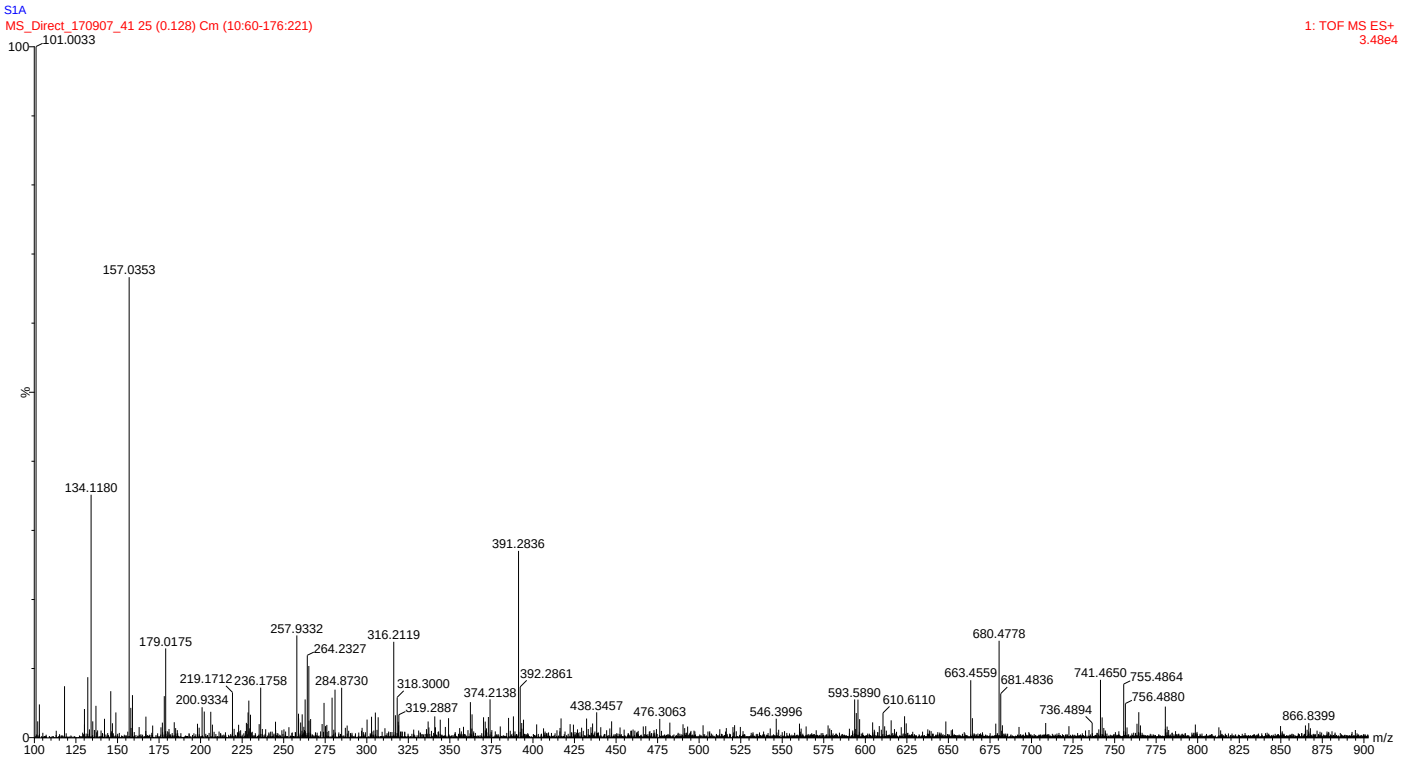


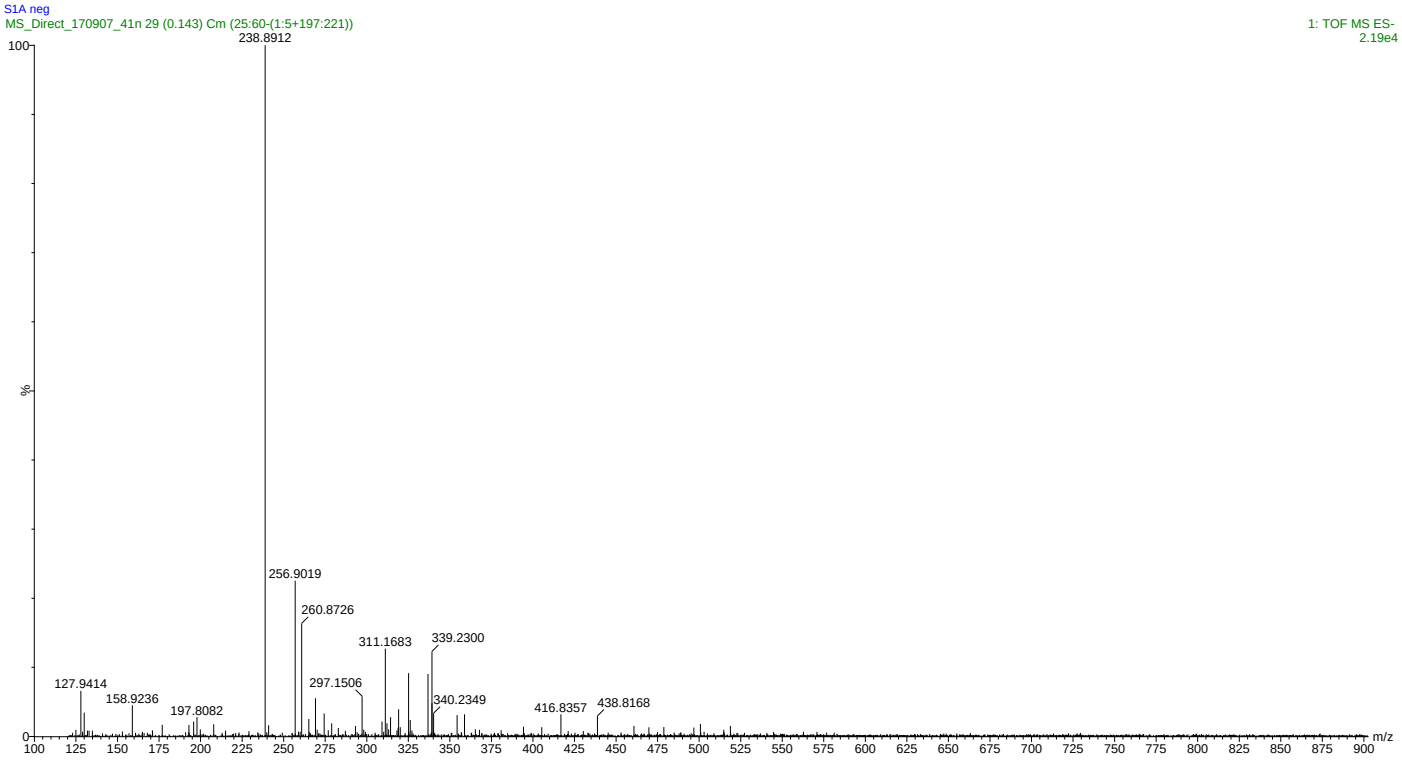


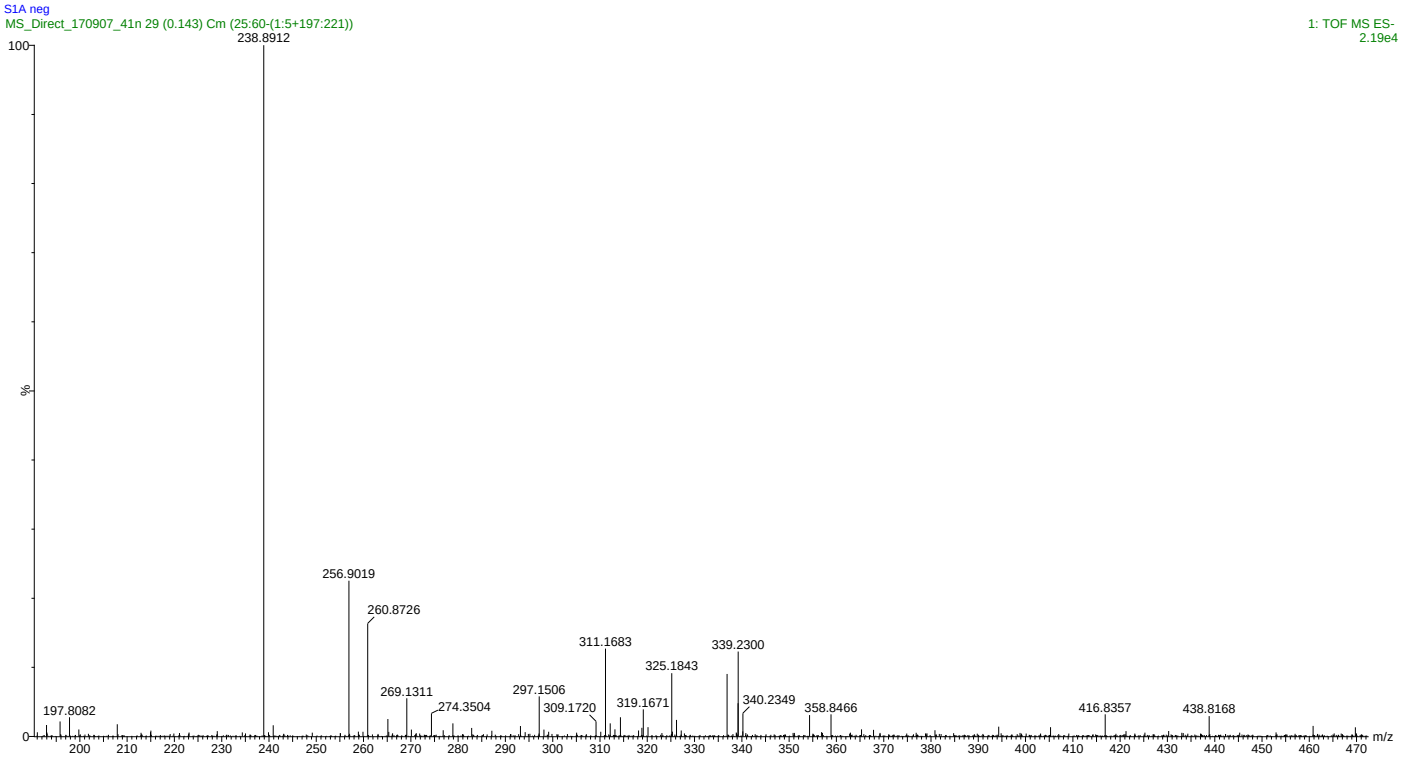




Appendix 2 Mass spectroscopy




S1A neg
MS_Direct_170907_41n 29 (0.143) Cm (25:60-(1:5+197:221))
1: TOF MS ES-
2.19e4
100
%
0
238.8912
256.9019
260.8726
311.1683
339.2300
297.1506
340.2349
127.9414
158.9236
197.8082
416.8357
438.8168
m/z
100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700 725 750 775 800 825 850 875 900


S1A neg
MS_Direct_170907_41n 29 (0.143) Cm (25:60-(1:5+197:221))
1: TOF MS ES-
2.19e4
100
%
0
238.8912
256.9019
260.8726
311.1683
339.2300
325.1843
269.1311
297.1506
340.2349
197.8082
274.3504
309.1720
319.1671
358.8466
416.8357
438.8168
200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470
m/z

Appendix 3 FTIR