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Bioactivity and ^{1}H NMR-based metabolomics analysis of polyherbal formulations used traditionally for the treatment and management of sexually transmitted infections

By

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Submission Declaration

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Dedication

"In memory of my dearly beloved mother Jagriti Baba"

(1964-2021)

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ABSTRACT

Ethnopharmacological relevance: Sexually transmitted diseases (STDs) is a major global concern of an ever-growing population. The daily rate of infectious STDs increases by an estimate of one million infections. The development of antimicrobial resistance pathogens is a further concern. Complementary and alternative medicines (CAM) is investigated to provide aid/relief from this resistance. Medicinal plants are used traditionally as an alternative form of medicine.

Aim: The aim of this study was to evaluate four medicinal plants (*Dicoma anomala* sbsp *anomala*, *Elephantorrhiza elephantina*, *Eucalyptus cinerea* and *Kigelia africana*) used in combination and individually for their antimicrobial activity against sexually transmitted diseases and to determine the similarities of these combinations based on their chemical constituents. Furthermore, the study determined the cytotoxicity effects and mycotoxin presence of the most active extracts.

Methodology: Aqueous extracts of various different combinations and individual plant were prepared, and stored at various conditions (includes: temperatures of 4˚C, 25˚C and 37˚C for 7 and 14 days respectively). The extracts were evaluated for their antimicrobial properties against a bacterium *Neisseria gonorrhoea* and a fungus *Candida albicans* using the microtiter dilution assay. The fractional inhibitory concentration index of the combinations was also determined against both pathogens. The chemical profile of these samples was analysed using a metabolomic approach via nuclear magnetic resonance and liquid-chromatography mass spectrometry methods (^{1}H NMR and LC-MS). Cytotoxicity studies were conducted to determine the level of toxicity of noteworthy samples against non-cancerous Vero cell lines. Furthermore, various mycotoxin levels present were determined via high-performance liquid chromatography.

Results: Antimicrobial activity of polyherbal formulations containing four individual plants (*Dicoma anomala* sbsp *anomala Elephantorrhiza elephantina*, *Eucalyptus cinerea* and *Kigelia africana*) in combination, which were stored at 25˚C for 7 and 14 days respectively, exhibited noteworthy activity against *N. gonorrhoea*. Eight of the ninety-eight extracts displayed a minimum inhibitory concentration (MIC) value of 0.39 mg/mL. ^{1}H NMR metabolomic analysis of all the combinations and individual plants exhibited prominent signal peaks in the aliphatic

region (0.8- 3.00 ppm), carbohydrate region (3.00- 6.00 ppm) and some combinations yield signals within the aromatic region (5.00- 9.00 ppm). Thus, resulting in a similarity between phytoconstituent amongst the various different combinations irrespective of their storage conditions and antimicrobial activity. The similarity within the different types of combinations is displayed via distinct grouping on the PCA and OPLS-DA score plots. Concerning cytotoxicity studies eight polyherbal formulations were tested, cytotoxicity levels against non-cancerous Vero cell lines ranged from slightly cytotoxic to non-cytotoxic (289.12 ± 21.23 µg/mL to 866.47 ± 22.41 µg/mL). Polyherbal formulations subjected to mycotoxin analysis displayed non-detectable mycotoxin levels.

Conclusion: The biological and chemical activity observed amongst the different plant combinations supports the traditional usage of polyherbal formulations in the treatment and management of sexually transmitted diseases.

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Abbreviations:

µg/µL	microgram/microliter
ATM	African traditional Medicine
CAM	Complementary and alternative medicine
CD_3OD	deuterated methanol
CFU	colony forming units
CO_2	Carbon dioxide
DMSO	dimethylsulphoxide
DON	Deoyxnivalenol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Media
EO	Essential oils
FB	Fumonisin B_n
FBS	Fetal Bovine Serum
FIC	Fracitional inhibitory concentration
HPLC	High Performance Liquid Chromatography
HPV	Human papillomavirus
IC_{50}	half maximal inhibitory concentration
IV	intravenously
LC-MS	Liquid Chromatography Mass Spectrometry
MIC	Minimum inhibitory concentration
MDA	Multivariate Data Analysis
mg/mL	milligram/millilitre
NIV	Nivalenol
NMR	Nuclear Magnetic Resonance
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
PBS	Phosphate Buffer Saline
PCA	Principle Component Analysis
PSF	Penicillin, Streptomycin Fungizone
SD	Standard deviation
STDs	Sexually transmitted diseases
STIs	Sexually transmitted infections
TCM	Traditional Chinese medicine
UPLC	Ultra-Performance Liquid Chromatography
UTIs	Urinary tract infections
UV	Ultraviolet
VD	Venereal diseases
WHO	World Health Organization
ZEA	Zearalenone

Chapter 1: Introduction

Chapter 1: Introduction

1.1. Problem statement

Living organisms such as plants and animals have an adapted mechanism of action to coexist in nature (Gradstein & Kerp, 2012). Plants can be used as a source of food, fuel, clothing, recreational and medicinal purposes. In ancient times human beings relied on traditional medicines, often called complementary and alternative medicine (CAM) (Jamshidi-Kia, Lorigooini, & Amini-Khoei, 2018). There are multiple forms of traditional medicines, which include Chinese or Oriental traditional medicine (TCM), African Traditional Medicine (ATM) as well as acupuncture, Ayurveda and homeopathy. Modern technology has advanced the field of medicine.

Plants originated around the mid-Palaeozoic era; about 480 to 360 million years ago (Kenrick & Crane, 1997). Traditional and herbal medicines are made based on prior knowledge, skills and practices and are used in diagnosis, treatment, prevention of physical and mental illnesses. Traditional medicine vary amongst different cultures, locality and theories between traditional healers and their traditional knowledge (Mahomoodally, 2013). African traditional medicine is known as one of the oldest therapeutic systems that is still used in modern times. Herbal medicines include herbal materials and herbs that are made into herbal preparations containing active ingredients of various plants parts, as well as other plant materials. However, in some preparations there may be animal and mineral matter included (Jamshidi-Kia et al., 2018). Traditional medicinal practice relies on a more holistic manner whereby traditional healers tend to treat the patients on a more personal manner as compared to medical doctors. Traditional healers act as an intermediary between a visible and an invisible world, in others words they are a link between the living and the dead. The patients are sometimes brought into contact with spirits and to bring a person back into harmony with their ancestors (Abdullahi, 2011).

Due to the ever emerging population, 80% of the world's population both in developed and developing countries rely on traditional or herbal medicine (Jamshidi-Kia et al., 2018; Mahomoodally, 2013; World Health Organization [WHO], 2019). Currently there is an estimated 200 000 to 300 000 traditional healers in South Africa. The traditional healer to patient ratio being 1:500-1200 as opposed to the ratio of a medical doctor to a patient being 1:40000 (Osheledi

& Sibanda, 2018). The vast majority of people within South Africa rely on traditional medicine is mainly due to the lack of access to allopathic medicine and Western medical treatment. Besides the lack of access and shortage of medical doctors, Western treatment is more expensive than traditional medicine (Mahomoodally, 2013).

Plant diversity varies greatly in Sub-Saharan Africa, with approximately 45000 plant species spanning across 29 million km^2 (Linder, 2014), of which 5000 plant species are used medicinally (Mahomoodally, 2013). Species richness varies greatly across Sub-Saharan Africa, with the lowest species richness indicated in yellow and the highest indicated in red and purple (Figure 1.1). The countries indicating higher species richness are the coastal regions of West Africa; the Cameroon-Gabon region, the in-lands of east Africa and the southern tip of Africa (Linder, 2014).

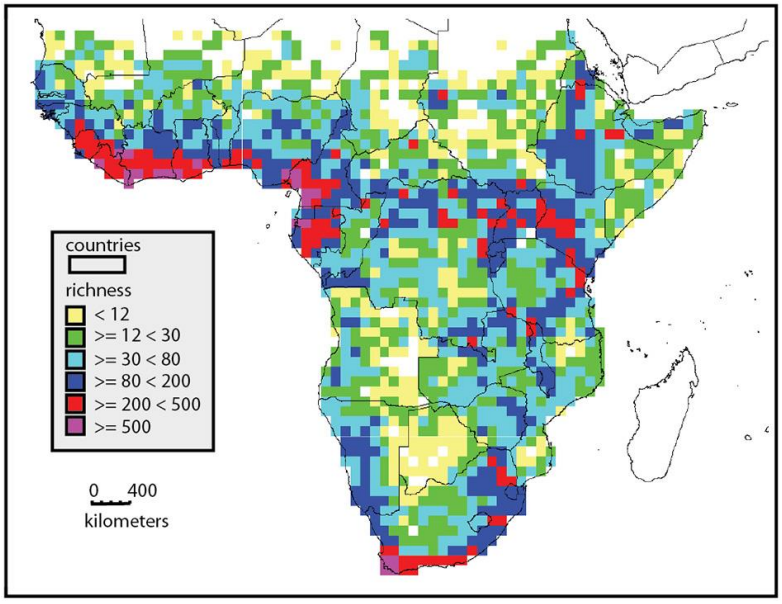

Figure 1.1: The distribution of species across the Sub Saharan African based on 5881 species mapped to a grid of 1˚ (Linder, 2014).

Medicinal plants provide a great alternative to Western medicine due to its properties. Various bioactive phytochemicals are beneficial to the plant as they provide plant protection against diseases and pest damage, as well as contribute to factors such as colour, aroma, and flavour. These phytochemicals protect the plant against drought, pollution, pathogenic attack, stress, and UV exposure. The phytochemistry of medicinal plants includes: alkaloids (18%), which act as a mood enhancer, flavonoids, glycosides, tannins, phenolics, which acts as an antioxidant and

venotonics (45%), saponins (27%), terpenoids (27%) which acts as an antibacterial and antifungal agent (Figure 1.2) (Saxena, Saxena, Singh, & Gupta, 2013). The phytochemicals present may act individually, additively, or synergistically. The synergism of the phytochemicals provides an effect that could surpasses the effect of individual constituents (Mahomoodally, 2013).

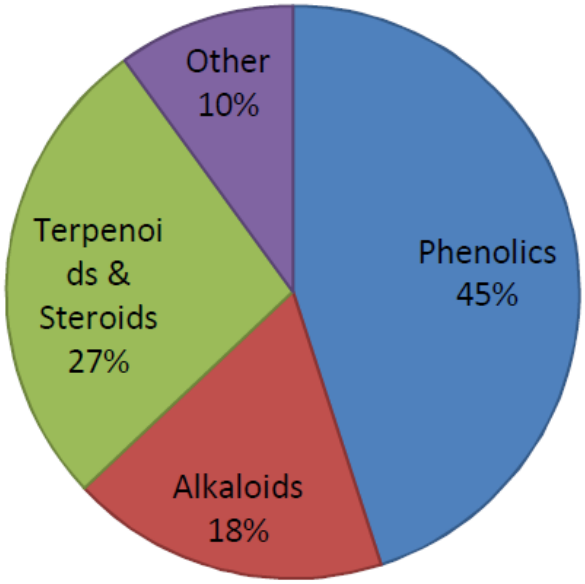

Figure 1.2: The percentages of phytochemicals present within medicinal plants (Saxena et al., 2013).

Sexually transmitted infections (STIs) or venereal diseases (VD) are infections that are transmitted from one individual to another via sexual contact; this includes contact via anal, oral, and vaginal sex. According to the World Health Organization (WHO), surpass of one million people contract an STIs every day and is an ever-growing issue in the developing countries (Shaukat & Kazmi, 2015). The four major STIs include *Neisseria gonorrhoeae* (Zopf & Trevisan) (Gonorrhea), *Treponema pallidum* (Schaudinn & Hoffmann) (Syphilis), Human papillomavirus (HPV), and *Candida albicans* (C-P. Robin) Berkhout (1923). These infections can be passed on to individuals who are sexually active that have engaged in unprotected sexual activities such as anal, oral, or vaginal sexual contact and skin to skin contact. Other transmissions of sexually transmitted diseases (STDs) may occur via the use of unsterilized needles and injections, as well as blood transfusions and maternofoetal transmissions (Shaukat & Kazmi, 2015).

Symptoms of STIs are dependent on the type of infection, although common symptoms include a discharge from the vagina in females and a penile discharge in males. Some individuals experience dysuria, which may lead to dyspareunia and pain in the pelvic region. The penetration region (oral cavity, genital, and anal region) may develop chancre (Shaukat & Kazmi, 2015).

1.2. Hypothesis

- The poly-herbal formulation based on ethnobotanical knowledge containing a combination of four medicinal plants will exhibit antimicrobial activity against sexually transmitted infections (STIs).

1.3. Aims and Objectives

1.3.1. Aims:

The main aim of this study is to evaluate the safety and antimicrobial potential of four medicinal plant species used traditionally in combinations for the treatment of sexually transmitted infections (STIs).

1.3.2. Objectives:

- To evaluate the antimicrobial activity of four individual plant species and their combinations stored at different temperature for seven or fourteen days.
- To evaluate the interaction efficacy of the plant species in combinations.
- To conduct a metabolomics analysis on the extracts to evaluate the quality of the formulations based on the similarity and differences of their chemical constituents.
- To determine the cytotoxicity of the most active extracts (individual plant and combinations).
- To determine the presence and type of mycotoxins present within the most active extracts (individual plant and combinations).

1.4. Importance of the study.

The importance of this study was to evaluate the bioactivity of selected South African medicinal plants in relation to the treatment of STIs. Sexually transmitted infections have a detrimental impact on the sexual and reproductive health of the population with an estimate of 82 million

new infections in 2020 according to the WHO (WHO, 2021). Although conventional synthetic drugs are available, multiple factors play a vital role in the use of traditional medicine; these include antimicrobial resistance, access to western doctors, conventional medicine may be expensive. A large percentage of the population in developing countries (80%) rely on complementary and alternative medicine (World Health Organization [WHO], 2019).

1.5. Scope of the dissertation

Chapter 1 outlines the problem statement regarding sexually transmitted infections (STIs) and their impact on human society. The hypothesis' aims and objectives of the dissertation are stated in this Chapter.

Chapter 2 outlines an extensive review of medicinal plants; *Dicoma anomala* sbsp *anomala* Sond., *Elephantorrhiza elephantina* (Burch.) Skeels, *Eucalyptus cinerea* F.Muell. ex Benth. and *Kigelia africana* (Lam). Benth are used in diagnosis, treatment, and its association with *Neisseria gonorrhea* and *Candida albicans.*

Chapter 3 provides a detailed analysis of the anti-microbial activity of four medicinal plant species and their combinations (*Dicoma anomala* sbsp *anomala*, *Elephantorrhiza elephantina*, *Eucalyptus cinerea,* and *Kigelia africana*).

Chapter 4 is an extensive metabolomic study to analyse the quality of the polyherbal formulations.

Chapter 5 assess the cytotoxicity and the presence of mycotoxins of the most active extracts.

Chapter 6 contains the general discussion, conclusion and outlines the future prospects of the study.

1.6. References

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Chapter 2:
Literature Review

Chapter 2: Literature review

2.1. Background

Medicinal plants have active ingredients that are used in drug development and synthesis. A percent of synthetic drugs is isolated and/or derived from natural or medicinal plants; a common example is Opium alkaloids (morphine, codeine thebaine, noscapine and papaverine) isolated from *Papaver somniferum L.* (Figure 2.1). Medicinal plants are used as traditional medicine in the treatment of acute and chronic conditions such as asthma, cancers, liver diseases, Human Immunodeficiency Virus (HIV), sexually transmitted diseases (STDs) and rheumatologic disorders (Keskin, 2018). In traditional medicine, medicinal plants are used either as a combination of various herbal plants (polyherbal) or as an individual plant. Traditional healers retain the knowledge and cultural practices of medicinal plants that have been passed on from prior generations to assist in remedies for treatment, commonly known as ethnobotany (Nolan & Turner, 2011).

Figure 2.1: A *Papaver somniferum* flower commonly known as Opium poppy or breadseed poppy. (Encyclopædia Britannica).

Polyherbal formulations are prepared in different forms such as tinctures, infusions, and decoctions that contain multiple medicinal plants and/ or herbs. The ratio of plant content within these formulations that contain two or more plants with different phytoconstituents varies depending on the potency required to achieve optimum therapeutic effects (Maurya & Kumar, 2019). The therapeutic effects of polyherbal formulations ranges from an effective low dosage to an adequate high dosage (Maurya & Kumar, 2019). The dosage range is determined by the effectiveness of the agent's antimicrobial activity.

2.1.1. Antimicrobial activity

Antimicrobial agents play a pivotal role in the advancement of medicine for example, the treatment of infectious diseases such as gonorrhea. Due to its pivotal role, antimicrobial agents have assisted in the life span of humans by changing the outcomes of microbial infections. Although beneficial, antimicrobial agents have been proven to be troublesome when increased and misused in treatment against diseases (C Reygaert, 2018). The misuse and increased use of synthetic antimicrobial agents has created a cause for concern for scientists due to antimicrobial resistance. Another factor of antimicrobial resistance is the inaccurate prescription of antimicrobial therapy (C Reygaert, 2018; Goossens, 2009; Griffith, Postelnick, & Scheetz, 2012; Yu, 2011). Antimicrobial resistance paves a channel for further research and development of alternative form of treatment.

Medicinal plants contain phytoconstituents that produce its therapeutic effects due to multiple medicinal plants present within these polyherbal formulations. The phytopharmacological activity of medicinal plants have adverse effects on the human body due to antagonistic or synergistic potentiative effects (Kiyohara, Matsumoto, & Yamada, 2004). The synergistic and antagonistic effects of medicinal plants against pathogens may be due to the activity of phytoconstituents present within an individual medicinal plant influencing the phytoconstituents of another medicinal plant (Kiyohara et al., 2004).

2.1.2 Synergism

Synergy is defined as the effect of an interaction between two or more constituents, components, or substances that are greater than the sum of their individual contributions (Pezzani et al., 2019; Williamson, 2001). The definition of synergy based on Mosby's Dictionary of Complementary and Alternative Medicine (CAM) is the effect of a combination of components interacting that produce new and different effects than the individual components (active constituents) (Jonas, 2005; Pezzani et al., 2019). There are four mechanisms to achieve effective synergistic effects: pharmacodynamics and pharmacokinetic synergism, synergistic multi-target effect, interference with resistance mechanisms, and the elimination or neutralization potential of the polyherbal formulation (Pezzani et al., 2019).

Pharmacodynamics synergism studies include the synergistic effect of active phytoconstituents that produce similar therapeutic effects which are targeted to a similar

receptor or physiological systems (Karole et al., 2019). Pharmacokinetic synergism studies the effects (absorption, distribution, metabolism, and elimination- ADME) of how active phytoconstituents of medicinal plants affect the human body; for example, the concentration of active phytoconstituents within the blood and the metabolizing of the active constituents that may be altered in the presence of other active phytoconstituents (Karole et al., 2019; Kiyohara et al., 2004). Phytoconstituents present within a medicinal plant may have multi-target synergistic effects such that the phytoconstituent does not act upon a single target, but affects multiple targets; for example, all the functional and structural cell constituents such as metabolites, receptors, enzymes, transporter proteins, nucleic acids, and ribosomes (Imming, Sinning, & Meyer, 2006; Pezzani et al., 2019).

Another mechanism of action can occur via the interference of resistance mechanisms. Microbial resistance occurs due to three reasons, namely: (i) a reduction in the affinity of active constituents binding to the active site due, to modifications, (ii) the synthesis of enzymes that selectively target and destroy or modify the antibiotics and (iii) the efflux of antibiotics within the cell (Hemaiswarya, Kruthiventi, & Doble, 2008; Pezzani et al., 2019; Sheldon, 2005). Polyherbal formulations have a combination of phytoconstituents present, which can act as modifiers and produce a therapeutic effect (Hemaiswarya et al., 2008). The elimination and neutralization of potential these toxic effects caused by phytoconstituents present within the poly herbal formulation reduces their mechanism of resistance. The phytoconstituents of one medicinal plant may have the ability to eliminate or reduce the toxic effects produced by phytoconstituents of another medicinal plant, resulting in an improved treatment used against ailments (Pezzani et al., 2019).

Antagonistic effects or antagonism is the opposite of synergism as the effect of an interaction between two or more constituents, components, or substances are less than the sum of their individual contributions thus producing a reduced effect (Pezzani et al., 2019; Williamson, 2001).

Mathematical calculations are used to explain the difference between antagonistic, synergistic and additive (no interaction) effects of multi-drug/poly herbal formulations. The Isobole method (Figure 2.2) is ideally used to present synergistic effects, as there are no assumptions of the behavior of an individual agent, but rather a combination of mixtures.

The interactions between phytoconstituents present within medicinal plants of poly herbal formulations may produce i) an additive effect (no interaction). In this instance, the individual medicinal plants produce the same effects as when the plants are combined producing a straight line (Figure2.2). Polyherbal formulations that produces synergistic effects indicates that the combined effect of the mixture is greater than that of their individual plant and results in a concave synergistic curve (ii). An antagonistic effect produces a convex antagonistic curve where the combined effect of the mixture is less than that of their individual plant (iii) (Bosgra, Van Eijkeren, & Slob, 2009; Pezzani et al., 2019; Williamson, 2001).

The equations of the Isobole methods derived mathematically are as follows:

I. $OE\ (da, db) = OE\ (da) + OE\ (db)$ (Additive)

II. $OE\ (da, db) > OE\ (da) + OE\ (db)$ (Syngerism)

III. $OE\ (da, db) < OE\ (da) + OE\ (db)$ (Antagonism)

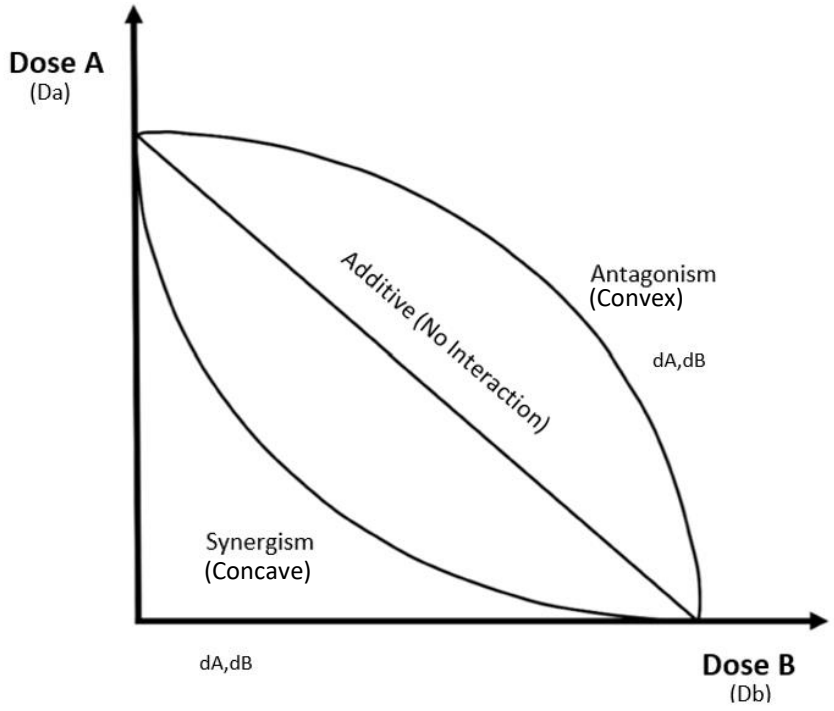

Figure 2.2: Isobole method; depicting antagonism, synergism and additive (no interaction) between active phytoconstituents of poly herbal formulations, Da and Db indicate the individual doses of a and b, da and db are the doses of a and b in a mixture (Pezzani et al., 2019; Williamson, 2001).

2.1.3 Quality control analyses via Metabolomics tools

Medicinal plants are complex as there are multiple active constituents that contribute to its' biological activity, in some instance producing synergistic effects (Pezzani et al., 2019). Multiple factors affect the production of secondary metabolites in medicinal plants. These factors include physiological (organ development, seasonal variation and mechanical or chemical injuries), environmental (climate, pollution, diseases, pests and edaphic factors) and graphical variation, as well as genetic factors, and storage of plants (Figueiredo, Barroso, Pedro, & Scheffer, 2008). Due to these factors, secondary metabolites may vary and thus the quality of plant extracts need to be examined and the standardization of these extracts is required to protect the coherence of these extracts for pharmaceutical quality (Heyman & Meyer, 2012).

Techniques such as chromatography and spectrometry are used to validate the quality of plant extracts. The use of metabolomics provides a standardized metabolic fingerprint of herbal medicine, which is used when examining other medicinal plants and their chemical constituents (Heyman & Meyer, 2012; Lee, Jeon, Lee, Lee, & Choi, 2017). The overall metabolic composition of primary and secondary metabolites or specific subset of metabolites is analyzed and is used to determine the quality of herbal medicine (Gilard, Balayssac, Malet-Martino, & Martino, 2010). NMR based metabolomics provides a qualitative and quantitative analysis of metabolites present in plants. Due to its' sensitivity metabolomic studies poses to be challenge in the field of science.

"Omics" is described as studying the entire biological system collectively rather than its' individual components. The field of "omics" is multidisciplinary; involving genomics, transcriptomics, proteomics and metabolomics. The field of metabolomics involves the study of the entire metabolome, focusing on the high-throughput identification of metabolites; low-molecular-compounds (< 15000Da) present within an organisms (Clish, 2015). Metabolic fingerprinting, profiling metabolomics and metabonomics are terms that are used interchangeable, these terms describe a comprehensive quantitative and qualitative analysis of all metabolites present in an organism (Nicholson, Connelly, Lindon, & Holmes, 2002). Metabolomics based mass spectrometry (MS), NMR and LC-MS plays an intricate role in the effects of drug development, discovery, toxicity and quality control

The principle of Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical chemistry technique whereby a nuclei spin is detected around a strong magnetic field. Once there is an external magnetic field applied; there is a creation of an energy gap between the base energy (spin-up state) and a higher energy (spin-down state). During the spin-up state the nuclei is aligned with the magnetic field and in a spin down state, it is in the opposite direction. An energy transfer takes place at a wavelength which corresponds to a radio frequency and when the spin returns to the base level it emits energy at the same frequency. The signal that matches the transfer is measured and processed to produce an NMR spectrum (Gerothanassis, Troganis, Exarchou, & Barbarossou, 2002).

NMR based plant metabolomics utilizes tools such as multivariate data analysis to reduce discrimination signals due to the complexity of ^{1}H NMR spectra. An unsupervised clustering method known as principal component analysis (PCA) separates that multivariate data into small principle component without any prior knowledge of the data, which also accounts for the greatest variation of the dataset and is advantageous as is displays outliers and trends within the dataset (Jollife & Cadima, 2016).

Methods that are more supervised such as partial least squares projection to latent structures (PLS) or orthogonal projection to latent structures discriminant analysis (OPLS-DA) are classified according to classes based on the samples that are being tested for example; active versus non-active extracts (Westerhuis, van Velzen, Hoefsloot, & Smilde, 2010). The method OPLS is similar to PLS however there are orthogonal signal corrections (OSC) that filter out NMR variation that is unrelated to the set class; for example that environment that the medicinal plant in this study was grown in (Gilard et al., 2010; Holmes, Tang, Wang, & Seger, 2006; Y. Wang et al., 2004). The processing of ^{1}H NMR spectra is required to produce an optimal statistical model. Processing the data involves multiple sub-processing stages, these stages include, binning and alignment of that dataset, this eliminates any noise and masks low-intensity peaks that may influence strong chemical signals in the vicinity. Normalization of datasets eliminates the dilution variable of each sample and masks any obscure interpretation of loading points (Worley & Powers, 2013).

Liquid chromatography-mass spectrometry (LC-MS) is the combination of two techniques that are used for metabolomics studies. The principle of LC-MS is the separation of a sample into individual components by ionization and separation of the ions is based on the mass/charge

ratio and differentiate compounds on the basis of their physical and chemical properties. The mass spectrum is created by sorting the ions according to their mass by using a mass analyzer (Parasuraman et al., 2014). LC-MS has three main ionization methods which are: 1. atmospheric ionization (API) that involves 2. electrospray ionization (ESI) and 3. chemical ionization (APCI) (Codrea, Jiménez, Heringa, & Marchiori, 2007; Dunn, 2008; Fenn, Mann, Meng, Wong, & Whitehouse, 1989). Methods used for LC-MS are atmospheric ionization that consists of electrospray ionization and atmospheric chemical ionization. Atmospheric ionization can be used as it doesn't require the use of volatile compounds and therefore heat-liable functional groups, chemically unstable structures, compounds that have high vapour points and high molecular weight can be examined (Okazaki & Saito, 2012).

2.1.4 Cytotoxicity and effects of drugs on a cell

Cells are the smallest living units on its own, which makes up organism (organs and tissues), and therefore is the fundamental requirement to carry out life processes. Therapeutic drugs whether synthetic or traditional pose a risk in deteriorating the viability of cells to conduct its function. The deterioration caused by the effects of these drugs on cells, may lead to the death of cells activating a process called cell death. There are four general mechanisms of cell death namely, apoptosis, autophagy, necrosis and necroptosis.

Apoptosis known as a programmed cell death that is regulated by the destruction of damaged or excess cells (Elmore, 2007). Autophagy is self-degradative process whereby cells cause destruction to itself by removing the nonfunctional components (Glick, Barth, & Macleod, 2010). Cell necrosis is the irreversible damage and injury caused to the cell which leads to a premature cell death through the process of autolysis (Golstein & Kroemer, 2007). Necroptosis is known as a programmed cell death which can be regulated by signal transduction pathways, thus can target the cellular process for its therapeutic effects (Fulda, 2013). Usage of synthetic and traditional medicine can cause toxic effects to the body at a cellular level.

Toxicity is the adverse side effects experienced by a living organism caused by the usage of a substance (therapeutic drugs), known as a drug-host interaction (Mückter, 2003). Substance toxicity induces severe effects through the whole organism (body) or can be specific to a component; for example, cells (cytotoxicity) or organs (organotoxicity). There are three classifications of toxicity, namely acute, chronic and subchronic effects (Parasuraman, 2011).

Acute toxicity occurs within 24 to 72 hours after exposure to a large single substance dosage. Chronic toxicity is the exposure to a substance over a long term period usually months or years inducing irreversible toxic effects (Mückter, 2003). Cell toxicity (cytotoxicity) refers to the ability of a drug (synthetic or medicinal) to interfere within processes of a cell; such as cell attachment, cell growth, proliferation of the cells and in extreme toxicity cause cell death (Riss, Moravec, & Niles, 2011).

There are multiple cell lines that are used in cytotoxicity studies these include various human cell lines; HeLa, HaCaT, and HEK293 (Wienkers & Heath, 2005). There are cancerous and non-cancerous cell lines, which are utilized in cytotoxicity studies to examine the toxic effects that medicinal plants, impose on cells. Cancerous cell lines are extensively used in screening studies that involve chemotherapeutic drug development and effectiveness (Ertel, Verghese, Byers, Ochs, & Tozeren, 2006). Non-cancerous cell lines are utilized in instances of examining toxic effects of medicinal plants on normal cells for the potential of non-chemotherapeutic drug development (Alibert, Goud, & Manneville, 2017). Vero cells are derived from the kidney of an African green monkey (*Chlorocebus aethiops,* formerly known as *Cercopithecus aethiops*) (vervet kidney-VK cells), the usage of these cells dated back to the mid-1960s (Ammerman, Beier-Sexton, & Azad, 2009). These cells are derived from normal kidneys and are used in cytotoxicity studies due to their cell contact inhibition properties and can resist cell death programmed by apoptosis (Wink, Ashour, & El-Readi, 2012).

2.1.5 Mycotoxins

Mycotoxins are low molecular weight products such as secondary metabolites produced by filamentous fungi (Bennett & Klich, 2003). Mycotoxins are found within food and beverage items as well as can grow on plants if they parasitize the host plant during the developmental stage or they grow as saprophytes on plants during harvest and storage (Fletcher & Blaney, 2015). Whilst harvesting and storage play a role in fungi development, mishandling and marketing of plants for traditional use affect the production of mycotoxins. Other factors such as inadequate storage facilities, rodents and insects, and conducive environmental conditions influences fungal growth (Iqbal, Bhatti, Asi, Bhatti, & Sheikh, 2011). Human exposure to mycotoxins occur via various ways, these include; dermal via skin contact, ingestion and inhalation resulting in either acute or adverse effects (Pitt, 2013). All mycotoxins are of fungal origin but not all toxic compounds produced by fungi are called mycotoxins.

Mycotoxins present on medicinal plants poses a major health risk to patients using traditional medicine. The increase usage of medicinal plants may lead to an increased uptake of mycotoxins leading to adverse health problems (Ashiq, Hussain, & Ahmad, 2014). Medicinal plant contamination can occur, pre-; post- and during harvest, influencing the quality of the plant (Zheng et al., 2017). The following mycotoxins will be discussed; Aflatoxin B_1, Deoxynivalenol (DEO), Zearaleonone (ZEA), Nivalenol; Orchratoxin A and Fumonisins.

Aflatoxins are a family of mycotoxins produced by molds of the *Aspergillus* species; like *A. flavus*, and *A. parasiticus* (Bennett & Klich, 2003). These *Aspergillus* species thrive in warm (15-40˚C) and moist conditions. There are four major aflatoxins produced by the fungi, *A. flavus* produces mainly aflatoxins B_1 and B_2, and *A. parasiticus* produces all four aflatoxins B_1, B_2, G_1 and G_2 (Fletcher & Blaney, 2015). These aflatoxins are found on crops such as cereals, figs, oil seeds, nuts, spices and tobacco (Diener et al., 1987). Contamination of some crops occur pre-harvest, however post-harvest crops are more susceptible to contamination due to favourable mold growth conditions (moisture content and humidity of storage rooms) (Bennett & Klich, 2003). Aflatoxin B_1 is carcinogenic in humans, and the synergistic interaction between AFB_1 and hepatitis B virus induces the onset of hepatocellular carcinoma (HCC). The mechanism of action causing liver cancer caused by AFB_1 occurs when Cytochrome P450 enzymes convert aflatoxins to a reactive product: 8,9-expoxide, that binds to DNA and proteins. Due to this binding property of 8,9-expoxide, DNA adducts arise resulting in genetic changes within the target cells, leading to DNA strand breakage, a mutation in DNA base (a transversion of G:C to T:A) and oxidative damage. The transversion occurs within the promoter region of the p53 tumour suppressor gene that may result in the onset of (HCC) (Bennett & Klich, 2003; Hamid, Tesfamariam, Zhang, & Zhang, 2013)

Deoxynivalenol (DEO) is a trichothecene mycotxin produced by the *Fusarium* genera mainly the species *F. graminearum* and *F. culmorum* (Bennett & Klich, 2003). Deoxynivalenol is detected in cereal crops; barley, oats, rye, wheat and maize. Optimal environmental conditions that favour the *Fusarium* species that produce DEO are low temperatures and high humidity (Sobrova et al., 2010). The ingestion of crops that contain DEO results in severe side effects that include acute temporary nausea, vomiting, diarrhoea, abdominal pain, headaches, dizziness and fever. These side effects aren't detrimental to humans; however

they may have minimal immunotoxic; genotoxic, cytotoxic and reproductive and teratological effects (Sobrova et al., 2010).

Another trichothecene; nivalenol (NIV) produced by the *Fusarium* genera; mainly *F. cerealis, F. poae F. graminearum* and *F. culmorum*. Nivalenol is found in cereal crops; barley, oats rye, wheat and grain based food products (Eriksen & Pettersson, 2004). The optimal environmental conditions for fungal growth that produce NIV are moist, cool and damp conditions. Although the mycotoxin NIV is understudied as opposed to other trichothecene mycotoxins, it is said to exhibit, immunotoxicity, haematoxicity and reproductive toxicity. The proposed mechanism of action of a combination of other trichothecenes and NIV inducing a reduced cell proliferation rate and induces apoptosis of cells in organs such as the small intestines, thymus, spleen, bone marrow and testes (Eriksen & Pettersson, 2004; Ryu et al., 1987).

A nonsteroidal mycotoxin; Zearaleonone (ZEA) produced by fungi within the *Fusarium* species, mainly *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. equiseti, F. crookwellense*, *F. semitectum*, *F. verticillioides*, *F. sporotrichioides*, *F. oxysporum* (Ropejko & Twarużek, 2021). Zearaleonone commonly occurs on crops such as barley, maize, millet, rice, sorghum, soybeans and wheat (Gupta, Mostrom, & Evans, 2018). The optimal environmental conditions for the growth of *Fusarium* species causing the production of ZEA is a 3-week period of moisture levels over 20% and temperatures between 20˚C to 25˚C. However when under stressful environmental conditions of temperatures between 8˚C to 15˚C the production of ZEA may still occur (Gupta et al., 2018; Ropejko & Twarużek, 2021). The structure of ZEA is similar to estrogens namely; estradiol, estrone, and estriol, therefore ZEA is commonly known as a phytoestrogen causing hyperestrogenism in species. After consumption the keto-group at the C-6’ position is reduced and converted to two major metabolites α-Zearalenol and β-Zearalenol (Pfeiffer, Hildebrand, Mikula, & Metzler, 2010). (Zhang, Feng, Song, & Zhou, 2018) conducted *in vivo* studies that indicated exposure to ZEA can lead to follicular growth disorder, early ovulation, and the onset of puberty.

Ochratoxin A is a mycotoxin produced by fungi within the *Aspergillus* genera mainly *Aspergillus ochraceus* (Pfohl-Leszkowicz & Manderville, 2007). Ochratoxin A can be found in crops, plants and trees such as cereals, barely, beans, coffee, oats, rye, and wheat. The mycotoxin can also be found in products such as dried fruits, wine and dried peanuts (Bennett & Klich, 2003). The optimal growing conditions for the fungus to grow are moist and warm

conditions. Ochratoxin A is a clinically important mycotoxin, as it has neurotoxic, genotoxic, carcinogenic, immunotoxic, nephrotoxic, hepatotoxic and teratotoxic effects. However, the main target organ is the kidney, causing nephrotoxic effects by disrupting the DNA, RNA and protein synthesis thus affecting the function of various enzymes (Fuchs & Peraica, 2005; Tao et al., 2018).

Fumonisins are mycotoxins produced by fungi within the *Fusarium* genera mainly by *Fusarium verticillioides*, and *F. proliferatum* (Bennett & Klich, 2003; Fletcher & Blaney, 2015). There are approximately 15 fumonisins however fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) are of health importance. The *Fusarium* species infect crops, including cereals, corn and wheat; these crops are infected when conditions for infection are at their optimal, which include high moisture content and average temperatures ranging from 20˚C to 25˚C (Bennett & Klich, 2003; Fletcher & Blaney, 2015). Comparative studies conducted in China and South Africa indicated that in humans, fumonisins mainly cause oesophageal squamous cell carcinoma, as individuals with high levels of corn consumption are at a higher risk of developing oesophageal squamous cell carcinoma than those who have a low level of consumption. Another defect caused by fumonisins is neural tube defects (NTDs) whereby embryonic defects of the brain and spinal cord results in non-closure of the neural tube (Stockmann-Juvala & Savolainen, 2008).

2.2. Selected pathogens associated with sexually transmitted infections

2.2.1 *Neisseria gonorrhoea*

2.2.1.1 Introduction

Neisseria gonorrhoea is a non-motile gram-negative diplococcus bacterium. It is an oxidase-positive and aerobic bacterium which falls within the beta proteobacteria phylogenetic group in the *Neisseriaceae* family (Figure 2.3A) (McSheffrey and Gray-Owen, 2014). The bacterium multiplies and rapidly grows within the mucous membranes of humans, causing gonococcal infections within the oral cavity, urogenital, anorectal, pharyngeal and conjunctival infections. The distinct identification of *N. gonorrhoea* from other *Neisseria* bacteria is done by using biochemical capacity, such that the bacterium tests positive for the following enzymes: oxidase, catalase, superoxol, and hydroxyprolilaminopeptidase activity but tests negative for DNase, beta-galactosidase and gamma-glutamyl aminopeptidase activity. Acid production

from the bacterium occurs from the oxidative metabolism of glucose, but not maltose, lactose, and sucrose (Figure 2.3B) (McSheffrey & Gray-Owen, 2014).

Gonorrhoea is classified as a major sexually transmitted infection as it possesses the risk of a coinfection with other sexually transmitted infections such as *Chlamydia trachomatis* (Chlamydia) and Human immunodeficiency virus (HIV) (Skinner et al., 2014). Gonorrhoea possesses a serious health risk to humans due to its transmissibility of organisms, the rate of effective partner exchange, the duration of infectiousness and antibiotic resistance (Tapsall, 2006).

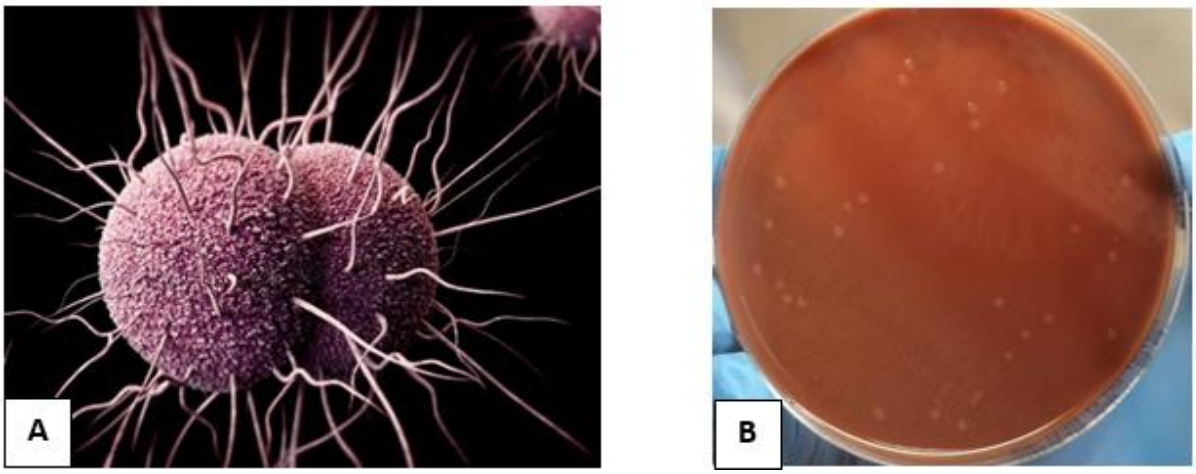

Figure 2.3: (A) A diplococcus bacteria, *Neisseria gonorrhoea*, (B) a culture of *Neisseria gonorrhoea* on chocolate agar.

2.2.1.2 Types of Infections, Signs, and Symptoms

Urogenital infections are infections that occur within the urinary tract and the genital organs within males and females. Urogenital infections in males result in acute infections of the urethra, are usually symptomatic, and appear two to five days post-infection. These symptoms include dysuria, testicular pain, and an abundant amount of purulent penile exudate. These infections result in epididymitis, orchitis, and sterility. More severe but rare complications include penile lymphangitis, penile edema, and periurethral abscesses (Mayor, Roett, & Uduhiri, 2012; McSheffrey & Gray-Owen, 2014).

Urogenital infections in females occur within the columnar epithelial cells of the endocervix, vagina, Bartholin's gland, skene glands and rectum. However, it often remains asymptotic in 30-80% of females. This may be due to the vagina having a higher pH and gonococcal

infections flourishing in acidic environments (pH 4). Symptoms in females may occur five to 10 days post-infection. These symptoms include dysuria, vaginal discharge, and abnormal bleeding. Symptoms and signs of bartholinitis include perilabila pain, oedema of the labia and enlargement and tenderness of the gland respectively. Due to the high rate of females that remain asymptomatic, they are often misdiagnosed. This leads to further complications once the bacteria have ascended to adjacent areas such as the uterus and fallopian tubes. Complications include ectopic pregnancies, pelvic inflammatory disease, and sterility (Mayor et al., 2012; McSheffrey & Gray-Owen, 2014).

Urogenital gonococcal infections lead to complications in pregnancies. Such complications include: inflammation of foetal membranes (amnion and chorion), premature ruptures of membranes, preterm births, low birth weight for the gestational age infants and spontaneous abortions in pregnant women (Donders, Desmyter, De Wet, & Van Assche, 1993; Heumann, Quilter, Eastment, Heffron, & Hawes, 2017; Larry Maxwell & Watson, 1992; Liu et al., 2013).

Pelvic inflammatory disease (PID) known as inflammation of the uterus, fallopian tubes and pelvic structures mainly caused by sexually transmitted microorganisms, gonorrhoea and *Chlamydia trachomatis*. The degree of complication of PID varies depending on the time and demographic of the STI prevalence (Barret & Taylor, 2005; McCormack, 1994). The sign and symptoms of PID are mainly associated with pain in the lower abdominal region, abnormal vaginal bleeding and discharge. Other symptoms include dysuria, pain during sexual intercourse, nausea, vomiting, and fever (McCormack, 1994).

Figure 2.4A shows the spread of the bacteria from the vagina and cervix into the upper genital tract. Individuals that have an increase risk to develop PID are women with endocervical infections and upper genital infection (Figure 2.4B) whereby the infection is associated with gonococci. Figure 2.4C shows the increase in bacterial vaginosis resulting in an overgrowth of bacteria. Due to the overgrowth of bacteria there is a spread of infection into the endometrium, fallopian tubes, and peritoneal cavity (Figure 2.4D) (Barret & Taylor, 2005; McCormack, 1994).

Figure 2.4: (A) Bacterial growth within the upper genital tract, (B) Bacterial growth spreads into the upper genital tract, (C) An overgrowth of bacteria; (D) Bacterial growth spreads further to other parts of the female reproductive structures.

Complications resulting from PID include tubal damage and scarring, chronic pelvic pain, irregular menstrual cycles, ectopic pregnancies, and infertility (McCormack, 1994). Management of PID includes immediate use of a combination of antibiotics however; the use of antibiotics depends on the severity of the disease. Other ways of managing the disease include disease control and preventative methods used in preventing STIs (Barret & Taylor, 2005). Anorectal infections occur within the anal region. Which is a result of anal receptive intercourse, which usually occurs in males who engage in sexual intercourse with other males. Other transmissions can occur with a spread of infection within the genital to the rectal region due to the proximity from each other (Assi, Hashim, Reddy, Einarsdottir, & Longo, 2014). Although infections may be latent and nonspecific, some symptoms include anal, rectal pain, mucopurulent discharge tenesmus, constipation, and anorectal bleeding (Assi et al., 2014; Mayor et al., 2012). Pharyngeal infections occur when gonorrhoea is transmitted via unprotected sexual interactions with the oropharynx. Coinfection may occur if an individual has cervical gonorrhoea. The individual has a higher probability of contracting oropharynx gonorrhoea. The signs of oropharynx include a sore throat, oropharyngeal erythema or exudates and cervical lymphadenopathy (Mayor et al., 2012).

Disseminated gonococcal infection (DGI) occurs when the bacteria disseminated into the bloodstream, resulting in an infection. These occur in two major forms, namely localized septic arthritis and arthritis-dermatitis syndrome. Disseminated gonococcal infection rarely occurs, however, it is more prominent within individuals that experience endocarditis and osteomyelitis. Disseminated gonococcal infection has a combination of symptoms, for example tenosynovitis, dermatitis (rash, papules, or petechiae), polyarthralgia, and purulent arthritis. Lesions such as erythematous pustular or vesiculopustular occur due to dermatitis, which is often painless. Other symptoms include fevers, chills, and general discomfort (Creighton, 2014; McSheffrey & Gray-Owen, 2014; Sherrard, 2010).

Gonococcal conjunctivitis (GC) not only occurs in neonates, but also in adults. However, GC occurs rarely in adults. Neonatal GC (ophthalmia neonatorum) occurs during childbirth,

whereby the bacteria is transmitted to babies once passing the birth canal. Adult GC occurs when bacteria are transmitted via genital secretions through hand contact and contaminated surfaces, such as towels and unwashed clothing from a sexual partner (M. Wang, Wang, & Li, 2019). The signs and symptoms that become visible 2-5 days post-infection (after birth) are a conjunctival exudate (Figure 2.5), redness, tenderness, and periorbital oedema (McSheffrey & Gray-Owen, 2014). Other symptoms include swelling of preauricular lymph nodes, corneal ulcers, scarring, and blurred vision (M. Wang et al., 2019).

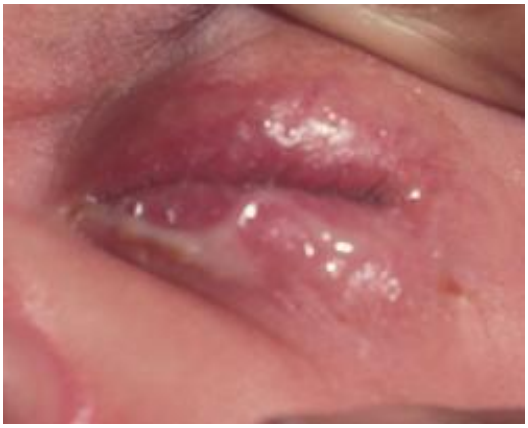

Figure 2.5: Symptoms; redness of the eye and conjunctival exudates of Gonococcal conjunctivitis in a neonatal patient.

2.2.1.3 Current treatment

Treatment for gonorrhoea is currently available to the public; however, a medical professional should do diagnosis, before any treatment is given. Treatment should be done to prevent further transmission and complications until the infection is completely eradicated. Drugs given for the treatment of gonorrhoea include spectinomycin (Figure 2.6A), ceftriaxone (Figure 2.6B), cefixime (Figure 2.6C) ciprofloxacin (Figure 2.6D), ofloxacin (Figure 2.6E), ampicillin (Figure 2.6F), and azithromycin (Figure 2.6G). Dosages of drugs are given according to the onset of the disease and are dependent on the severity and type of the infection (Sherrard, 2010; Suay-García & Pérez-Gracia, 2018). For uncomplicated gonococcal infections of the cervix, urethra and the rectum, a single dose of ceftriaxone 250 mg intramuscular and a single dose (IM/sd) can be given, whereas uncomplicated gonococcal infections of the pharynx are treated with ceftriaxone 250 mg (IM/sd).

Vulnerable patients such as pregnant women and individuals that are allergic to certain types of drugs should be provided with alternatives. Pregnant women should not be treated with quinolones and tetracycline due to its high risk to induce spontaneous abortions (Muanda, Sheehy, & Berard, 2017; Suay-García & Pérez-Gracia, 2018). If a patient is diagnosed with PID the patient should be hospitalized for treatment (Miller, 2006). Individuals that have a penicillin allergy are usually given cephalosporins and spectinomycins as treatment. Individuals that have a β lactam allergy are given spectinomycin or ciprofloxacin (Suay-García & Pérez-Gracia, 2018). Patients that have DCI should first be hospitalized to determine the severity and be evaluated for clinical symptoms of endocarditis and meningitis (Miller, 2006).

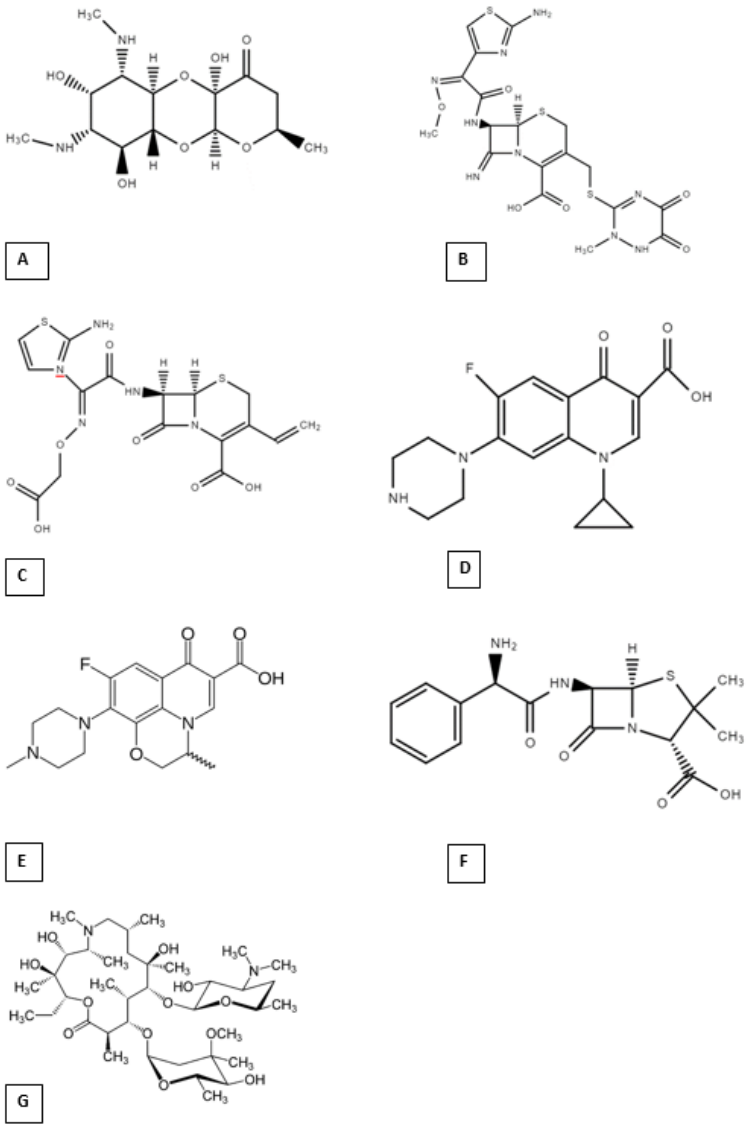

Figure 2.6: Chemical structures of (A) Spectinomycin; (B) Ceftriaxone; (C) Cefixime; (D) Ciprofloxacin; (E) Ofloxacin; (F) Ampicillin; (G) Azithromycin.

Due to the increase in cases worldwide every year, gonococcal antibiotic resistance is becoming a major concern in providing adequate and effective therapy. Research regarding complementary and alternative medicine (CAM) has increased over the years, which provides insight into the development of natural products (Peltzer, 2009).

2.2.1.4 Current medicinal treatment

Due to antibiotic resistance of synthetic medication over the many decades, medicinal plants have been under investigation to provide an alternative measure, which may be used in the treatment against gonorrhoea. Current medicinal treatment available for gonorrhoea is found within plants that are not endemic to South Africa and Africa alone, but within other continents of the world. Table 2.1 displays the current medicinal treatment derived from plants that is predominantly found in South Africa.

Table 2.1: A list of current medicinal plants used South African traditionally against sexually transmitted infection (STI) gonorrhoea caused by the bacteria *Neisseria gonorrhoea.*

Botanical Name	**Family Name**	**Common name**	**Vernacular Name**	**Plant part(s) used**	**Method of preparation**	**Reference**
Albizia adianthifolia (Schumach.)	Mimosaceae	flat-crown albizia	uSolu (Zulu)	Bark	Infusion	(Naidoo, Van Vuuren, Van Zyl, & H. De Wet, 2013; Van Puyvelde, Geiser, Rwangabo, & Sebikali, 1983)
Carica papaya L.	Caricaceae	Papaya	uPopo (Zulu)	Leaves and roots	Decoction	(H. De Wet, Nzama, & Van Vuuren, 2012)
Combretum molle R. Br. Ex G. Don	Combretaceae	Velvet bushwillow or velvet leaf willow	Mugwiti (Venda)	Leaves and roots	Decoction	(H. De Wet et al., 2012; Fyhrquist et al., 2002)
Bryophyllum pinnatum (Lam.) Oken	Crassulaceae	Cathedral bells	uMvuthuzi (Zulu)	Leaves	Decoction	(H. De Wet et al., 2012; Halberstein, 2005)
Ximenia caffra Sond. var. *caffra*	Olacaeae	Large sourplum	Morokologa (Northern Sotho)	Roots	Decoction	(H. De Wet et al., 2012)

Abrus precatorius L. ssp *africanus*	*Papilionaceae/Fabaceae*	Bead vine, coral bead plant, crabs eye, lucky bean creeper	Amabope (Nde) Nsimani (Shan) uMkhokha (Zulu)	Roots and leaves	Decoction	(Inga Hedberg et al., 1983)
Albizia adianthifolia (Schumach.) W. Wight var. *adianthifolia*	Papilionaceae/Fabaceae	Flat-crown albizia, rough bark flat-crown	umHlandlothi (Xhosa)	Leaves	Decoction	(H. De Wet et al., 2012; Van Puyvelde et al., 1983)
Dalbergia melanoxylon Guill. & Perr.	Papilionaceae/Fabaceae	Zebra wood, African blackwood, African ebony	Mpingo (Swati) Xipalatsi (Tsonga) Muuluri (Venda)	Roots and leaves	Decoction	(Inga Hedberg et al., 1983)
Lonchocarpus bussei Harms.	Papilionaceae/Fabaceae	Narrow lance pod, Small apple-leaf	Muvule (Nyamwezi)	Roots	Decoction	(Inga Hedberg et al., 1983)
Adenia gummifera (Harv.) Harms.	Passifloraceae	Snake climber	Impindamshaye (Zulu)	Roots	Decoction	(Hedberg et al., 1983)
Securidaca longepedunculata Fresen.	Polygalaceae	Violet tree, Fibre tree	Mpesu (Venda) Mmaba (Tswana)	Roots	Decoction	(Inga Hedberg et al., 1983)
Scutia myrtina (Burm. f.) Kurz	Rhamnaceae	Cat-thorn	iSiphingo (Zulu) Uqapula (Xhosa)	Roots	Decoction	(Inga Hedberg et al., 1983)

Crossopteryx febrifuga (Afzel. ex. G. Don) Benth.	Rubiaceae	Common crown-berry, Crystal Bark	Mukhobekwa (Venda)	Stem bark	Infusion	(Inga Hedberg et al., 1983)
Solanum incanum L.	Solanaceae	Thorn apple, Bitter apple, Bitterball, and Bitter tomato	Munhundurwa (Shona)	Roots and leaves	Decoction	(Inga Hedberg et al., 1983)
Steganotaenia araliacea Hochst.	Umbelliferae	Carrot tree	Mubanda (Shona)	Roots	Decoction	(Inga Hedberg et al., 1983)
Kigelia africana (Lam.) Benth	Bignoniaceae	Sausage tree	Muvevha (Venda)	Fruit	Decoction and infusion	(Inga Hedberg et al., 1983)

2.2.2 *Candida albicans*

2.2.2.1 Introduction

Candida albicans (C-P. Robin) Berkhout (1923) is a harmless opportunistic gram-positive yeast like pathogen amongst 200 known *Candida* species within the Saccharomycetaceae family. It is hosted in different tracts of the human body, for example, the gastrointestinal and genitourinary tracts that form part of the human microbiota (Kabir, Hussain, & Ahmad, 2012). *Candida albicans* remains harmless within approximately 50-70% of the human population, this is dependent on the population examined (Williams, Kuriyama, Silva, Malic, & Lewis, 2011). *Candida albicans* goes from a commensal pathogen to an invasive pathogen due to its ability to undergo rapid changes or responses to changes in the environment (Palmer, 2008). The *Candida* species is polymorphic and reproduces in unique forms as the morphology transitions between unicellular yeast (Figure 2.7A), filamentous hyphae (Figure 2.7B) and forms chlamydospores (Figure 2.7 C). The morphological transition plays an important role in the infection process (Dowd, 2011; Sudbery, 2011; Villar, Kashleva, & Dongari-Bagtzoglou, 2004).

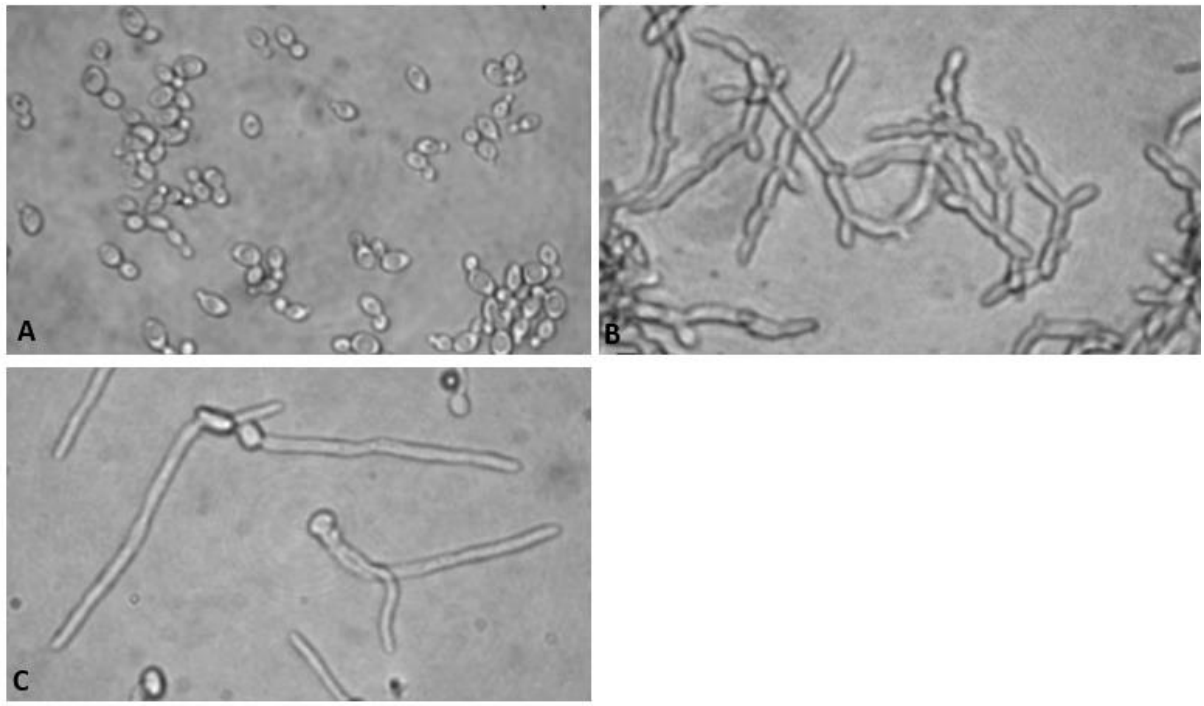

Figure 2.7: Photomicrographs of growth morphologies of *Candida albicans:* (A) pseudohyphae, (B) hyphae, and (C) chlamydospores. (Moran, Coleman, & Sullivan, 2012).

The pathogen resides in moist, damp regions of the human body; for example, in folds of skin of obese or overweight individuals. There are two different severities, mucosal and systemic infections (François L. Mayer, Duncan Wilson, & and Bernhard Hube, 2013). Mucosal infections are superficial infections that occur within the cells of the oral cavity (thrushes), esophagus, gastrointestinal tract, genitals (vulvovaginitis) and the respiratory system. Superficial infections also include cutaneous infections, which occur on the skin and nails of an individual. Systemic candidiasis is predominately present within individuals who are immunocompromised. For instance, individuals who are infected with HIV, transplant recipients, and chemotherapy patients, which can cause endocarditis, lung, kidney, and brain infections (Calderone & Fonzi, 2001; Kabir et al., 2012; Pfaller & Diekema, 2007).

2.2.2.2 Types of Infections, Signs, and Symptoms

Mucosal infections occur in the mucous membrane of an infected individual. This primarily leads to oral, oesophageal, gastrointestinal, and respiratory candidiasis. Other mucosal infections include candidal vulvovaginitis and balanitis, which mainly infects the reproductive tracts (Vazquez & Sobel, 2002). Oral candidiasis infects the mucous membrane of the mouth when there is an overgrowth of *Candida* species. There are three different classifications of oral candidiasis namely acute candidiasis, chronic candidiasis and angular cheilitis (stomatitis). Acute illness includes acute pseudomembranous (thrush) (Figure 2.8A) and acute atrophic (erythematous) candidiasis (Figure 2.8B). Chronic illness includes chronic hyperplastic (candidal leucoplakia), denture-induced (chronic atrophic-erythematous) candidiasis, and median rhomboid glossitis. Symptoms of acute pseudomembranous include whitish cream plaque-like lesions, a slight tingling sensation and a foul taste on the oral surfaces of labial and buccal mucosa, tongue, hard and soft palate, and oropharynx. Some infected individuals experience dysphagia and chest pains due to the infection progressing to the oesophageal region. Erythematous candidiasis symptoms include lesions on the dorsum of the tongue and the palate, which often become painful lesions. Candidal leukoplakia signs and symptoms include white plaque-like and erythematous nodular lesions that appear on the bilateral regions of the buccal mucosa and the lateral region of the tongue.

Denture candidiasis (denture stomatitis) typically appears on the mucosa of the denture region and tends to be dominant in individuals with poor oral hygiene practice, nocturnal denture wear and ill-fitting prostheses. The lesions, which appear as pinpoint hyperaemia,

diffuse erythematous on the underside of the denture base and inflammatory granular/papillary hyperplasia of the palate. Infected individuals experience a burning sensation and soreness in the infected area (Kossioni, 2011).

Median rhomboid glossitis predominately affects individuals who are tobacco smokers and inhalation-steroid users. The affected areas appear in the middle of the dorsum of the tongue. The lesions on the tongue appear as a smooth symmetrical depapillated area arising anterior to the circumvallate papillae. Some individuals experience persistent pain, irritation and pruritus. Angular cheilitis usually appears on one or both ends of the mouth as erythematous fissures and are associated with bacteria *Staphylococcus* and *Streptococcus* (Akpan & Morgan, 2003; Kossioni, 2011; Patil, Rao, Majumdar, & Anil, 2015; Vazquez & Sobel, 2002).

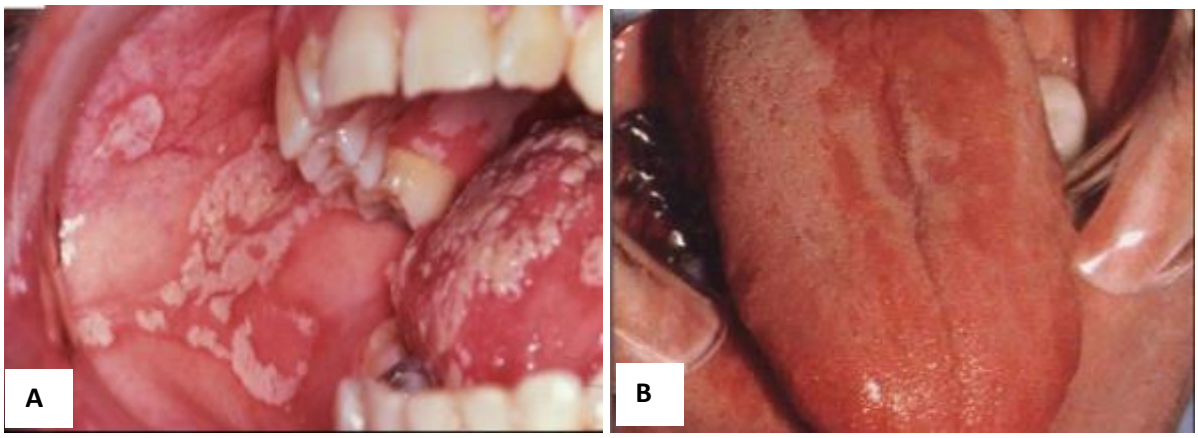

Figure 2.8: Clinical manifestations of oral candidiasis, (A) *Pseudomembranous candidiasis* possessing whitish/cream lesions on the tongue and buccal mucosa and (B) Erythematous candidiasis, exhibiting red lesions on the tongue (Jabra-Rizk et al., 2016).

Vulvovaginal candidiasis occurs within the epithelial cells of the vagina after the colonization of hyphae within these cells. The signs and symptoms of vulvovaginal candidiasis include pruritus and a cottage cheese-like, cream/whitish, watery, or homogenous thick vaginal discharge. Other symptoms include vaginal sores, pain, and redness. Symptoms that are more predominant include vulvar burning, dysuria, and dyspareunia. Erythema and swelling of the labia and vulva are often observed upon examination, often with fissures and pustulopapular peripheral lesions. The cervix usually remains normal and only the vulvar and vagina are mainly infected (Eckert et al., 1998; Sobel, 2007; Spinillo, Capuzzo, Acciano, De Santolo, & Zara, 1999).

Cutaneous infections occur as secondary infections on the nails and skin, which could be sub-acute or chronic infections. Infections that have affected the nails and skin can be localized or generalized to the nail and skin. Infections on the skin can lead to lesions on the face, neck, ears, shoulders, and scalp and often in the groin region. Symptoms of infection on the nails include lesions on the fingernails~~,~~ that vary is discoloration and dystrophy of the nails. Other symptoms include nail crusting and hyperkeratotic horns (Vazquez & Sobel, 2002).

Systemic infections can occur within the blood and spread through the bloodstream to other organs including the eyes, kidney, liver and brain, leading to invasive candidemia. Symptoms of candidemia include fever or chills, a skin rash, muscle aches, eye infections or visual impairments, headaches and neurological deficits and abdominal pain (Kotthoff-Burrell, Fahy, Lareau, Hage, & Sockrider, 2019).

2.2.2.3 Current treatment

Once the fungus *Candida albicans* is identified using the diagnostic mechanisms, the appropriate medical treatment is provided after an examination is done by a medical professional. The treatment used to eradicate the fungus mainly contains –azoles known as Clotrimazole (Figure 2.9A), Fluconazole (Figure 2.9B), Isavuconazonium (Figure 2.9C), Itraconazole (Figure 2.9D), Ketoconazole (Figure 2.9E), Miconazole (Figure 2.9F), and Voriconazole (Figure 2.9G).

Imidazoles compounds, namely miconazole, clotrimazole and ketoconazole, consist of a five-membered ring structure containing two nitrogen atoms where there is a side chain that is attached to one of the nitrogen atoms. Fluconazole and itraconazole are triazoles containing three nitrogen atoms within the ring structure. Other triazole antifungals include~~;~~ posaconazole, ravuconazole, and voriconazole (Casalinuovo, Di Francesco, & Garaci, 2004). The treatment used for candida infections are type-dependent and are prescribed accordingly.

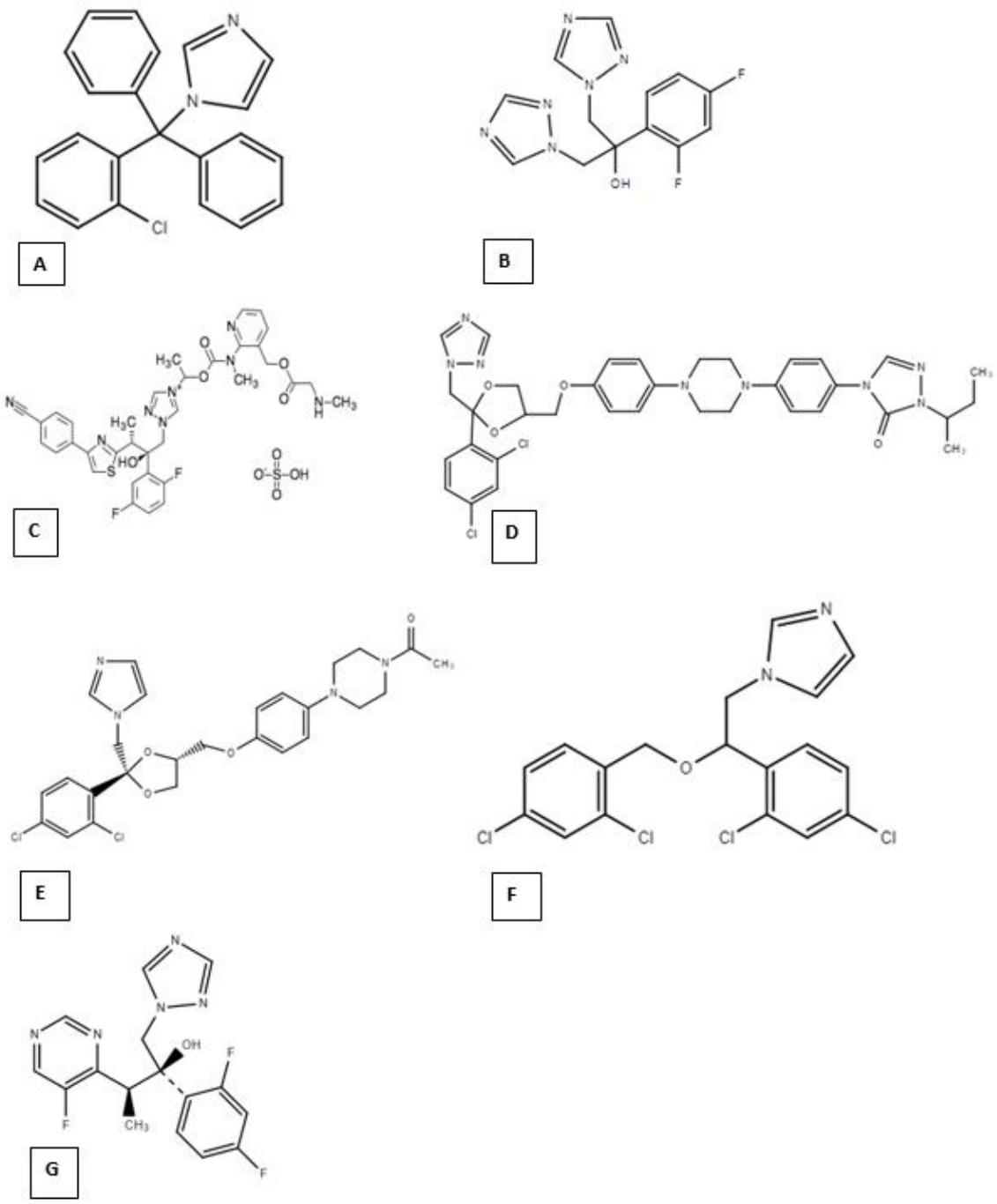

Figure 2.9: Anti-fungal medication containing mainly -azoles; these anti-fungal include (A) Clotrimazole, (B) Fluconazole, (C) Isavuconazonium, (D) Itraconazole, (E) Ketoconazole, (F) Miconazole and (G) Voriconazole.

Fluconazole is a bis-triazole antifungal drug, with pharmacokinetic properties such as, metabolic stability and relatively high water solubility, which contributes to its therapeutic effects (Grant & Clissold, 1990). Fluconazole's mechanism of action is its inhibition of the lanosterol demethylase enzyme (or 14α-sterol demethylase) which disrupts the conversion

of lanosterol to ergosterol. This is achieved by the removal of 14α-sterol demethylase from lanosterol. The enzyme 14α-sterol demethylase is a cytochrome P450-dependent enzyme (P440-Erg11p or Cyp51p). Due to the enzyme inhibition, the accumulation of 14α-methylated sterols results in a defected cell membrane and altered permeability of the fungal cell. Fluconazole is given to infected individuals whether the infection is superficial or systemic (Figure 2.10) (Casalinuovo et al., 2004; Grant & Clissold, 1990; Lewis, Lund, Klepser, Ernst, & Pfaller, 1998).

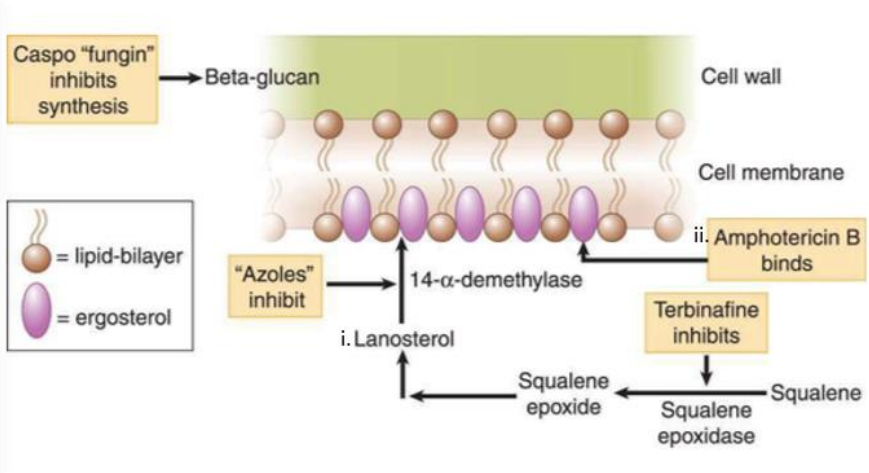

Figure 2.10: A schematic diagram depicting the different mechanisms of actions of antifungals against *Candida* species.

Amphotericin B (Figure 2.11A) is an antifungal drug given to infected individuals intravenously that have an invasive fungal infection. The mechanism of action of amphotericin B involves it binding to ergosterol in the cell membrane. Due to the binding, there are ion channels (pores) that develop. The ion channel development accommodates a rapid leakage of monovalent ions (K^+, Na^+, and Cl^-) and protons (H^+) resulting in depolarization and subsequent cell concentration death (Laniado-Laborin & Cabrales-Vargas, 2009; Lewis et al., 1998).

Fluconazole and amphotericin B are currently used as a treatment for candidal infections. Due to the worldwide increase in the incidence of candidal infections, research continues for an improved antifungal drug. After an extensive search for an improved antifungal drug, scientist were lead to the discovery of echinocandins. Echinocandins namely anidulafungin (Figure 2.11B), caspofungin (Figure 2.11C), and micafungin (Figure 2.11D) inhibit the enzyme β (1,3)-D- glucan synthase, influencing the integrity of the fungal cell wall (Grover, 2010). The

mechanism of action involves the disruption of the cell wall where echinocandins act as a non-competitor of the β (1,3)-D- glucan synthase, which affects the rigidness of the cell resulting in osmotic instability and ultimately the cell lysis (Grover, 2010; Onishi et al., 2000; Sucher, Chahine, & Balcer, 2009).

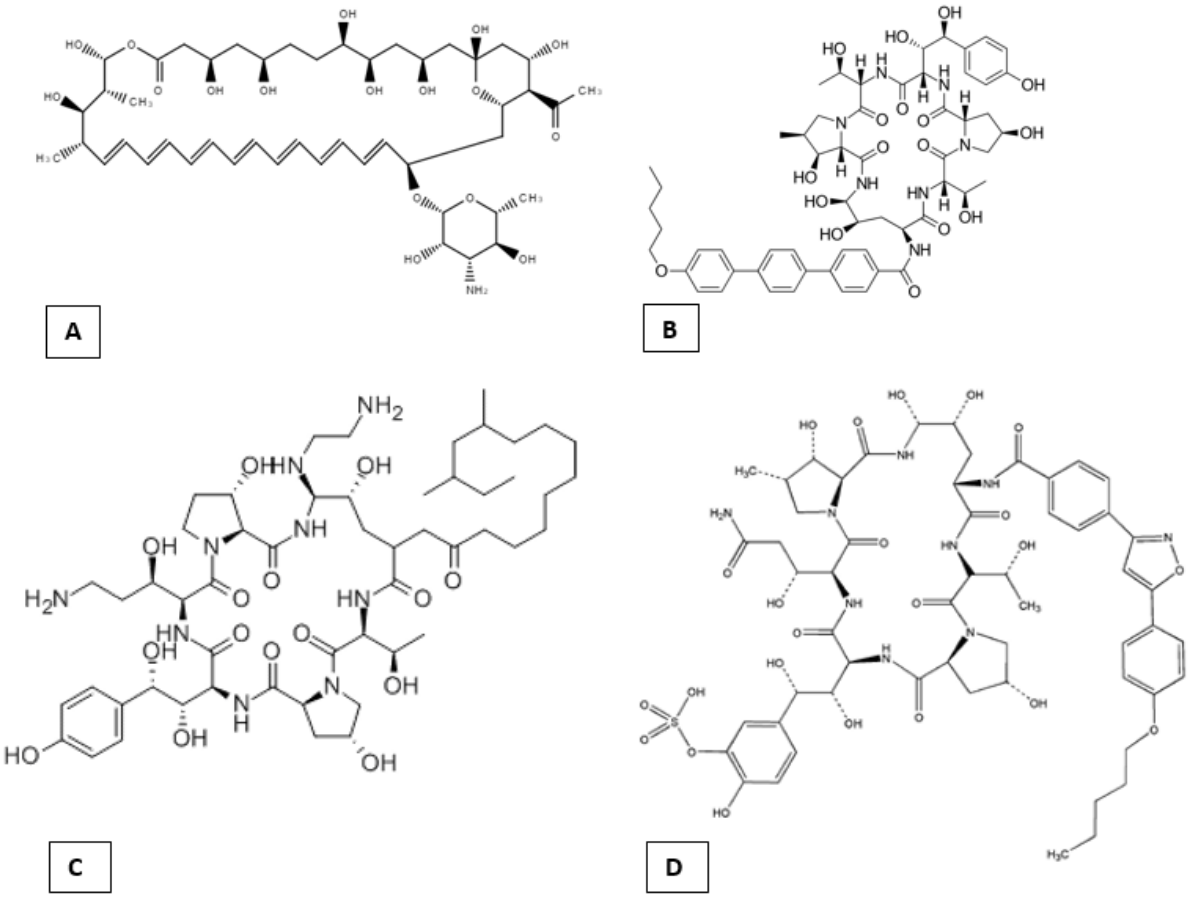

Figure 2.11: Chemical structure of antifungal drugs; (A) Amphotericin; (B) Anidulafungin; (C) Caspofungin; and (D) Micafungin

2.2.2.4 Current medicinal treatment

Due to antimicrobial resistance of antifungal agents, alternative treatment has been investigated. Medicinal plants (Table 2.2) illustrates alternative medicinal treatment, dosage and preparation of these plants used to treat various candidal infections. Some of these medicinal plants can be found in South Africa but are not endemic to Africa.

Table 2.2: A list of medicinal plants used in South African that was documented for the treatment of sexually transmitted infection (STI) Candida caused by the fungus *Candida albicans.*

Botanical Name	Family Name	Vernacular Name	Plant part(s) used	Method of preparation	Route of administration	Condition	Reference
Carica papaya L.	Caricaceae	uPopo (Zulu)	Leaves and roots	Grind roots of *C. papaya* and *Psidium guajava* in hot water.	Oral, a cup is taken 3 times a day or douching.	Vaginal candidiasis	(Masevhe, McGaw, & Eloff, 2015; Deborah K.B. Runyoro, Matee, Ngassapa, Joseph, & Mbwambo, 2006)
Carpobrotus edulis (L.) L. Bolus	Mesembryanthemaceae	uMgongozi (Zulu)	Bark	Boil grounded bark (powder form) for 20-30 minutes	Oral mouthwash; 3 times a day for a week.	Oral thrush	(Masevhe et al., 2015; Thring & Weitz, 2006)
Dodonaea augustifolia L.f.	Sapinadaceae	Mutata-vhana (Venda)	Fresh leaves	Crushed fresh leaves are macerated in water with bark of *Dovyalis zeyheri*	Oral (1/2 cup); macerate the plant material together.	Oral candidiasis	(Masevhe et al., 2015; Patel & Coogan, 2008)
Elaeodendron transvaalense (Burtt Davy)	Celastraceae	uMgugudo (Zulu)	Roots	Dried powder boiled in water	Oral, ½ cup of the decoction; 3 times a day	Candidiasis	(Bessong et al., 2005; Masevhe et al., 2015)

Botanical Name	Family Name	Vernacular Name	Plant part(s) used	Method of preparation	Route of administration	Condition	Reference
Elephantorrhiza elephantina (Burch.) Skeels	Fabaceae	Gumululo (Venda)	Bark Rhizomes	Ground dry bark	Topical application: twice a day Oral, decoction	Candidal infections	(Maroyi, 2017a; Masevhe et al., 2015)
Knowltonia bracteata Harv. Ex J.Zahlbr.	Ranunculaceae	Umvuthuza (Zulu)	Roots	Roots are pulverized and soaked in water for at least two days	Oral, 1 cupful maceration taken 3 times a day	Candidal infections	(Buwa & Van Staden, 2006; Masevhe et al., 2015)
Sclerocarya birrea (A. Rich.) Hochst sups. *caffra* (Sond.) Kokwaro	Anacardiaceae	Mufula (Venda)	Root and stem bark	Decoction is made	Oral, glass is taken 3 times a day	Oral and oesophageal candidiasis	(Naidoo et al., 2013; D. K.B. Runyoro, Ngassapa, Matee, Joseph, & Moshi, 2006)

2.3. Medicinal plant selection

The four medicinal plants used in this study was selected based on their antimicrobial properties in the treatment and management against sexually transmitted infections. Traditional healers from a village in the Jongilanga tribal council within the Mpumalanga region use these medicinal plants as a treatment. Traditionally the medicinal plants are prepared according to the type of disease in question and plant parts used, as well as preparation methods. Voucher specimens of the plants selected were identified and deposited at the HGJW Schweickerdt Herbarium of the University of Pretoria.

2.3.1 *Dicoma anaomala Sond. subsp anomala*

Dicoma anomala Sond. subsp. a*nomala* is taxonomically classified within the Asteraceae family and is commonly known as fever bush and stomach bush (Eng.). It is also known by other names, such as maagbitterwortel, aambeibos, koorsbossie, gryshout and kalwerbossie (Afr.), Hloenya and Mohasetse (Sotho), and Umuna (Zulu). The fever bush is endemic to South Africa, however is widely distributed throughout sub-Saharan Africa. *Dicoma anomala* is widely distributed throughout the nine provinces namely the Eastern Cape, Free State, Gauteng, KwaZulu Natal, Limpopo, Mpumalanga and North-West (Balogun, Omotayo, & Ashafa, 2016; Raimondo et al., 2005). The natural habitat of the fever bush is stony grasslands, hillsides or flat grasslands, as well as the savannah region (Maroyi, 2018).

2.3.1.1 Description

The phytomorphology of this herb is characterized by its hairy thin stems and white flower heads which are at the terminal position (Figure 2.12). *Dicoma anomala* stems range from 5 cm to 60 cm, consist of an erect or suberect branches and are decumbent. These stems are branched, yellowish in colour and are subterranean. This herb is a perennial shrub that consists of semi-woody tubers at the end of the hairy woody stem. The leaves are simple, narrow, and lanceolate in shape. The leaves have an alternate arrangement on the stem. The underside of the leaf has fainted uneven margins and is white in colour with fine hairs. The upper side of the leaf contains a prominent central vein along the leaf, which folds inwards. It is olive green in colour and has a smooth texture. Terminal flower heads are pinkish-white in colour and are cone shaped (Maroyi, 2018; Mnegwane & Koekemoer, 2007).

Figure 2.12: *Dicoma anomala Sond*. plant used in medicinal therapy.

2.3.1.2 Ethnomedicinal uses

Ethnomedicinal uses of *D. anomala* have been used in countries such as Botswana, Lesotho, Malawi, Namibia, South Africa, Swaziland, Tanzania and Zimbabwe. South Africa has the highest number of ethnomedicinal uses to treat human ailments, namely 37 (Table 2.3). These ailments are treated by preparing concoctions using various parts of *D. anomala*. Different ethnic groups within South Africa use different concoctions for treatment of various ailments. Some of the commonly treated human ailments include gastrointestinal disorders such as diarrhoea, dysentery, stomach problems, pains, sores and wounds. Other ailments which can be treated by this herb include colds, cough, sore throats, fever, blood circulation problems and sexually transmitted infections (STIs) (Maroyi, 2018; Seleteng Kose, Moteetee, & Van Vuuren, 2015).

Table 2.3: Ethnomedicinal uses of *Dicoma anomala* used by traditional healers mainly in South Africa and surrounding countries.

Plant part	Ethnomedicinal uses	References
Bark	Coughs	(Gerstner, 1939; Maema, Mahlo, & Potgieter, 2017; Roberts, 1990)
Leaves	Diabetes	(Balogun, Tshabalala, & Ashafa, 2016; Moteetee & van Wyk, 2011; Seleteng Kose et al., 2015)
	Infertility	(Moteetee & Seleteng Kose, 2016; Panganai & Shumba, 2016; Steenkamp, 2003)
Roots	Bladder problems	(Monakisi, 2007)
	Blood circulation diseases	(Maroyi, 2018; S. S. Semenya & Potgieter, 2013)
	Enema	(Monakisi, 2007)
	Fever	(Mugomeri, Chatanga, Raditladi, Makara, & Tarirai, 2016; Roberts, 1990)
	Genital problems	(Monakisi, 2007)
	Gonorrhoea	(Marekerah, 2015)
	Indigestion during pregnancy	(A Hutchings, Scott, Lweis, & Cunningham, 1996; Varga & Veale, 1997)
	Intestinal worms	(Roberts, 1990)
	Kidney problems	(Monakisi, 2007)
	Measles	(S. S. Semenya, Potgieter, & Tshisikhawe, 2013)

	Pneumonia	
	Prostrate problems	
	Respiratory problems	(Gerstner, 1939)
	Skin lesions	(Maema et al., 2017; Moteetee & Kose, 2017; Shale, Stirk, & Van Staden, 1999)
	Sexually transmitted infections	(S. S. Semenya et al., 2013)
	Swollen legs	(S. S. Semenya et al., 2013)
	Venereal diseases	(Roberts, 1990)
Tubers	Acne	(Mabona, Viljoen, Shikanga, Marston, & Van Vuuren, 2013; Madzinga, Kritzinger, & Lall, 2017; Maroyi, 2017a)
	Asthma	(Maema et al., 2017)

Dicoma anomala can be used in combination with other medicinal plants to treat various ailments. Neighbouring countries such as Lesotho, Malawi and Zimbabwe have used other medicinal plant species such as *Scabiosa columbaria, Helichrysum caespititium*, combinations of *S. columaria* and *Zantedeschia albomaculata, Trichodesma physaloides (Fensl) Aspilia pluriseta Schweinf* and *Cymbopogon* spp. Infusions are made using combinations of various medicinal plants for the treatment of ailments such as menstrual pains and backaches. These infusions are also used to initiate labour in order to facilitate easier childbirth (Maroyi, 2018).

2.3.1.3 Phytochemistry

Phytochemical analysis of *D. anomala* includes secondary metabolites such as acetylenic compounds, diterpenes, flavonoids, phenolic acids, phytosterols, saponins, sesquiterpene lactones, tannins and terpenoids (Maroyi, 2018). Phytochemicals present within the aerial parts and roots are illustrated in Table 2.4. Biological activities such as, antimicrobial, anti-inflammatory, antioxidant, antiplasmodial, anticancer, toxicity, cytotoxicity, antihyperglycaemic and hepatoprotective have been stated in literature where *D. anomala* was used (Becker et al., 2011; Cindy, 2012; Setshogo & Mbereki, 2011). Phytochemical constituents play a vital role in antimicrobial activity. The constituents present within the aerial and root parts include stigmasterol, β-sitosterol, lupeol, and albicolide. Other constituents include germacrene D, taraxasterol, lupenone, crisimaritin and scutellarein.

Table 2.4: Phytochemical constituents present in various plant parts of *Dicoma anomala.*

Classification	Phytochemical compound	Plant Part	Reference
Sesquiterpenes	Germacrene D	Aerial and root parts	(Zdero & Bohlmann, 1990)
	Lupenone		
Sesquiterpene lactones	Eudesmanolides	Aerial and root parts	(Bohlmann, Singh, & Jakupovic, 1982)
	Melampolides		
	Germacronolides		
	Guaianolides		
Germacranolide and guainolide-type sesquiterpene lactones	albicolide and 14-acetoxydicomanolide with 6,7-lactone closure		(Becker et al., 2011; Rademeyer, Van Heerden, & Van Der Merwe, 2008)
Melampolide-type sesquiterpene lactones			(Marekerah, 2015)
Phytosterols/sterols	β-sitosterol		(Bohlmann, Singh, & Jakupovic, 1982, Bohlmann & le van, 1978, Mukanganyama, Munodawafa, Chagonda, & Moyo, 2013, Bezabih, et al., 2011, van der Merwe, 2008)
	Lupeol		
	Stigmasterol,		
	Taraxasterol		

2.3.2 *Elephantorrhiza elephantina*

Elephantorrhiza elephantina (Burch.) Skeels belongs to the Fabaceae family and is commonly known as elephant's root (Eng.). Other common names include olifantswortel, leerbossie and baswortel (Afr.); mupangara (Shona); mositsane (Sotho, Tswana) and intolwane (Xhosa, Zulu). The plant flourishes in hot, dry grassland regions and is endemic to the southern regions of Africa. *Elephantorrhiza elephantina* is widely spread in the southern parts of Angola, Botswana, Lesotho, Mozambique, Namibia, Zimbabwe, and South Africa. In South Africa, it is commonly found in eight of the nine provinces, namely Limpopo, North-West, Gauteng, Mpumalanga, Free State, KwaZulu-Natal, Northern Cape and Eastern Cape (Maroyi, 2017a).

2.3.2.1 Description

Elephant's root is characterized by its low growing, unbranched and unarmed aerial stems. The bark and young branchlets are dark reddish/brown in colour. The plant has green bipinnately compound leaves that are arranged from 2 to 17 opposite or sub-opposite pairs of pinnae. Flowers are arranged in an axillary, solitary cluster of racemes that vary from a golden yellow to a pale yellow and protrude from the ground level (Figure 2.13). The flowering season usually occurs between September and December; it does not require a rainy season for flowering. The fruits are a dark reddish-brown in colour (Stone, Raine, Prescott, & Willmer, 2003).

Figure 2.13: The flower arrangement of *Elephantorrhiza elephantina.*

2.3.2.2 Ethnomedicinal uses

Structural organs such as rhizomes, roots, leaves and stems have medicinal properties and are currently being used as traditional medicine. *Elephantorrhiza elephantina is* used as a traditional medicine is the highest within South Africa and is used for the treatment of various ailments (Table 2.5). Some of these ailments include, blood pressure, kidney problems, peptic ulcers and sexually transmitted infections. The concoction used may include combinations of *E. elephantina* as well as other medicinal plant species.

Table 2.5: Ethnomedicinal uses of *Elephantorrhiza elephantina* used by traditional healers mainly in South Africa and surrounding countries.

Plant part	Ethnomedicinal uses	References
Rhizomes	Blood pressure	(Monakisi, 2007)
	Cleaning the womb after an abortion	(Aaku, Office, Dharani, Majinda, & Motswaiedi, 1998; Pujol, 1990)
	Clearing the air canal	(Monakisi, 2007)
	Itching	(Monakisi, 2007)
	Kidney failure	(Monakisi, 2007)
	Sores	(Nciki, Vuuren, van Eyk, & de Wet, 2016)
	Tonsillitis	(Monakisi, 2007)
Roots	Chest pains	(Gerstner, 1939)
	Dysentery	(Lall & Kishore, 2014; S. S. Semenya & Potgieter, 2013; B. Van Wyk, Van Oudtshoorn, & Gericke, 1997)
	Fever	(Gerstner, 1939; Ribeiro, Romeiras, Tavares, & Faria, 2010)
	Haemorrhoids	(Mpofu, Msagati, & Krause, 2014)
	Hypertension	(Mpofu et al., 2014)
	Peptic ulcers	(A Hutchings et al., 1996)
	Rheumatic heart disorder	(A Hutchings et al., 1996)

	Sexually transmitted infections	(Aaku et al., 1998; Bandeira, Gaspar, & Pagula, 2001)
	Shingles	(Helene De Wet, Nciki, & van Vuuren, 2013)
	Syphilis	(Guillarmod, 1971; Lall & Kishore, 2014; Seleteng Kose et al., 2015; B. Van Wyk et al., 1997)
Rhizomes and Roots	Acne	(Felhaber & Mayeng, 1997; Lall & Kishore, 2014; Pujol, 1990; B. Van Wyk et al., 1997)
	Eczema	(Felhaber & Mayeng, 1997; Pujol, 1990)
	Erectile dysfunction	(Mukanganyama, Ntumy, Maher, Muzila, & Kerstin, 2011; S. Semenya, Maroyi, Potgieter, & Erasmus, 2013; S. S. Semenya & Potgieter, 2013)
	Intestinal disorder	(Felhaber & Mayeng, 1997; Lall & Kishore, 2014; Seleteng Kose *et al.*, 2015; B. Van Wyk et al., 1997)
	Sunburn	(Lall & Kishore, 2014; B. Van Wyk et al., 1997)
Rhizomes, Roots, Leaves and Stems	Diarrhoea	(Bisi-Johnson, Obi, Kambizi, & Nkomo, 2010; B. Van Wyk et al., 1997)
	Menstrual problems	(Bisi-Johnson et al., 2010; I Hedberg & Stuagard, 1989)

2.3.2.3 Phytochemistry

Metabolomics studies conducted on rhizomes, indicated the following classes of phytochemicals: anthocyanidins, anthraquinones (Figure 2.14A), esters (Figure 2.14B), fatty acids (Figure 2.14C), flavonoids (Figure 2.14D), gylcosides (Figure 2.14E), phenolic compounds (Figure 2.14F), polysterols (Figure 2.14G), saponins (Figure 2.14H), sugars (Figure 2.14I), tannins and triterpenoids (Figure 2.14J). Major phytochemical classes include fatty acids (39.13%), phenolic compounds (26.09%) and esters (13.04%). The remaining classes comprised of less than 10% (Maroyi, 2017a). (Table 2.6) indicate the major phytochemicals present. The isolation, detection, quantification and purification of phytochemicals present have been done using various spectrometric techniques.

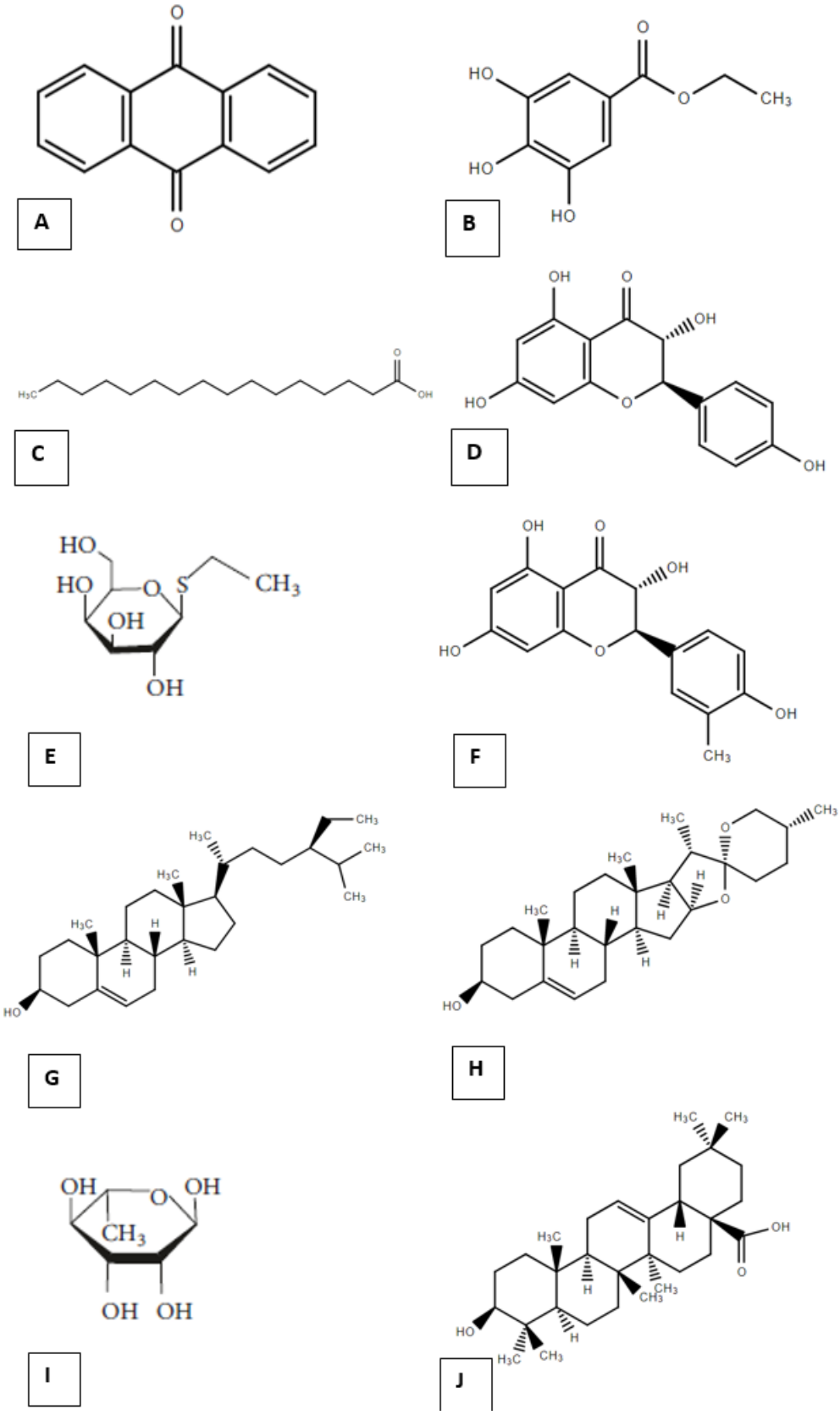

Figure 2.14: Chemical structure of (A) anthraquinone, (B) ethyl gallate, (C) hexadecanoic acid, (D) dihydrokaempferol, (E) ethyl-1-0-β-D-galactopyranoside, (F) catehcin, (G) β-sitosterol, (H) diosgenin, (I) rhamnose and (J) oleanolic acid.

Table 2.6: Phytochemical constituents present in various plant parts of *Elephantorrhiza elephantina.*

Classification	**Phytochemical compounds**	**Plant part**	**Reference**
Anthraquinone	Anthraquinone	Leaves, roots and rhizomes	(Mpofu et al., 2014)
Esters	Ethyl gallate		(Aaku et al., 1998)
	Butanedioci acid		(Msimanga, Fenstermacher, Levitz, Najimudeen, & Wysocki, 2013)
	Benzoic acid		(Msimanga et al., 2013)
	3-phenyl-2-propenoic acid		(Msimanga et al., 2013)
	Nonanedioic acid		(Msimanga et al., 2013)
	Methyl 3-(3,5-di,tert-butyl-4-hydroxy-phenyl)propionate		(Msimanga et al., 2013)
Fatty acids	Hexadecanoic acid		(Msimanga et al., 2013)

	9,12-Octadecadienoic	Leaves, roots and rhizomes	(Msimanga et al., 2013)
	9-Octadecenoic		(Msimanga et al., 2013)
	Octadecanoic acid		(Msimanga et al., 2013)
	Tridecanoic acid		(Msimanga et al., 2013)
	Methyl pentadecanoate		(Msimanga et al., 2013)
	Methyl hexadec-9-enoate		(Msimanga et al., 2013)
	Methyl hexadecanoate		(Msimanga et al., 2013)
	Cis-10-heptadecenoic acid		(Msimanga et al., 2013)
	Methyl heptadecanoate		(Msimanga et al., 2013)
	Methyl octadecanoate		(Msimanga et al., 2013)

	Cis-5,8,11,14,17-escosapenta-enoic acid	Leaves, roots and rhizomes	(Msimanga et al., 2013)
	Eicosanoic acid		(Msimanga et al., 2013)
	Methyl tetracosanoate		(Msimanga et al., 2013)
	Pentacosanoic acid		(Msimanga et al., 2013)
	Hexacosanoic acid		(Msimanga et al., 2013)
	Methyl octacosanoate		(Msimanga et al., 2013)
	Tetradecanedioic acid		(Msimanga et al., 2013)
Flavonoids	Dihydrokaempferol		(Aaku et al., 1998)
	Kaempferol		(Aaku et al., 1998; Mpofu, Msagati, & Krause, 2015)
Glycosides	Ethyl-1-O-β-D-galactopyranoside		(Aaku et al., 1998)

Phenolic compounds	2-(3,4-Dihydroxyphenyl) ethanol	Leaves, roots and rhizomes	(Aaku et al., 1998)
	Catechin		(Aaku et al., 1998; Mthembu, 2007)
	Gallic acid		(Aaku et al., 1998; Mthembu, 2007)
	4-Hydroxybenzoic acid		(Aaku et al., 1998)
	Quercetin 3-O-β-D-glucopyranoside		(Aaku et al., 1998; Mthembu, 2007)
	Epigallocatechin gallate		(Mpofu et al., 2015)
	Quercetin		(Mpofu et al., 2015)
	Epicatechin gallate		(Mpofu et al., 2015)
	Methyl gallate		(Mthembu, 2007)
	3-O-Galloyl-3,3’,5,5’,7-pentahydroxyflavone		(Mthembu, 2007)

	Taxifolin-3’-O-β-D-glucoside	Leaves, roots and rhizomes	(Mthembu, 2007)
	Epicatechin		(Mpofu et al., 2015; Mthembu, 2007)
Phytosterols	β-Sitosterol		(Mthembu, 2007)
Saponin	Diosgenin		(Mpofu et al., 2014)
Sugar	Rhamnose		(Mpofu et al., 2014)
	Glucuronic acid		(Mpofu et al., 2015, 2014)
	Arabinose		(Mpofu et al., 2015, 2014)
Triterpenoid	Oleanolic acid		(Mpofu et al., 2014)

2.3.3 *Eucalyptus cinerea*

Eucalyptus cinerea taxonomically belongs to the Myrtaceae family and is commonly known as Silver Dollar Gum, Argyle Apple and Mealy Stingybark. There are approximately 700 *Eucalyptus* species, which is native to Australia, and a number of them to the Pacific Islands. Therefore, it is invasive in South Africa (Rejmanek & Richardon, 2011; Soliman, Fathy, Salama, & Saber, 2014).

2.3.3.1 Description

Argyle Apple is characterized by the smoothness of its bark, as well as its fibrous leaves with oil glands. The growth formation of *E. cinerea* varies from shrubs to tall trees (Figure 2.15A). The trees have a single stem or mallees, that grow directly from the ground, that are multi-stemmed and do not grow taller than 10 meters. The foliage of *E. cinerea* are oval in shape, with a camphoraceous fragrance and is silver green in colour. The stem however is reddish brown in colour (Soliman et al., 2014) (Figure 2.15B).

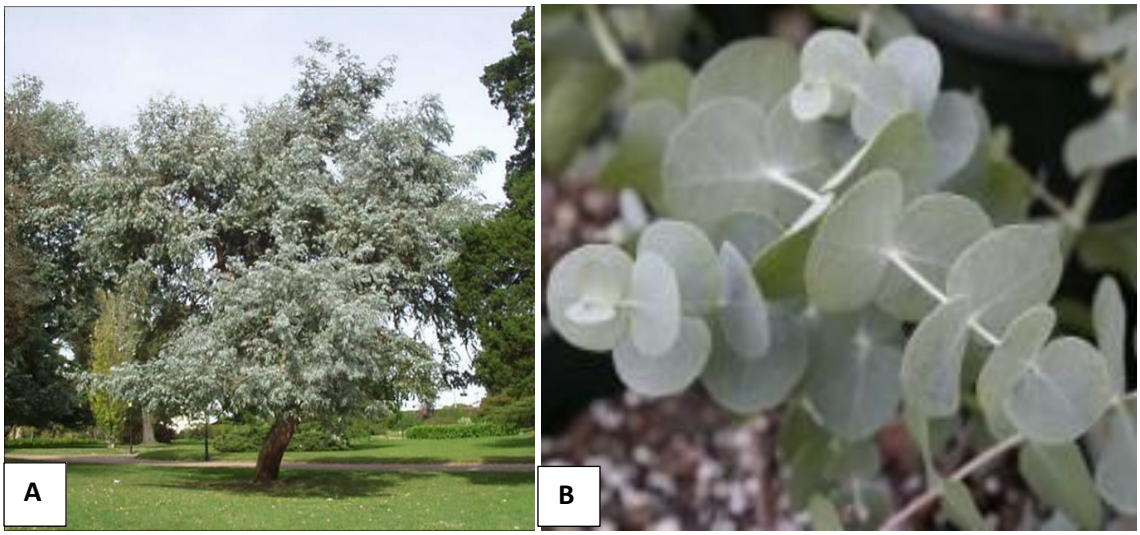

Figure 2.15: (A) *Eucalyptus cinerea* tree and (B) the foliage colour of *Eucalyptus cinerea.*

2.3.3.2 Ethnomedicinal uses

Different parts of the plant such as the leaves, bark, and stems have been used to make essential oils. Essential oils from the leaves of *E. cinerea* are used for its medicinal properties, such as its astringent and antiseptic properties (Soliman et al., 2014). Biological activities include antimicrobial, fungicidal, insect repellent, fumigant, and pesticidal activities (Batish, Singh, Kohli, & Kaur, 2008; Soliman et al., 2014). Other ethnomedicinal uses include the healing of wounds such as burns, cuts, abrasions, sores and scrapes. It can also help treat respiratory

issues such as relieving a cough, helping to expel mucus and relaxing respiratory muscles. *E. cinerea* can be used as a pain killer, a nerve blocker and an anti-inflammatory (Dhakad, Pandey, Beg, Rawat, & Singh, 2018; Vuong, Chalmers, Jyoti Bhuyan, Bowyer, & Scarlett, 2015). Table 2.7 indicates which part of the plant is used to treat which ailment.

Table 2.7: Ethnomedicinal uses of *Eucalyptus cinerea* used by traditional healers.

Plant part	**Ethnomedicinal use**	**References**
Leaves and inner bark	Flus, colds and fever	(Gómez-Estrada et al., 2011; Lamorde et al., 2010; Locher & Currie, 2010; Vuong et al., 2015)
Leaves, stem and bark	Aching, sore and internal pain	
Leaves	Heals sores and cuts	
Bark	Toothache and oral infection	
Fresh leaves and stems	Childbirth	

2.3.3.3 Phytochemistry

Phytochemical constituents of *E. cinerea* play a major role in the antimicrobial, anti-inflammatory and anticancer properties of the plant. There are nonvolatile compounds and volatile compounds such as apigenin (Figure 2.16A), ellagic acid (Figure 2.16B) and gallic acid (Figure 2.16C) (Table 2.8) present. The volatile compounds include Eucalyptol (1,8-cineole) (Figure 2.16D) which accounts for 70% of the oil mass and is responsible for the camphor-like smell. Other volatile compounds include (-)-limonene (Figure 2.16E) and (+)-α-terpineo (Figure 2.16F) (Murata et al., 2013). Nonvolatile compounds include mostly phenolic compounds, which play a role in the antioxidant properties of the plant. These compounds include apigenin, catechin, ellagic acid, epicatechin, gallic acid, myricetin, naringenin, pyranoside, quercetin and rutin (Bhuyan et al., 2016).

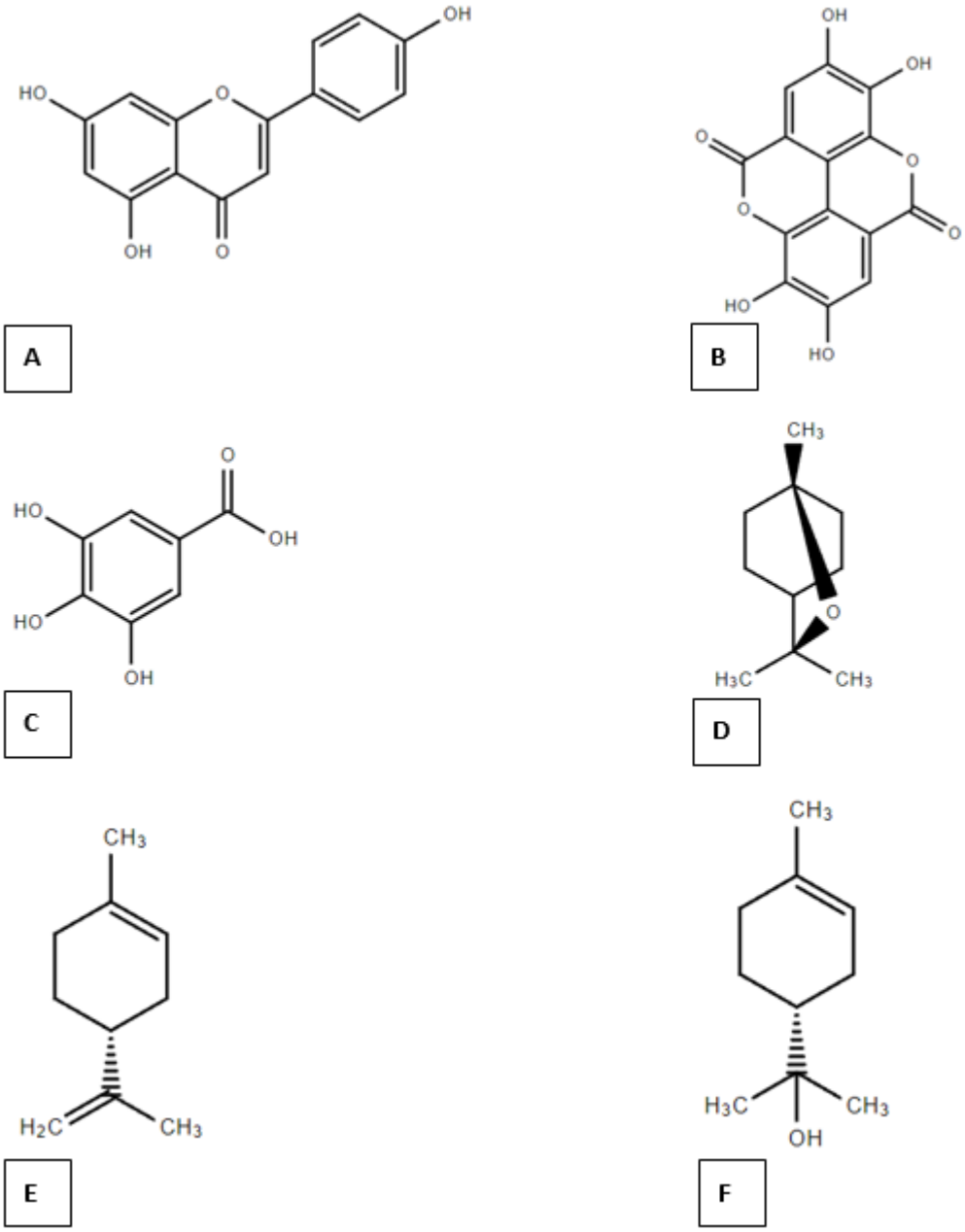

Figure 2.16: Chemical structures of (A) apigenin, (B) ellagic acid, (C) gallic acid, (D) eucalyptol (1,8-cineole), (E) (-)- limonene and (F) (+) α-terpineol.

Table 2.8: Phytochemical constituents present in various plant parts of *Eucalyptus cinerea.*

Classification	Phytochemical compound	Plant part	Reference
Nonvolatile compounds (Phenolic compounds)	Apigenin	Leaves	(Bhuyan et al., 2016; Gutiérrez & del Río, 2001; Kim et al., 2001; Lagrange, Jay-Allgmand, & Lapeyrie, 2001; Mongkholrattanasit et al., 2013; Vuong et al., 2015)
	Catechin		
	Ellagic acid	Stem bark	
	Epicatechin	Leaves	
	Gallic acid		
	Myricetin		
	Pyranoside	Stem bark	
	Quercetin	Leaves	
	Rutin	Roots	
Volatile compounds	Eucalyptol (1,8-cineole)	Leaves	(Murata et al., 2013; Vuong et al., 2015)
	(-)-limonene		
	(+)-α-terpineol		

2.3.4 *Kigelia africana*

Kigelia africana (Lam.) Benth. belongs to the Bignoniaceae family and is commonly known as the sausage tree (Eng.), worsboom (Afr.), umVunguta, umFongothi (Zulu), modukguhlu (North Sotho) and Muvevha (Venda). The tree is native to and widely distributed throughout Sub-Saharan Africa and is found in South Africa. *Kigelia africana* is found in four of the nine provinces in South Africa, namely Gauteng, KwaZulu Natal, Limpopo and Mpumalanga. Optimal environmental conditions for *K. africana* are open woodlands, along riverbanks, streams and flood lines (Bello, Shehu, Musa, Zaini Asmawi, & Mahmud, 2016).

2.3.4.1 Description

The sausage tree can grow up to 25 m in height and has a dense crown (Figure 2.17A). Towards the crown, the leaves are found in an opposite arrangement or in whorls of three and has a terminal leaflet. The flower arrangements are long open sprays of about 5-12 flowers. The petals are long and deep red or maroon in colour (Figure 2.17B). The flowers are pollinated by bats, insects and sun-birds (Namah, Midgley, & Kruger, 2019; Osman, Ali, Chitiboyina, & Khan, 2017). The fruit, typically shaped like a sausage, can grow up to approximately 1 m in length and 18 cm in diameter, and weighing about 12 kg (Figure 2.17C). They appear greyish brown in colour when ripe and contain a pulp with multiple seeds embedded in the fruit (Bello et al., 2016; Olatunji & Olubunmi, 2009).

Figure 2.17: (A) Leathery and waxy leaves; (B) flower arrangement and (C) pigmentation of *Kigelia africana.*

2.3.4.2 Ethnomedicinal uses

There are multiple uses of *K. africana*. Various structural organs including the bark, roots, flowers and leaves are used medicinally to treat illness such as anemia, epilepsy, kwashikor and liver ailments (Glew et al., 2010). *Kigelia africana* is used as a traditional medicine for the

treatment of ulcers, sores and venereal diseases. Due to the potency of fresh fruits causing blisters in the mouth and on the skin, the best route to administer it is orally in the form of crushed dried fruits (Saini, Kaur, Verma, Singh, & Singh, 2009). Different structural organs can be used for the treatment of various ailments. The root and unripe fruit are used as an anthelmintic medicine and as a treatment for haemorrhoids and rheumatism (Irvine, 1961). The fruit and bark-are usually boiled in water and taken orally, as a traditional medicine, for the treatment of gynecological disorders, skin illness, tumors and male infertility (Osman et al., 2017; Owolabi, Omogbai, & Obasuyi, 2002). Table 2.9 summarizes the ethnomedicinal uses of *K. africana*

Table 2.9 Ethnomedicinal uses of *Kigelia africana* used by traditional healers mainly in South Africa and surrounding countries.

Plant part	Ethnomedicinal use	Reference
Bark	Infertility	(Bello et al., 2016; H. de Wet & Ngubane, 2014; B. Van Wyk et al., 1997)
Bark and fruit	Gynecological and obstetric conditions	(Bello et al., 2016; H. de Wet & Ngubane, 2014; B. E. Van Wyk, 2011)
Bark and leaves	Boils, eczema, psoriasis, leprosy, skin cancer, sexually transmitted infections	(Bello et al., 2016; H. de Wet & Ngubane, 2014; B. E. Van Wyk, 2011)
Fruit	Penile enlargement	(Bello et al., 2016; Neuwinger, 1996)
Leaves	Jaundice, Sexually transmitted infections	(Bello et al., 2016; H. De Wet et al., 2012; Eldeen & Van Staden, 2007; Neuwinger, 1996)
Roots	Gynecological complaints, constipation and tapeworms	(Bello et al., 2016; Fouche et al., 2008)
Seeds	Pneumonia, malaria, diabetes antifungal, eczema and waist pain.	(Bello et al., 2016; Fomogne-Fodjo, Van Vuuren, Ndinteh, Krause, & Olivier, 2014; Saini et al., 2009)

2.3.4.3 Phytochemistry

Phytochemical analysis of structural organs, such as leaves, stem, bark, fruit and flowers of *K. africana.* Analysis indicates that there are approximately 150 phytoconstituents, which include alkanes (Figure 2.18A), coumarins (Figure 2.18B), quinones (Figure 2.18C) and iridoids (Figure 2.18D). Other phytoconstituents include phenolic compounds (Figure 2.18E), triterpenes (Figure 2.18F), diterpenes (Figure 2.18G), sterols (Figure 2.18H), unsaturated fatty acids (Figure 2.18I) and esters (Figure 2.18J). Table 2.10 indicates the phytoconstituents and chemical structures found within the fruit~~s~~ of the sausage tree (Bello et al., 2016).

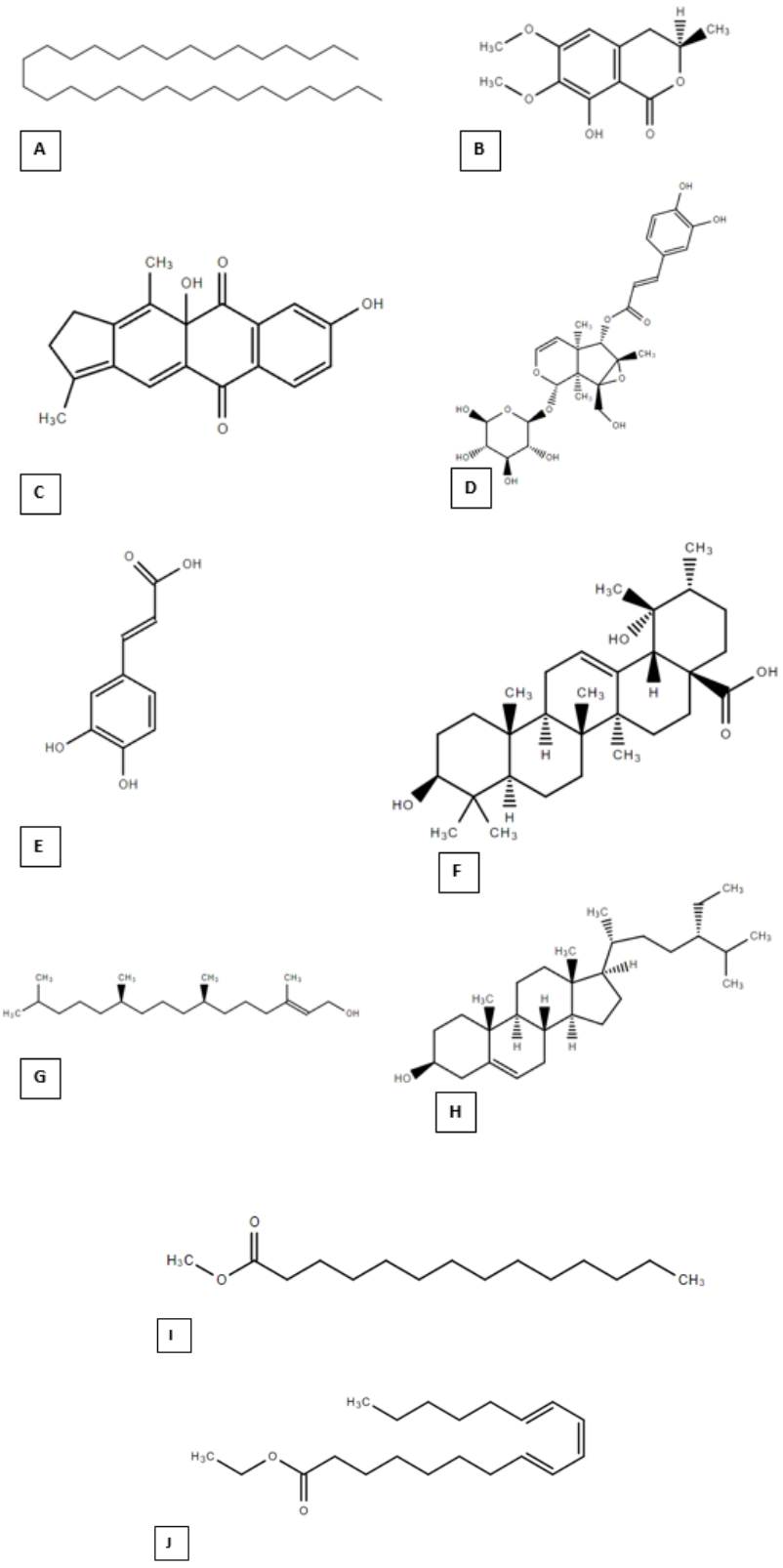

Figure 2.18: Chemical structures of (A) tritriacontane, (B) kigelin, (C) kigelinol, (D) verminoside, (E) caffeic acid, (F) pomolic acid, (G) phytol, (H) γ-sitosterol, (I) methyl-12-methyltetradecanoate and (J) ethyl-linoleoate.

Table 2.10: Phytochemical constituents present in various plant parts of *Kigelia africana.*

Classification	Phytochemical compound	Plant parts	References
Alkanes	n-hentriacontane	Leaves	(Dhungana, Jyothi, & Das, 2016)
	11-(2,2-dimethylpropyl) heneicosane		
	2,6,10-trimethyldodecane		
	Heneicosane		
	4,4-dimethylundecane		(Clarkson et al., 2004; Houghton, 2002)
	1-iodexadecane		(Houghton, 2002)
	1-iododecane		
	Tritriacontane		(Gouda et al., 2003)
	Hentriacontane		
	Nonacosane		
Coumarins	Kigelin	Roots, stem bark, leaves, wood	(Sidjui et al., 2014)
	Isokigelin	Stem bark	(Dhungana et al., 2016; Saraswathy, Purushothaman, Patra, Dey, & Kundu, 1992)
	1,3-dimethylkigelin	Stem bark	(Akande, Aboaba, & Flamini, 2018; Govindachari, Patankar, & Viswanathan, 1971)
	6-Demethylkigelin	Roots, stem bark	(Akande et al., 2018)
	6-Methoxymellein		
	8-hydroxy-6,7-dimethoxy-3-methyl-3,4-dihydroisocoumarin	Roots	(Adam, 2021; Dhungana et al., 2016)
Diterpenes	Phytol	Leaves	(Atolani, Olatunji, Fabiyi, Adeniji, & Ogbole, 2013)
	3-Hydro-4,8-phytene		
Esters	Pentafluoro-N-heptadecyl	Leaves	(Dhungana et al., 2016)
	2-ethylhexyloctadecyl sulphurous acid		
	2-(4-hydroxyphenyl) ethyl ester	Bark	(Sidjui et al., 2014)
	Ethyl linoleoate	Leaves, flowers	(Akande et al., 2018)

Iridoids	7-Hydroxyviteoid II	Fruits	(Atawodi & Olowoniyi, 2015; Bharti, Singh, Naqvi, & Azam, 2006; Dhungana et al., 2016; Gouda et al., 2003)
	7-hydroxy-10-deoxyeucommiol		
	Jiofuran		
	3-(2-hydroxyethyl)-5-(2-hydroxypropyl)-4,5-dihydrofuran-2(3H)-one		
	7-hydroxyeucommic acid		
	7-hydroxy eucommiol	Twigs, roots, leaves, wood	(Gouda et al., 2003; Grace et al., 2002)
	Jioglutolide		
	1-Dehydroxy-3,4-dihydroaucubigenin		
	Des-p-hydroxy benzoyl kisasagenol B		
	Ajugol		
	6-Trans-ca-eoyl ajugol		
	Verminoside	Stem bark, fruits, twig leaves, roots	(Adam, 2021; Agyare et al., 2013; Akunyili & Houghton, 1993; Bharti et al., 2006; Gouda et al., 2003; Olubunmi, Adeyemi, Akpan, Adeosun, & Olatunji, 2011; Sainadh, Nagarathna, Vasantha, & Kulkarni, 2013; Saini et al., 2009; B. E. Van Wyk, de Wet, & Van Heerden, 2008)
	Specioside	Stem bark	(Akunyili & Houghton, 1993; Gouda et al., 2003; Zorn et al., 2001)
	Minecoside		(Dhungana et al., 2016)
Phenolic compounds	p-Coumaric acid	Stem bark, fruits, roots	(Jackson, Houghton, Retsas, & Photiou, 2000; Sidjui et al., 2015, 2014)
	Caffeic acid	Stem bark, fruits, roots	(Sidjui et al., 2015, 2014)
	Ferulic acid	Stem bark, fruits	(Akunyili & Houghton, 1993; Binutu, Adesogan, & Okogun, 1996; Dhungana et al., 2016; Houghton, 2002; Jackson et al., 2000; Saraswathy et al., 1992)

	Atranorin	Stem bark	(Zofou et al., 2011)
	Noncosanoic acid, 2-(4-hydroxyphenyl) ethyl ester	Stem bark	(Sidjui et al*.,* 2015, 2014)
	Luteolin	Roots, leaves, wood	(Adam, 2021; Njogu, Arika, Machocho, Ngeranwa, & Njagi, 2018)
	Luteolin 7-O-glucoside	Leaves	(Houghton, 2002)
	6-hydroxyluteolin	Roots, leaves, wood	(Njogu et al., 2018)
	6-p-coumaroyl-sucrose	Fruit	(Olaokun, Alaba, Ligege, & Mkolo, 2020)
	Kigeliol	Wood	(Akande et al., 2018; Dhungana et al., 2016; Jackson et al., 2000; Saraswathy et al., 1992)
	Balaphonin	Stem bark	(Adam, 2021; Govindachari et al., 1971; Grace et al., 2002; Njogu et al*.*, 2018; Singh, Kumari, Singh, & Singh, 2018)
Quinones	Lapachol	Stem bark, fruits, roots, heartwood	(Binutu et al., 1996; Houghton, 2002; Sidjui et al., 2015, 2014)
	Dehydro α-lapachol	Stem bark, fruits, roots, heartwood	(Akande et al., 2018; Govindachari et al., 1971; Sidjui et al., 2014)
	2-acetylfuro-1,4-naphthoquinone	Stem bark	(Binutu et al., 1996; Sidjui et al., 2015, 2014)
	Kigelinol	Stem bark, fruits, roots	(Sidjui et al., 2015, 2014)
	Kigelinone	Stem bark	(Akunyili & Houghton, 1993; Moideen, Houghton, Rock, Croft, & Aboagye-Nyame, 1999; Olatunji & Olubunmi, 2009)
	Isokigelinol	Stem bark, fruits, roots	(Grace et al., 2002; Lamorde et al., 2010)
	Pinnatal	Stem bark, fruits, roots	(Akunyili & Houghton, 1993; Jackson et al*.,* 2000; Moideen et al*.,* 1999; Olatunji & Olubunmi, 2009)

	Isopinatal	Stem bark, fruits, roots	(Akunyili & Houghton, 1993; Olatunji & Olubunmi, 2009)
	Norviburtinal	Stem bark, fruits, roots	(Adam, 2021; Jackson et al., 2000)
	Sonovoburtinal	Root bark	(Jackson et al., 2000)
	2-(1-Hydroxyethyl)-naphtho[2,3-b] furan-4,9-quinone	Roots, stem bark	(Moideen et al., 1999)
	Kigelinone	Stem bark, fruits, roots, heartwood	(Binutu et al., 1996; Saraswathy et al., 1992)
	2-acetylnaphtho[2,3-b] furan-4,9-quinone	Stem bark, roots	(Grace et al., 2002)
	2-(1-hydroxyethyl)-naphtho[2,3-b] furan-4,9-dione		
	Tecomaquinone-I	Heartwood	(Atawodi & Olowoniyi, 2015)
	Kojic acid	Stem bark	(Sidjui et al., 2015)
Sterols	β-Sitosterol	Stem bark, fruits, heartwood, roots	(Govindachari et al., 1971; Saraswathy et al., 1992; Sidjui et al., 2014)
	Stigmasterol	Stem bark, roots, heartwood	(Grace et al., 2002; Idris, Al-tahir, & Idris, 2013)
	γ-Sitosterol	Stem bark, fruits	(Grace et al., 2002; Jackson et al., 2000)
Triterpenes	Oleanolic acid	Stem bark	(Grace et al., 2002; Sidjui et al., 2015, 2014; Zofou et al., 2011)
	Pomolic acid		
	2β,3β,19α-Trihydroxy-urs-12-en-28-oic acid		
Unsaturated Fatty acids	(9ζ,12ζ)-Methyl octadeca-9,12-dienoate	Leaves	(Olatunji & Olubunmi, 2009)
	Vernolic acid	Stem bark, roots, leaves, heartwood	(Adam, 2021; Grace et al., 2002; Njogu et al., 2018)
	Methyl-12-methyltetradecanoate	Leaves	(Grace et al., 2002; Houghton, 2002)
	Palmitic acid or hexadecanoic acid	Leaves and flowers	(Akande et al., 2018)

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Chapter 3:
Antimicrobial activity of four medicinal plants used traditionally in combination to treat sexually transmitted infections

Chapter 3: Antimicrobial activity of four medicinal plants used traditionally in combination to treat sexually transmitted infections

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Abstract

Antimicrobial resistance has become a global concern as scientists seek alternative medicine to combat diseases and infections caused by microbes. Medicinal plants provide an alternative source to combat synthetic drug resistance. This study aimed to investigate the *in vitro* antimicrobial potential and assess the interaction of four medicinal plants used traditionally in combination to treat sexually transmitted infections. Aqueous extracts of four plants species (*Dicoma anomala sbsp anomala*, *Elephantorrhiza elephantina*, *Eucalyptus cinerea* and *Kigelia africana*) and ten different combinations stored at different temperature for 7 and 14 days were prepared. Antimicrobial activity of the extracts was investigated using the minimum inhibitory concentration (MIC) assay against *Neisseria gonorrhea* and *Candida albicans*. Fractional inhibitory concentration (∑FIC) was used to assess the interaction of the combinations. From the ten different combinations and individual plants studied, eight combinations exhibited the highest microbial inhibition (0.39 mg/mL) against *Neisseria gonorrhea*. These combinations were stored at 25˚C for 7 and 14 days respectively. The combinations that exhibited noteworthy antimicrobial activity, displayed non-interactive interactions.

Keywords: Antimicrobial; medicinal plants; sexually transmitted infections

3.1 Introduction

Natural products are commonly used in the treatment of infectious diseases including sexually transmitted infections. Traditional medicine is commonly used by about 80% of the population within developing countries (Nascimento, Locatelli, Freitas, & Silva, 2000). Medicinal plants contain bioactive phytoconstituents, these include alkaloids, flavonoids, glycosides, tannins, saponins, phenolics and terpenoids have been shown to have antimicrobial properties which has been confirmed by scientific research (Saxena et al., 2013).

An important secondary metabolite group is phenolics, the hydroxyl (-OH) group plays a vital role in its' antimicrobial activity. This group induces inhibitory action by causing a disruption in the cell membrane and leakage of cellular components (Gyawali & Ibrahim, 2014). The disruption occurs due to the –OH group that delocalizes electrons to mimic secondary active transport system and cause a reduction in the –OH gradient of the cell wall's cytoplasmic membranes. Cell death occurs when there is a collapse of a proton motive force and a depletion of ATP which is caused by a reduced –OH gradient (Ultee, Bennik, & Moezelaar, 2002).

Polyherbal formulations that consist of multiple medicinal plants, contain various phytoconstituents which results in various types of interactions The fractional inhibitory concentrations index is a standard used to determine the type of interactions that occur in different combinations (Bassolé & Juliani, 2012; Burt, 2004; Goñi et al., 2009; Pei, Zhou, Ji, & Xu, 2009; Suliman, Van Vuuren, & Viljoen, 2010). Synergistic effects occur via various mechanisms of action; these include pharmacodynamic and pharmacokinetic synergy; the elimination of adverse effects and via the interference of disease resistance mechanisms (Caesar & Cech, 2019)(Caesar & Cech, 2019; Pezzani et al., 2019).

The aim of this study was to determine the antimicrobial activity of four medicinal plants individually and in combination. Synergistic effect of combinations were investigated.

3.2 Methods

3.2.1 Plant collection

Four medicinal plants (*D. anomala, E. elephantina, E. cinerea*, and *K. africana*) were collected from Mashishing, Mpumalanga province (South Africa) as guided by Mr James Mahore (Traditional healer). The voucher specimens of the plant's species were deposited and identified at the HGJW Schweickerdt Herbarium, Department of Plant and Soil Sciences at the University of Pretoria.

3.2.3 Preparation of extracts

Different plant parts were dried in the shade for four weeks and then ground down. Decoctions of *D. anomala* (whole plant), *E. cinerea* (leaves), *E. elephantina* (roots) and *K. africana* (fruits) were prepared using 100 g of the ground plant material in 500 mL of distilled water. Similarly, various combinations as described in Table 3.1 were prepared using an equal amount of individual plant material (25 g/individual plant in a total of 500 mL). The extracts were divided into seven equal proportions of 40 mL and stored in 440 mL plastic bottles for 7 days and 14 days at different temperature (4 ˚C, 25 ˚C and 37 ˚C). Upon the completion of the storage period, the extracts were filtered using WhatMan's filter paper No 3. Some combinations contained bacteria/fungal growth; these colonies were removed and cultured on potato dextrose agar (PDA). The samples were subjected to lycophilization (freeze drying) for 7 days and were tested on the 7th day

Table 3.1: The various combinations that the decoction has been prepared.

Combinations
D. anomala (A) + *E. elephantina* (B) + *E. cinerea* (C) + K. africana (D)
D. anomala (A) + *E. elephantina* (B) + *E. cinerea* (C)
D. anomala (A) + *E. elephantina* + *E. elephantina* (B) + *K. africana* (D)
E. elephantina + B *E. cinerea* (C) + *K. africana* (D)
D. anomala (A) + *E. elephantina* (B)
D. anomala (A) + *E. cinerea* (C)
D. anomala (A) + *K. africana* (D)
E. elephantina (B) + *E. cinerea* (C)
E. elephantina (B) + *K. africana* (D)
E. cinerea (C) + *K. africana* (D)

3.2.3 Culturing of pathogens

Bacterial pathogen *N. gonorrhoea* was cultured from KWIKSTIK™ ATCC No: 19424 on Thermo Scientific™-selected media chocolate blood agar for 48 hours in a CO_2 enriched environment. *Neisseria gonorrhoea* culture was inoculated in Merck-Mueller-Hinton broth on a shaking incubator (Labotec, South Africa) for 24 hours in a CO_2 enriched environment. Fungal pathogen *C. albicans* was cultured from KWIKSTIK™ ATCC No: 10231 on Merck-Tryptone Soy Agar for 48 hours. Fungal cultures were inoculated in Merck Tryptone Soy Broth on a shaking incubator (Labotec, South Africa) for 24 hours.

3.2.4 Minimum inhibitory concentration determination (MIC)

Antimicrobial activity of the samples was determined in triplicates using a micro titration method on a 96-well microtiter plate (Eloff, 1998). The microorganisms were standardized to an optical density (0.08-0.1) using a DU® 720 UV/Vis Spectrophotometer. Microorganisms (*N. gonorrhoea* and *C. albicans*) were grown in sterile broth and sampled at a density of 1.5x 10^8 colony forming units CFU per mL (CFU/mL) according to the 0.5 McFarland standard. The plant samples were dissolved in dimethylsulfoxide (10% DMSO) and made up to a final stock concentration of 50 mg/mL. Ciprofloxacin (2 mg/mL) was used as a positive control (*N. gonorrhoea*); fluconazole (10 mg/mL) was used as a positive control for *C. albicans* and 10% DMSO as a negative control.

To perform the serial dilution, 100 µL of sterile broth was added to each well of the ELISA plate. Thereafter 100 µL of plant extracts in row A in triplicates, (row A 1-3 consists of one type of plant extract, etc.). Once serially diluted 100 µL of bacteria/fungi was added to each well and incubated for 24 hours at 37 ˚C. Presto Blue™ Cell viability reagent (ThermoFisher Scientific) was used as a visual agent to determine the minimum inhibitory concentration (MIC). If microbial growth had occurred there would have been a change in the colour dye. It would go from being blue in colour to being pink in colour. For the determination of MIC values 20 µL of PrestoBlue® was added to the incubated 96 well plates and further incubated for 30 minutes and thereafter visually examined. Interaction test methods were conducted using a checkerboard method. This method requires the usage of MIC values to determine the fractional inhibitory concentration (FIC).

3.3 Results and Discussion

3.3.1 MIC determination assay

Antibacterial agent ciprofloxacin is a broad-spectrum fluoroquinolone agent that is used in the treatment of various infections caused by Gram-negative pathogens. These type of infections include urinary tract infections (UTIs), sexually transmitted infections (STIs) (gonorrhoea and chancroid) skin and bone infections, lower respiratory infections and infections associated with individuals that are immunocompromised (Campoli-Richards et al., 1988; Davis, Markham, & Balfour, 1996). Ciprofloxacin is an ideal antibacterial agent, as the mechanism of action of these agents inhibit the function of bacterial DNA gyrase, which affects bacterial DNA replication. Another mechanism of action is the inhibition of topoisomerase IV; which functions similarly to DNA gyrase (Campoli-Richards et al., 1988). The use of ciprofloxacin in antimicrobial studies arises from its antibacterial activity as it has an MIC value of 0.094 mg/mL which is below the standardized MIC value of natural products (0.1 mg/mL) (Sueke et al., 2010). Antifungal agent, fluconazole is a broad-spectrum agent used in treatment against pathogenic and opportunistic fungi. Fluconazole is a systemic azole agent; and the mechanism of action of the antifungal agent is the disruption ergosterol synthesis via the binding of the azole compound to cytochrome P-450. The binding of fluconazole interferes in the demethylation step required for the conversion of 14-α-methylsterols to ergosterol which is necessary for fungal growth. Studies indicate that the MIC range of fluconazole is between 0.025-0.032 mg/mL (Eksi, Gayyurhan, & Balci, 2013).

3.3.1.1 MIC determination assay *(Neisseria gonorrhoea)*

The results of antimicrobial activity of 98 plant species extracts (in combinations or individually) against *Neisseria gonorrhoea* indicated that the MIC values range from 12.5 mg/mL to 0.19 mg/mL (Table 3.2). Minimum inhibitory concentration values of plant extracts need to be standardized to create uniform standard for natural products and thus a standard MIC value of ≤1 mg/mL is considered noteworthy (Shirinda, Leonard, Candy, & van Vuuren, 2019; van Vuuren, 2008). Plant extracts that exhibited MIC values of >8 mg/mL is considered to have poor activity (Shirinda et al., 2019), however plant extracts that have a MIC value of 8 mg/mL is considered to have partial antimicrobial activity, indicating the reason of the highest starting concentration of 12 mg/mL (van Vuuren, 2008).

Antimicrobial activity of eight different combinations of plant extracts displayed noteworthy MIC values against *Neisseria gonorrhoea* ATCC 19424 (Table 3.2) (FigureA.1 in Appendix A displays the trend of MIC values). A minimum inhibitory concentration of 0.39 mg/mL (Table 3.2) was observed when analyzing the samples. The MIC value (0.39 mg/mL) was observed in eight different combination, these combinations include the plants; *Elephantorrhiza elephantina* (B), *Eucalyptus cinerea* (C) and *Kigelia africana* (D). Plant combinations BCD, BC, CD and C, which had been stored at 25˚ C for 7 and 14 days respectively, had an MIC value of 0.39 mg/mL.

Combinations that contain *Dicoma anomala* exhibited relatively higher MIC values as opposed to the other observed combinations ranging from 6.25 mg/mL to 12.5 mg/mL. The plant parts used for *D. anomala* in the study were the leaves, flowers, and tubers and there is no literature regarding the use of the leaves and flowers against *N. gonorrhoea*. However, Maroyi, 2018 reported that the use of a decoction containing roots of *D. anomala* is traditionally used against *N. gonorrhoea.* (Anne Hutchings & van Staden, 1994).

Plant combinations BCD, BC, CD and C containing, *E. elephantina* (B), *E. cinerea* (C) and *K. africana* (D), whereby the following plant parts were used in the decoction for plant B was the roots, plant C, the leaves and plant D, the fruit. These combinations functioned either synergistically or individually.

A decoction of *E. elephantina* roots in combination with other plant species have indicated that there is antimicrobial activity against *N. gonorrhoea*, however there isn't any previous studies conducted on *E. elephantina* using the roots as a decoction against *N. gonorrhoea.* Various other plant parts including the roots of *E. elephantina* has been used for the treatment of syphilis, another sexually transmitted infection, not included in this study. A study conducted by (Maroyi, 2017b) indicated that the roots of the species *Elephantorrhiza goetzei* (Harms) is used as a decoction traditional medicine against gonorrhoea in Zimbabwe. The phytochemistry within species from the same genus exhibited similar phytochemistry; *E. elephantina* and *E. goetzei* have a number of phenolic compounds that are similar for example, catechin, gallacatechin, epigacatechin, gallic acid, methyl gallate and 2-(3',4'-Dihydroxyphenyl) ethanol (Refer to Literature review, Section 2.4.2.3) (Maroyi, 2017b). The similarity of phenolic compound may attribute to the antimicrobial activity of *E. elephantina* as *E. goetzei* exhibits positive antimicrobial activity (Maroyi, 2017b; Ultee et al., 2002).

Currently there are no studies conducted on a decoction containing *E. cinerea* against sexually transmitted infections in South Africa. However, a study conducted in Brazil using *E. globulus's* leaves as an infusion to treat upper respiratory infection was conducted (Agra, 2008).

Traditionally the fruits of *K. africana* has been used as a treatment against *N. gonorrhoea.* The fruits of *K. africana* contain classes of the following phytochemical constituents; alkaloids, steroids, terpenoids, flavonoids, saponins, phenolic compounds and tannins (Bhramaramba, Babu, Teja, Kumari, & Rathna, 2012). Phenolic compounds are responsible for antimicrobial activity therefore, the phenolic compounds ρ-Coumaric acid, caffeic acid and 6-ρ-coumaroyl-sucrose isolated from fruits of *K. africana* could be responsible for the antimicrobial activity exhibited (Ultee et al., 2002).

Table 3.2: Minimum Inhibitory Concentration (MIC) values (mg/mL) of different type of combinations of plant extracts against the bacteria *Neisseria gonorrhoea.*

Types of Combinations	DAY 0	7 DAYS STORAGE			14 DAYS STORAGE		
	Room Temperature	Day 7 4˚C	Day 7 25˚C	Day 7 37˚C	Day 14 4˚C	Day 14 25˚C	Day 14 37˚C
ABCD	1.56	1.56	1.56	3.13	1.56	1.56	3.13
ABC	0.19	0.78	1.56	1.56	0.39	1.56	3.13
ABD	12.5	12.5	6.25	6.25	12.5	12.5	6.25
BCD	0.39	0.39	**0.39**	0.39	0.39	**0.39**	0.78
AB	1.56	6.25	12.5	6.25	6.25	6.25	6.25
AC	1.56	3.13	3.13	3.13	3.13	6.25	3.13
AD	12.5	12.5	6.25	6.25	6.25	3.13	3.13
BC	0.78	0.91	**0.39**	0.78	0.39	**0.39**	0.39
BD	12.5	3,13	6,25	6,25	6,25	1,56	1,56
CD	0.39	0.78	**0.39**	0.39	0.39	**0.39**	0.78
A	6.25	6.25	12.5	12.5	12.5	12.5	6.25
B	0.78	3.13	1.56	3.13	1.56	0.39	0.78
C	1.56	0.78	**0.39**	0.78	0.39	**0.39**	0.39
D	12.5	6.25	6.25	6.25	6.25	6.25	6.25

* MIC values in bold indicated noteworthy values

A-*Dicoma anomala* B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana*

*Reference (Bassolé & Juliani, 2012; Hall, Middleton, & Westmacott, 1983; Meletiadis, Pournaras, Roilides, & Walsh, 2010; Suliman et al., 2010)

3 replicates were conducted for each combinatio

Interactions amongst the different type of combinations conducted in this study include additive, antagonistic, non-interactive and synergist effects. Polyherbal formulations that resulted noteworthy MIC values against *N. gonorrhoea* displayed additive (BCD), non-interactive (BC and CD) effects (Table 3.3). Multiple types of interactions amongst combinations is observed as mechanisms of interactions of traditional medicine is unknown and complicated (Caesar & Cech, 2019).

Table 3.3: Fractional inhibitory concentration (FIC) index of different types of combinations against *Neisseria gonorrhoea.*

∑Fractional inhibitory concentration (FIC) (mg/mL)							
Types of Combinations		**7 DAYS STORAGE**			**14 DAYS STORAGE 1**		
	Room Temperature	**4˚C**	**25˚C**	**37˚C**	**4˚C**	**25˚C**	**37˚C**
ABCD	3.37[c]	3.00[c]	5.37[b]	5.76[b]	5.37[b]	8.37[b]	13.04[b]
ABC	0.40[d]	1.37[c]	5.12[b]	2.62[c]	1.28[c]	8.12[b]	12.54[b]
ABD	19.02[b]	8.00[b]	5.51[b]	3.50[c]	12.01[b]	36.05[b]	10.01[b]
BCD	0.78[a]	0.69[a]	**1.31[c]**	0.69[a]	1.31[c]	**2.06[c]**	1.56[c]
AB	2.25[c]	3.00[c]	9.01[b]	2.50[c]	4.51[b]	16.53[b]	9.01[b]
AC	1.25[c]	4.50[b]	8.28[b]	4.26[b]	8.27[b]	16.53[b]	8.53[b]
AD	0.37[d]	4.00[c]	1.50[c]	1.50[c]	1.50[c]	0.75[a]	1.00[a]
BC	1.5[c]	1.46[c]	**1.25[c]**	1.25[c]	1.25[c]	**2.00[c]**	1.50[c]
BD	17.03[b]	3.13[c]	6.25[b]	6.25[b]	6.25[b]	1.56[c]	1.56[c]
CD	0.28[d]	2.12[c]	**1.06[c]**	0.56[a]	1.06[c]	**1.06[c]**	2.12[c]

[a]-Additive (≤ 0.5)
[b]-Antagonistic (0.5-1.0)
[c]-Non-additive (1.0-≤4)
[d]-Synergistic (>4.0)
FIC values in bold indicated noteworthy values based on MIC values
A-*Dicoma anomala* B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana*
3 replicates were conducted for each combination

3.3.1.2 MIC determination assay *(Candida albicans)*

The results of antimicrobial activity of two combinations of different polyherbal formulations from 98 different plant extracts against *C. albicans* ATCC 10231 indicated that the MIC values range from 1.56 mg/mL to ≥12.5 mg/mL (Table 3.4) (FigureA.2 in Appendix A displays the trend of MIC values). The negative control 10% DMSO had a MIC value of >12.5 mg/mL. The positive control fluconazole had a MIC value of <0.02 mg/mL.

There is no literature studies conducted on the usage of *D. anomala* leaves against *C. albicans* as a decoction. However there are studies conducted on the tubers of *D. anomala* as a decoction which exhibited an MIC value of 8.00 mg/mL (Mabona et al., 2013). Cindy, 2021 examined the phytochemicals present in leaves and the roots of *D. anomala* which resulted in the presence of steroids, saponins, flavonoids, tannins and terpenoids, the similarity in phytochemistry between the two structural organs could account for the antimicrobial activity in the current conducted study on the leaves.

Elephantorrhiza elephantina roots have moderate to weak MIC values ranging from 3.13 mg/mL to 12.5 mg/mL (Table 3.4); depending whether tested in combination with other plant species or individually. Previous published results of antimicrobial activity of a decoction made from *E. elephantina* roots against *C. albicans* have an MIC value of 2 mg/mL which is below the noteworthy MIC of 1 mg/mL of natural products (Nciki et al., 2016).

Antimicrobial activity of *E. cinerea* leaves resulted in a MIC value of 6.25 mg/mL when sampled individually and in combination with other plant species the MIC values range from 3.13 mg/mL to 6.25 mg/mL (Table 3.4). *Eucalyptus cinerea* resulted in moderate activity against *C. albicans*, eucalyptol exhibited an MIC value of 4.8 µl/mL when examined by Soliman et al., 2014, although an oil was examined which is insoluble in water, the leaves contain approximately 70% eucalyptol (Murata et al., 2013).

The fruit extracts of *K. africana* individually and in combinations with other plant species exhibited high antimicrobial activity against *C. albicans* ranging from 1.56 mg/mL to ≥12.5 mg/mL (Table 3.4). Mobona et al., 2013 conducted an antimicrobial study on aqueous fruit extracts of *K. africana* that supported high MIC values of >16.00 mg/mL against *C. albicans* which resulted in the current study. Although the study conducted by Mobona et al., used

amphotericin B as a positive control the mechanism of action is similar to that of fluconazole (Santos et al., 2012).

Table 3.4: Minimum Inhibitory Concentration (MIC) values (mg/mL) of different type of combinations of plant extracts against the fungi *Candida albicans.*

Types of Combinations	DAY	7 DAYS STORAGE			14 DAYS STORAGE		
	Day 0	Day 7 4˚C	Day 7 25˚C	Day 7 37˚C	Day 14 4˚C	Day 14 25˚C	Day 14 37˚C
ABCD	**1.56**	3.13	6.25	>12.5	>12.5	6.25	6.25
ABC	12.5	3.13	3.13	3.13	>12.5	3.13	6.25
ABD	3.13	3.13	3.13	3.13	3.13	3.13	3.13
BCD	3.13	3.13	3.13	3.13	3.13	3.13	3.13
AB	12.5	>12.5	12.5	>12.5	12.5	>12.5	>12.5
AC	6.25	6.25	6.25	6.25	6.25	3.13	3.13
AD	>12.5	>12.5	6.25	6.25	>12.5	3.13	6.25
BC	6.25	3.13	6.25	3.13	3.13	3.13	3.125
BD	12.5	12.5	12.5	12.5	12.5	3.13	12.5
CD	12.5	3.13	12.5	3.13	**1.56**	6.25	3.13
A	>12.5	>12.5	>12.5	>12.5	>12.5	6.25	6.25
B	6.25	>12.5	6.25	3.13	3.13	6.25	6.25
C	6.25	6.25	6.25	6.25	6.25	6.25	6.25
D	6.25	6.25	6.25	6.25	6.25	6.25	6.25

* MIC values in bold indicated noteworthy values

A-*Dicoma anomala* B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana*

*Reference (Bassolé & Juliani, 2012; Hall et al., 1983; Meletiadis et al., 2010; Suliman et al., 2010)

3 replicates were conducted for each combination

Polyherbal formulations examined against *C. albicans* results in different type of interactions amongst the observed combinations. These interactions include additive, antagonistic and non-interactive effects. Combination ABCD exhibited an additive effect and ABC an antagonistic effect. Antimicrobial activity of *Candida albicans* did not exhibited noteworthy results, which may be confirmed as majority of the combinations tested against this pathogen resulted in non-interactive interactions

Table 3.5: Fractional inhibitory concentration (FIC) index of different types of combinations against *Candida albicans.*

∑Fractional inhibitory concentration (FIC) (mg/mL)							
Types of Combinations	**DAY 0**	**7 DAYS STORAGE)**			**14 DAYS STORAGE**		
	Room Temp	**4˚C**	**25˚C**	**37˚C**	**4˚C**	**25˚C**	**37˚C**
ABCD	**0.87[a]**	1.50[c]	3.50[c]	9.00[b]	9.00[b]	4.00[c]	4.00[c]
ABC	5.00[b]	1.00[a]	1.25[c]	1.75[c]	7.00[b]	0.72[a]	3.00[c]
ABD	1.25[c]	1.25[c]	1.25[c]	1.75[c]	1.75[c]	1.50[c]	1.50[c]
BCD	1.50[c]	1.25[c]	1.50[c]	2.00[c]	2.00[c]	1.50[c]	1.50[c]
AB	3.00[c]	2.00[c]	3.00[c]	5.00[b]	5.00[b]	4.00[c]	4.00[c]
AC	1.50[c]	1.50[c]	1.50[c]	1.50[c]	1.50[c]	1.00[a]	1.00[c]
AD	3.00[c]	3.00[c]	1.50[c]	1.50[c]	3.00[c]	3.13[c]	2.00[c]
BC	2.00[c]	0.75[a]	2.00[c]	1.50[c]	1.50[c]	1.00[a]	1.00[a]
BD	4.00[c]	3.00[c]	4.00[c]	6.00[b]	6.00[b]	1.00[a]	4.00[c]
CD	4.00[c]	1.00[a]	4.00[c]	1.00[a]	**0.50[a]**	2.00[c]	1.00[a]

[a]-Additive (≤ 0.5)
[b]-Antagonistic (0.5-1.0)
[c]-Non-interactive (1.0-≤4)
[d]-Synergistic (>4.0)
MIC values in bold indicated noteworthy values based on MIC values
A-*Dicoma anomala* B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana*
3 replicates were conducted for each combination

Stafford, Jäger, & Van Staden, 2005 conducted an experiment on the effect of storage on the chemical composition and biological activity of medicinal plants, in the study it is evident that storage of plant extracts has an effect on some of the polyherbal formulations based on their antimicrobial activity against the pathogen *N. gonorrhoea*. Extracts that showed noteworthy antimicrobial activity whereby storage of the plant extracts had an effect on are the combinations BC and C. The antimicrobial activity of these extracts increased between Day 0 and Day 7 at 25˚C of storage. The MIC value of combination BC decreased from 0.78 mg/mL to 0.39mg/mL respective of the storage days. The MIC value of combination C decreased from 1.56 mg/mL to 0.39mg/mL from day 0 to day 7 at 25˚C. The compounds responsible for antimicrobial activity appear to form compounds that are more active during storage.

Antimicrobial results of *N. gonorrhea* that had noteworthy MIC values exhibited that temperature does not influence the activity of plant extracts drastically. The MIC values of the polyherbal formulations BCD and CD does not have much variation irrespective of the temperature, the values range between 0.39 mg/mL to 0.78 mg/mL. Polyherbal formulations BC and C display a wider range of MIC values ranging from 0.39 mg/mL to 1.56 mg/mL. A resistance to the influence of a variation of temperature of plant extracts may be due to the phytoconstituents present. The secondary metabolites provide a defense against abiotic and biotic stresses in plants. A rise in temperature is an abiotic stress, due to this defense mechanism plants can withstand elevated temperatures thus the phytoconstituents withstand these temperatures (Doughari, 2006; Jamloki, Bhattacharyya, Nautiyal, & Patni, 2021). Antimicrobial results of *N. gonorrhea,* which had noteworthy MIC values exhibited*,* that temperature does not influence the activity of plant extracts drastically. The MIC values of the polyherbal formulations BCD and CD did not have much variation irrespective of the temperature that the extracts were stored at, the values range between 0.39 mg/mL to 0.78 mg/mL. Polyherbal formulations BC and C display a wider range of MIC values ranging from 0.39 mg/mL to 1.56 mg/mL. A resistance to the influence of temperature variation of plant extracts may be due to the phytoconstituents present. The secondary metabolites provide a defense against abiotic and biotic stresses in plants. A rise in temperature is an abiotic stress, due to this defense mechanism, plants can withstand elevated temperatures thus it is proposed that the phytoconstituents can withstand these temperatures. Medicinal plants

used by traditional healers is usually prescribed as a decoction thus they are boiled at high temperature, for a long period preserving the activity of their phytoconsituents (Doughari, 2006; Jamloki et al., 2021; Netshiluvhi & Eloff, 2019).

3.4 Conclusion

After screening the selected plants for their inhibitory activities against selected sexually transmitted infections; *N. gonorrhoea* and *C. albicans* it can be concluded that eight polyherbal formulations against *N. gonorrhoea* displayed noteworthy MIC values. Polyherbal formulations BCD and CD exhibited a consistent MIC trend irrespective of the number of storage days and fluctuating temperature. These eight combinations of polyherbal formulations should be further examined as they could lead to potential new treatment as natural products against bacteria such as *N. gonorrhoea*. Based on their antimicrobial activity, storage period and temperature variation combinations that exhibited noteworthy MIC values at day 7 and day 14 at 25˚C were further examined.

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Chapter 4:
A metabolomic analysis of four medicinal plants used traditionally in combination to treat sexually transmitted infections

Chapter 4: A metabolomic analysis of four medicinal plants used traditionally in combination to treat sexually transmitted infections

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Abstract

Phytoconstituents of medicinal plants may vary depending on multiple factors affecting the quality of polyherbal formulations. These factors are caused by abiotic and/or biotic induced stresses. Metabolomics plays a pivotal role in design, development quality control of drugs; either synthetic or herbal. The study aimed to evaluate the quality of four medicinal plants used traditionally in combinations and individually via the usage of metabolomic as an analysis tool. The use of metabolomics techniques such as liquid chromatography mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry to determine the quality of polyherbal formulations based on the changes and similarities of the combinations chemical profile. An observation of ^{1}H NMR spectra yielded prominent signals in the aliphatic region (0.8- 3.00 ppm) and carbohydrate region (3.00- 6.00 ppm). Some combinations yield signals within the aromatic region (5.00- 9.00 ppm). Principle component analysis (PCA) and orthogonal partial least square- discriminant analysis (OPLS-DA) models display separation between groups and similarities within groups observed.

In conclusion, the polyherbal formulations analyzed in this study exhibit a good standing of the quality of plant extracts used as traditional medicine, however further ^{1} H NMR based metabolomics and LC-MS/MS (characterization of noteworthy extracts) studies need to be conducted for cross validation.

Keywords: Metabolomics; traditional medicine; medicinal plants; quality control.

4.1 Introduction

Polyherbal formulations that have been used as antimicrobial agents tend to have enhanced antimicrobial activity (Chassagne et al., 2021). The activity of these agents are induced due to various factors such as the type of medicinal plants used a traditional medicine, the conditions that these plants are subjected to and the preparation of the medicine (WHO, 2013). Medicinal plants that are grown in multiple types of environments may influence the secondary metabolite production thus affecting the antimicrobial activity.

Herbal medicine contains either a single or multiple medicinal plant, commonly known as polyherbal formulations. Herbal and polyherbal formulations are traditionally stored in a fridge or at room temperature, at various humidity levels, different light intensities ((WHO), 1998). The factors become a challenge to regulate for traditional healers, as most traditional healers' practice from a 'consulting room' on their premises.

The quality of traditional medicine may be affected if subjected to multiple conditions/factors. These factors include abiotic and biotic stresses experience throughout the process of developing traditional or herbal medicine (Rahman et al., 2019). A medicinal plant can experience stress during the growth phase or during storage, which alters the secondary metabolite product affecting the quality. According to the World Health Organization (WHO), traditional or herbal medicine should conform to uniformity and standardization amongst traditional or herbal medicine (WHO, 2013). Metabolomic studies are conducted on formulations that contain medicinal plants to determine the quality of the traditional medicine.

Metabolomics or a metabolomic analyses can be used as a tool for the quality control of polyherbal formulation. The qualification and quantification of tradition medicine (a single or multiple medicine plant formulation) using metabolomics provides a more comprehensive and detail overview of fingerprint and profile of the medicinal plant (Wolfender, Rudaz, Hae Choi, & Kyong Kim, 2013). The use of a full metabolomics approach of polyherbal formulations include the development of an authentic chemical fingerprint (using NMR techniques) of medicinal plants under multiple stresses. Thereafter the secondary metabolites should be identified via untargeted metabolite analysis using LC-MS techniques. Once untargeted metabolites are identified, marker metabolites, based on literature needs to be identified.

Further isolation and purification is required and finally the quantification of marker compounds within polyherbal formulations can be achieved (Rahman et al., 2019).

Nuclear magnetic resonance is a metabolomic tool used to examine the chemical profile of polyherbal formulations. This technique possesses various advantages and disadvantages, however is required to investigate the quality of single and polyherbal formulations. The use of NMR is advantageous as it doesn't require any pre-treatment as with column chromatography and derivatization which provides a high-throughput, robust and reliable analytical tool for metabolomics. There is minimal time needed for sample preparation when using NMR as an analysis tool. The diagnostic tool is non-destructive and selective in its detection mechanism and provides information on the structure of the compounds detected (metabolite imaging). Although a disadvantage of NMR is that method is sensitive to the pH of extractions, because there is a chemical shift that could take place if there is a change in the pH, therefore a buffer could be used to stabilize the pH (Chatham & Blackband, 2001; Okazaki & Saito, 2012). Another disadvantage is that NMR is less sensitive than other molecular techniques.

The use of liquid chromatography mass spectrometry as a metabolomic tool provides a quantified analysis of secondary metabolites. Advantages of LC-MS include sensitivity, specificity, and precision due to analysis occurring at a molecular level. Other advantages of LC-MS include; automated sample testing and minimal sample volume is needed for testing. Optimal separation is highly achievable due to different compositions of the mobile phase, their pH adjustments and pressure pumps amongst other factors. LC-MS analysis has the ability to identify most organic and some inorganic molecules. Although advantageous, the use of LC-MS as a metabolic analysis tool has disadvantages, as it is destructive to the sample and the sample cannot be recovered once tested. The analysis is qualitative rather than quantitative and usually requires separation of the sample, as well as it is less robust than Gas-chromatography-liquid-chromatograph (GC-MS) (Wishart, 2016). Previous studies conducted indicate that LC-MS analysis is easier to understand and analyze (Y. Z. Liang, Xie, & Chan, 2004).

The aim of this study is to use a metabolomic approach to analyze the quality of the polyherbal formulations in question that were examined against sexually transmitted infections.

4.2 Methods

4.2.1 Sample Preparation (Nuclear Magnetic Resonance) (Appendix B)

Test samples that showed the best anti-microbial activity against *N. gonorrhea* and *C. albicans* were subjected to ^{1}H NMR analysis. A mass of 8 mg of sample was used and dissolved in 700 µL of deuterated methanol CD_3OD. Samples were transferred to a 5 mm diameter NMR tube. The samples were analyzed using a Bruker AVII-600 (Bruker, Rheinstetten, Germany) 400 MHz NMR spectrometer at a temperature of 304.6 K with a total sum of 128 scans per samples done by Dr. M.A. Selepe.

4.2.1.1 Multivariate data analysis (MDA)

The raw data was processed using the MestReNova software; all samples were referenced to deuterated methanol 3.30 ppm. Thereafter the spectra were further processed, using the automatic phase correction and the Whittaker smoother baseline correction. To observe the difference between the peak intensities, all the spectra were normalized to the largest peak. An internal standard known as trimethylsilylpropanoic acid (TSP) was used. The chemical shift regions were specified to a bin range of 0.02 ppm.

The chemical shift ranges between 0.00-10.00 ppm of each spectrum. The regions between 3.16-3.40 ppm and 4.72-5.00 ppm were excluded from the analysis as these regions were residuals of methanol and water signals respectively. The data was imported into the SIMCA software (version 14.1, Umetrics, Umeå, Sweden) an independent modelling of class analysis to determine the multivariate data analysis. The data was Pareto scaled and assigned according to the different polyherbal combinations. An unsupervised principal component analysis (PCA) model was generated to observe separation clusters. A supervised orthogonal partial least square discriminant (OPLS-DA) was generated to determine if there is a difference between the polyherbal formulations based on their phytochemistry.

4.2.2 Sample extraction-LC-MS/MS

Polyherbal formulations that exhibited noteworthy antimicrobial activity were subjected to LC-MS analysis. A mass of 1 mg was used and dissolved in LC-MS grade Acetonitrile and water (1:1) and sonicated. A two-fold serial dilution was conducted and the samples were centrifuged resulting in a 0.1 µg/mL concentration. For sample, testing 100 µL of the supernatant was used and the samples were analyzed using a Waters Acquity UPLC Synapt

G2 High Definition Mass Spectrometer, the sample testing was conducted by Ms. M. Wooding.

4.2.2.1 LC-MS/MS Analysis (Appendix B)

Compound separation and detection from eight polyherbal formulations that exhibited noteworthy antimicrobial activity against the bacterium *Neisseria gonorrhoea* a was performed using a Waters® Synapt G2 high definition mass spectrometry (HDMS) system (Waters Inc., Milford, Massachusetts, United States of America (USA)). This system comprises of a Waters® Acquity Ultra Performance Liquid Chromatography (UPLC) that is hyphenated to a quadruple-time- (QTOF) instrument. The system is operated with MassLynx™ software, which is used for fine-tuning of the machine. The injection volumes were set at 5 µL using A Kinetex® 1.7 µm EVO C18 100 Å (2.1 mm ID x 100 mm length) column.

Operating parameters of the liquid chromatography component were set according to the following elution gradient. The elution gradient of the mobile phase consists of two mobile phases A and B, 95% H_2O (0.1% formic acid) and 100% acetonitrile (0.1% formic acid) respectively. The infusion flow rate was 20 µL/min with the following linear gradient: 0–14 min 97% to 3% A:B, 14–16 min 100% to 0% A:B:, and 16.5– 20 min return to 97% to 3% A:B. The operating parameters for the mass spectrometry component were as follows: the capillary voltage for ESI was 2.6kV and 2.0 kV for the positive and negative ionization respectively. The source temperature was set at 120˚C, with the sampling cone voltage at 25.0 V, the extraction cone voltage at 4.0 V and cone gas (nitrogen) flow at 10.0 L/Hr. The capillary voltage was set at 2.6 kV and an ion energy of 1 with a trap collision energy of 4. The desolvation temperature was set at 300˚C with a gas (helium) flow of 600.0 L/Hr. The same separation conditions were used to collect both positive and negative ion mass spectra with each sample having run time of 20 minutes. The acquisition mass range is between 50 to 1200 Da.

4.3 Results and Discussion

To determine the quality of the polyherbal formulations used in this study, 98 different polyherbal formulations were subjected to ^{1}H NMR analysis. NMR spectra were processed using MestReNova software to reduce any background noise that could have been present during sample testing. Further processing of NMR spectra was required to produce

multivariate data analysis; used for quality control purposes. Polyherbal formulations contain comprised of the following plants *Dicoma anomala* (A) (leaves and stems), *Elephantorrhiza. elephantina* (B) (roots), *Eucalyptus. cinerea* (C) (leaves) and *Kigelia. africana* (D) (fruit). This study comprises of ten different polyherbal combinations and four individual medicinal plants based a traditional remedy provided by a traditional healer to treat and manage sexually transmitted infections. These formulations were subjected to various conditions, which includes the storage of these for 7 days and 14 days at different temperature (4 ˚C, 25 ˚C and 37 ˚C).

The observed NMR spectra of 98 polyherbal (Figures 4.1-4.14) formulations exhibited prominent signals within the aliphatic regions (0.5-3.00 ppm), and within the carbohydrate region (3.00-6.00 ppm). Aromatic regions (5.00-9.00 ppm) were exhibited within majority of the spectra however not in all the spectra (Perin et al., 2013). Polyherbal formulations that exhibited prominent carbohydrate region signals are combinations BC and C, where combinations ABC, BCD, AC, AD, CD and B contain weak signals (Refer to table 3.1 for types of combinations). Figure4.1 to Figure4.7 depicts spectra of various combinations.

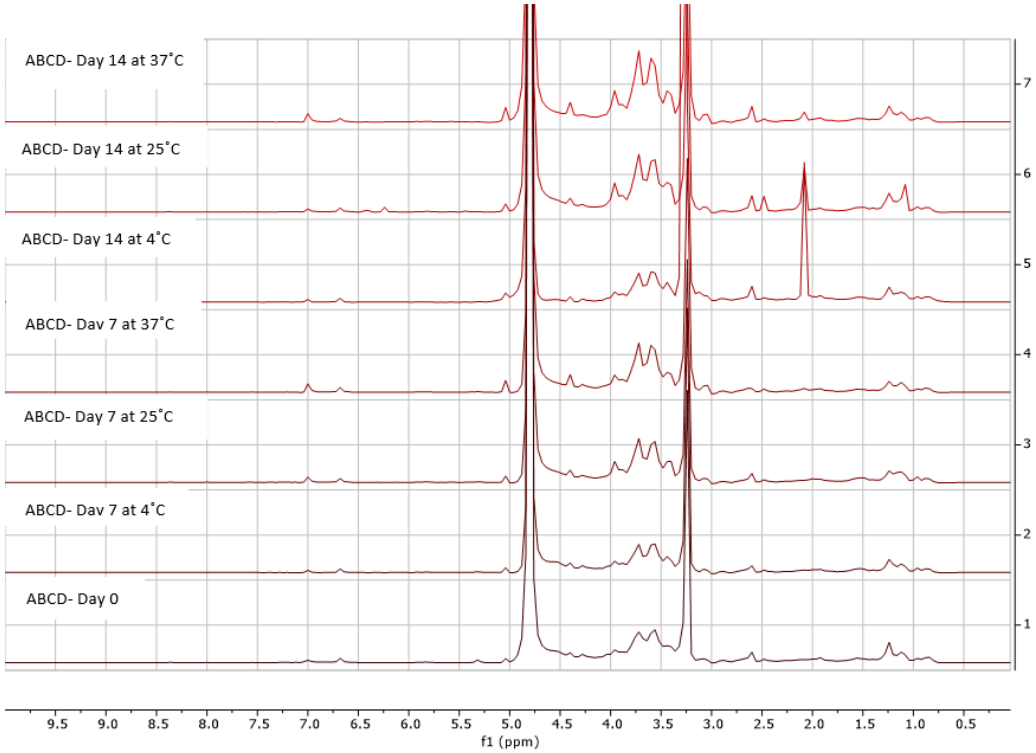

Figure 4.1: Zoomed in stacked ^{1}H NMR spectra of the polyherbal formulation containing plants ABCD, containing combinations that were stored at various temperatures over two different storage periods (stored for 7 days and 14 at 4˚C, 25˚C and 37˚C respectively).

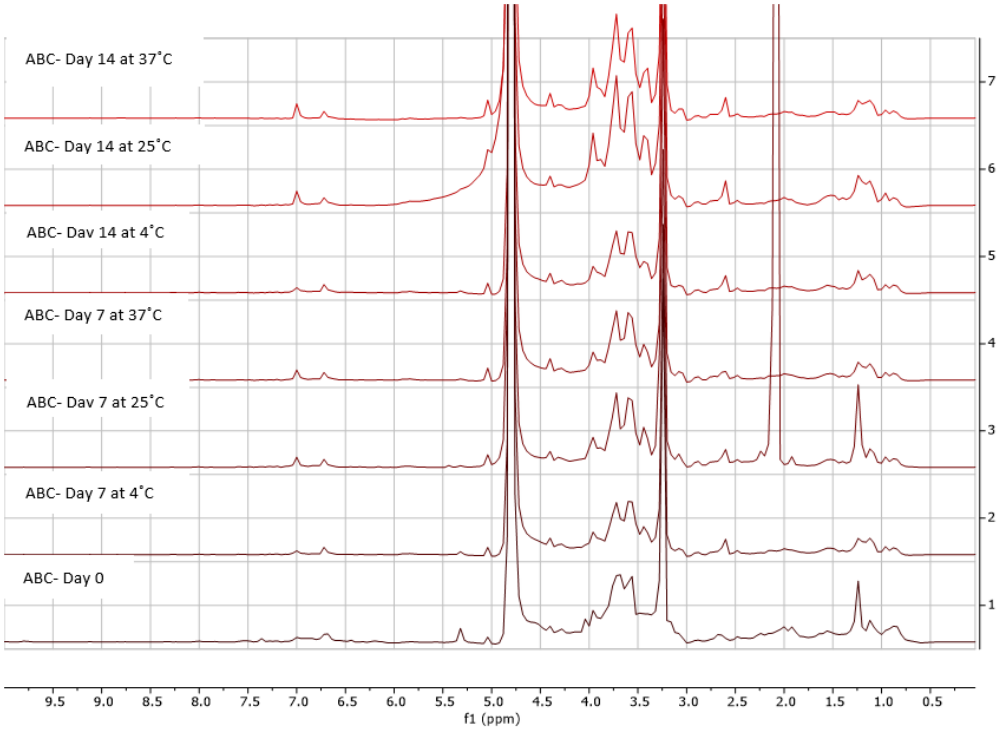

Figure 4.2: Zoomed in stacked ^{1}H NMR spectra of the polyherbal formulation containing plants ABC, containing combinations that were stored at various temperatures over two different storage periods (stored for 7 days and 14 at 4˚C, 25˚C and 37˚C respectively).

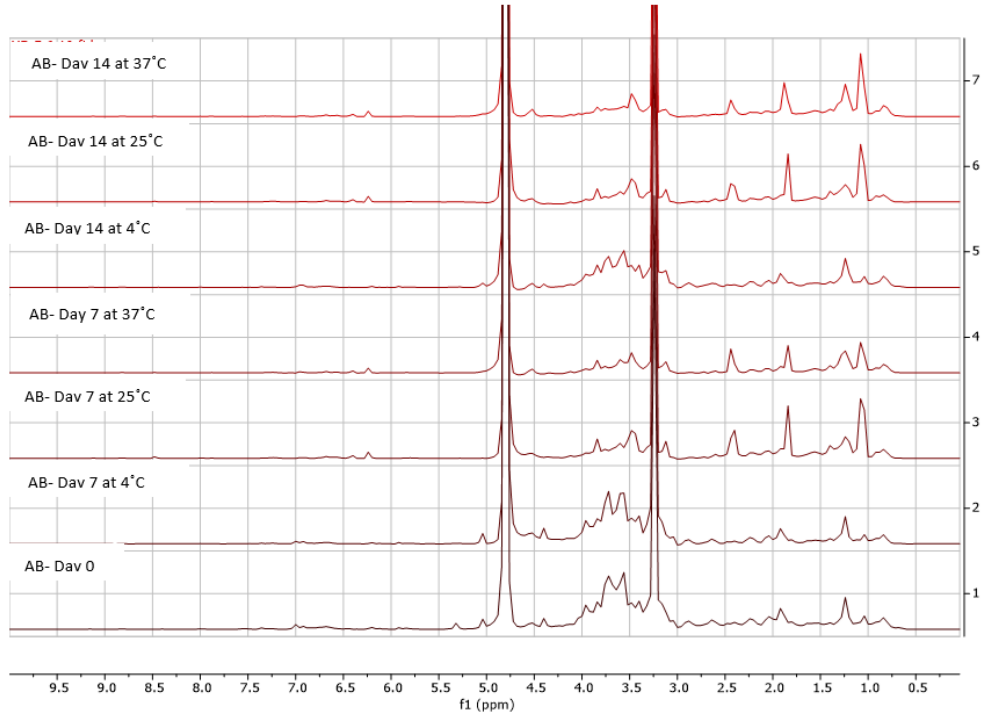

Figure 4.3: Zoomed in stacked ^{1}H NMR spectra of the polyherbal formulation containing plants AB, containing combinations that were stored at various temperatures over two different storage periods (stored for 7 days and 14 at 4˚C, 25˚C and 37˚C respectively).

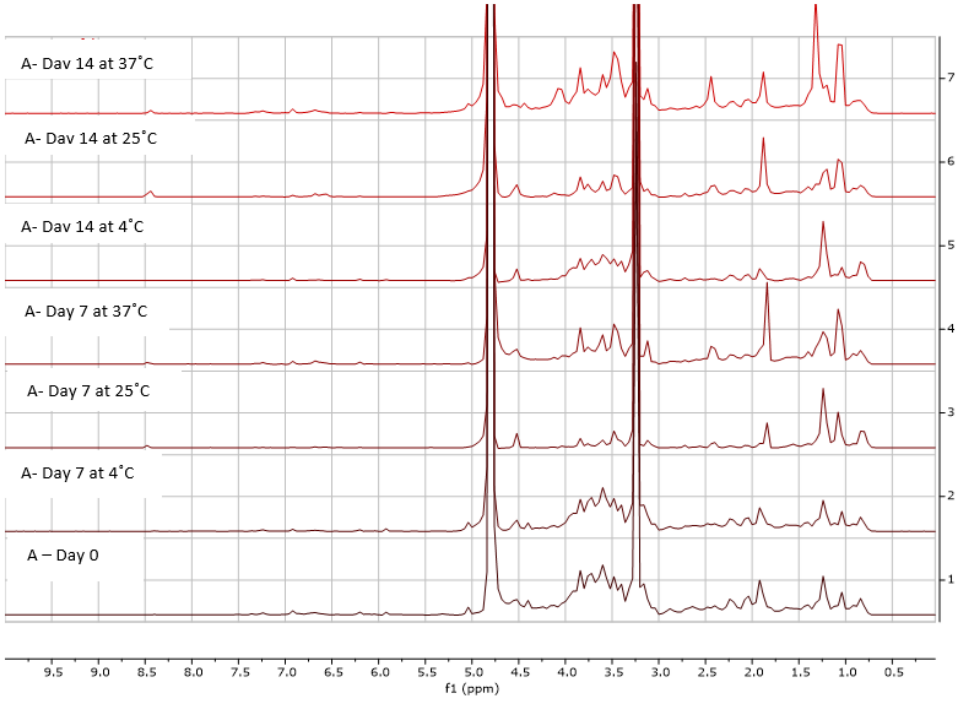

Figure 4.4: Zoomed in stacked ^{1}H NMR spectra of the herbal formulation containing plant A, *Dicoma anomala subsp anomala* containing combinations that were stored at various temperatures over two different storage periods.

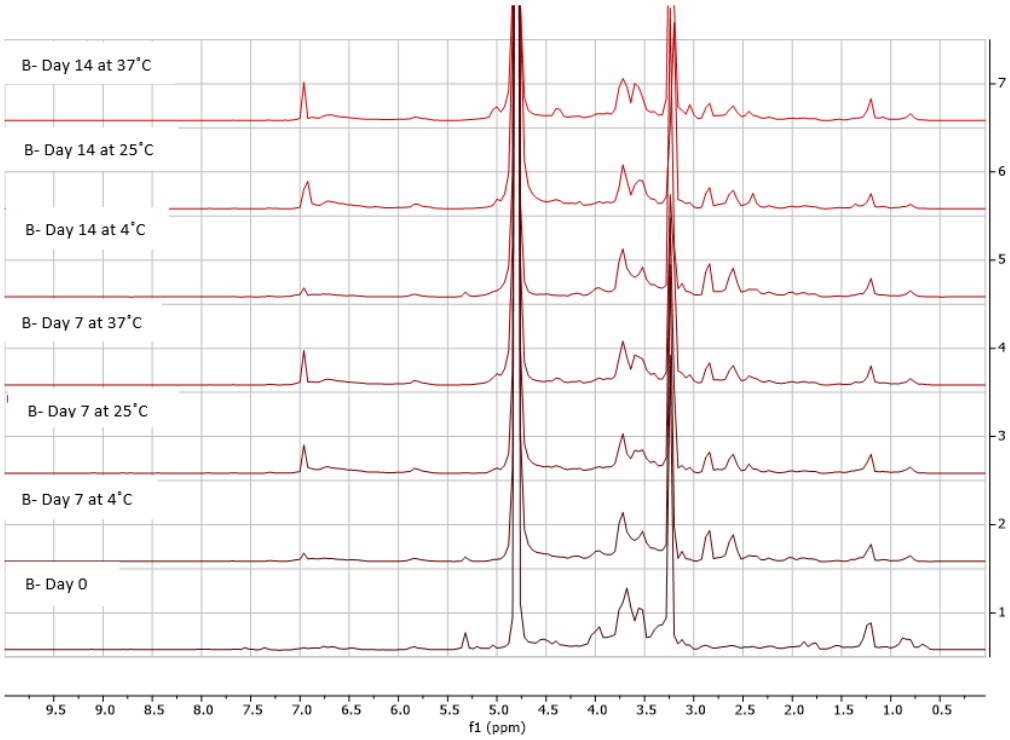

Figure 4.5: Zoomed in stacked ^{1}H NMR spectra of the herbal formulation containing plant B, *Elephantorrhiza elephantina* containing combinations that were stored at various temperatures over two different storage periods (stored for 7 days and 14 at 4˚C, 25˚C and 37˚C respectively).

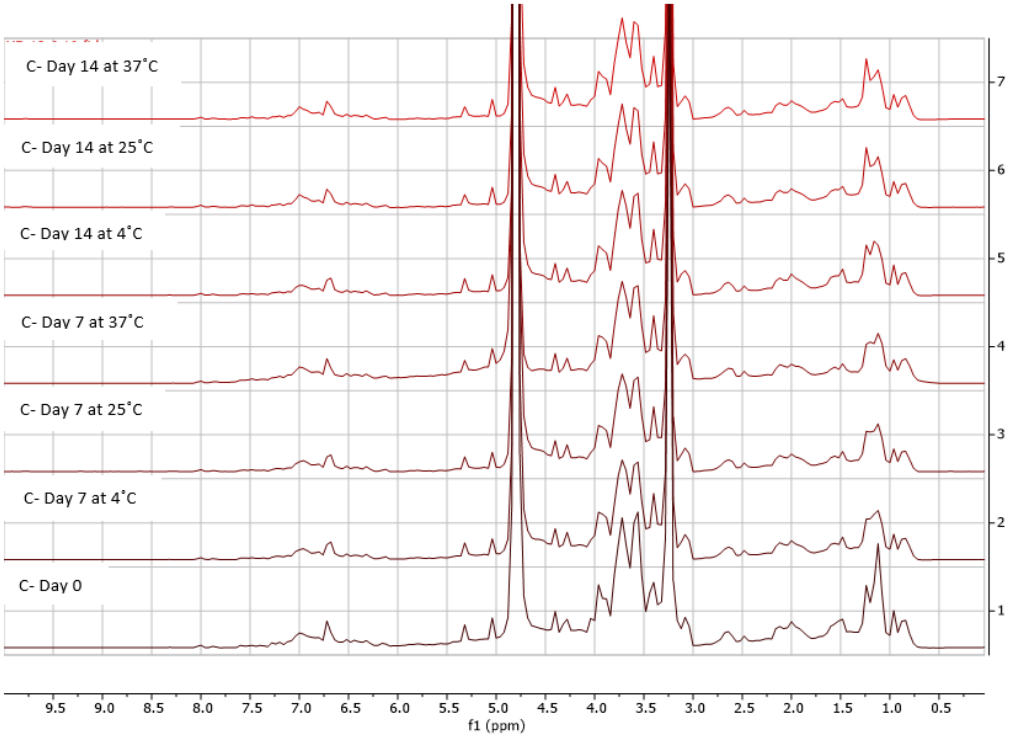

Figure 4.6: Zoomed in stacked ^{1}H NMR spectra of the herbal formulation containing plant C, *Eucalyptus cinerea* containing combinations that were stored at various temperatures over two different storage periods (stored for 7 days and 14 at 4˚C, 25˚C and 37˚C respectively).

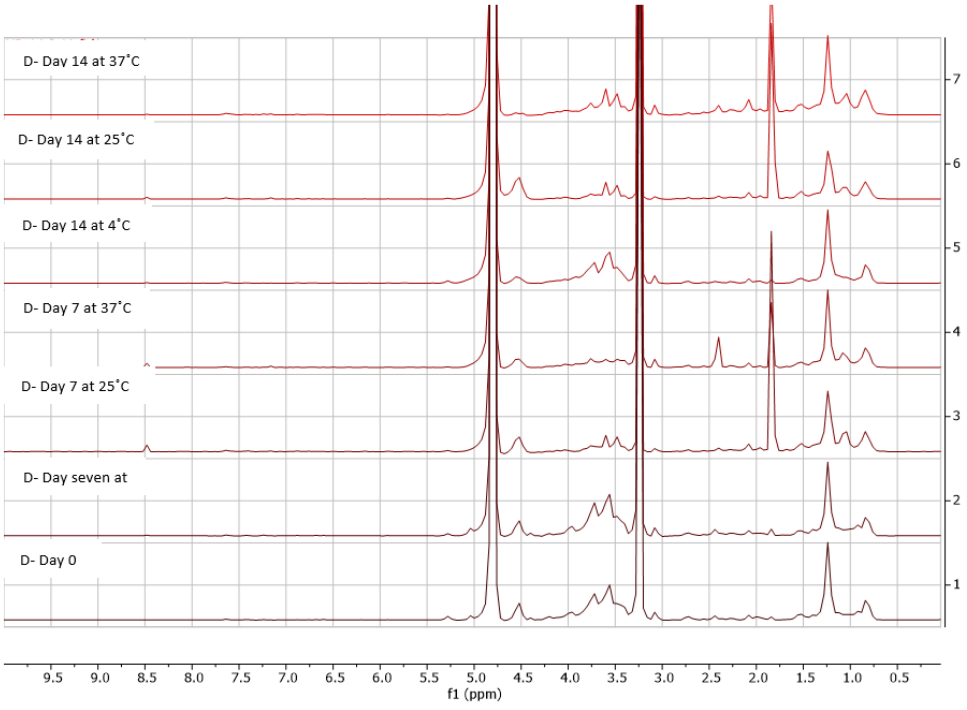

Figure 4.7: Zoomed in stacked ^{1}H NMR spectra of the herbal formulation containing plant D, *Kigelia africana* containing combinations that were stored at various temperatures over two different storage periods (stored for 7 days and 14 at 4˚C, 25˚C and 37˚C respectively).

Further analysis of NMR spectra is required to reduce any discriminating data and dimensions of the dataset. Reducing the dimensions of the dataset using multivariate data analysis provides a more comprehensive study that displays the similarities and differences between the samples. Computational methods, such as principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) are techniques used to depict the similarities and differences (Worley & Powers, 2013).

The principal component analysis (PCA) (Figure 4.8) generated from the ^{1}H NMR spectra from the 98 polyherbal formulations exhibit similarities and differences within the 14 classes. These classes were based on the different type of polyherbal formulations and the individual medicinal plants use in this study. The classes coincide with the aims of the study, which is to investigate the uniformity of the polyherbal formulations that were subjected to various conditions. When classes form a cluster it depicts that there are chemical similarities between the samples of the specific class. The outliers within and between each combination of the polyherbal formulations is visible especially within the polyherbal formulation ADB that was not stored and that which was stored at for 7 days at 4˚C. The difference in chemistries between the outliers and the rest of the polyherbal formulation ABD is accounted when examining the NMR spectra, there is a variation within the aromatic region (5.5 ppm). This could be due to a human error, contamination or there is a distinct difference in chemistries between the polyherbal formulations. Other errors include a disturbance in the analysis method (shimming of the spectra) (Sokolenko & Aucoin, 2015). The unsupervised PCA model displays the preserved variation within the dataset without transforming the dimensions of the data and the variables and separation of combinations is based on the difference and similarities in chemical constituents of the test samples (Bartholomew, 2010). Observations of the PCA models indicate that contain a single medicinal plant, namely B and C have a similarity within the chemistries irrespectively of the various conditions as clusters. Polyherbal formulations that contain two or more medicinal plants that for clusters include; formulations ABCD, BCD, AC, BC and CD. Polyherbal formulations ABC, AB, AD, and BD do not have distinct clusters, and there is no distinct chemical variation visible on the NMR spectra within the different polyherbal formulation. Single medicinal plant formulations that contain the medicinal plant A and D individually do not form distinct cluster, however the formulations that contain the medicinal plant D moderately form a cluster.

The statistical values R^2 and Q^2 of PCA and OPLS-DA indicate the robustness of the models, the R^2 value of the model statistically is defined as the fraction of variance of each component. Collectively these values indicate the validation of the model, with the Q^2 value exhibiting the predictability (Blasco et al., 2015). The unsupervised PCA model had an R^2 value of 0.808, which means that about 80.8% of the variation in the dataset has been accounted. The relative predictability; Q^2 value is 0.642 (64.2%) which indicates that the model can be used as a predictive tool (Blasco et al., 2015).

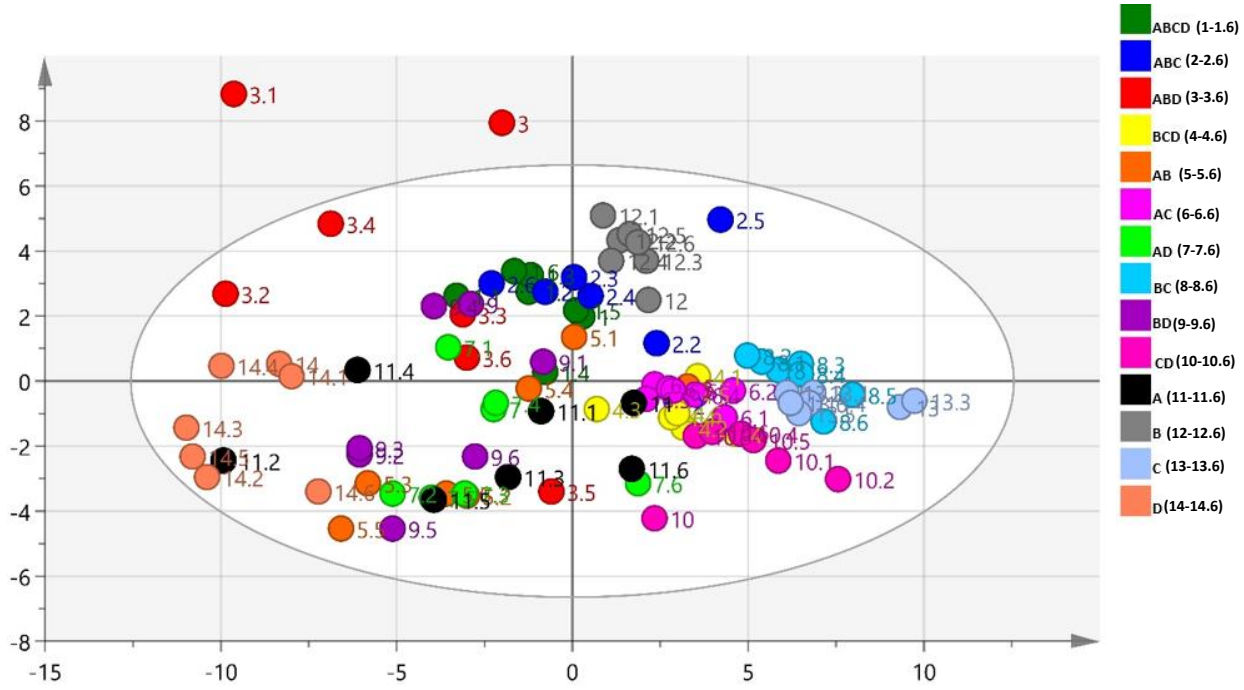

Figure 4.8: Principal component analysis (PCA) score plot generated from ^{1}H NMR spectra of ten polyherbal formulations containing the medicinal plants A-*Dicoma anomala* B-*Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana* and the individual plants.

* A-*Dicoma anomala* B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana*

The supervised orthogonal partial least square discrimination analysis (OPLS-DA) plot depicts more distinct grouping of the ten different polyherbal combinations and four individual medicinal plants than the PCA plot (Figure 4.9). The grouping of different classes polyherbal formulations, is based on the differences and similarities of chemical constituents of the datasets being tested. The relative robustness of the models is derived from the statistical values R2Y and Q2Y of the model. The R2Y of the OPLS-DA model is 0.815, which indicates that 81.5% variation is explained by the dataset. The predictability of the model is 48.1% with a Q2Y value of 0.481. The PCA and OPLS-DA models are considered to be robust as the

threshold to determine the relative robustness of a model is that the $R^2 \geq 50\%$ and the $Q^2 \geq 40\%$ (Blasco et al., 2015). The metabolomics data analysed is unique to this study and no previous studies have conducted on this magnitude. A cross validation of R^2 and Q^2 values is confirmed by the analysis of variance (ANOVA) with a P value = 0.023 which is significant ($p \leq 0.05$).

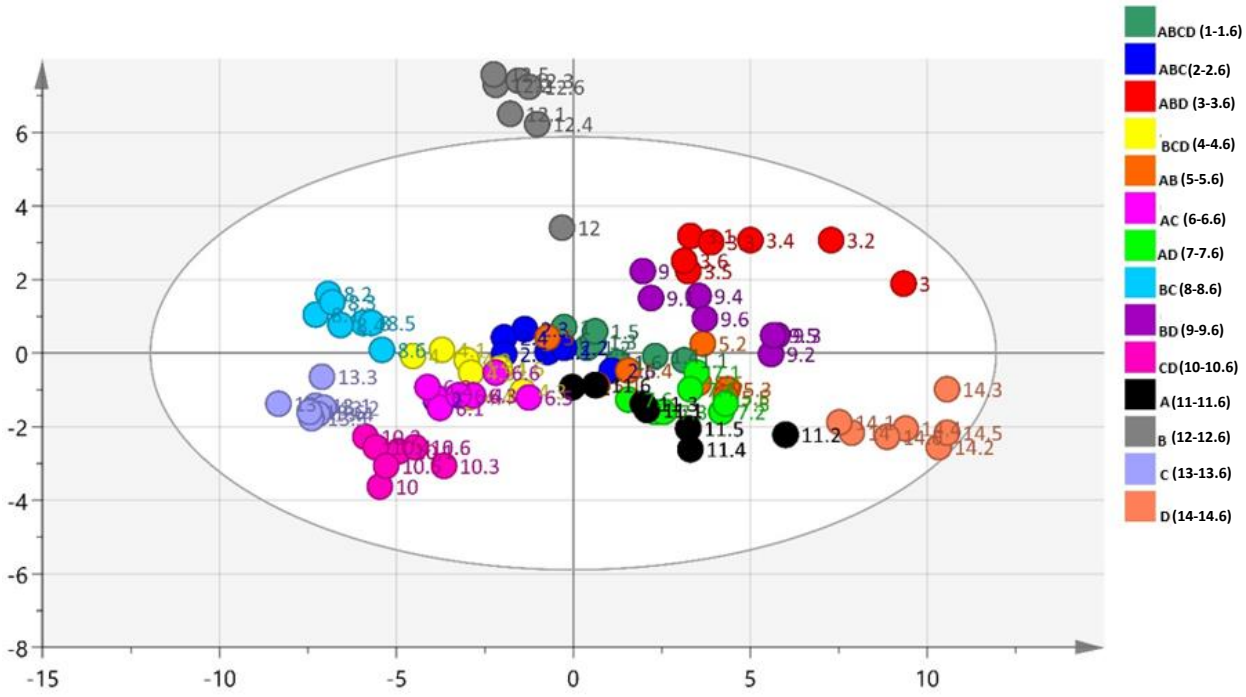

Figure 4.9: Orthogonal partial least square discriminant analysis (OPLS-DA) score plot generated from ^{1}H NMR ten polyherbal formulations containing the medicinal plants A-*Dicoma anomala* B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana* and the individual plants.

* A-*Dicoma anomala* B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana*

The reliability of the PCA and OPLS-DA model predicts that the quality of the different polyherbal formulations remains constant irrespective of the temperature and storage period. A contribution plot of polyherbal formulation ABD was generated to identify variables or regions (red) in the NMR spectra that can contribute to the separation of the polyherbal formulation that was not stored and that which was stored for 7 days at 4˚C (Figure 4.10). The variation in temperatures could account for the variation in chemical signal peaks (Figure 4.10) (Shamloo et al., 2017). The major bins that contribute to the greatest variation is 0.67-1.31 ppm, which correspond to metabolites such as lipids, fatty acids, terpenoids and steroids. Other prominent bins include from 6.03- 6.67 ppm, which are characteristics of

proanthocyanidins (Fatimah, 2020; Verpoorte, Choi, & Kim, 2007). Signals in the aromatic region are prominent and contribute to the variation in chemical constituents between the outliers and rest of the polyherbal formulation within the combination.

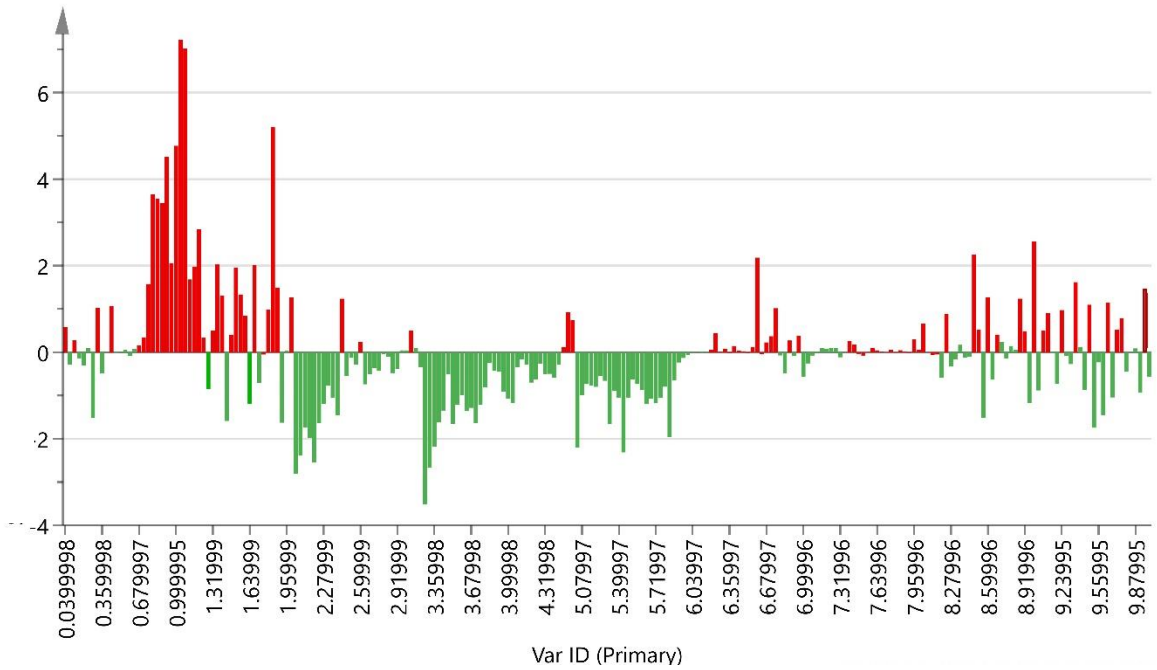

Figure 4.10: A contribution plot showing the major region in the ^{1}H NMR spectra (polyherbal formulation (ABD) which was not subjected to any condition: polyherbal formulation (ABD) stored for 7 days at 4˚C) bins that contribute to the outliers in the PCA plot.

Visual observations of ^{1}H NMR spectra and robustness of the metabolomic studies propose the class of chemical constituents. Cross validation can be obtained using liquid chromatography mass spectrometry (LC-MS/MS). Polyherbal formulations that exhibited noteworthy antimicrobial activity against *Neisseria gonorrhoea* were further subjected to LC-MS/MS analysis. The positive and negative ionisation modes were analysed (Figures 4.11-4.12). The spectra obtained from the analysis were further analysed from the identification of compounds responsible to antimicrobial activity. After an extensive analysis of these spectra (blank samples and test samples), the results obtained were inconclusive, which may be due to the high sensitivity of LC-MS/MS analysis (Ohno & Yamashita, 2015). However, mishandled samples and inaccurate method development could prevent the detection of compounds. Background noise causes interferences, which causes limitations and disturbance in quantitation and quantification of samples. The mobile phase can cause the background noise when factors such as the purity of the water, solvents and buffers aren't of quality (Elavarasi, Averal, & Ignatius, 2019). Characterisation and derivatization of crude extracts

is required to obtain more accurate results of the active chemical constituents (Deattu, Suseela, & Narayanan, 2013).

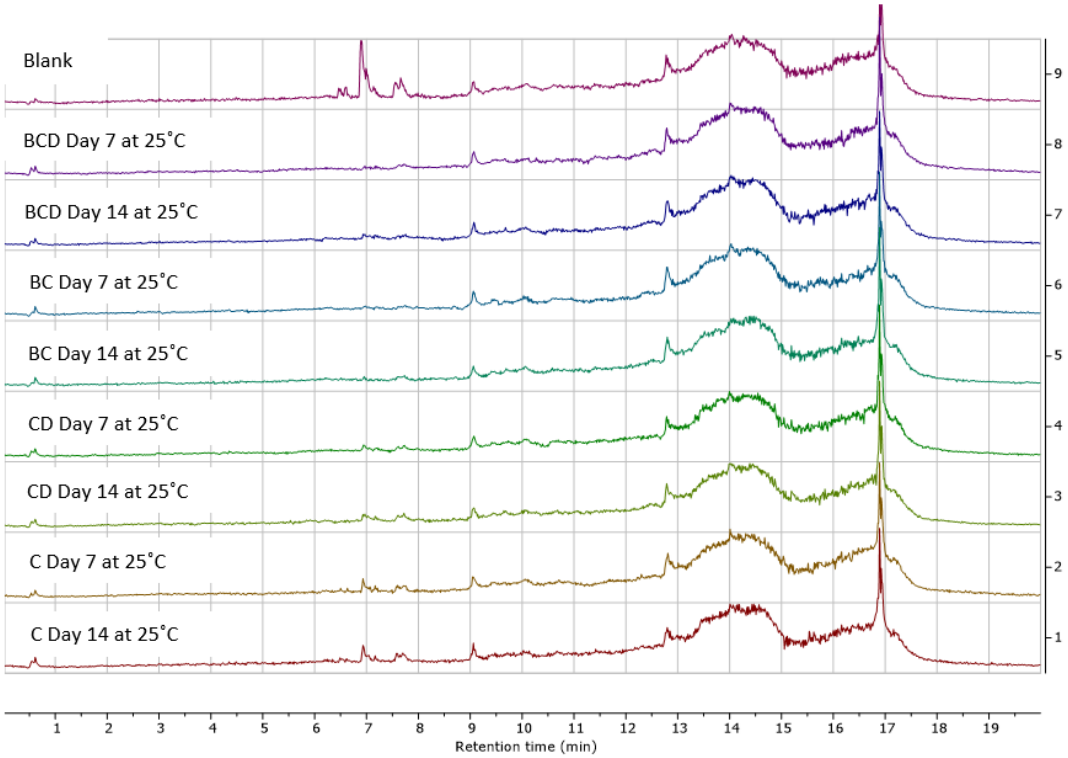

Figure 4.11: Zoomed in stack UPLC MS/MS positive ion mass spectra of eight polyherbal formulations that exhibited noteworthy antimicrobial activity against *Neisseria gonorrhoea*.

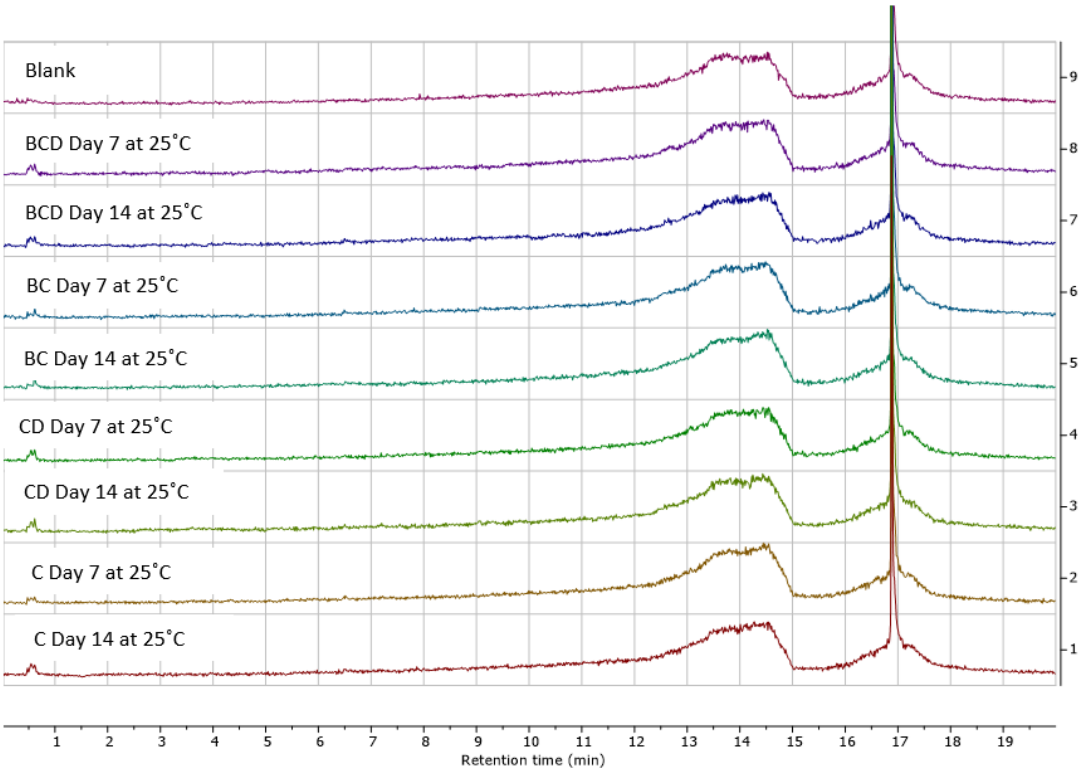

Figure 4.12: Zoomed in stack UPLC MS/MS negative ion mass spectra of eight polyherbal formulations that exhibited noteworthy antimicrobial activity against *Neisseria gonorrhoea*.

4.4 Conclusion

The quality control of the polyherbal formulations was extensively examined based on ^{1}H NMR metabolomics and LC-MS/MS. The quality remains relatively constant throughout the ten different polyherbal formulations and individual medicinal plants irrespective of the subjected conditions. The proposed signals visible on the ^{1}H NMR prosed chemical constituents. The clusters of different polyherbal formulations overlap in instance of mixtures depending on the type of plant used in each combination. The limitations incurred during LC-MS/MS analysis, requires further development of the method used to analyse the samples and to minimize any impurities.

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Chapter 5:

A cytotoxicity and mycotoxin analysis of the most active combinations of selected medicinal plants used in the treatment against *Neisseria gonorrhoea*

Chapter 5: A cytotoxicity and mycotoxin analysis of four medicinal plants used traditionally in combination to treat sexually transmitted infections.

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Abstract

The detrimental effects of prescribing medicinal plants without any scientific evidence is adverse. Antiproliferation (cytotoxicity) and mycotoxin studies are conducted to determine the safety of medicinal plants by examining potential toxicity to cells and the levels of mycotoxins present. Two types of cell lines can be examined depending on the type of traditional usage, as for cancer treatment, cancerous cell lines should be examined and for non-cancerous diseases, non-cancerous cell lines should be examined. Most mycotoxins present in medicinal plants have detrimental effects. The aim investigated in this study was to determine the level of cytotoxicity and mycotoxins exhibited by polyherbal formulations that resulted in noteworthy antimicrobial activity against sexually transmitted infections.

The antiprofileration activity of eight different polyherbal formulations that exhibited noteworthy antimicrobial activity *Neisseria gonorrhoea* were evaluated using cytotoxicity assay on kidney epithelial (Vero) cells. Mycotoxin levels present within the polyherbal formulations were analyzed via liquid chromatography mass spectrometry. All eight polyherbal formulations exhibited low to moderate toxicity against Vero cells. The IC_{50} ranged between 289.12-± 21.23 µg/mL to 866.47-± 22.41 µg/mL respective of the type of combination and storage period. The mass spectra analyzed exhibited non-detectable levels of mycotoxins present within the polyherbal formulations

In conclusion the toxicity of the polyherbal formulations increase slightly over a long period of time and should be given to patients with the utmost caution due to its rate of toxicity.

Keywords: Anti-proliferation, mycotoxins; toxicity; IC_{50}; Safety

5.1 Introduction

The efficacy of polyherbal formulations is enhanced as multiple active ingredients have the ability to target multiple pathways via additive or synergistic interactions (Abutaha et al., 2021). Although the efficacy of polyherbal formulations is enhanced, the toxic effect of traditional medicine is more detrimental than synthetic drug toxicity, as synthetic drug have a single active ingredient and is easier to identify than a drug containing multiple active ingredients (George, 2011). Some traditional medicine produced from medicinal plants have beneficial effects as a treatment and management in diseases, however are produced from toxic medicinal plants (Mensah et al., 2019).

Herbal drugs have a broad spectrum of action resulting in a more complex system to identify the toxicity of each active compound (Nasri & Hedayatollah, 2013). The safety and toxicity of medicinal plant extracts need to be verified through examining of its' toxic effects on organs and cells. The active ingredient or secondary metabolite present in medicinal plants that are responsible for biological activity, this influences the toxic effects it has on the individual utilizing the medicinal plant (Madariaga-Mazón, Hernández-Alvarado, Noriega-Colima, Osnaya-Hernández, & Martinez-Mayorga, 2019). Secondary metabolites such as alkaloids, phenolics and terpenoids may play a vital role in chemotherapeutic drugs and therefore influence the toxicity of medicinal plants (Wink et al., 2012).

Medicinal plant toxicity have adverse side effects, these effects are usually haemolytic, neurotoxic, spermatotoxic, nephrotoxic, hepatotoxic and genotoxic (Boukandou Mounanga, Mewono, & Aboughe Angone, 2015). Factors such as over- and incorrect usage of traditional medicine as well as preparation methods and routes of administration influences toxicity of the medicine. Other factors include microbial, heavy metals and mycotoxins (Cassileth et al., 2014).

The contamination of traditional medicine by fungi that produce mycotoxins possess a major health risk to patients. The fungus producing the mycotoxins does not necessarily influence the quality deterioration and cause discolouration, however causes detrimental effects (Gautam, Sharma, & Bhadauria, 2010). Various types of mycotoxins cause detrimental effects to the human body; these include: disrupting hormonal systems, damage organs, compromise the immune system or cause cancer. Classification of mycotoxins are complicated and

therefore classified according to the organ they effect. An example is if the mycotoxins effect the brain they are called neurotoxins, other classes of mycotoxins include; heptotoxins (liver); nephrotoxins (nephrons) and immunotoxins (immune system) (Bennett & Klich, 2003). The degree of severity caused by mycotoxins is dependent on the type and source, exposure and dose, species susceptibility and underlying conditions of the subject.

The risks involved in the use of traditional medicine that is contaminated by high levels of mycotoxins possess a major challenge to communities and individuals. According to the maximum mycotoxin limit permitted according to the regulations in South Africa and internationally (Table 5.1) the polyherbal formulations examined in this study is deemed safe for traditional use against the sexually transmitted infection; *Neisseria gonorrhoea*.

Table 5.1: Summary of the maximum mycotoxin limit permitted on plants according to the South African and International government regulations.

Mycotoxin	**Analytical detection method**	**Maximum limit permitted**	**Reference**
Aflatoxin B_1	HPLC, LC-MS	5 µg/kg	(Government Notice, 2004; Misihairabgwi, Ezekiel, Sulyok, Shephard, & Krska, 2019)
Deoxynivalenol (DON)	HPLC, TLC	1000 µg/kg	(Government Notice, 2004; Misihairabgwi et al., 2019)
Zearaleonone (ZEN	HPLC, LC-MS	1000 µg/kg	(FAO, 1997)
Nivalenol	HPLC	*	(FAO, 1997)
Orchratoxin A	HPLC, LC-FL, LC-MS	5 µg/kg	(FAO, 1997)
Fumonisin B1	HPLC, LC-MS	*	(Misihairabgwi et al., 2019)
Fumonisin B2	HPLC, LC-MS	*	(Misihairabgwi et al., 2019)
Fumonisin B3	HPLC, LC-MS	*	(Misihairabgwi et al., 2019)

The aim of this study was to investigate the levels of toxicity (cytotoxicity and mycotoxins) exhibited by polyherbal formulations that displayed noteworthy antimicrobial activity against *Neisseria gonorrhoea.*

5.2 Methods

5.2.1 Cytotoxicity

5.2.1.1 Cell culture preparation

Vero cells (ATCC CCL-81) (Figure 5.1) were prepared to evaluate the toxicity of plant extracts using African green monkey kidney cells. Culturing of cells was prepared in a Corning® CellBIND® Surface cell culture flask using Eagle's Minimum Essential Media (EMEM), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and 1% antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin D, and 250 µg/mL fungizone) (PSF). The cells are incubated to 80% confluency in a CO_2 humidified incubator (5% CO_2/ 95% filtered air) at 37˚C (Berrington & Lall, 2012; Lall et al., 2019; Rademan, Anantharaju, Madhynapantula, & Lall, 2019).

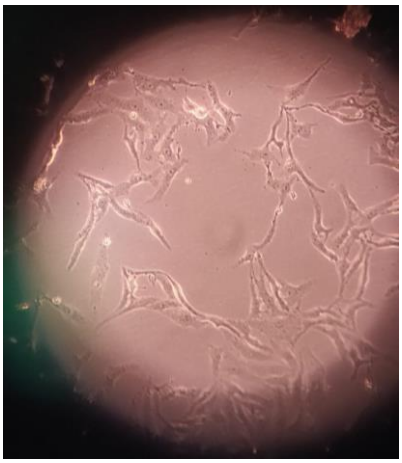

Figure 5.1: A microscopic image of a culture of Vero cells (ATCC CCL-81) using Eagle's Minimum Essential Media (EMEM) as a growth medium.

Adherent cells loosened from the bottom surface and then were rinsed three times with 10% Phosphate buffer saline (PBS). The detachment process of cells achieved via the addition of trypsin-EDTA (0.25% trypsin containing 0.01% EDTA) (Ethylenediaminetetraacetic acid). Flasks were incubated for 5 minutes at 37˚C. To inhibit the degradation of cells from the trypsin, fresh EMEM was added to the flasks. The flask's content was transferred to a falcon tube and centrifuged for 5 minutes at 900 rpm. The supernatant was discarded and the precipitate was resuspended in 1 mL fresh EMEM. Cell viability was determined using a Countess 3 FL Automated cell Counter. To determine the toxicity of plant extracts, each well contained approximately 10000 cells per well, the final concentration of cells was 1.0 x 10^5 cells per mL. Mircotiter plates were prepared by transferring 100 µL of mixed cells and fresh EMEM to each well (B2-B11 up until G2-G11) (Berrington & Lall, 2012; Lall et al., 2019; Rademan et al., 2019).

5.2.1.2 Cell viability

Plant extracts (4 mg) that displayed the best anti-gonococcal activity (<1 mg/mL) were dissolved in 5% DMSO in order to make a final stock of 4 mg/mL. A twofold serial dilution was conducted with a starting test concentration of 1 mg/mL for each plant sample, Actinomycin D (0.20 mg/ml) was used as a positive control at a starting test concentration of 0.05 mg/ml and 1% DMSO was used as a vehicle control. The plant samples and controls were actively prepared in a separate 48-well microtitre plate and 100 µL of content from each well was transferred to its respective well in the plates containing the cells. The 96-well plates were placed in the CO_2 humidified incubator (5% CO_2/ 95% filtered air) at 37˚C for 3 days. Upon the completion of the incubation period, 10 µL of Presto Blue™ Cell viability reagent (ThermoFisher Scientific) was added to the wells and further incubated for 2 hours. Cell viability was detected using the program Victor Nivo Prestoblue Cell Viability to analyze the plates. The program used to determine the half-maximal inhibitory concentration (IC_{50}) values for each sample was PRISM 4.

5.2.2 Mycotoxins

5.2.2.1 Sample preparation

The same ten samples that displayed the best anti-gonococcal activity were prepared for mycotoxin analyse. Sample preparation involved the preparing of 1 g of each plant sample and aliquots of mycotoxin standards (50 µg/ml); Alfatoxin B_1, Deoyxnivalenol (DON), Nivalenol, Orchratoxin A, Zearalenone (ZEA), FB_1, FB_2 and FB_2.

5.2.2.2 Multi Mycotoxin analyse

High performance liquid chromatography (HPLC) analyse was conducted by Mr Erick van Schalkwyk at the Central Analytical Facilities, Univeristy of Stellenbosch. A full detailed method can be found in the Appendix C.

5.3 Results and discussion

5.3.1 Cytotoxicity

Medicinal plants that exhibited antimicrobial activity against various diseases, including cancer, heart diseases and sexually transmitted infections (STIs) play a vital role in drug development, however the toxicity of these medicinal plants play a crucial role in their efficacy. Low toxicity and high efficacy of medicinal plants is significant when analyzing plant

extracts. Secondary metabolites found within medicinal plants influence its activity. The activities include biological; antimicrobial, antiviral, anthelminthic and cytotoxicity (Madariaga-Mazón et al., 2019).

The highest test concentration was 1000 µg/mL and the lowest 31.25 µg/mL. Positive (Actinomycin D) and vehicle (1% DMSO) controls were used to eliminate any biases from this study. The positive control used was Actinomycin D (1.56 x 10^{-3} µg/mL to 0.05 µg/mL), this drug is used in anti-proliferation studies as a positive control due to its' positive effects on cancerous and non-cancerous cells (Lu et al., 2015). The vehicle control (1% DMSO) does not influence the cell in a negative manner.

Actinomycin D (Figure 5.2) exhibits high levels of antibacterial and anticancer activity and is commonly used as a chemotherapeutic drug, as a treatment against various types of cancers. The drug is a natural chromopeptide which composes of a heterocyclic chromophore and two cyclic pentapeptide lactone rings and is the most well studied drug within the actinomycin family (Avendaño & Menéndez, 2008). Actinomycin D is one of the oldest anticancer drug used in the treatment of Wilms tumor, Ewings' sarcoma and gestational trophoblastic disease (GTD) (Lu et al., 2015). The mechanism of action of actinomycin D occurs via the intercalation of DNA thus disrupting the elongation process by affecting the RNA polymerases which results the inhibition of RNA synthesis (Lu et al., 2015; Sobell, 1985).

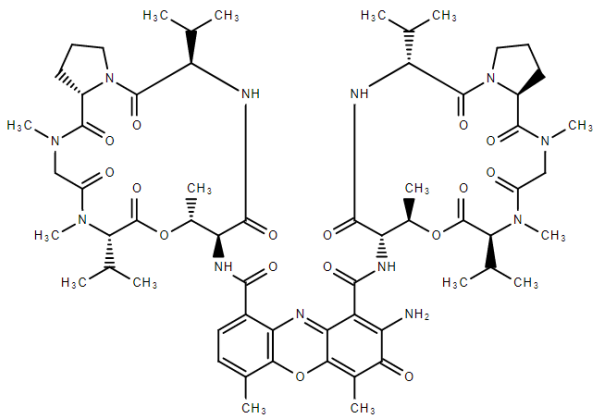

Figure 5.2: Chemical structure of antibacterial and anticancer drug, Actinomycin D.

The toxicity of eight polyherbal combinations that exhibited the best antimicrobial activity against bacteria *Neisseria gonorrhoea* was investigated against non-cancerous Vero cells. The IC_{50} values range between 289.12 µg/mL to 866.47 ± 22.41 µg/mL (Figure5.3) and the IC_{50}

value of the positive control was 0.02 ± 0.14 µg/mL. The standard toxicity threshold for plant extracts examined against non-cancerous cell lines are as follows; if the IC_{50} values is lower than 100 µg/mL (IC_{50} ≥ 100 µg/mL) the plant extracts are deemed to be significantly toxic. Plant extracts that exhibited IC_{50} values between 100 µg/mL and 300 µg/mL are considered to be moderately toxic and from 300 µg/mL to 1000 µg/mL to have low toxicity. IC_{50} values above 1000 µg/mL (IC_{50} ≤ 1000 µg/mL) exhibited no toxicity in in vitro studies (Berrington & Lall, 2012; Kuete & Efferth, 2015). The standard deviation range observed throughout the different polyherbal formulations is small and therefore variation for the IC_{50} values have been taken into account (Livingston, 2004). A noticeable trend observed is that the longer the polyherbal formulation has been stored the slightly more toxic the formulation tends to become.

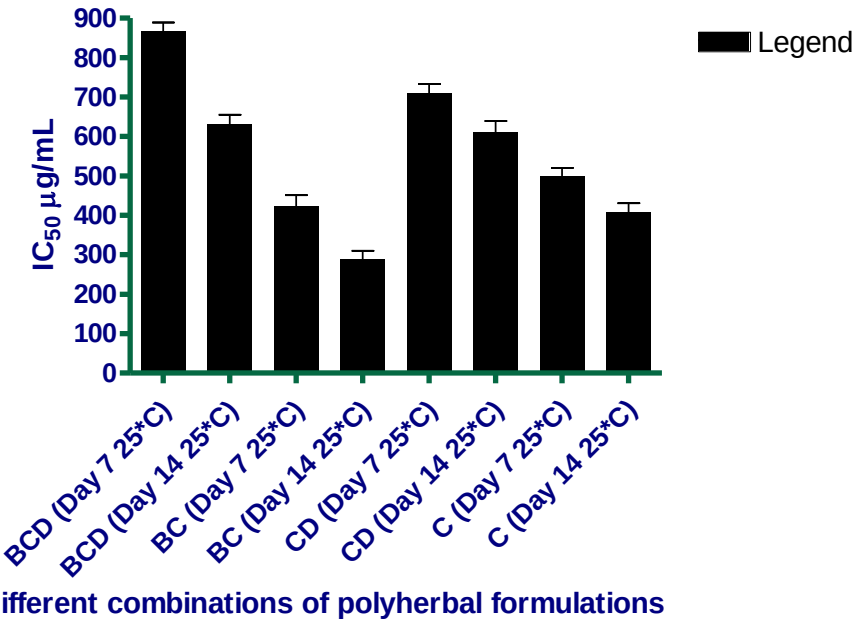

Figure 5.3: Graphical description of the half maximal inhibitory concentrations (IC_{50}) and standard deviation of Cytotoxic analysis of eight polyherbal formulations that exhibited the best antimicrobial activity against sexually transmitted infections (STIs) on Vero cell line.

The polyherbal formulations examined in this study did not exhibit IC_{50} values that are significantly toxic to non-cancerous cells (Table 5.2). Polyherbal formulation BC that was stored for 14 days at 25˚C exhibited an IC_{50} value of 289.12 ± 21.23 µg/mL which is considered to moderate toxic as per the standard threshold. Combinations BCD that has been stored at 25˚C for 7 and 14 days, exhibited an IC_{50} value of 866.47 ± 22.41 µg/mL and 631.28 ± 23.57 µg/mL respectively and is considered to exhibited low toxicity. Polyherbal combinations BC

(stored for 7 days at 25˚C), CD (stored at 25˚C for 7 and 14 days) and C (stored at 25˚C for 7 and 14 days) exhibited IC_{50} values of 421.5 ± 29.78, 709.33 ± 23.57, 611.05 ± 28.12, 499.65 ± 20.48 and 408.08 ± 22.83 µg/mL respectively.

Table 5.2: The half maximal inhibitory concentrations (IC_{50}) determination using Cytotoxic analysis of eight polyherbal formulations that exhibited the best antimicrobial activity against sexually transmitted infections (STIs) on Vero cell line.

Combinations of polyherbal formulations	IC_{50} (µg/mL) ± SD
BCD (Day 7 25˚C)	866.47 ± 22.41
BCD (Day 14 25˚C)	631.28 ± 23.57
BC (Day 7 25˚C)	421.5 ± 29.78
BC (Day 14 25˚C)	289.12 ± 21.23
CD (Day 7 25˚C)	709.33 ± 23.57
CD (Day 14 25˚C)	611.05 ± 28.12
C (Day 7 25˚C)	499.65 ± 20.48
C (Day 14 25˚C)	408.08 ± 22.83

*SD-Standard deviation

B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana*

3 replicates were conducted for each combination

All the different polyherbal formulations contain the medicinal plant C-*Eucalyptus cinerea*, four of the eight combinations contain *Elephantorrhiza elephantina*, and the other four contain *Kigelia africana.* A study conducted by Elaissi e al., the activity of essential oils from *E. cinerea* against Vero cells exhibited low toxicity, it was proposed that the eucalyptol (1,8-cineole) accounts for the low toxicity. Polyherbal formulations that contain *K. africana* exhibited low toxicity levels irrespective of their storage period and is supported by a study which exhibited that water extracts of *K. africana* fruit resulted in low toxicity to Vero cells (Nabatanzi, Nkadimeng, Lall, Kabasa, & McGaw, 2020).

Although the positive control used was doxorubicin, the mechanism of action of Actinomycin D and doxorubicin is similar (Lu et al., 2015; Sobell, 1985; Thorn et al., 2011). According to another study done, *E. elephantina* exhibited low levels of toxicity, above 100 µg/mL at two different concentration (Sigidi et al., 2017). Phytochemicals namely terpenoids; has been proposed influence the storage of medicinal plant extracts, thus indicating that the longer the polyherbal formulations have been stored the more toxic the formulations becomes. Monoterpenoids exhibit acute toxicity to biological components (Brari & Thakur, 2015; Zárybnický, Boušová, Ambrož, & Skálová, 2018; Zielińska-Błajet & Feder-Kubis, 2020).

5.3.2 Mycotoxins

Data was obtained via HPLC analysis based on calibration curves, chromatograms and calibration curves of both sample extracts and standards (Appendix C). Chromatograms and calibration curves of standards revealed peaks at their respective retention times. According to the results,c mycotoxins were not detected in any of the eight polyherbal combinations.

Due to increase usage of medicinal plants by traditional healers as medicine, their usage safety, quality control and efficacy requirements need to be examined. Mycotoxins present on medicinal plants pose a serious health risk to patients of traditional healers. Contamination of medicinal plants with various fungi that produce mycotoxins take place during soil contamination, harvesting, storage, distribution and mishandling of the plants (Ashiq, Hussain, & Ahmad, 2014; Calixto, 2000).

Areo, Phoku, Gbashi, & Njobeh, 2020 conducted a mycotoxin analysis on the roots of *Elephantorrhiza elephantina* which resulted in the non-detection of aflatoxin B_1. The chemical composition of *Eucalyptus cinerea* and *E. globulus* is similar as both plants contain eucalyptol, an essential oil the composition of eucalyptol in *E. cinerea* is approximately 84% and in *E. golobulus* 48% (Jerbi, Derbali, Elfeki, & Kammoun, 2017; Soliman et al., 2014). Essential oil (EO); eucalyptol of *E. globulus* was used in a study to test its effect against mycotoxin production. The EO reduced the production of fumonisins and the inhibition of aflatoxin B_1 production by using 500 µL of the EO (López-Meneses et al., 2015; Soares, Damiani, Moreira, Stefanon, & Vassallo, 2005). Due to the potency of the EO present it can be proposed that formulations containing *E. cinerea* may contribute to the non-detectable levels of certain mycotoxins. There are no studies conducted on effects of mycotoxin production on the fruit of *Kigelia africana*. Although the polyherbal formulations were stored at the optimum temperatures and storage days for the production of fungi that produce the mycotoxins. It could be that the plant parts did not have a fungal contamination before the study was conducted and thus no mycotoxins were produced.

5.4 Conclusion

The usage of medicinal plants by traditional healers required scientific validation in multiple effects, for example its' activity against pathogens, the chemical constituents and toxic effects. The polyherbal formulations used in this study exhibited low levels of toxicity towards non-cancerous Vero cells. The various polyherbal formulations exhibited a range of IC_{50} and

displayed a narrow standard deviation range. The limitation in this study involves the usage of non-cancerous cell lines, as these cell lines have an indefinite replication rate causing an overgrowth of normal cells (Alibert et al., 2017). Further studies should involve the examination of polyherbal formulations against both cancerous and non-cancerous cell lines.

Studies conducted on mycotoxins found on medicinal plants in South Africa is limited. Areo et al., 2020 conducted a mycotoxin study on 36 medicinal plants found in South Africa, including the root of *E. elephantina*. The following mycotoxins were analyzed aflatoxin, zearalenone and ochratoxin A using HPLC as an analysis tool. This limitation is of concern as medicinal plants are widely used by traditional healers to an ever-growing population of patients. The polyherbal formulations used in this study did not contain detectable levels of mycotoxins. Although previous studies conducted on *E. elephantina* exhibited non-detectable levels of aflatoxin B_1, and eucalyptol has displayed inhibition of fumonisin growth there is a demand for more studies to be conducted on different types of mycotoxins. Due to the limitations, no studies have been conducted on the mycotoxin present in the fruit of *K*. *africana*.

5.4 Reference

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Chapter 6:

General discussion and conclusion

6 Chapter 6: General discussion and conclusion

6.1 General discussion and conclusion

Sexually transmitted infections (STIs) is of concern amongst the world's population, and the prevalence of contracting an infection amongst women remain high between the age group 15-24 years. Gonorrhoea accounts for 1.4% to 8.9% of STIs amongst this age group (Francis et al., 2018). When infected and left untreated, STIs are fatal, in the year 2000 STIs accounted for 26% of all deaths in South Africa (H. De Wet et al., 2012). The antimicrobial resistance (AMR) of *Neisseria gonorrhoea* over the past ≥75 years has been ever growing and additional measures should be investigated to reduce or combat the infections rate (Kenyon, Laumen, Van Den Bossche, & Van Dijck, 2019). Medicinal plants provide an alternative source of treatment to synthetic drugs.

Numerous of medicinal plants are formulated into water-based and alcohol-water based preparations. Water-based preparations include decoctions and infusions; a decoction is a freshly made formulation using either dried or fresh plant material. The plant material is placed in in a vessel with water and brought to a boil. An infusion is either a cold or hot water formulation, the plant material is submerged in either cold or hot water, and however the material is not subjected to the boiling process. Methods of administration vary greatly from inhalation to the rubbing of medicinal plants on the effected body part and the ingestion by taking it in as a drink, porridge or solid foods (Shumba et al., 2009).

This study was conducted to determine the antimicrobial activity, quality control, chemical constituents, cytotoxicity and mycotoxin levels of selected medicinal plants used for the management and treatment of sexually transmitted infections (STIs) in the Venda community. The four selected medicinal plants *Dicoma anomala* sbsp. *anomala* (A), *Elephantorrhiza elephantina* (B), *Eucalytpus cinerea* (C) and *Kigelia africana* (D) were investigated individually and as a polyherbal formulation of various combination types.

In vitro antimicrobial studies conducted on two pathogens a bacterium, *Neisseria gonorrhoea* and a fungus, *Candida albicans* using a microtiter dilution assay (chapter 3). The polyherbal formulations contain various types of plant organs (leaves, rhizomes, roots and fruits), using different ratios of plant material for the different type of combinations. The plant extracts exhibited poor antimicrobial activity against *Candida albicans*. Plant extracts that are

exhibited a minimum inhibitory concentration (MIC) value of ≤ 1mg/mL is considered noteworthy (van Vuuren, 2008).Polyherbal formulations that exhibited noteworthy activity (0.39 mg/mL) against *Neisseria gonorrhoea* include BCD, BC, CD and C, which had been stored at 25˚C for 7 and 14 days respectively.. The fractional inhibitory concentration (FIC) index is used to determine the various types of interactions; additive, antagonism, synergism and non-interactive effects. Combination BCD resulted in an additive effect, meaning the total activity of the polyherbal formulation is greater than the activity of each single plant. Non-interactive effects were observed during the examination of combination BC and synergistic effects of combination CD.

The phytochemistry of a medicinal plant varies depending on multiple factors, these include, seasonal, geographical and environmental factors (Ncube, Finnie, & Van Staden, 2011). Metabolomic studies were conducted to determine the similarities between the phytochemistry of the various combinations of polyherbal formulations (chapter 4). Prominent signal peaks were observed on the ^{1}H Nuclear magnetic resonance (NMR) spectra in the aliphatic region (0.8- 3.00 ppm), carbohydrate region (3.00- 6.00 ppm) and aromatic region (5.00- 9.00 ppm). The PCA and OLPS-DA models determine the robustness and relative goodness of fit of the model. The R^2 and Q^2 values of the unsupervised PCA model is 0.808 and 0.642 respectively, whereby 80.8% variation in the dataset has been accounted for and has a 64.2% predictable accuracy. The supervised OPLS-DA model has an R^2 and Q^2 value of 0.815 and 0.481 respectively. Approximately 81.5% variation of the supervised model has been accounted for and 48.1 predictability of future models. The identification of compounds present in the eight noteworthy antimicrobial results against *N. gonorrhoea* were determine via liquid-chromatography mass spectrometry. The analysis produced inconclusive results, and therefore further characterization and derivatization of the plant sample is required to determine semi-pure compounds.

Furthermore, cytotoxicity and mycotoxin (chapter 5) analysis of the eight polyherbal formulations were conducted to determine the level of toxicity towards non-cancerous vero cells. The different combinations of polyherbal formulations resulted in range of half-maximal inhibitory concentration (IC_{50}) values. The range is between 289.12 µg/mL ± 21.23 and 866.47 µg/mL ± 22.41, from moderately toxic to low toxicity levels. These polyherbal formulations can be utilized by traditional healers, however further analysis involving the usage of

cancerous cell lines should be conducted. Mycotoxin analysis of polyherbal formulations resulted in low to no levels of mycotoxin detection.

In conclusion, the eight different combinations of polyherbal formulations selected in the treatment and management of against sexually transmitted infections *Neisseria gonorrhoea* and *Candida albicans* exhibited noteworthy results. The objectives were achieved through various biological and chemical analysis. However, furthermore chemical analysis is required to isolate and determine the bioactive compounds responsible for the activities.

6.2 Perspective future work

- Further studies should be conducted on the combinations of polyherbal formulations that resulted in noteworthy activity. These include:
- Isolation of active compounds using characterization and derivatization of samples via accurate LC-MS method development, based on the nature of samples.
- The mycotoxin examined in this study are mainly found on crops, however there are other mycotoxins that result in detrimental effects that can be studied such as patulin which need to be examined.

6.3 References

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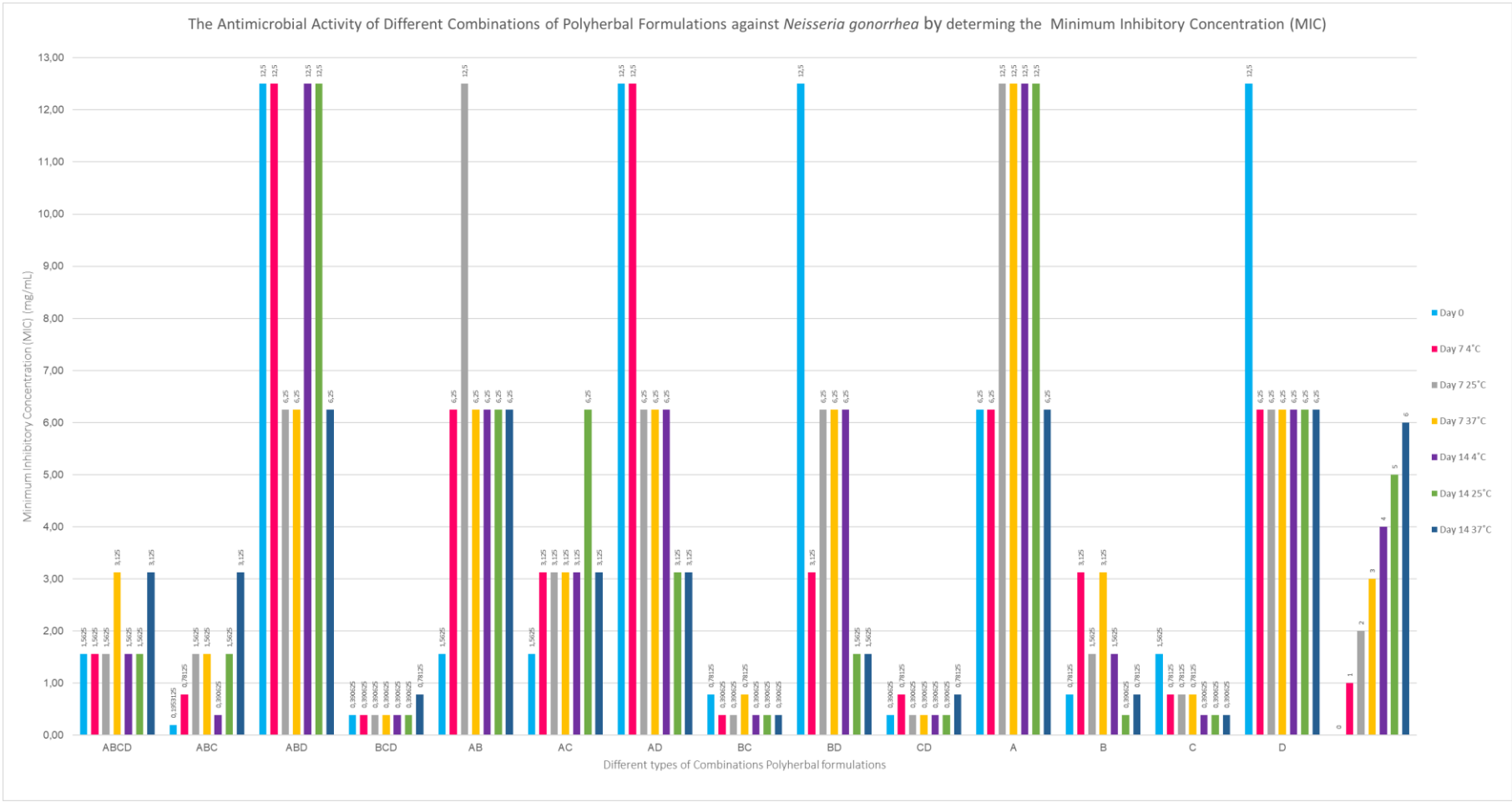
Appendices

Appendices

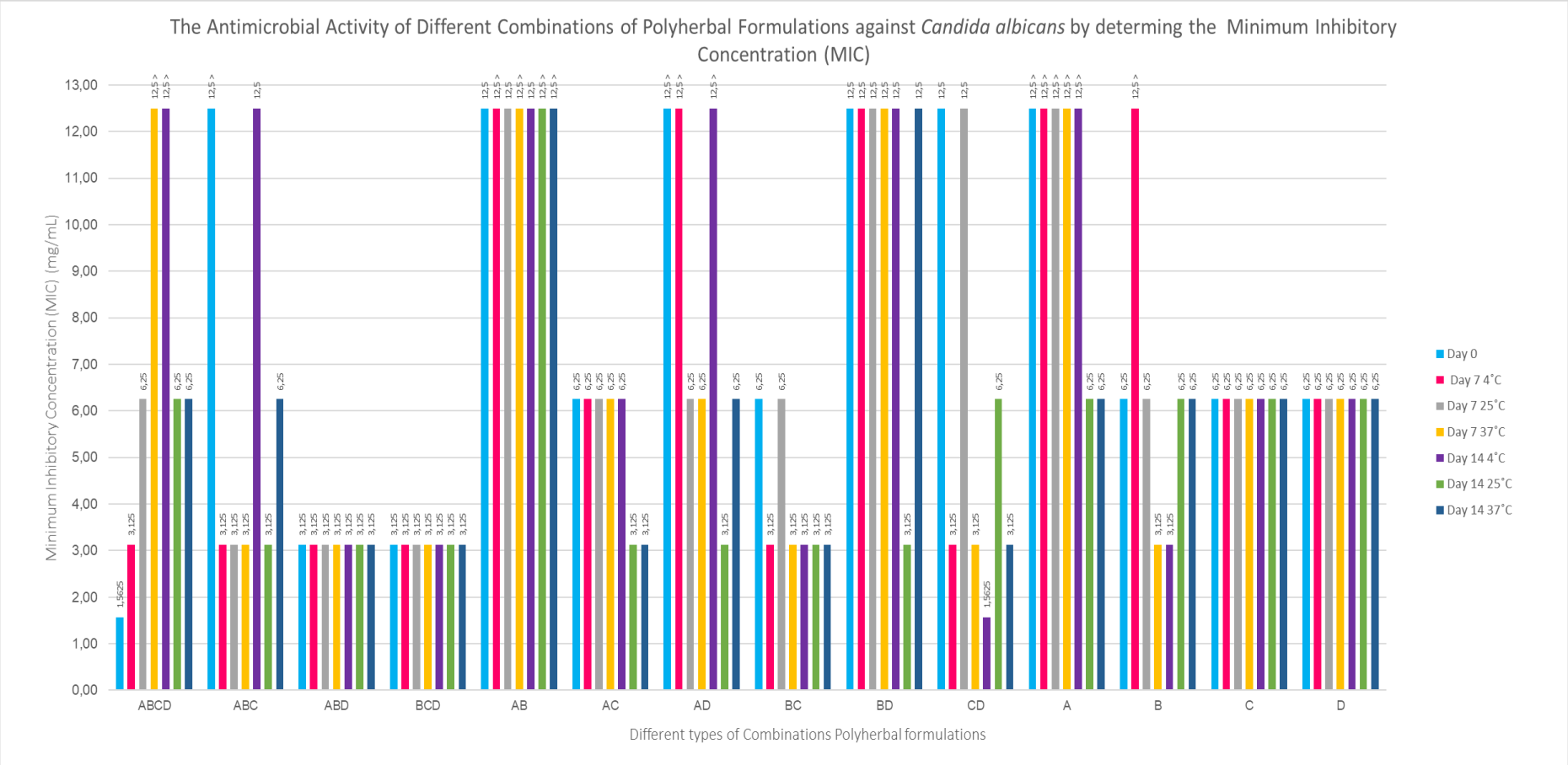
Appendix A

Chapter 3: Antimicrobial activity of four medicinal plants used traditionally in combination to treat sexually transmitted infections



*A-*Dicoma anomala* B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana*

Figure A.1: Minimum Inhibitory Concentration (MIC) values (mg/mL) of different type of combinations of plant extracts against the bacteria *Neisseria gonorrhoea.*



*A-*Dicoma anomala* B- *Elephantorrhiza elephantina* C- *Eucalyptus cinerea* D- *Kigelia africana*

Figure A.2: Minimum Inhibitory Concentration (MIC) values (mg/mL) of different type of combinations of plant extracts against the fungus *Candida albicans.*

Chapter 4: A metabolomic analysis of selected plants used in the treatment against *Niessseria gonorrhoea* and *Candida albicans*

Descriptive method of Ultra Performance Liqud Chromatography (MS/MS) (UPLC-MS/MS).

Ultra-Performance Liquid Chromatography (MS/MS) Analyses

Compound separation and detection was performed using a Waters® Synapt G2 high definition mass spectrometry (HDMS) system (Waters Inc., Milford, Massachusetts, United States of America (USA)). The system comprises of a Waters® Acquity Ultra Performance Liquid Chromatography (UPLC) hyphenated to a quadrole-time-of0fkight (QTOF) instrument. The system is operated with MassLynx™ (version 4.1) software (Waters Inc., Milford, Massachusetts, USA) for the acquisition and processing. An internal lock mass control standard, 2 ng/µL solution leucine enkephaline was indirectly infused into the source through a secondary orthogonal electrospray ionization (ESI) probe allowing intermittent sampling. The internal control was used for stability and to compensate for the instrumental drift, to enable good mass accuracy throughout the runs. Calibration of the instrument is done by using sodium formate clusters and Intellistart functionality. Resolution of 20 000 at m/z 200 and mass error within 0.4 mDa were obtained.

The operating parameters were as follows: the capillary voltage for ESI was 2.6kV and 2.0 kV for the positive and negative ionization respectively. The source temperature was set at 120˚C, the sampling cone voltage at 25 V, extraction cone voltage at 4.0 V and cone gas (helium) flow at 10.0 L/Hr. The desolvation temperature was set at 350˚C with a gas (helium) flow of 600.0 lL/Hr.

Quantitative data-independent acquisition (DIA) was done using two simultaneous acquisition functions with low and high collision energy (MS^E approach) with a QTOF instrument. Fragmentation was performed using high energy collision induced dissociation (CID). The fragmentation energy was set at 2 V and 3 V for the trap and collision energy respectively. The ramping was set from 3 V to 4 V and 20 V to 40 V for trap and transfer collision energy respectively. Mass spectral scans were collected every 0.3 seconds, raw data was collected in a continuous profile form. Mass to charge ratio (m/z) between 50 and 1200 Da were collected.

Separation was completed using a reverse phase step gradient elution scheme from 95% H_2O (0.1% formic acid) to 100% acetonitrile (0.1% formic acid). Formic acid (99+% purity) was added to the solution as a buffer (pH correction); preservative and proton source for ionization. The gradient started with an isocratic flow (hold 0.1 min) followed by a linear increase to 100% ACN. The column is subsequently washed for 1 min followed by conditioning and re-establishing of initial conditions to allow for equilibration, before the start of the next run for the complete elution scheme. Column temperature was kept constant at 40˚C and the flow was set at 0.4 mL/min for each run; totaling a 20 min time for each run. Injection volumes were set at 5 µL using A Kinetex® 1.7 µm EVO C18 100 Å (2.1 mm ID x 100 mm length) column. The same separation conditions were used to collect both positive and negative ion mass spectra. Solvents used for separation was ultra-purity; Liquid Chromatography methanol/water/acetonitrile (Romil-UpS™,Microsep, South Africa)

Proton NMR (^{1}H NMR) spectra of 98 extracts of different combinations of polyherbal formulations

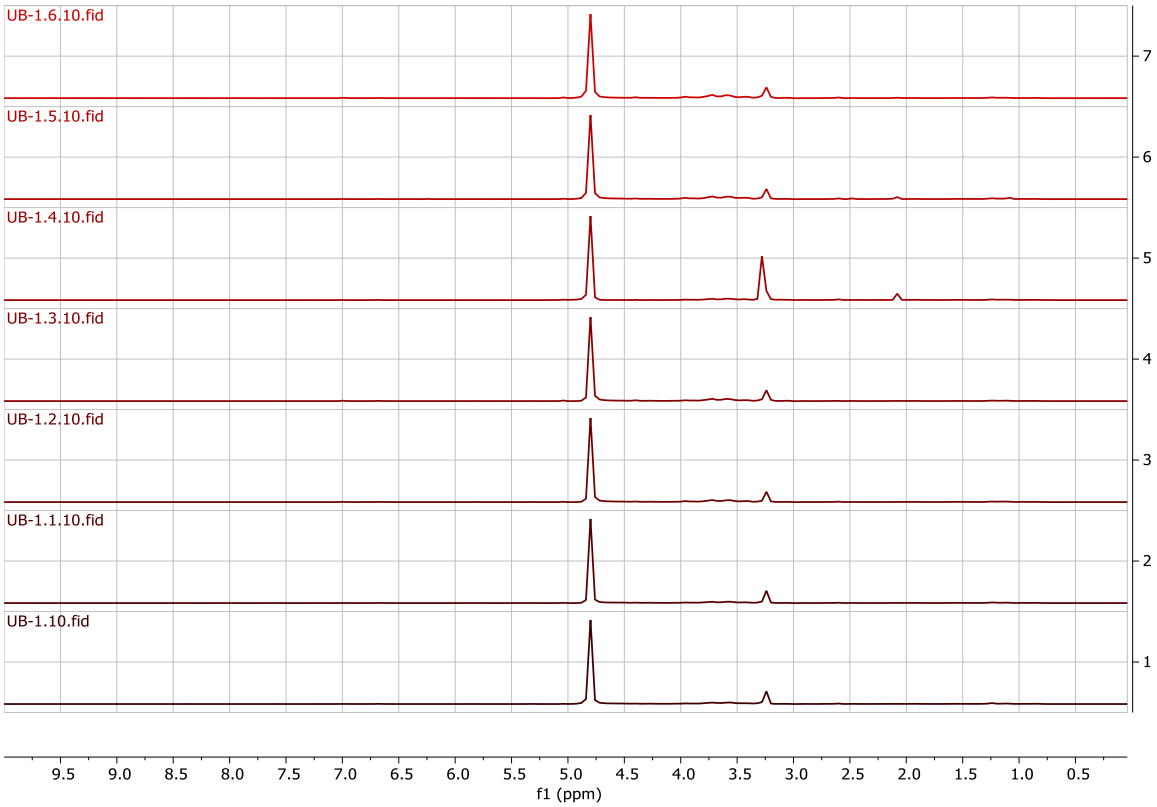


Figure A.3: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants ABCD.

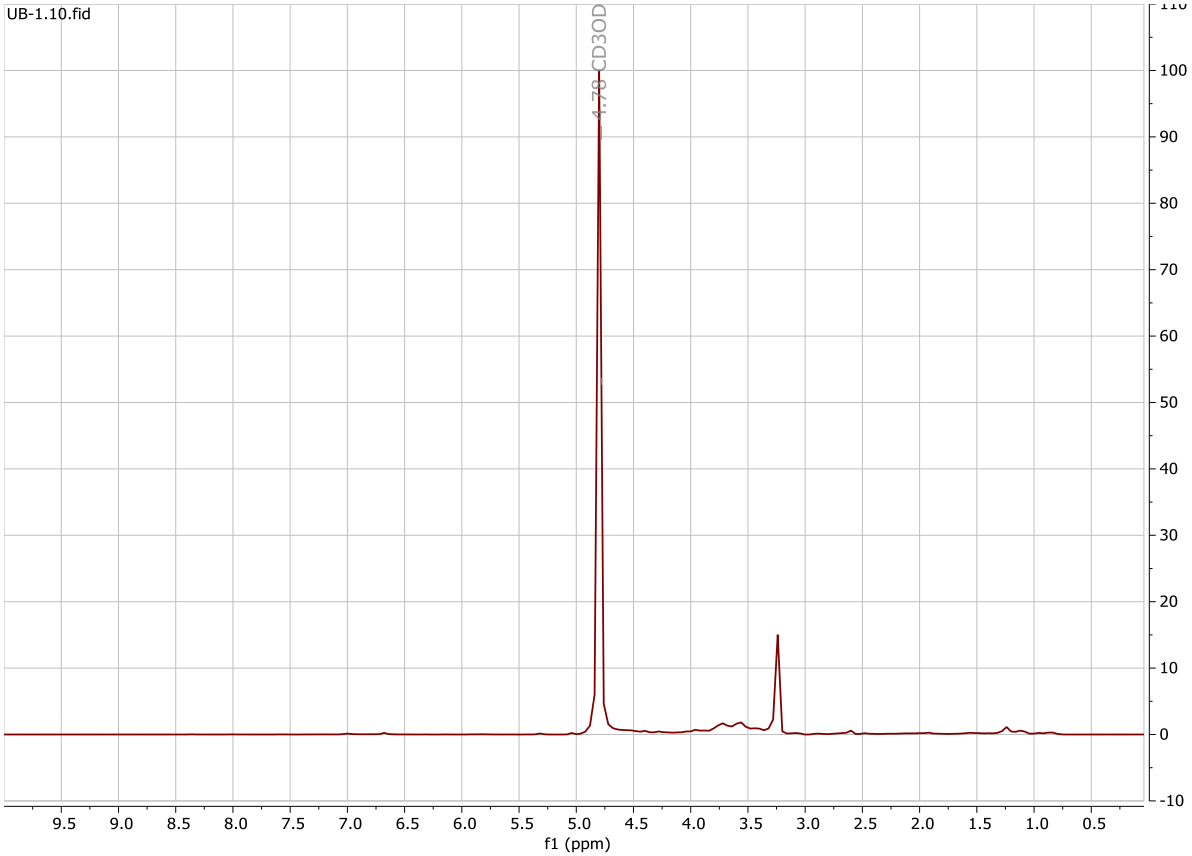


Figure A.4: ^{1}H NMR spectrum of the polyherbal formulation ABCD, which was not stored over a period.

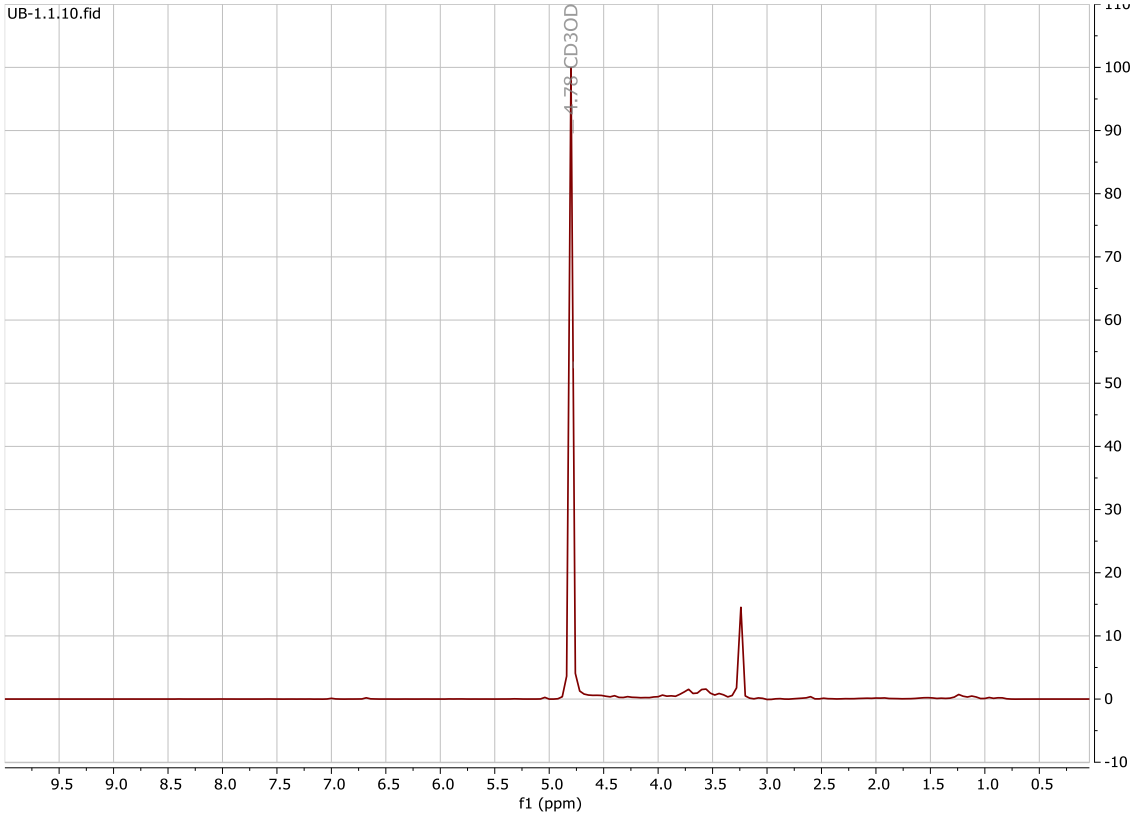


Figure A.5: ^{1}H NMR spectrum of the polyherbal formulation ABCD, which was stored over a period 7 days at 4˚C.

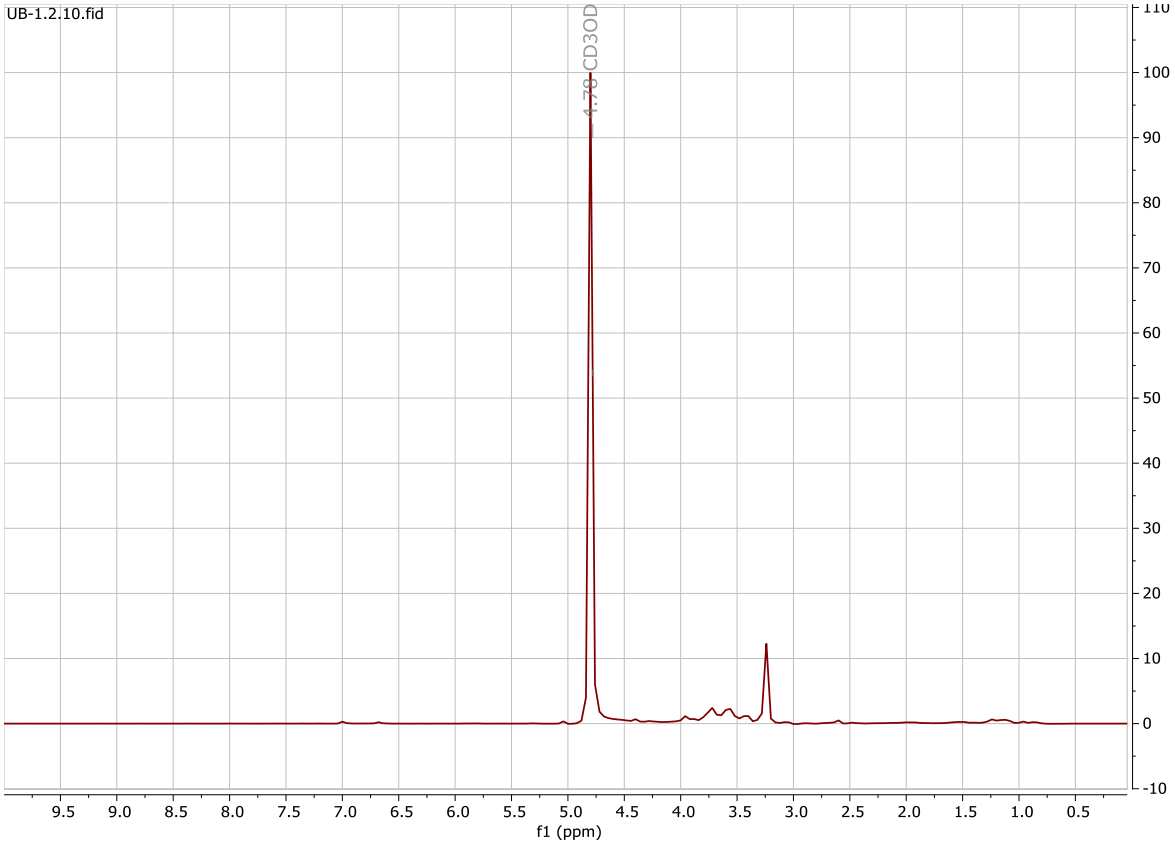


Figure A.6: ^{1}H NMR spectrum of the polyherbal formulation ABCD, which was stored over a period 7 days at 25˚C.

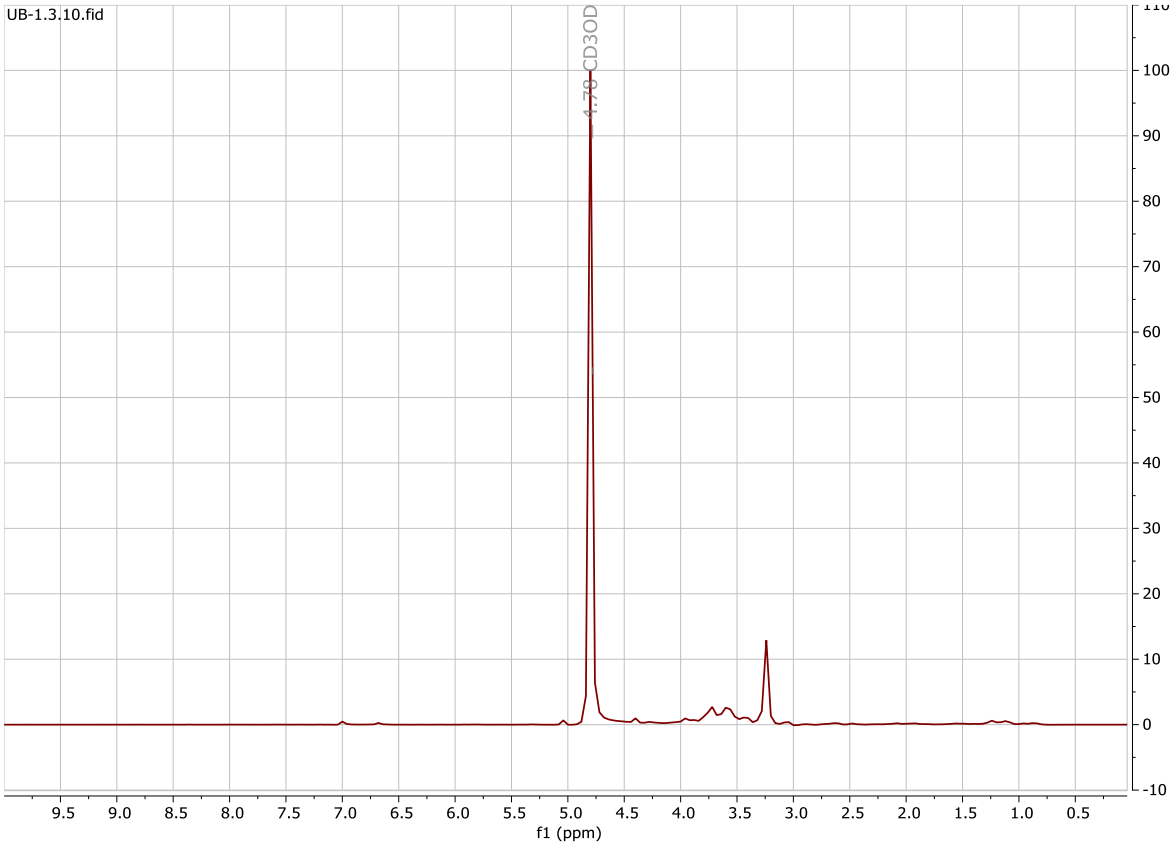


Figure A.7: ^{1}H NMR spectrum of the polyherbal formulation ABCD, which was stored over a period 7 days at 37˚C.

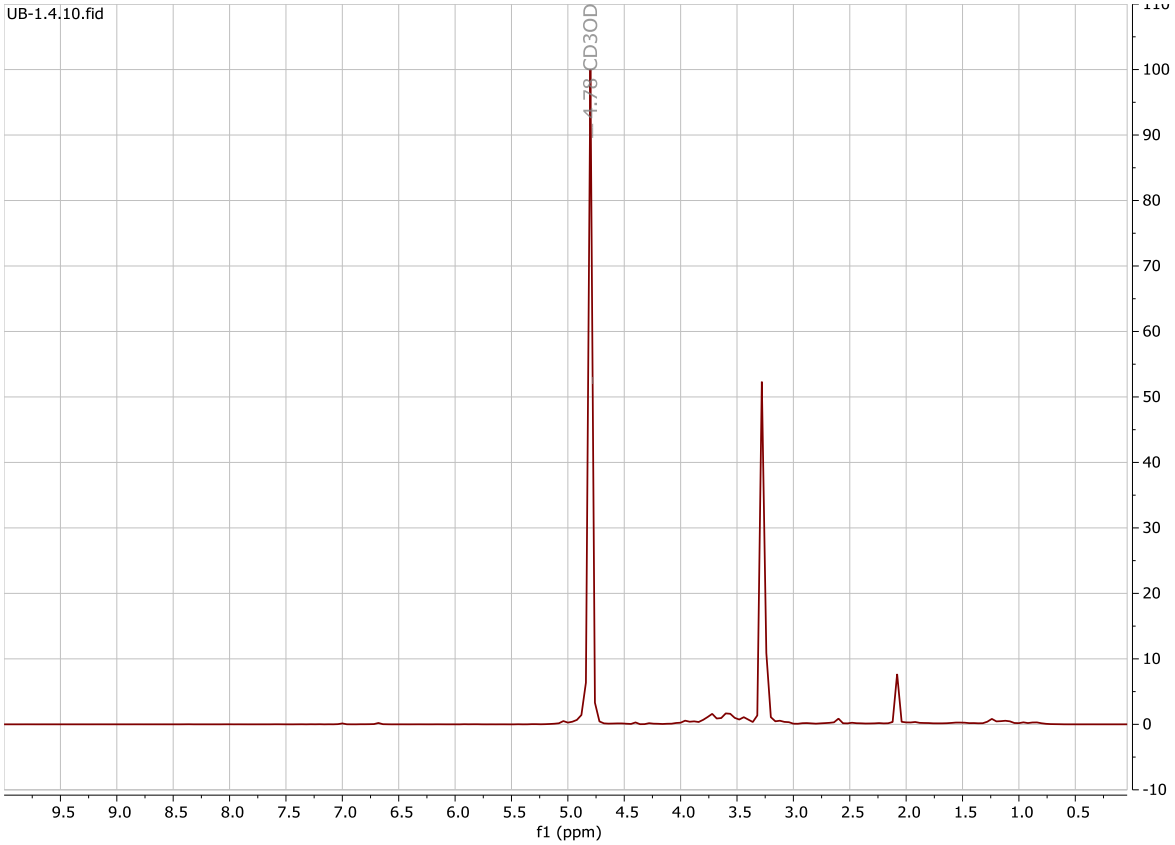


Figure A.8: ^{1}H NMR spectrum of the polyherbal formulation ABCD, which was stored over a period 14 days at 4˚C.

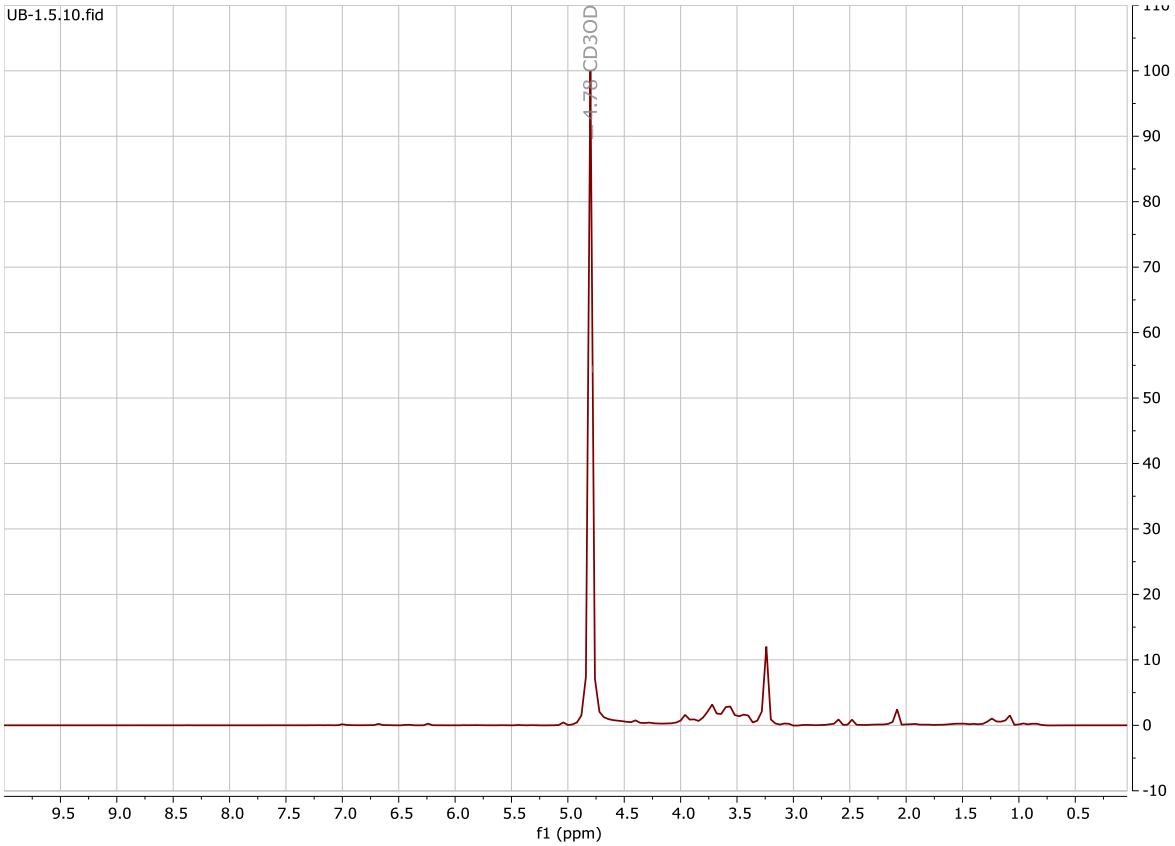


Figure A.9: ^{1}H NMR spectrum of the polyherbal formulation ABCD, which was stored over a period 14 days at 25˚C.

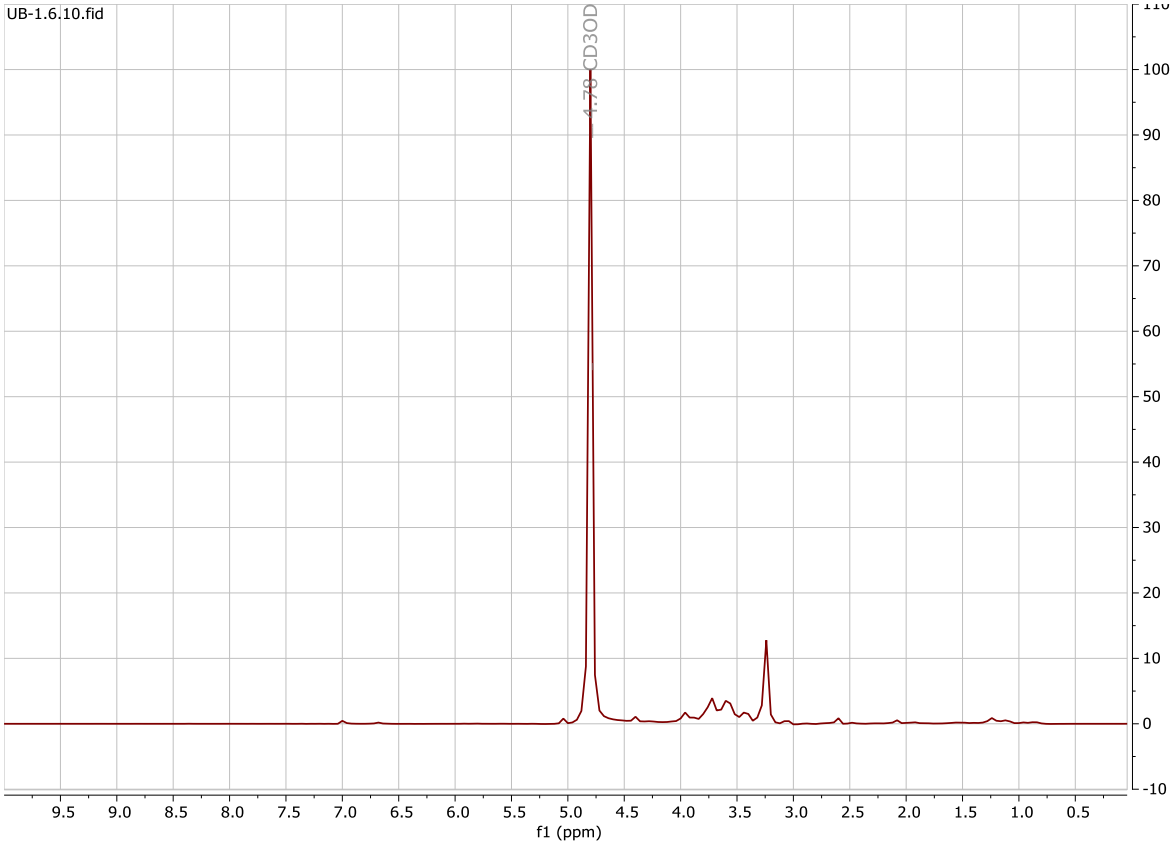


Figure A.10: ^{1}H NMR spectrum of the polyherbal formulation ABCD, which was stored over a period 14 days at 37˚C.

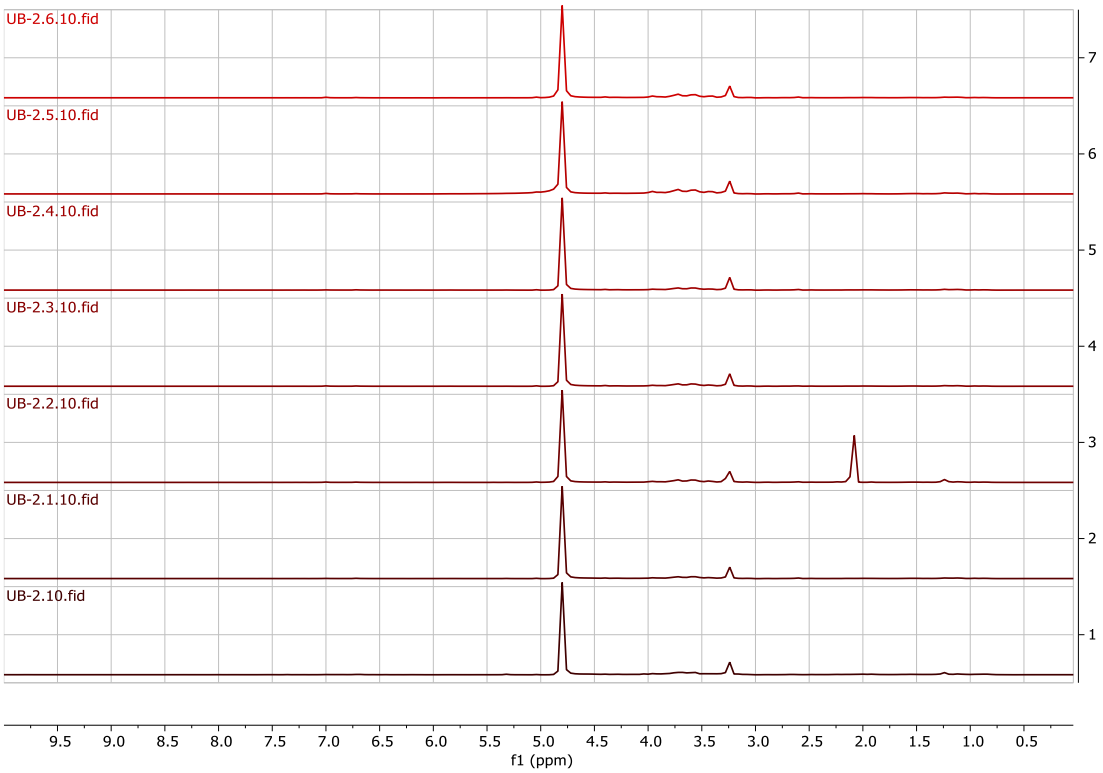


Figure A.11: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants ABC.

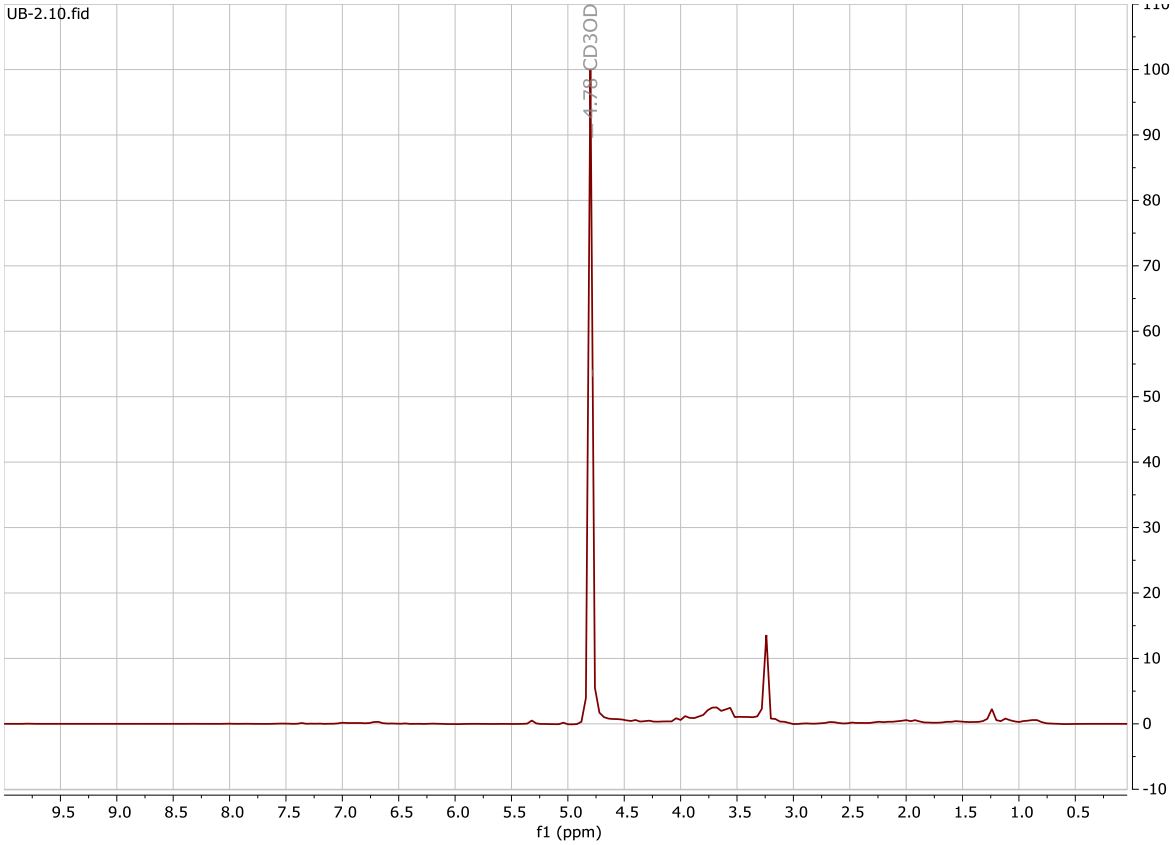


Figure A.12: ^{1}H NMR spectrum of the polyherbal formulation ABC, which was not stored over a period.

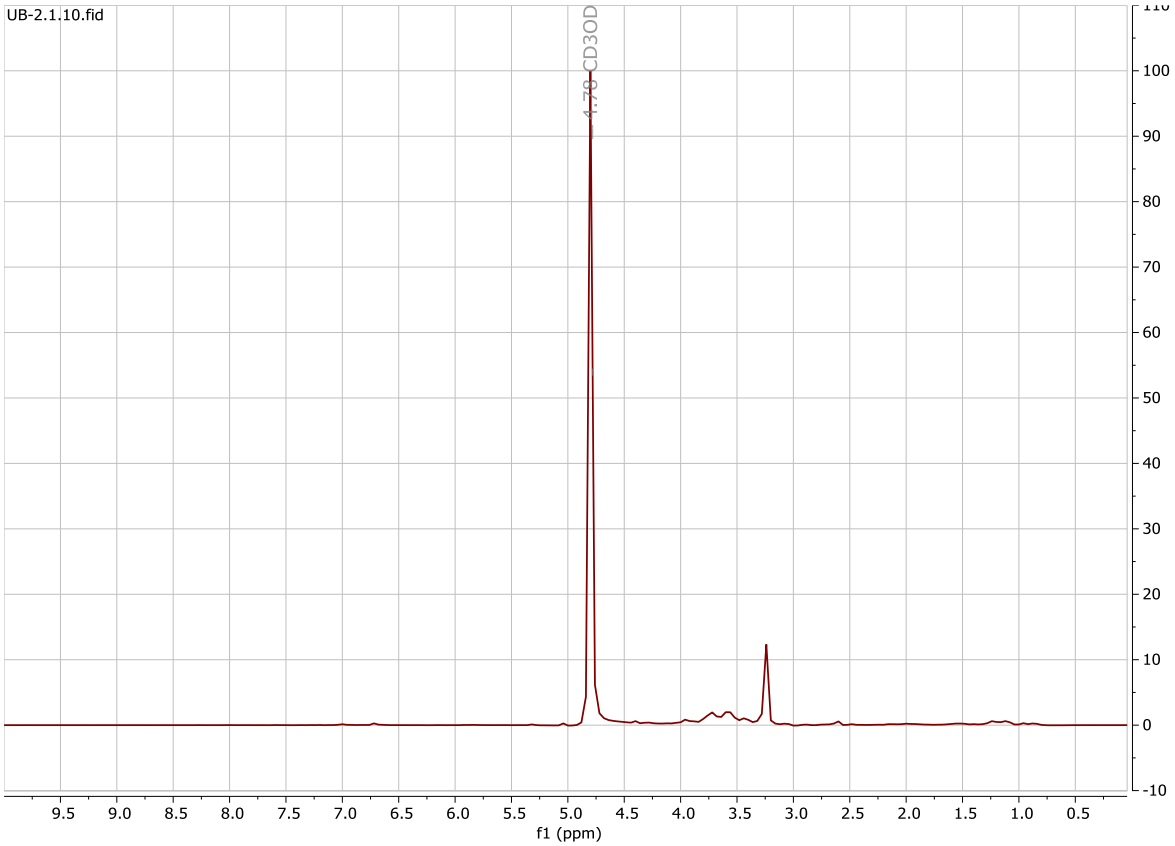


Figure A.13: ^{1}H NMR spectrum of the polyherbal formulation ABC, which was stored over a period 7 days at 4˚C.

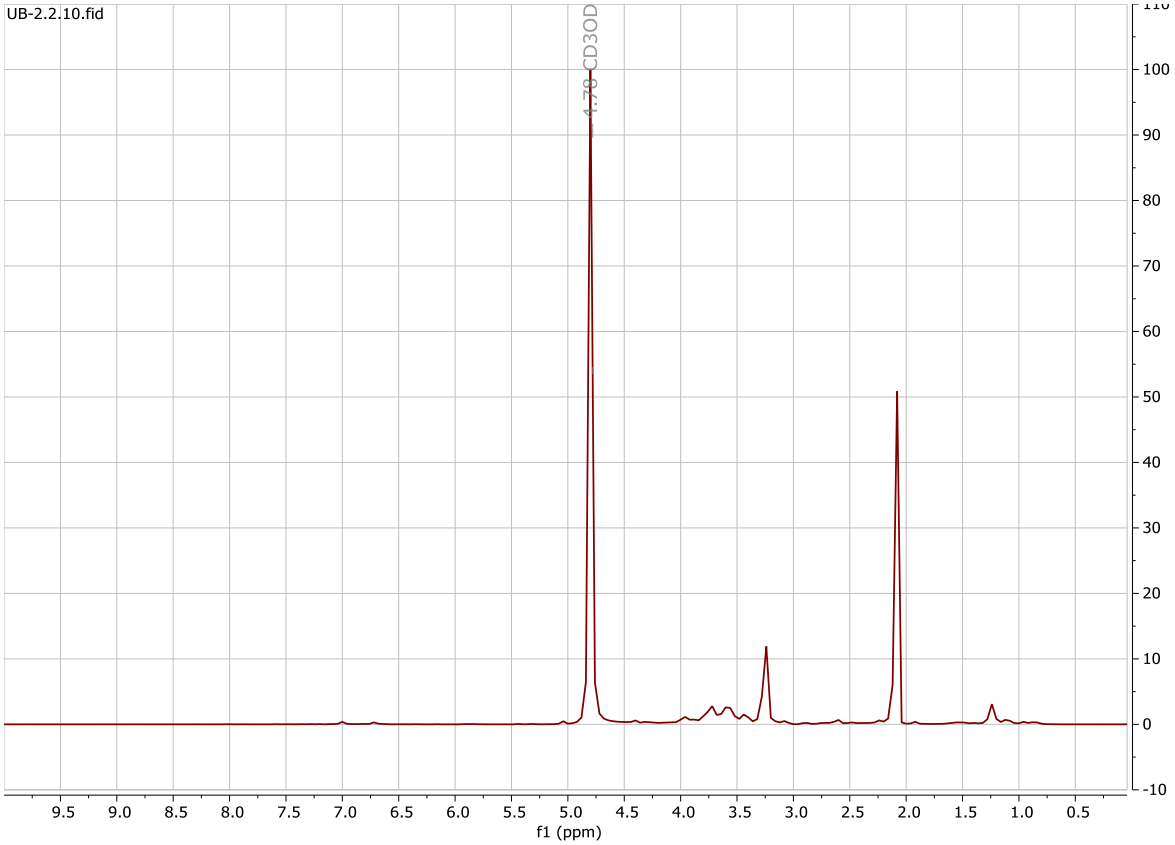


Figure A.14: ^{1}H NMR spectrum of the polyherbal formulation ABC, which was stored over a period 7 days at 25˚C.

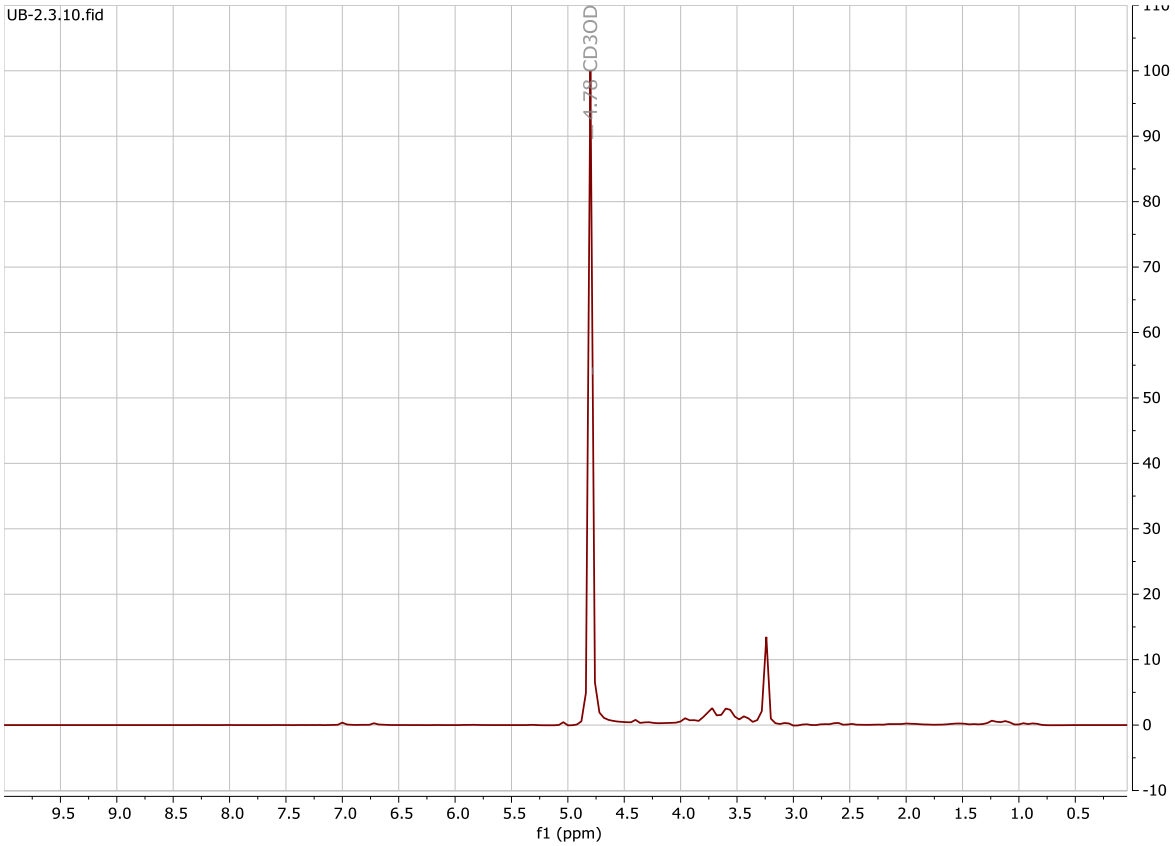


Figure A.15: ^{1}H NMR spectrum of the polyherbal formulation ABC, which was stored over a period 7 days at 37˚C.

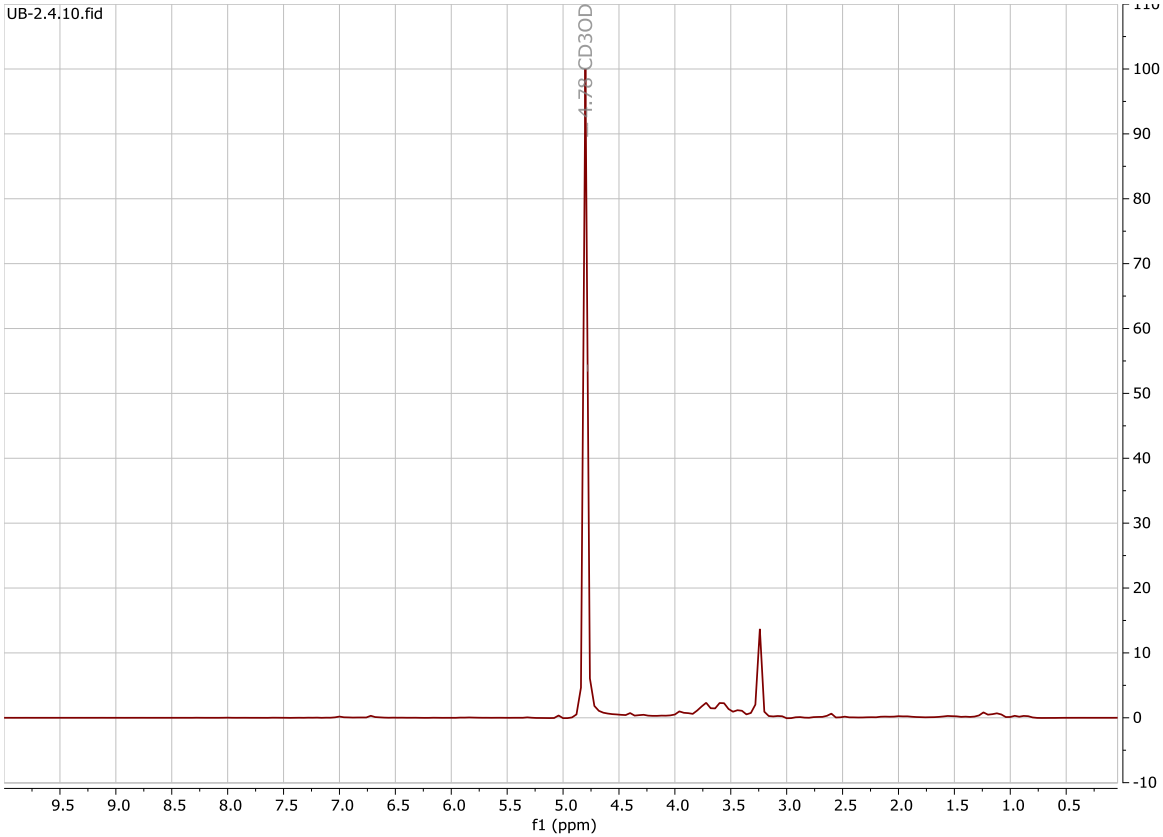


Figure A.16: ^{1}H NMR spectrum of the polyherbal formulation ABC, which was stored over a period 14 days at 4˚C.

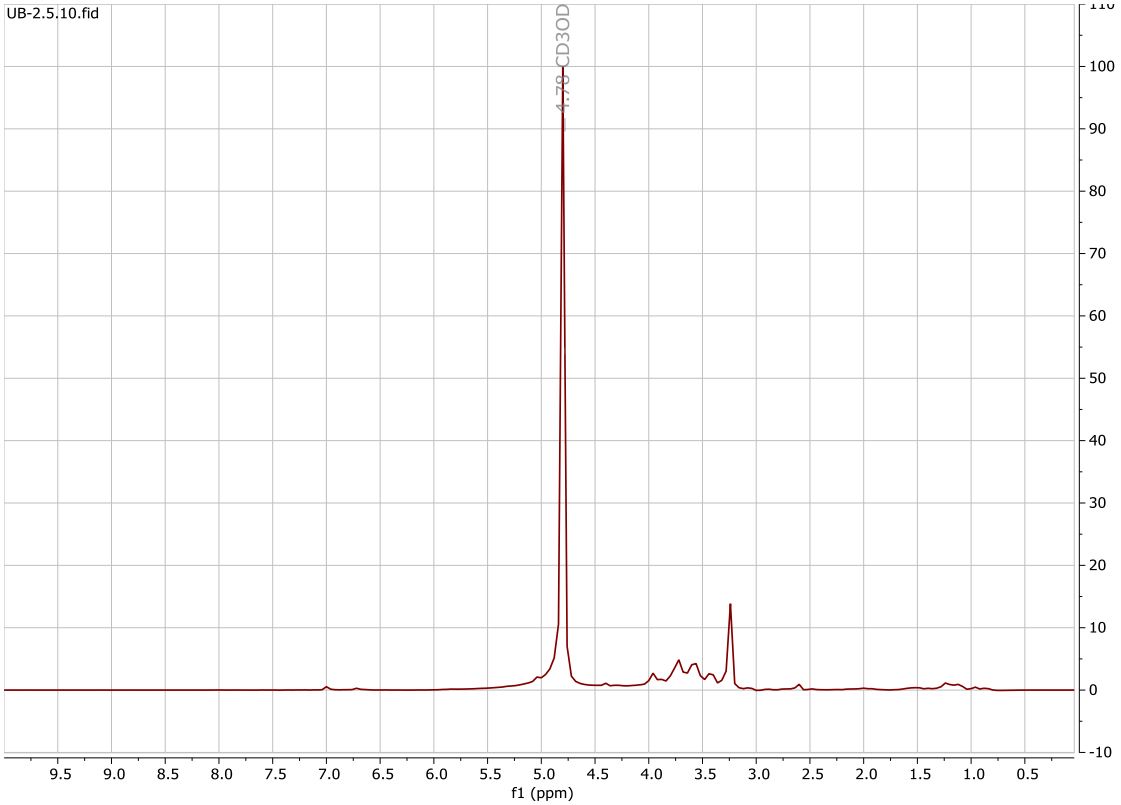


Figure A.17: ^{1}H NMR spectrum of the polyherbal formulation ABC, which was stored over a period 14 days at 25˚C.

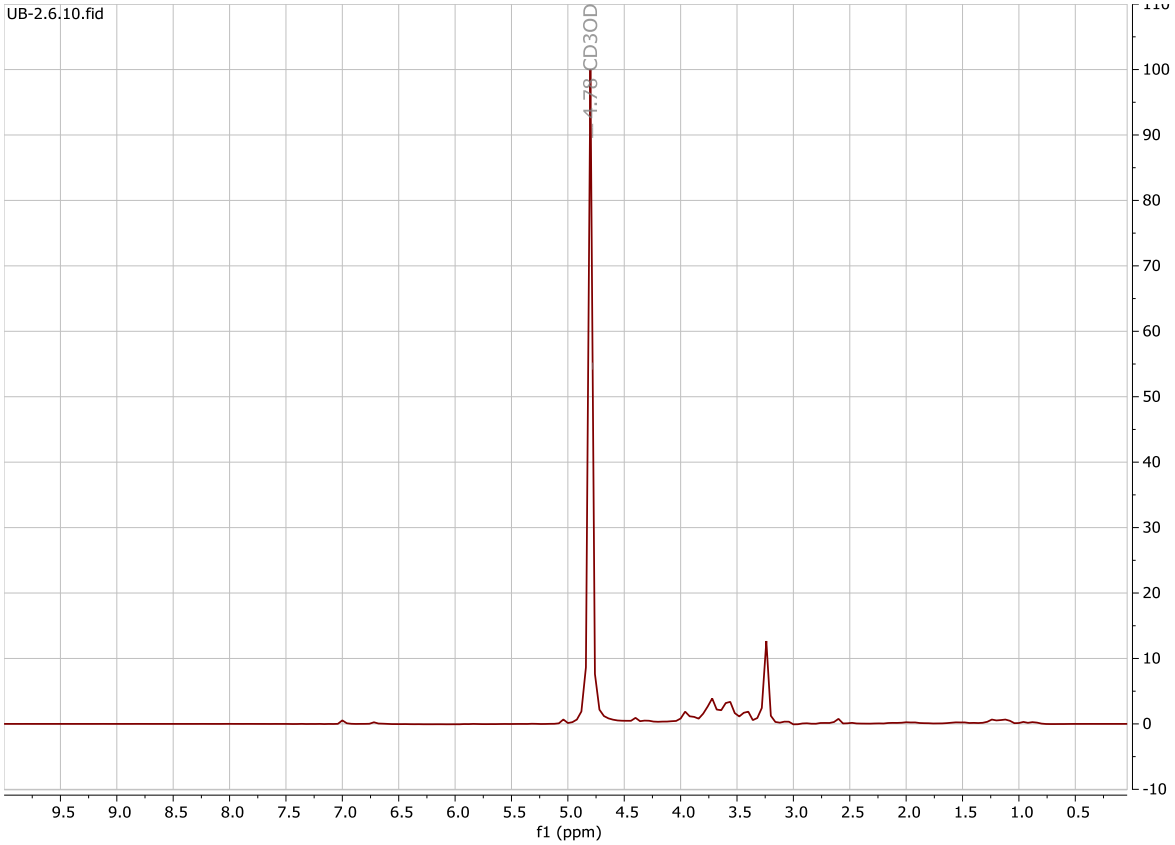


Figure A.18: ^{1}H NMR spectrum of the polyherbal formulation ABC, which was stored over a period 14 days at 37˚C.

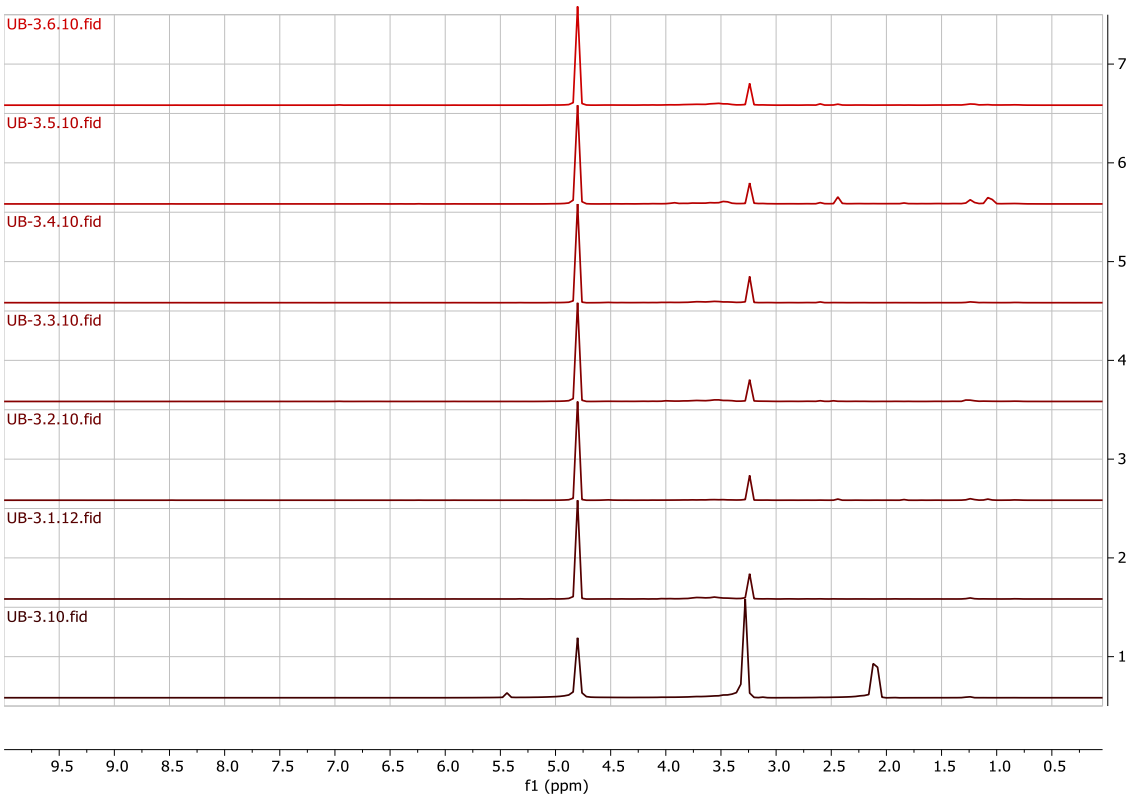


Figure A.19: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants ABD.

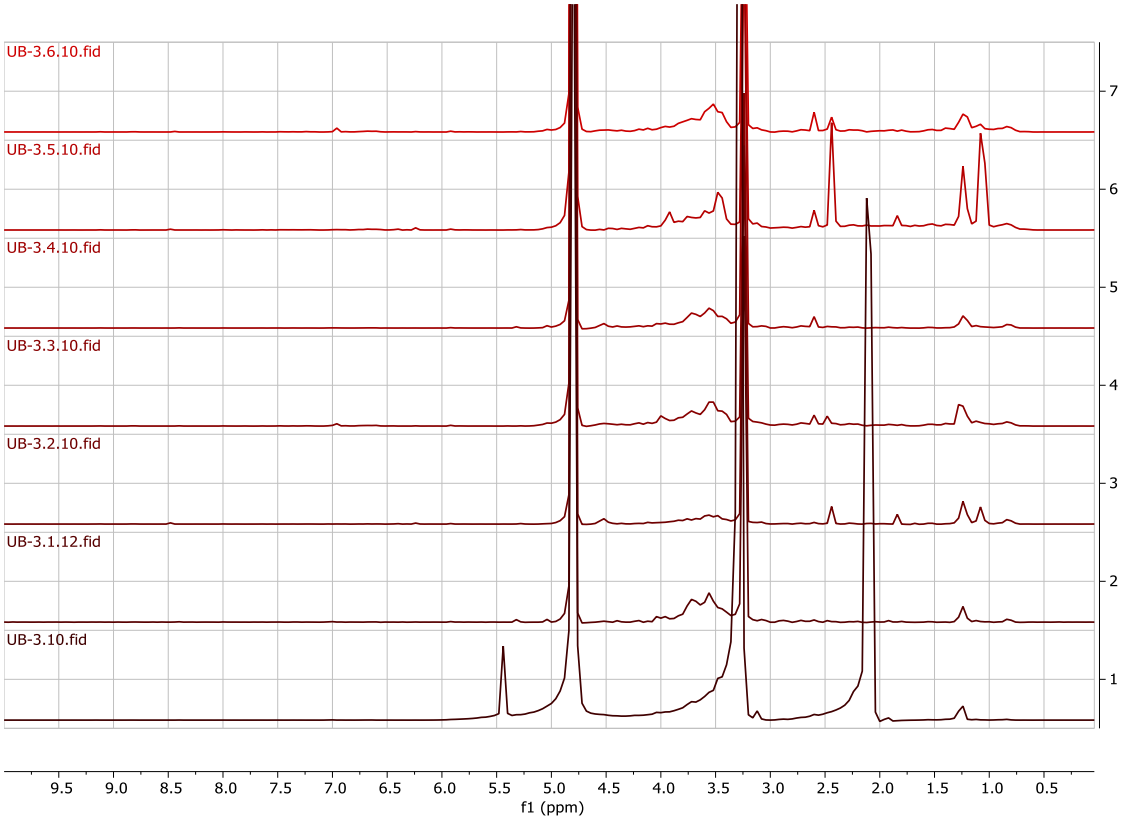


Figure A.20: Zoomed in stacked ^{1}H NMR spectra of the polyherbal formulation containing plants ABD, containing combinations that were stored at various temperatures over two different storage periods.

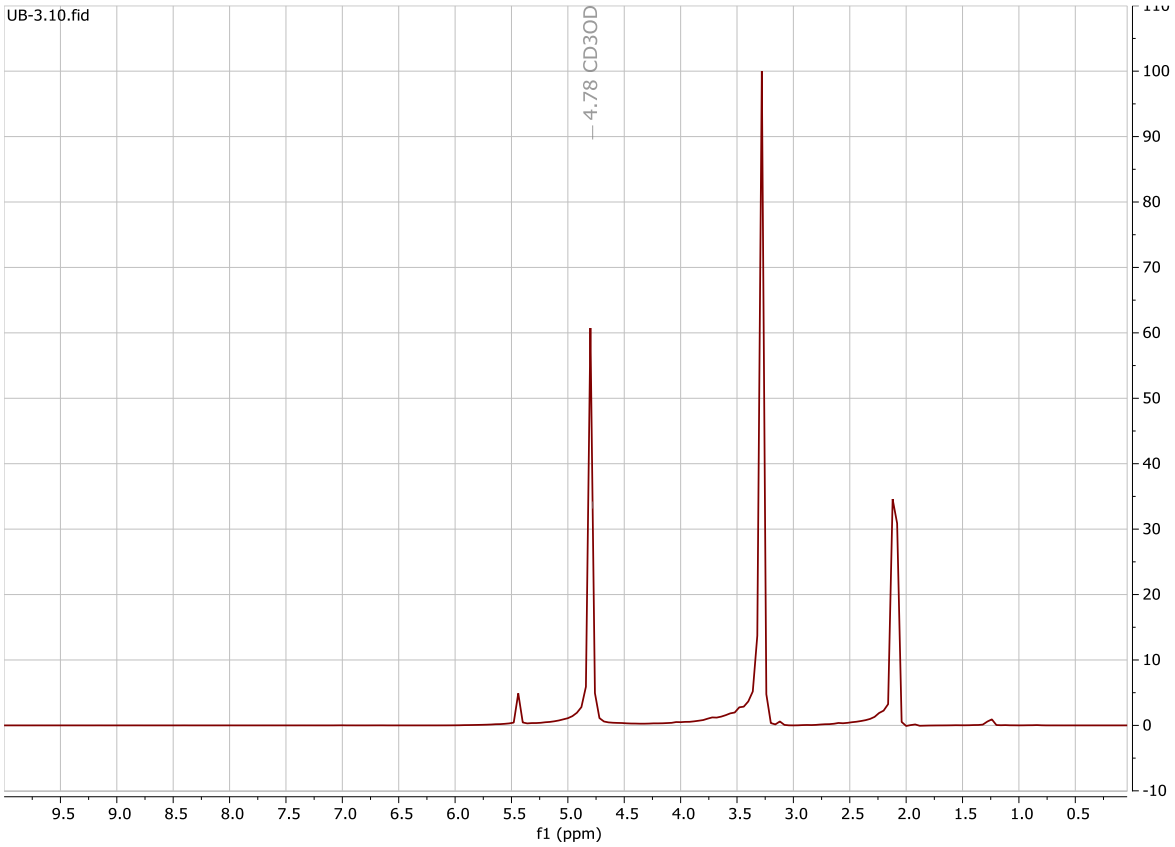


Figure A.21: ^{1}H NMR spectrum of the polyherbal formulation ABD, which was stored not over a period.

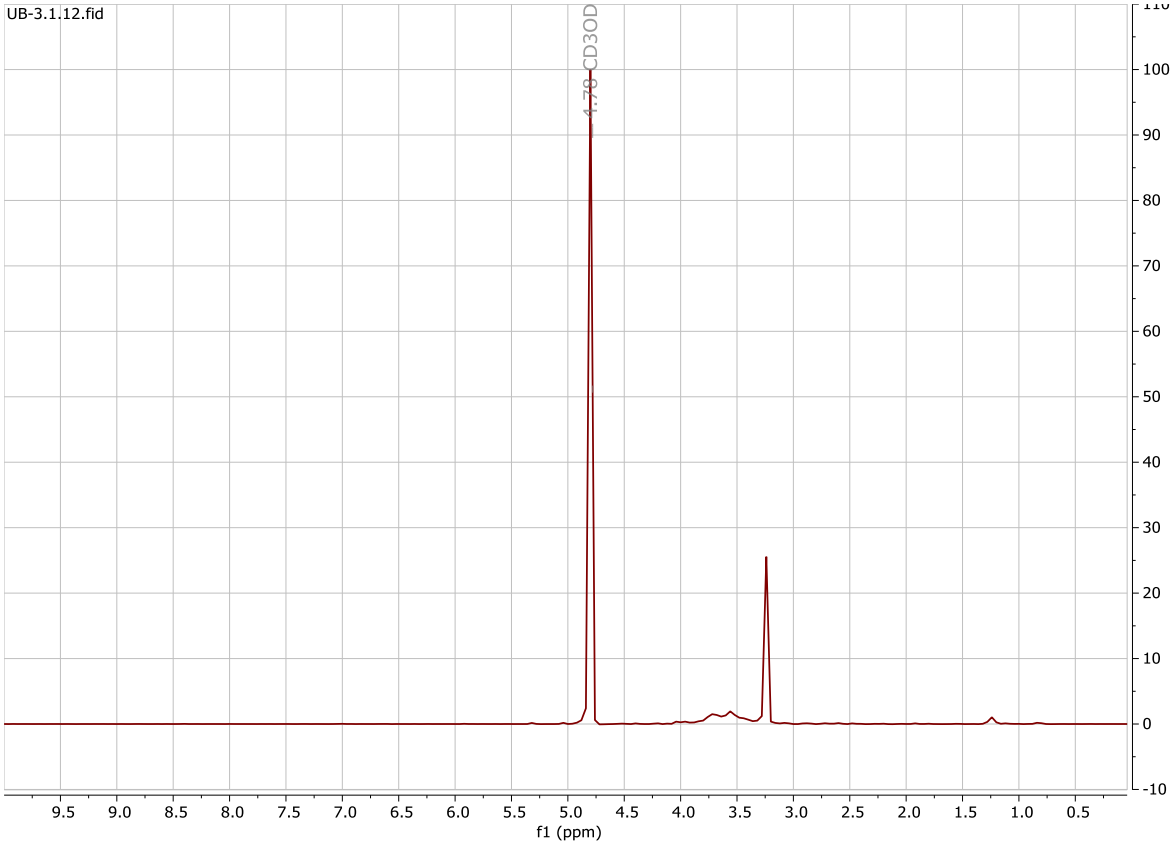


Figure A.22: ^{1}H NMR spectrum of the polyherbal formulation ABD, which was stored over a period 7 days at 4˚C.

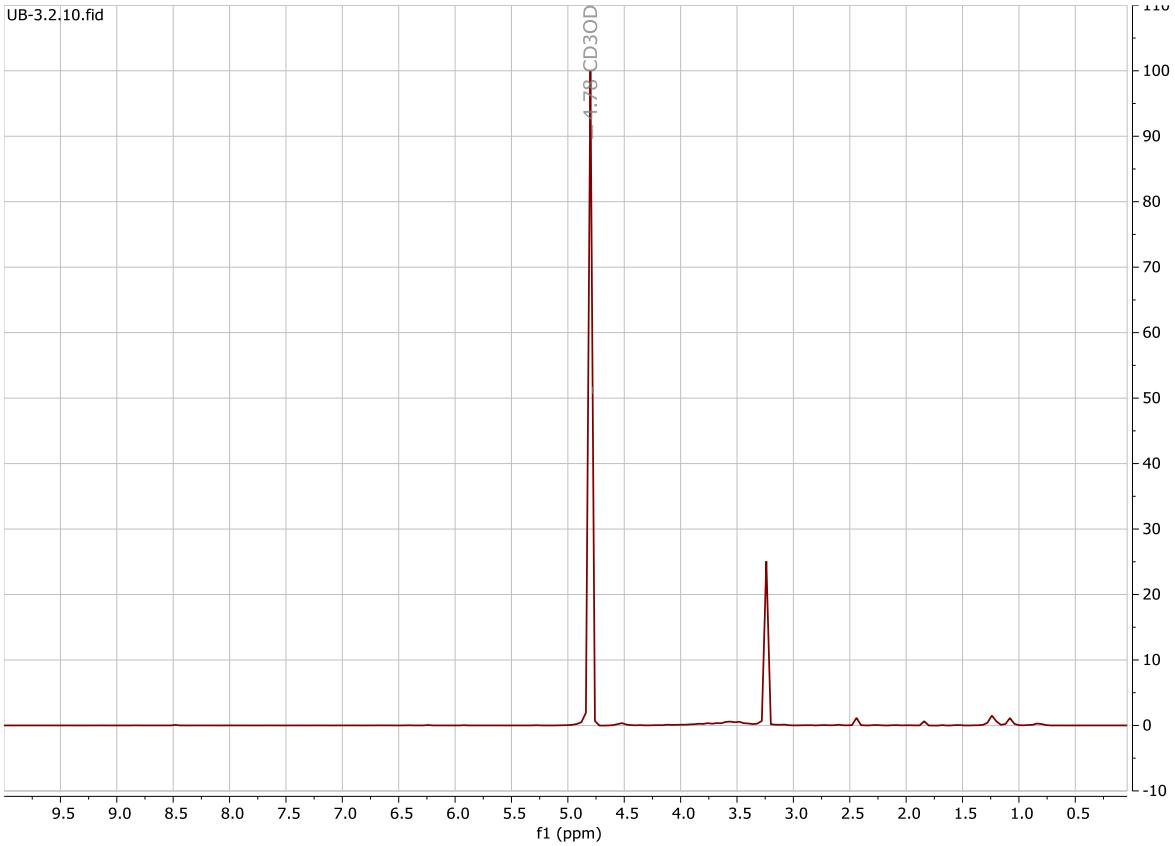


Figure A.23: ^{1}H NMR spectrum of the polyherbal formulation ABD, which was stored over a period 7 days at 25˚C.

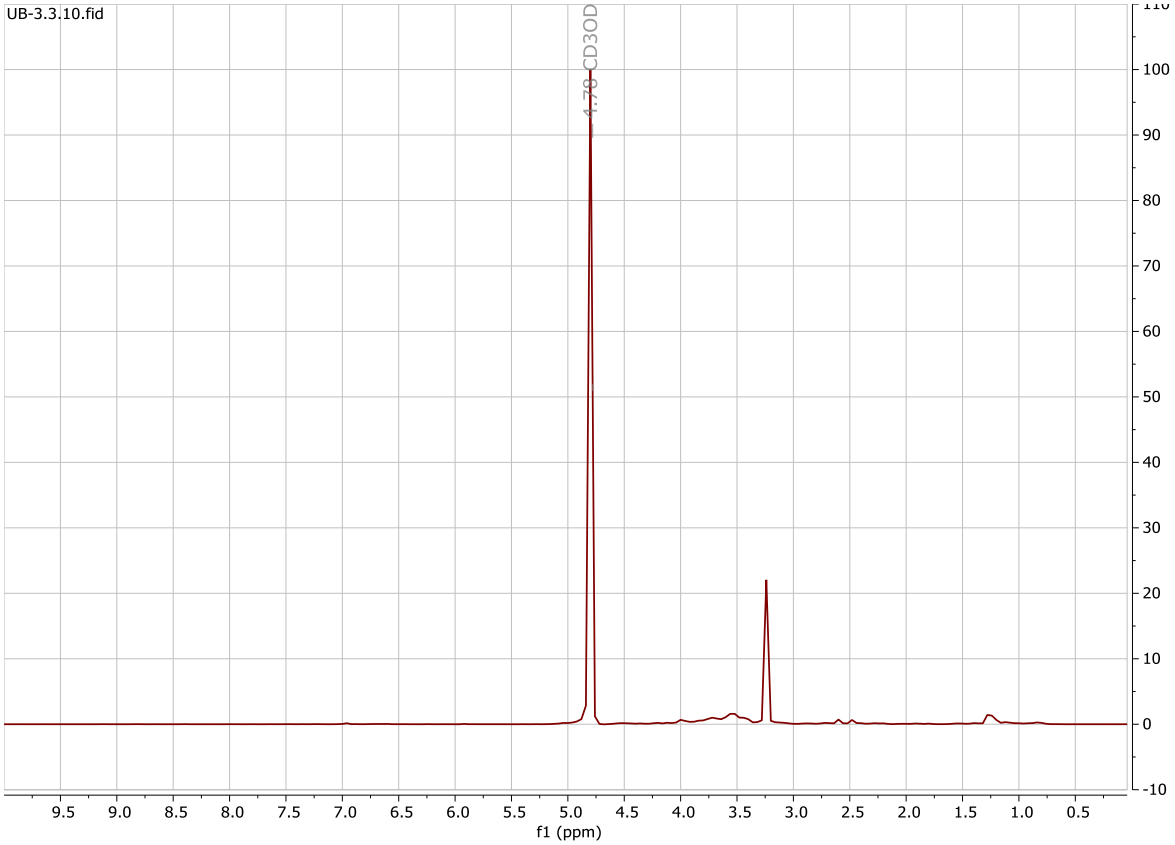


Figure A.24: ^{1}H NMR spectrum of the polyherbal formulation ABC, which was stored over a period 7 days at 37˚C.

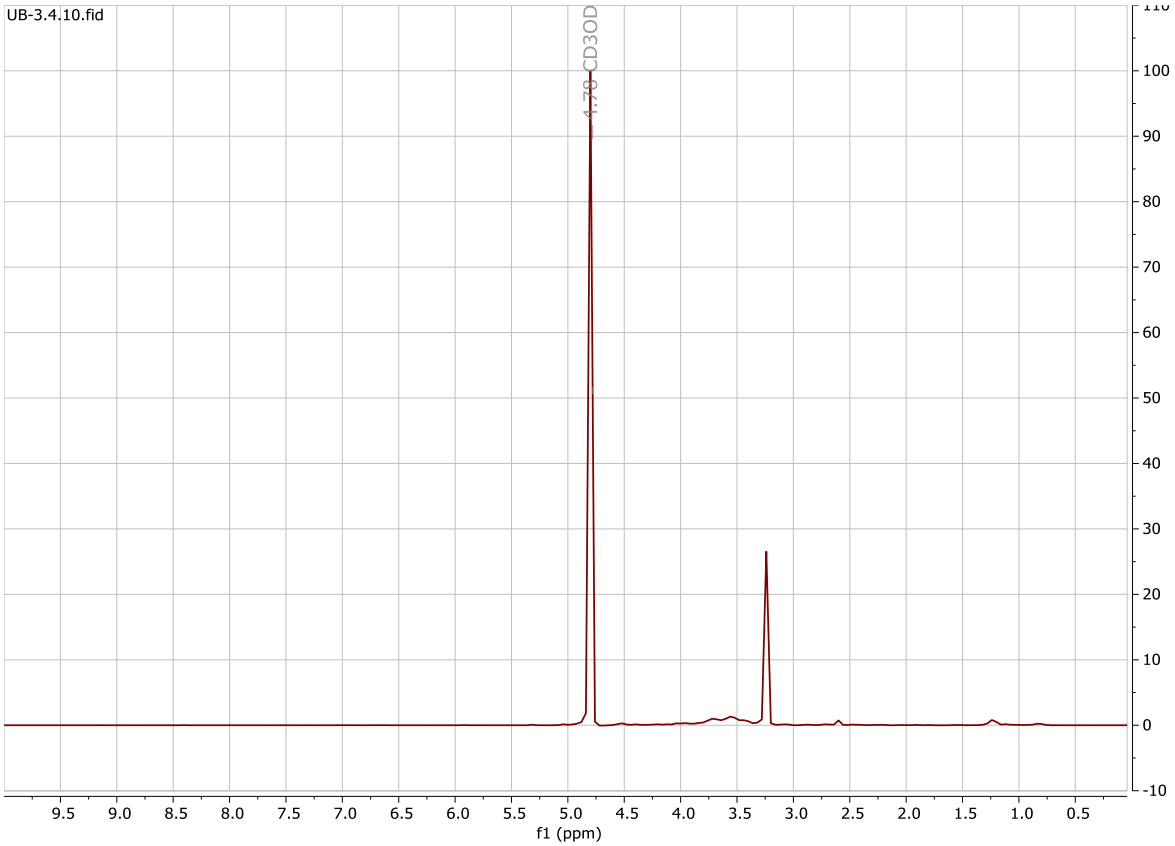


Figure A.25: ^{1}H NMR spectrum of the polyherbal formulation ABD, which was stored over a period 14 days at 4˚C.

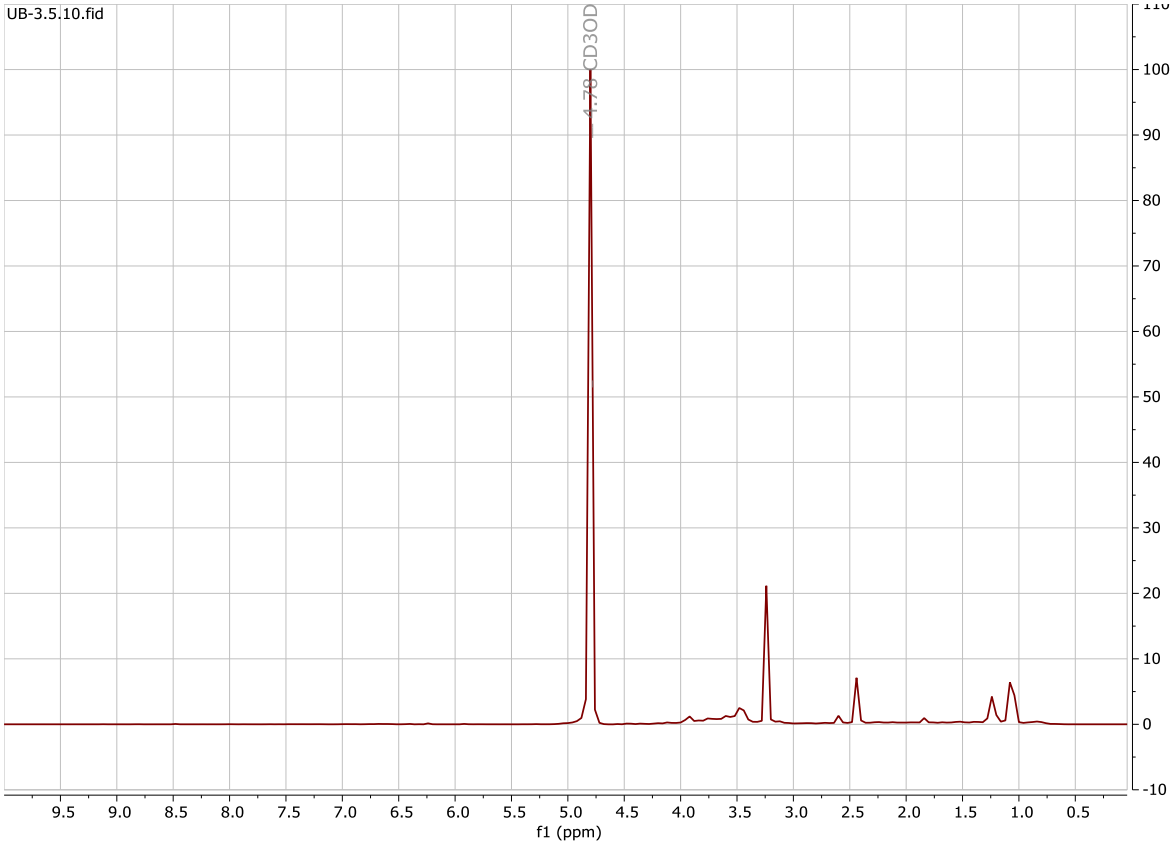


Figure A.26: ^{1}H NMR spectrum of the polyherbal formulation ABD, which was stored over a period 14 days at 25˚C.

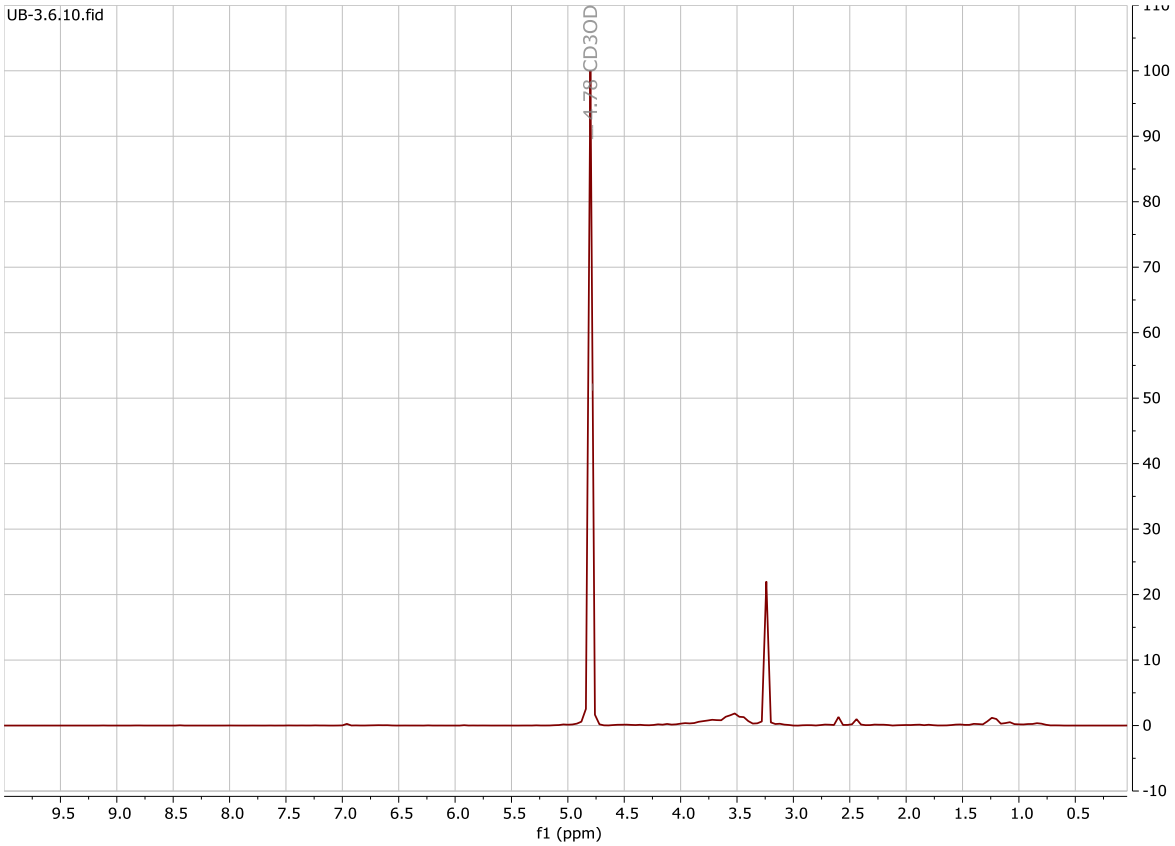


Figure A.27: ^{1}H NMR spectrum of the polyherbal formulation ABD, which was stored over a period 14 days at 37˚C.

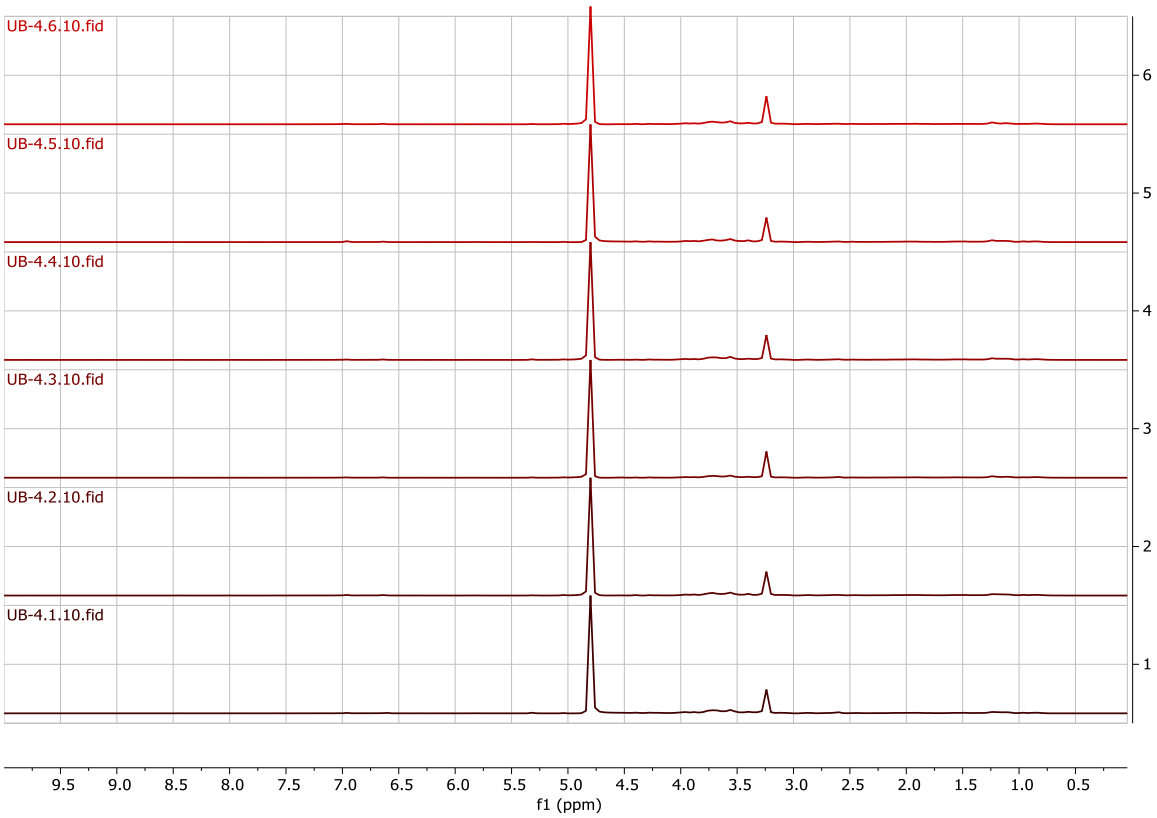


Figure A.28: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants BCD

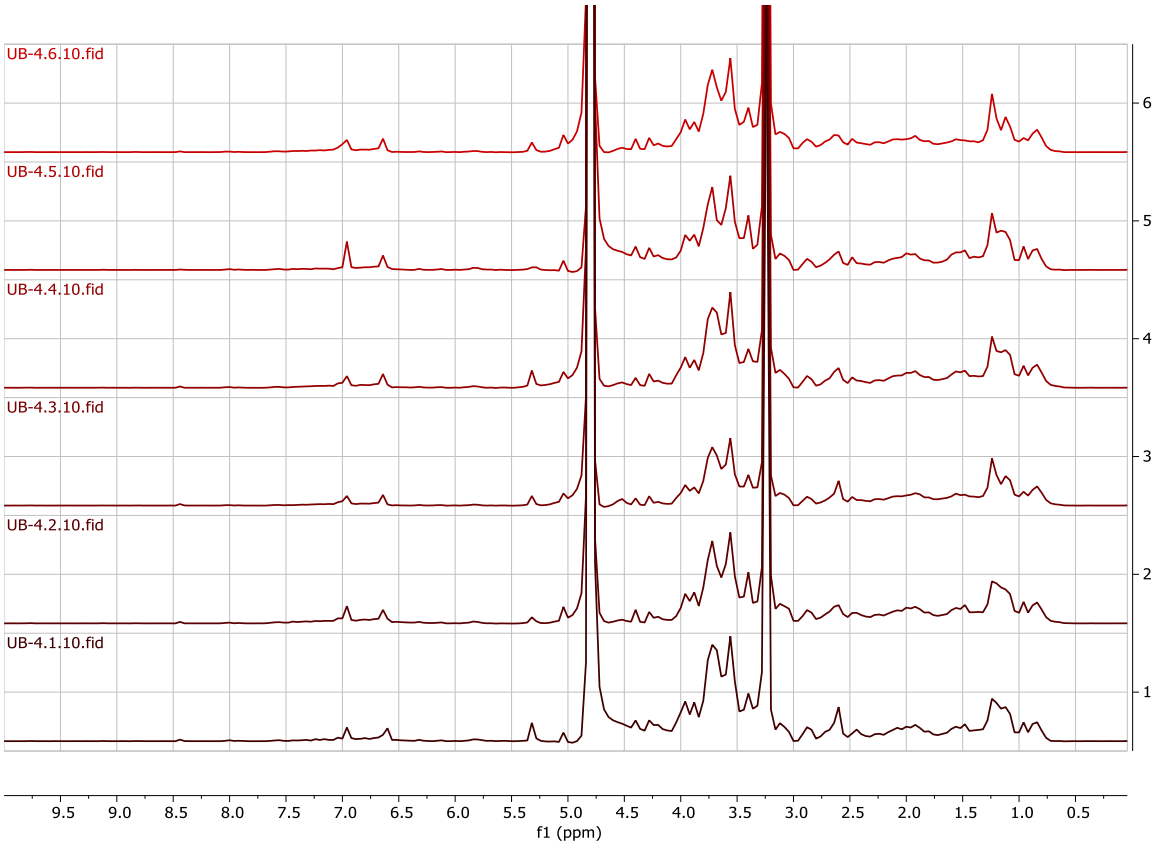


Figure A.29: Zoomed in stacked ^{1}H NMR spectra of the polyherbal formulation containing plants BCD, containing combinations that were stored at various temperatures over two different storage periods.

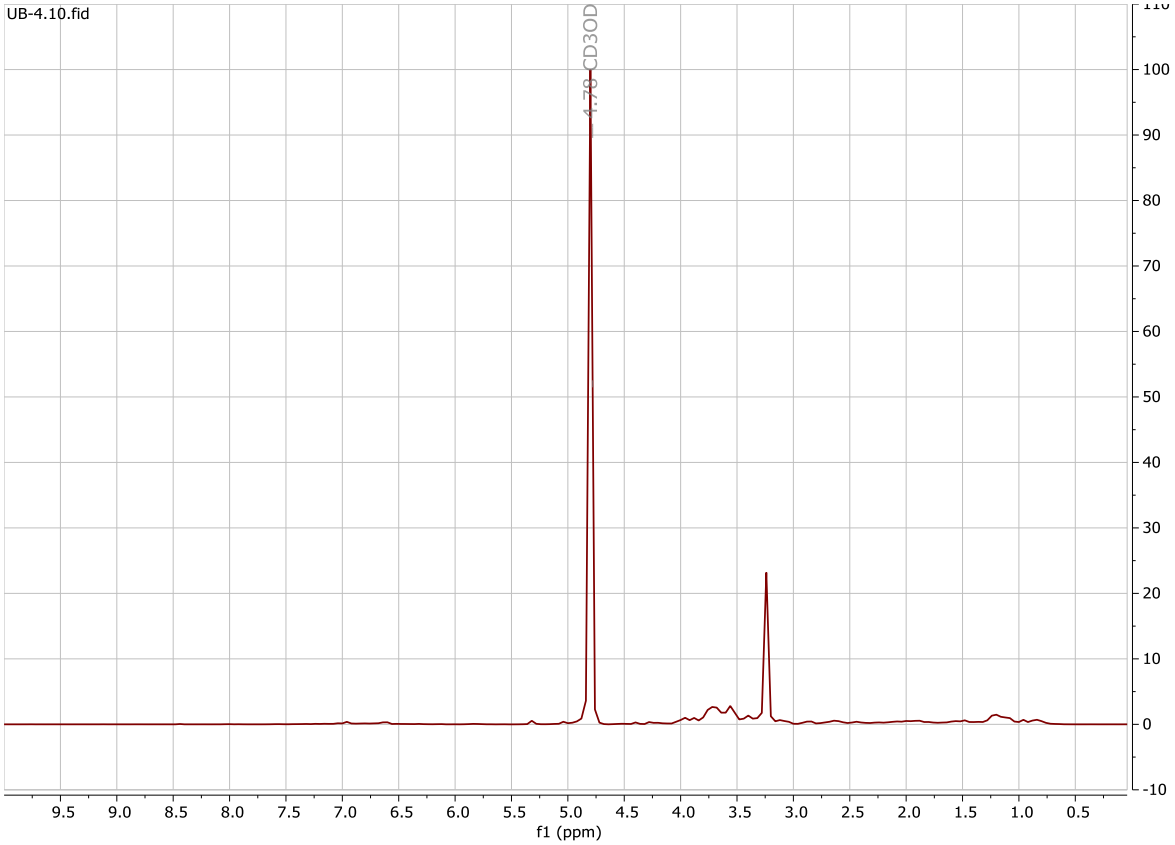


Figure A.30: ^{1}H NMR spectrum of the polyherbal formulation BCD, which was not stored over a period.

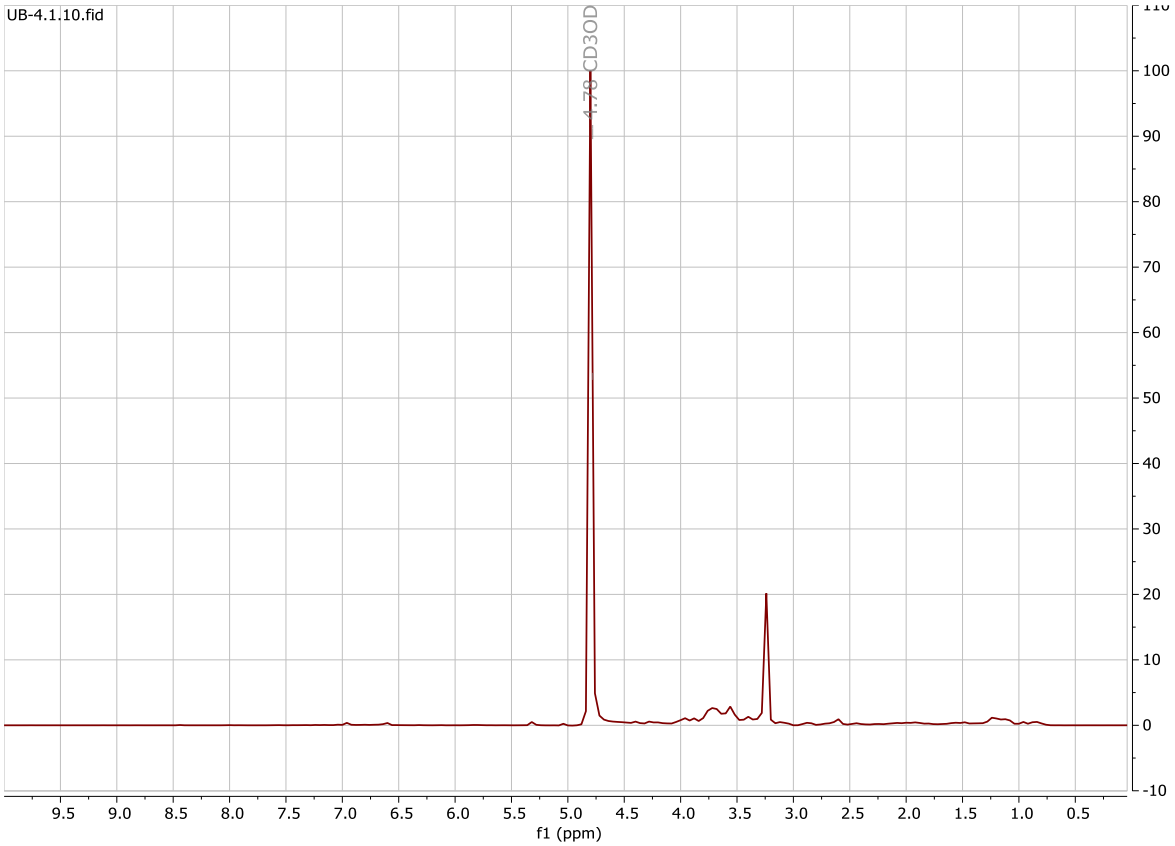


Figure A.31: ^{1}H NMR spectrum of the polyherbal formulation BCD, which was stored over a period of 7 days at 4˚C.

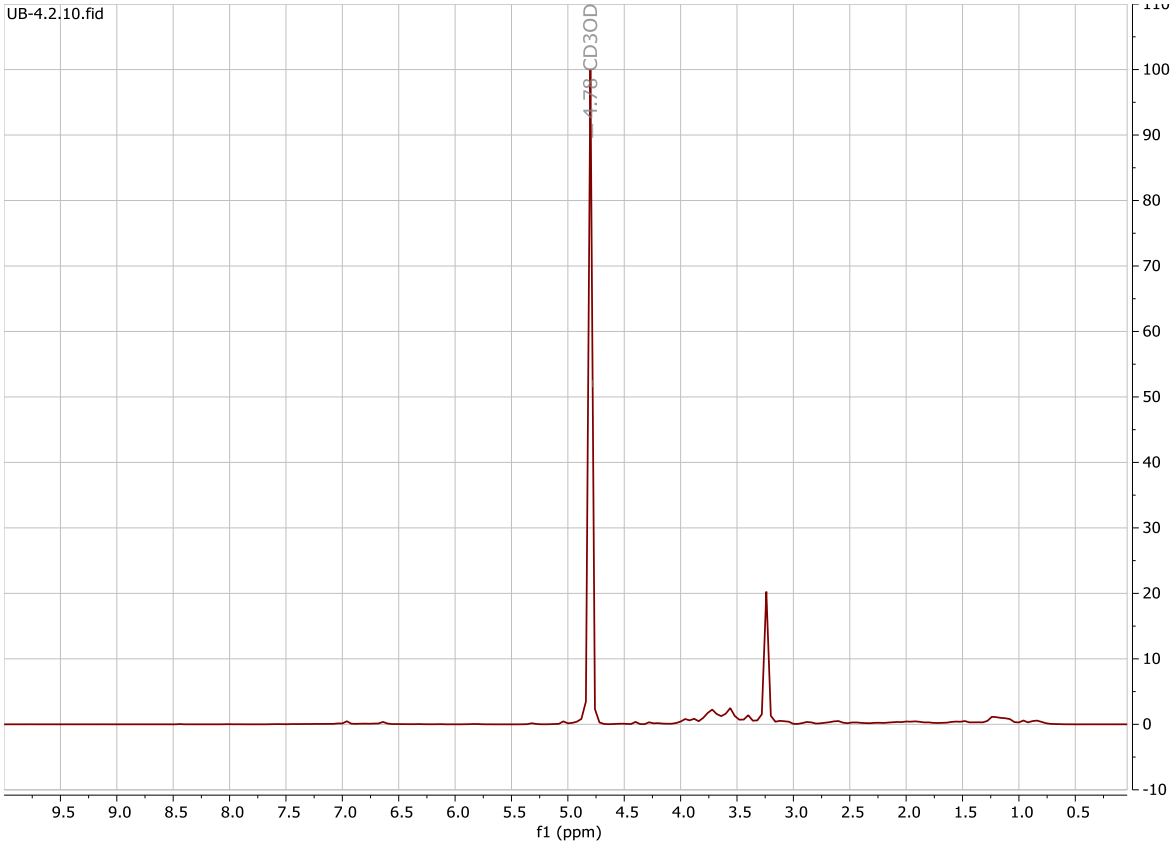


Figure A.32: ^{1}H NMR spectrum of the polyherbal formulation BCD, which was stored over a period of 7 days at 25˚C.

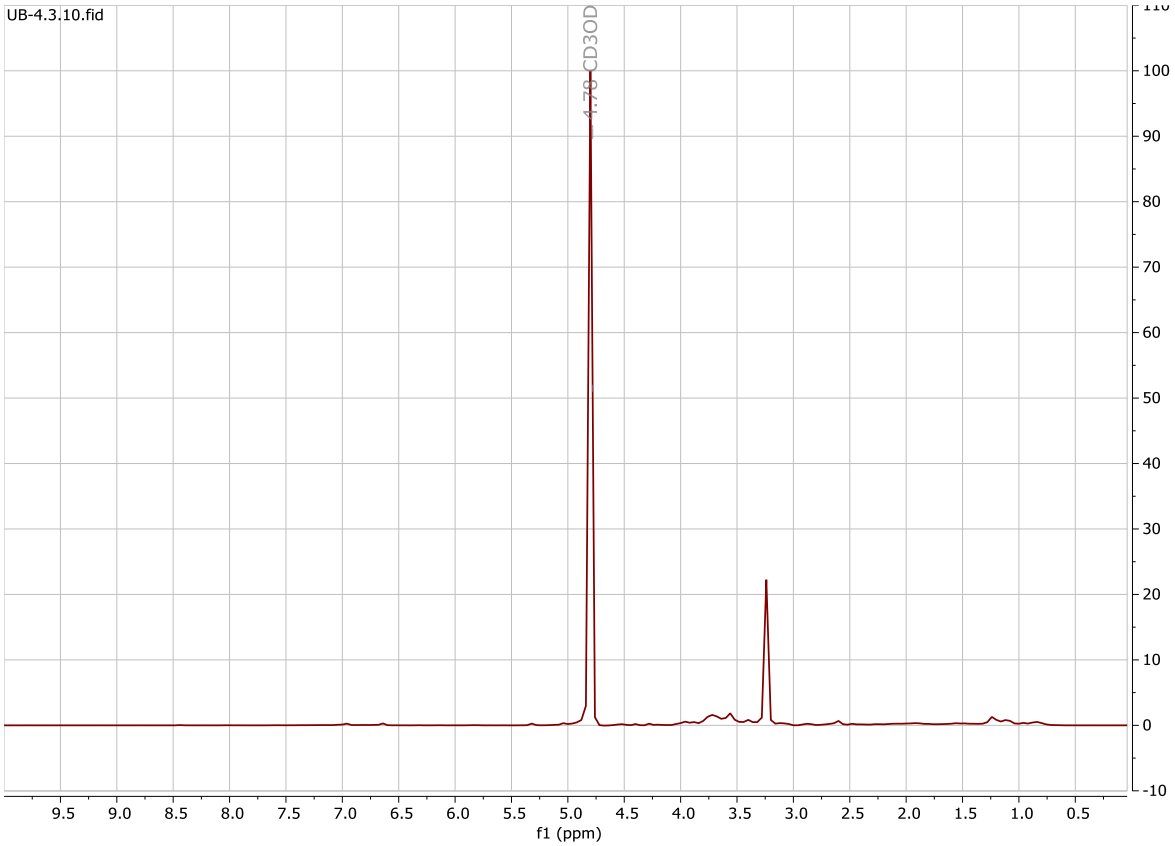


Figure A.33: ^{1}H NMR spectrum of the polyherbal formulation BCD, which was stored over a period of 7 days at 37˚C.

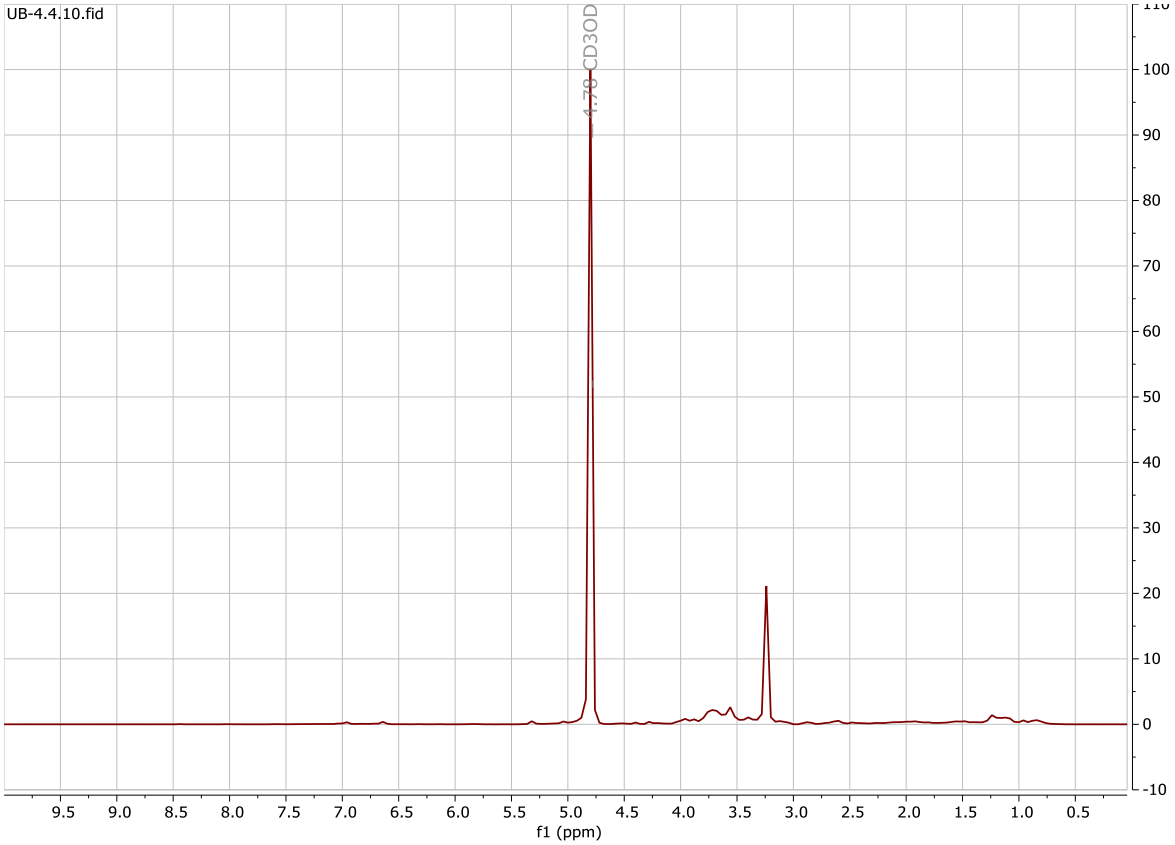


Figure A.34: ^{1}H NMR spectrum of the polyherbal formulation BCD, which was stored over a period of 14 days at 4˚C.

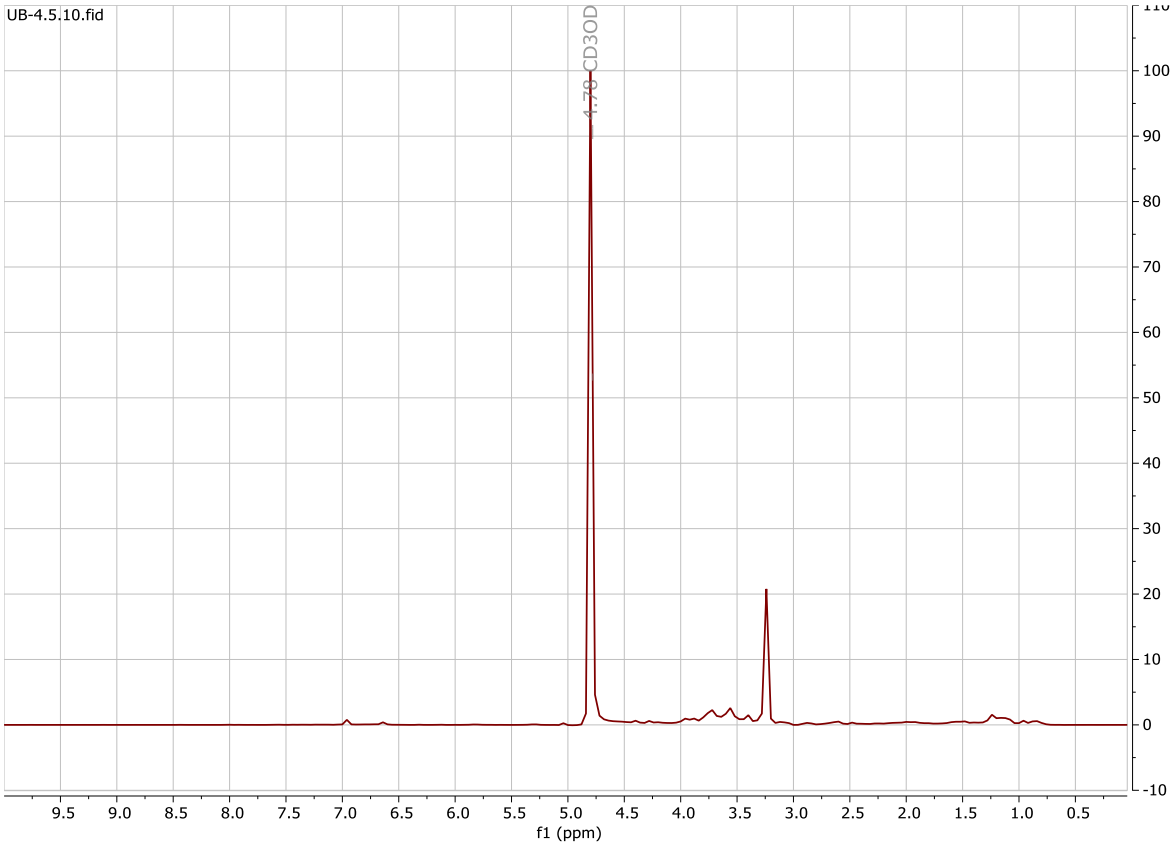


Figure A.35: ^{1}H NMR spectrum of the polyherbal formulation BCD, which was stored over a period of 14 days at 25˚C.

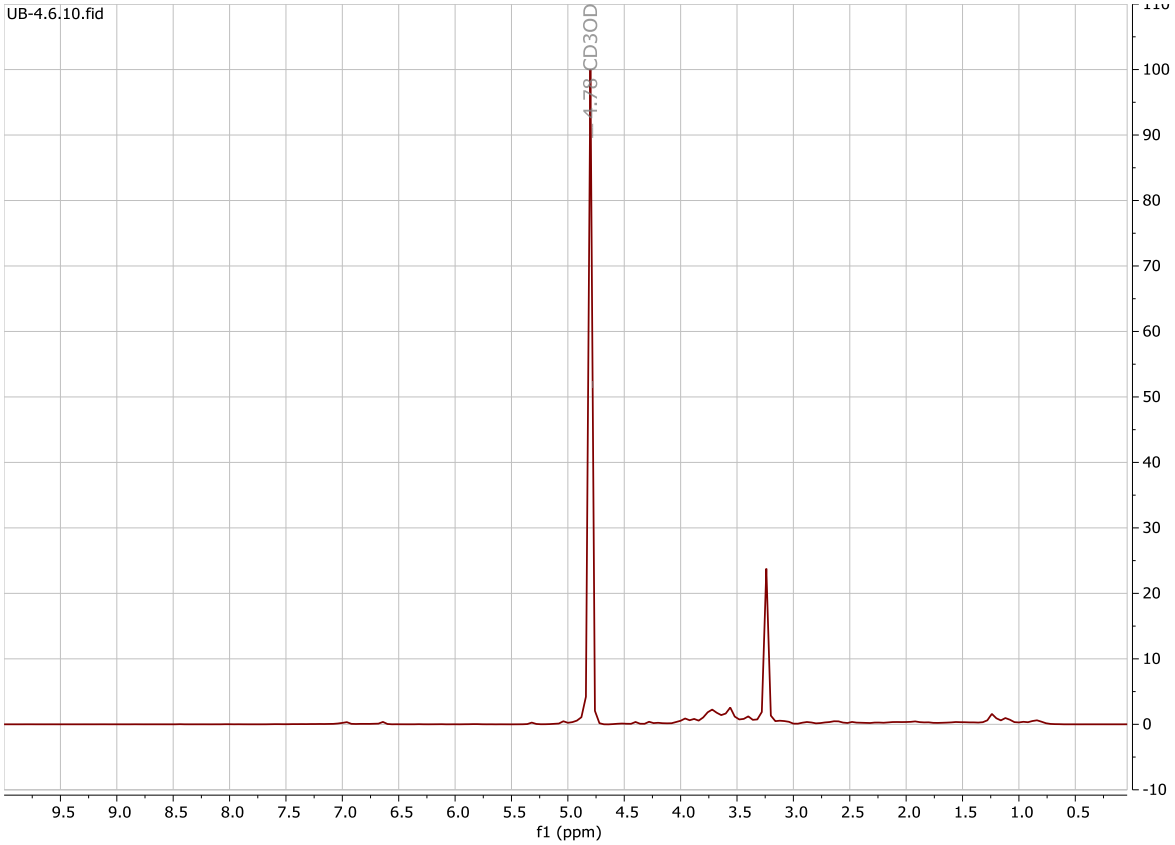


Figure A.36: ^{1}H NMR spectrum of the polyherbal formulation BCD, which was stored over a period of 14 days at 37˚C.

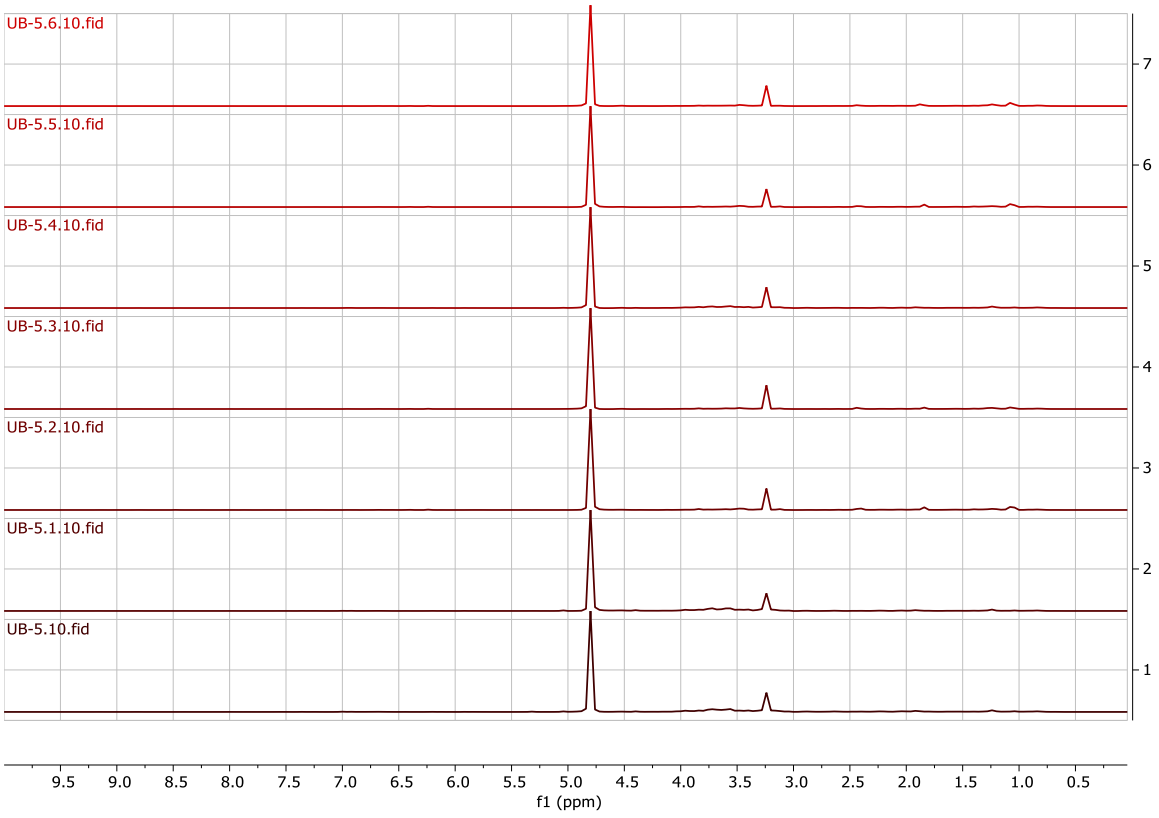


Figure A.37: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants AB

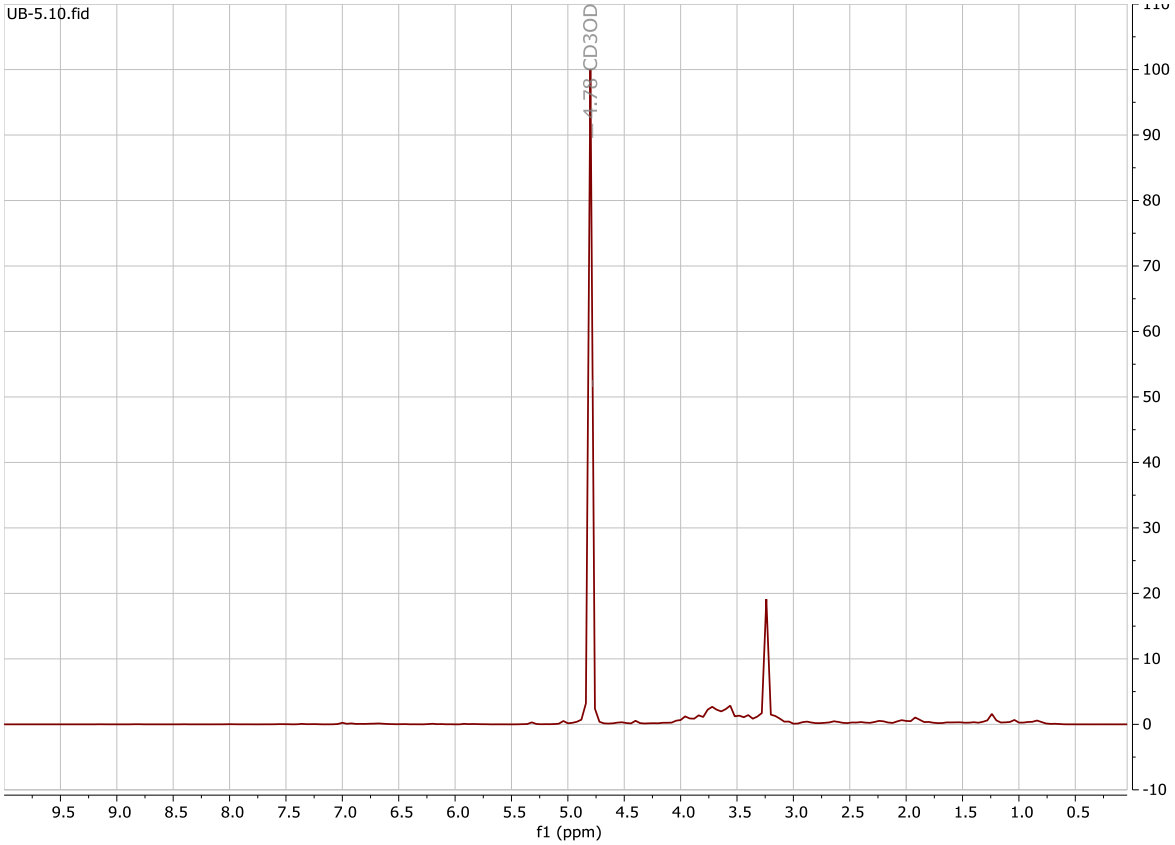


Figure A.38: ^{1}H NMR spectrum of the polyherbal formulation AB, which was not stored over a period.

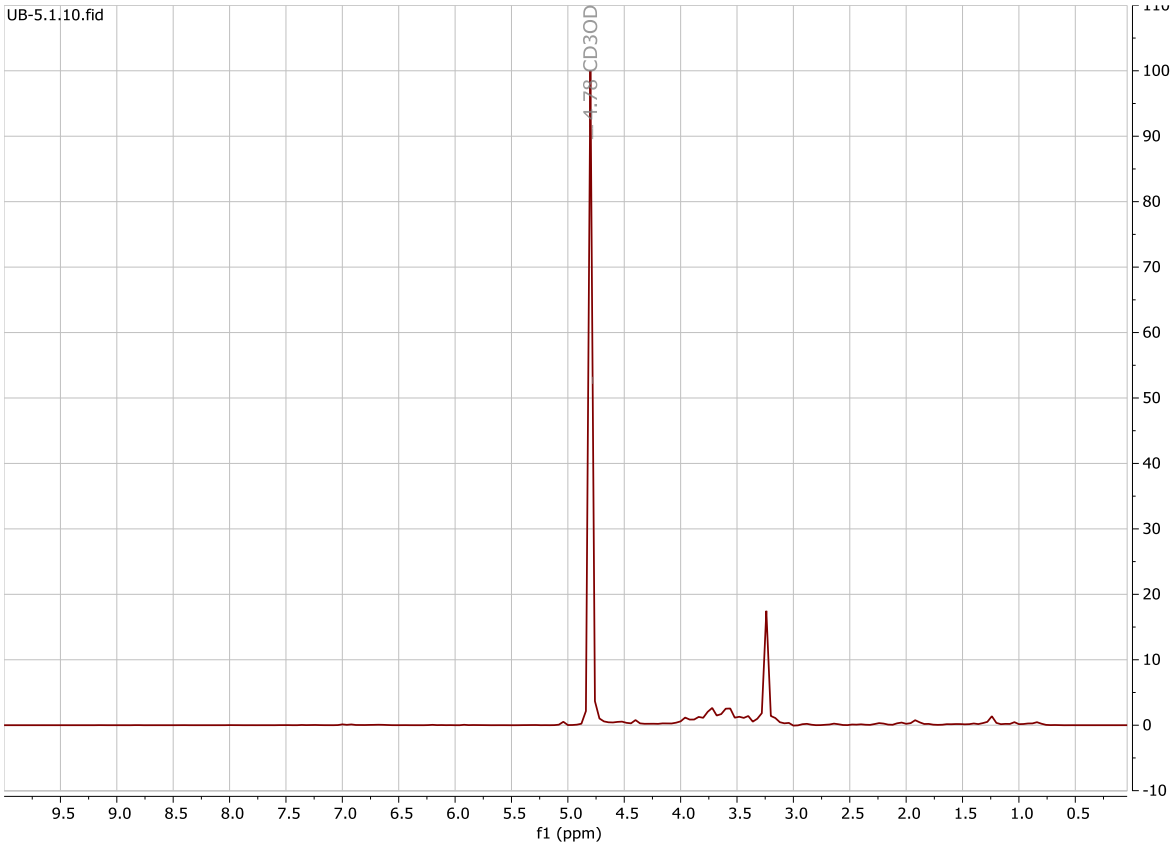


Figure A.39: ^{1}H NMR spectrum of the polyherbal formulation AB, which was stored over a period of 7 days at 4˚C.

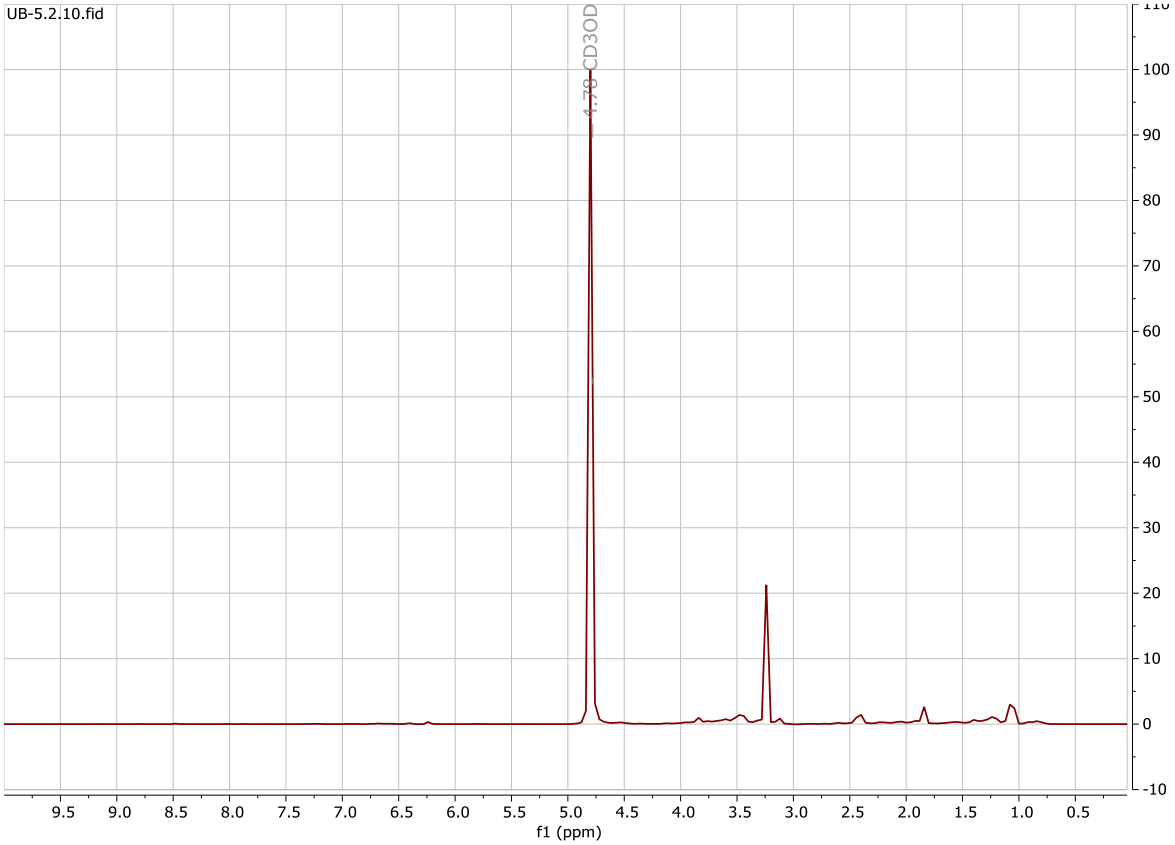


Figure A.40: ^{1}H NMR spectrum of the polyherbal formulation AB, which was stored over a period of 7 days at 25˚C.

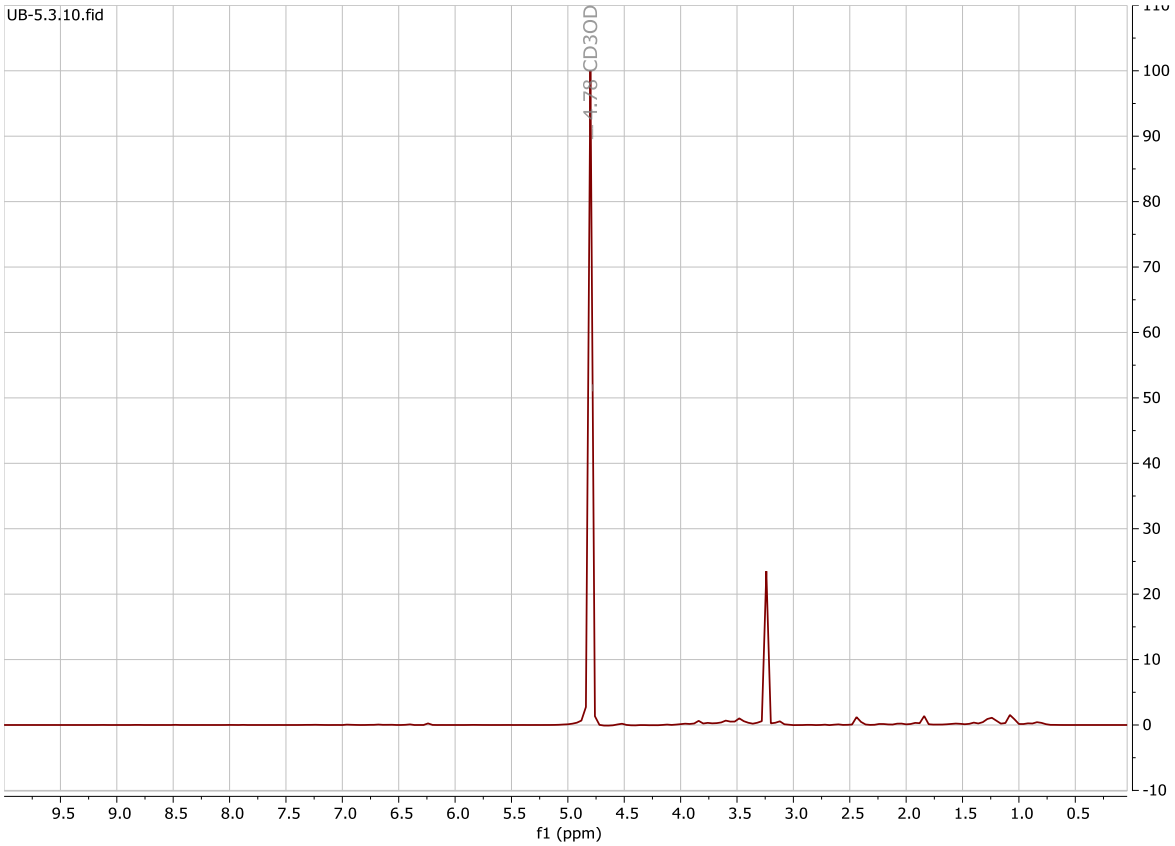


Figure A.41: ^{1}H NMR spectrum of the polyherbal formulation AB, which was stored over a period of 7 days at 37˚C.

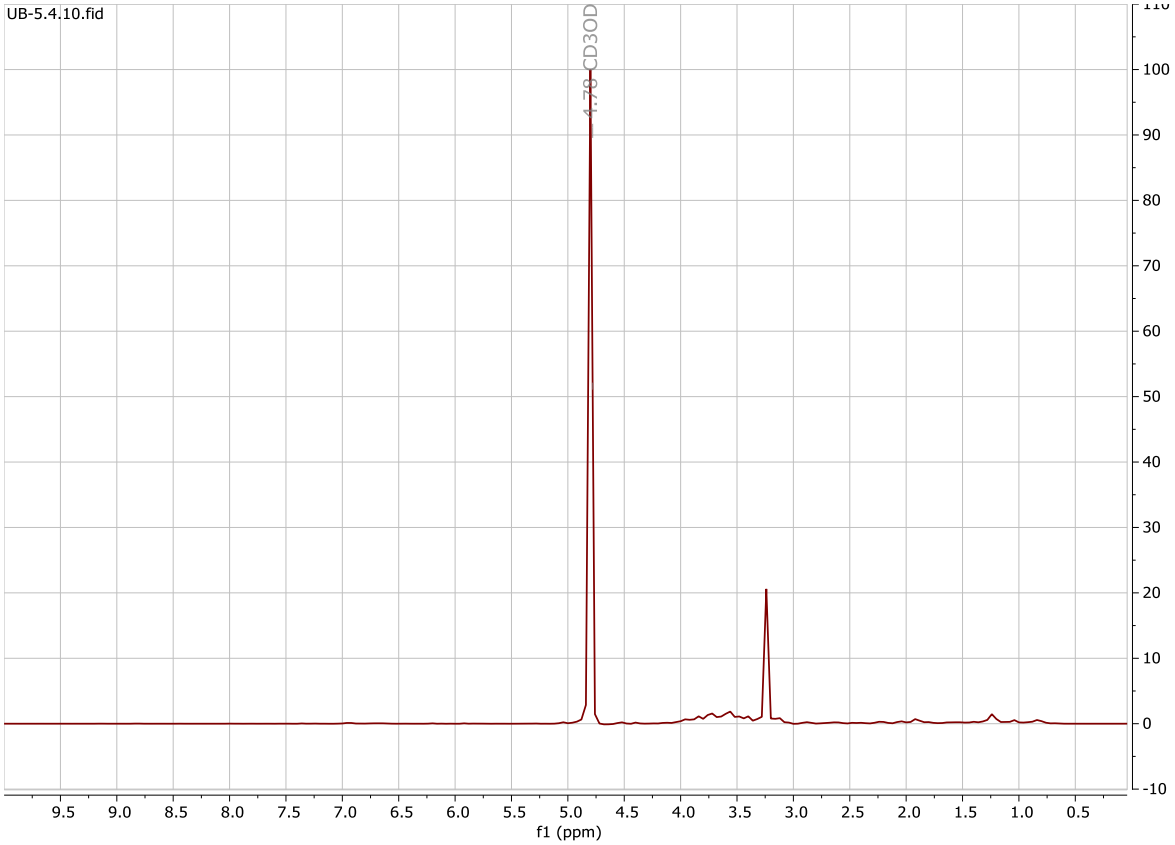


Figure A.42: ^{1}H NMR spectrum of the polyherbal formulation AB, which was stored over a period of 14 days at 4˚C.

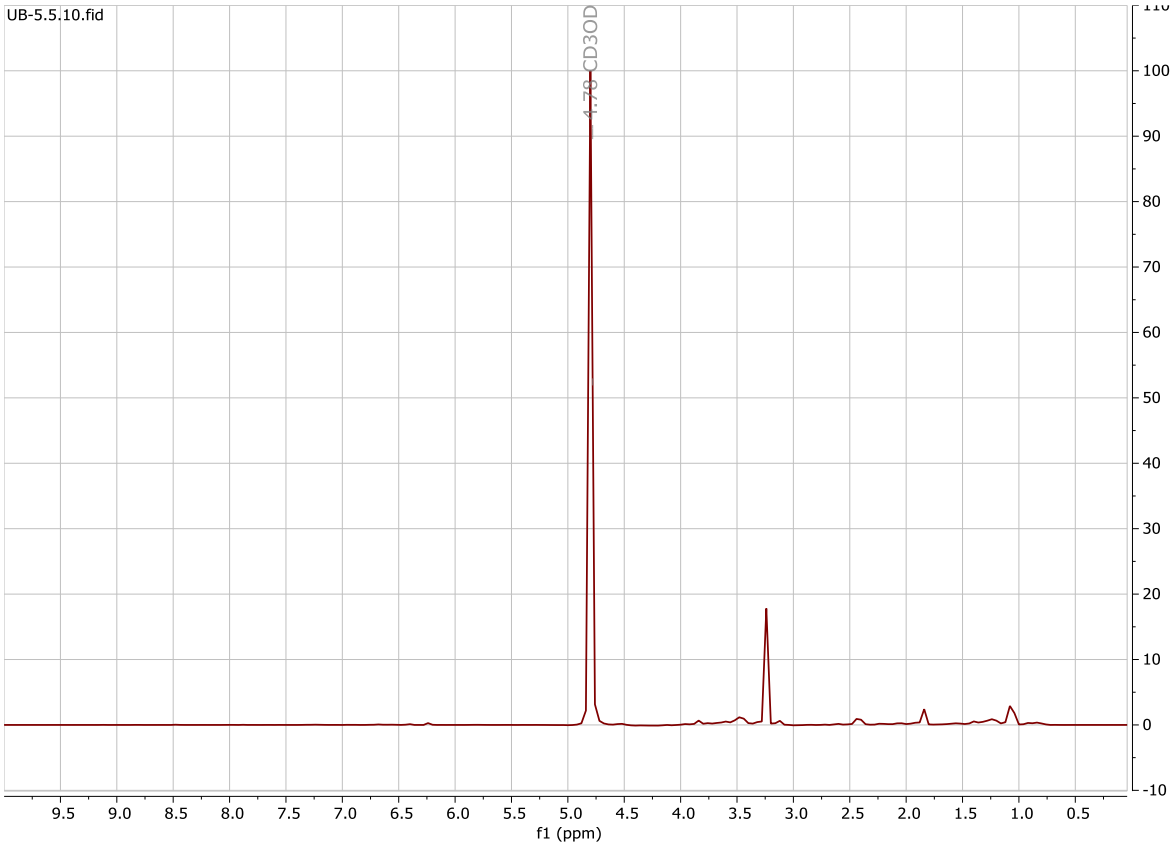


Figure A.43: ^{1}H NMR spectrum of the polyherbal formulation AB, which was stored over a period of 14 days at 25˚C.

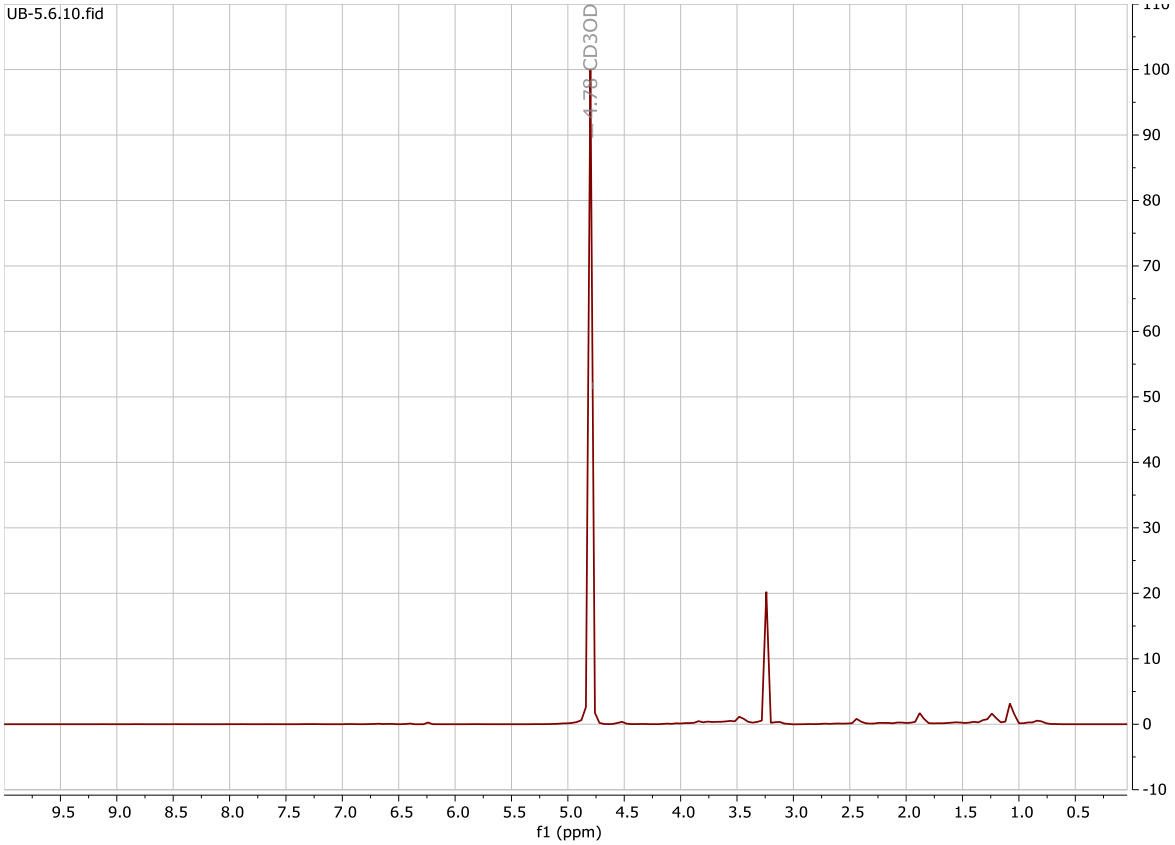


Figure A.44: ^{1}H NMR spectrum of the polyherbal formulation AB, which was stored over a period of 14 days at 37˚C.

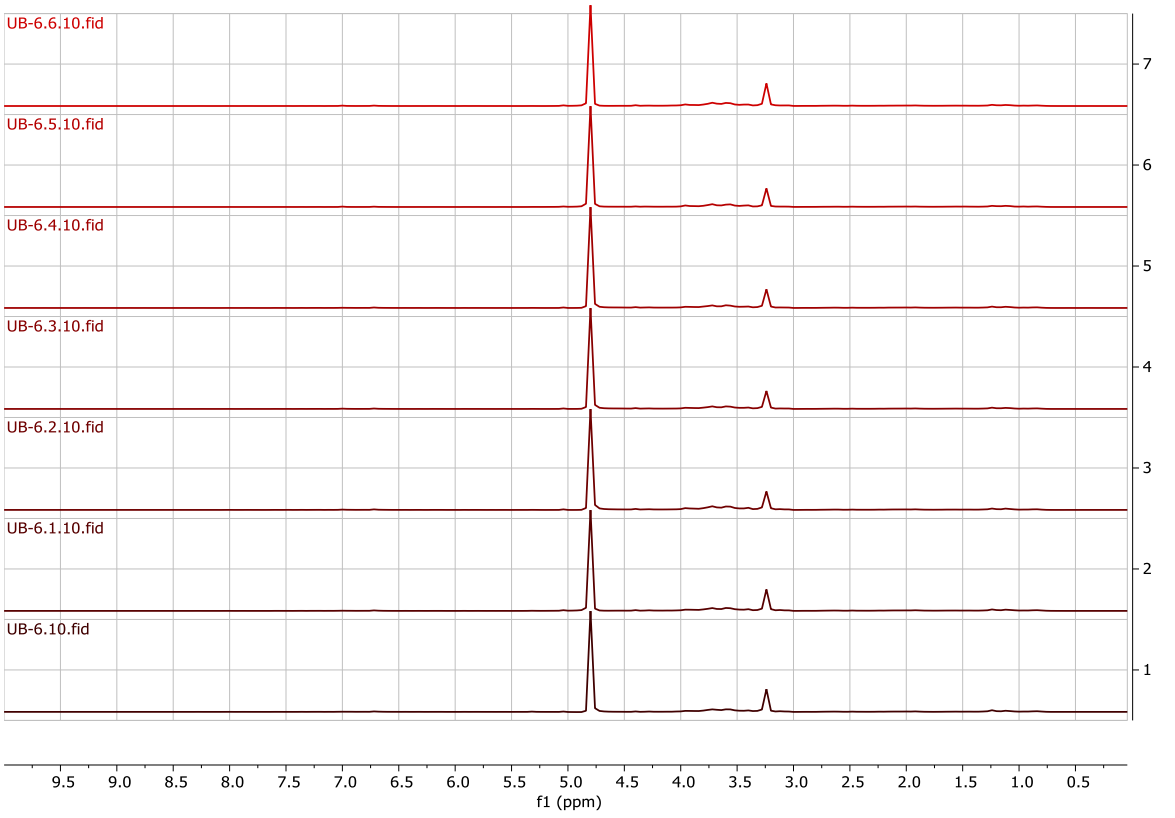


Figure A.45: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants AC.

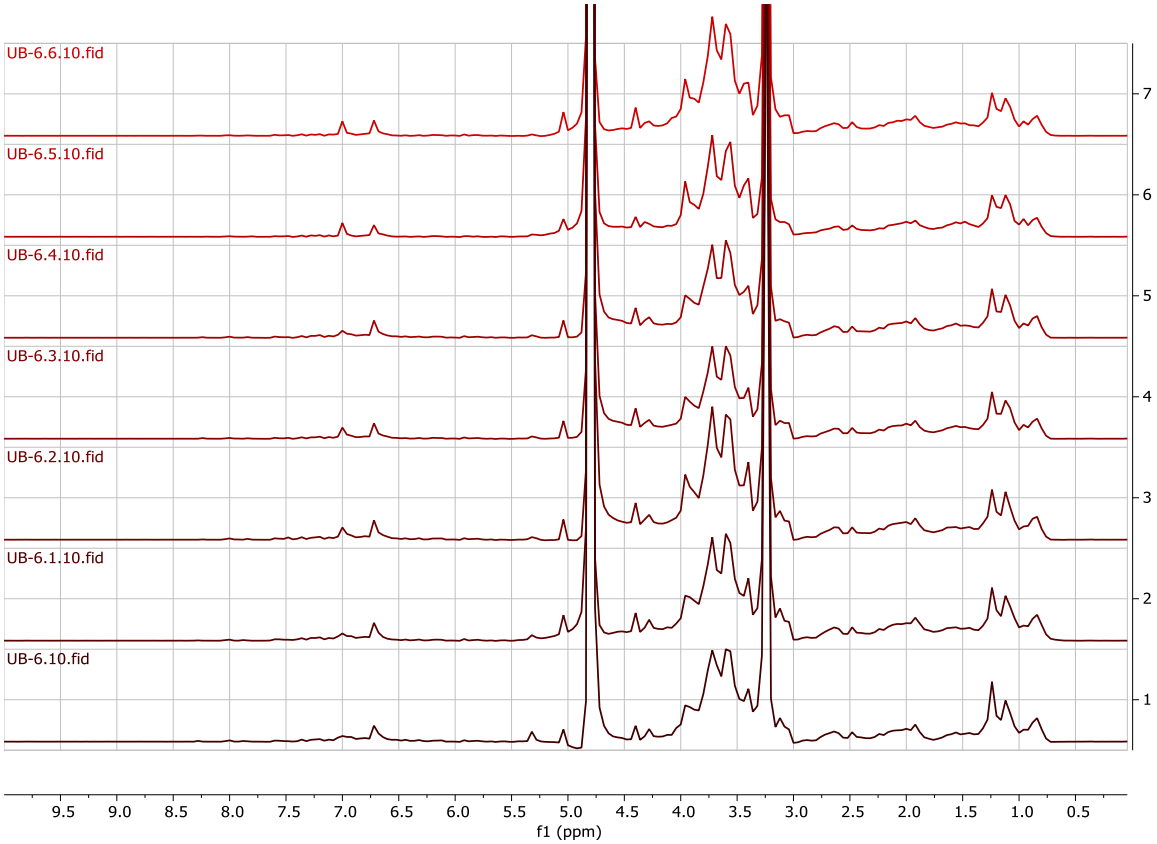


Figure A.46: Zoomed in stacked ^{1}H NMR spectra of the polyherbal formulation containing plants AC, containing combinations that were stored at various temperatures over two different storage periods

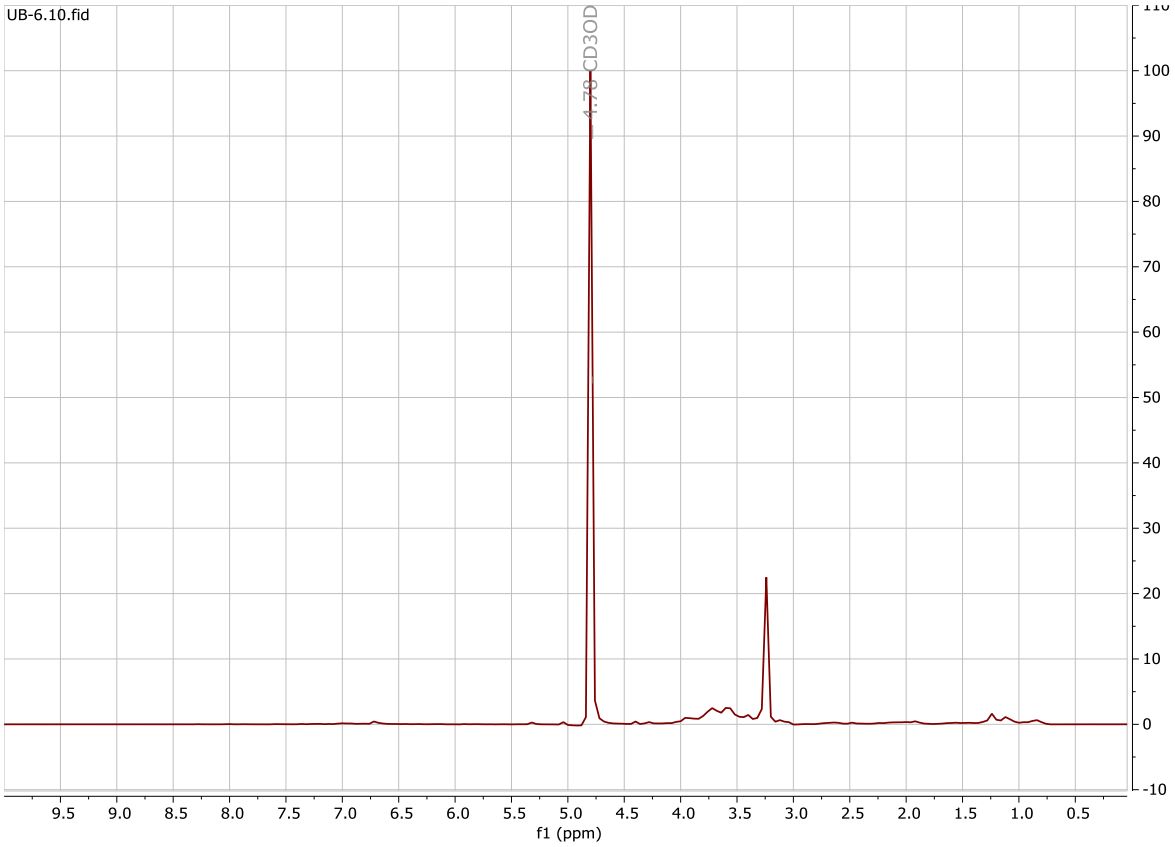


Figure A.47: ^{1}H NMR spectrum of the polyherbal formulation AC, which was not stored over a period.

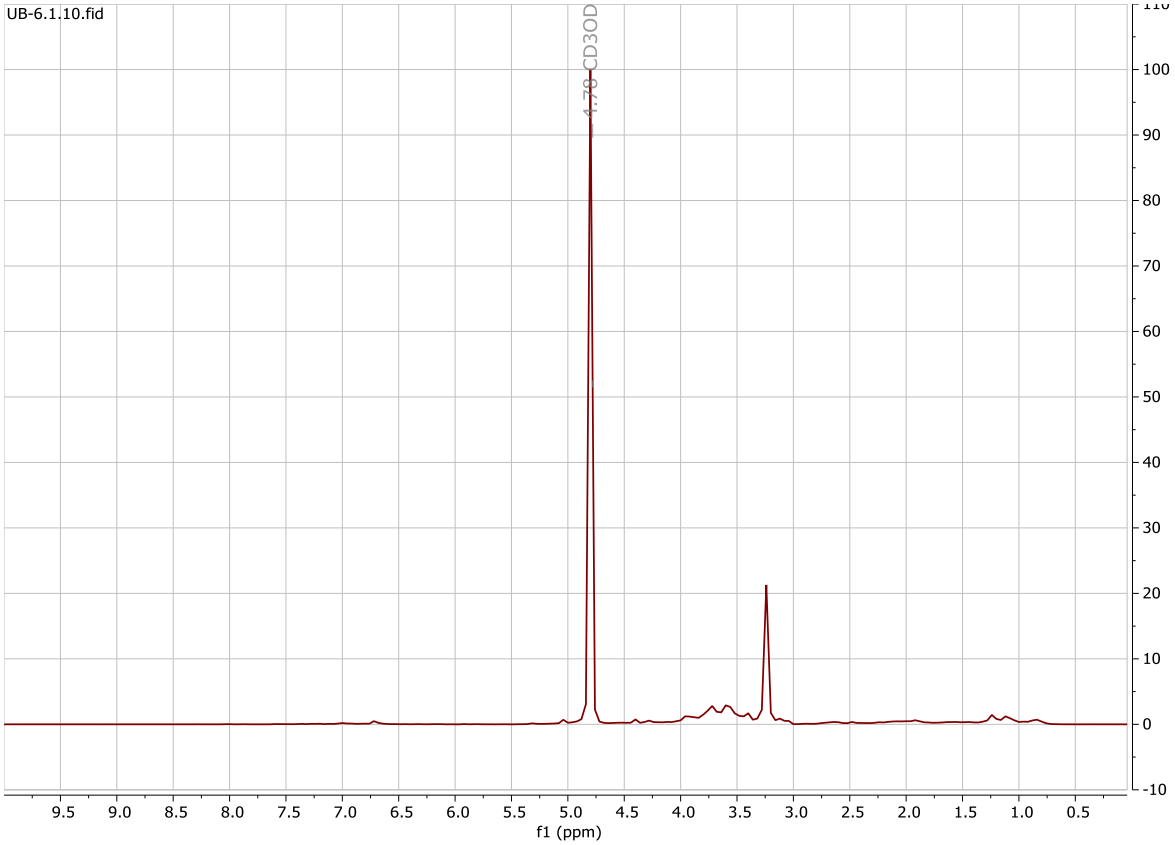


Figure A.48: ^{1}H NMR spectrum of the polyherbal formulation AC, which was stored over a period of 7 days at 4˚C.

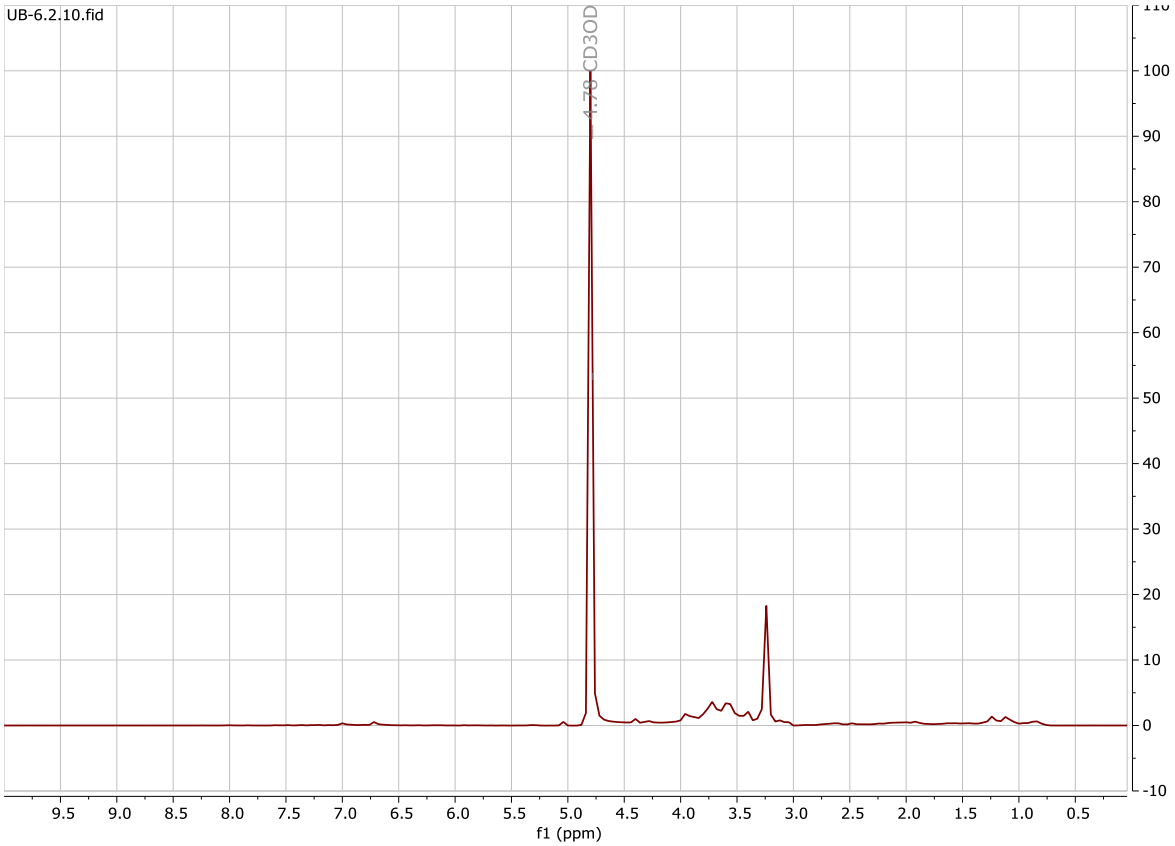


Figure A.49: ^{1}H NMR spectrum of the polyherbal formulation AC, which was stored over a period of 7 days at 25˚C.

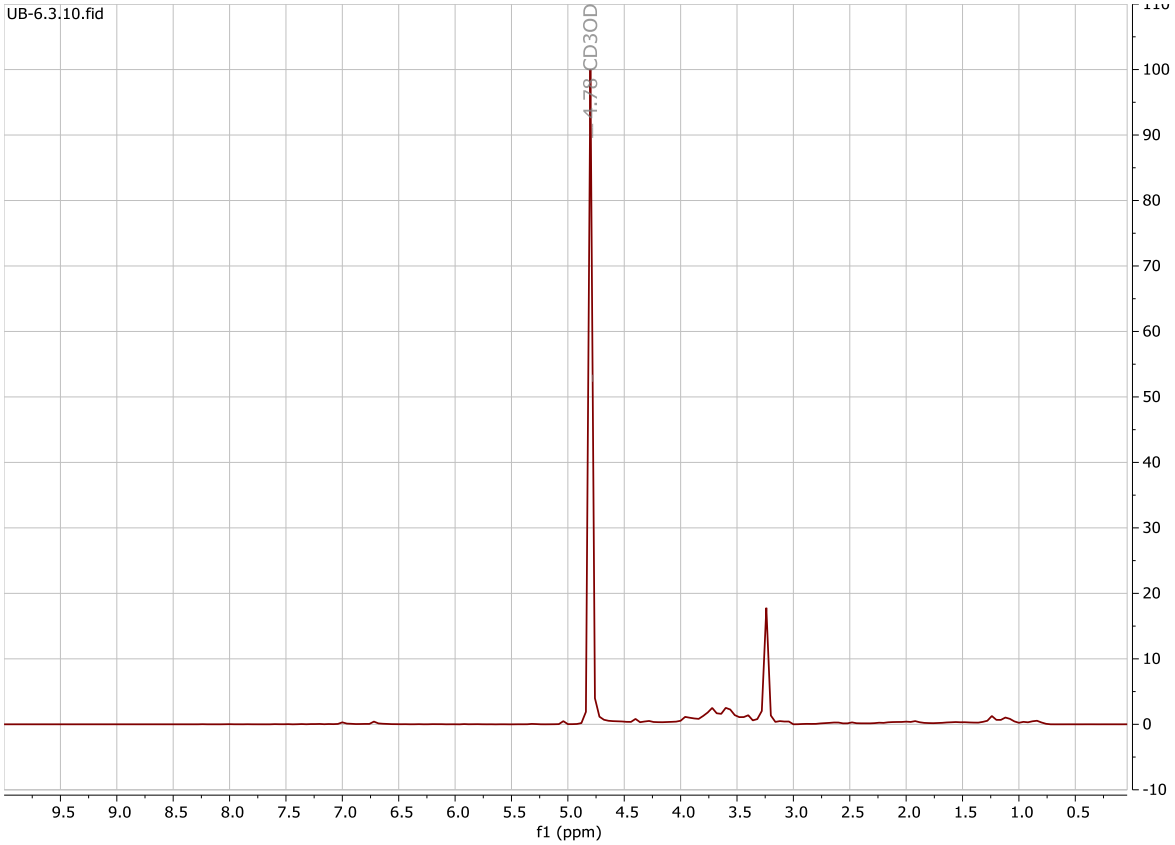


Figure A.50: ^{1}H NMR spectrum of the polyherbal formulation AC, which was stored over a period of 7 days at 37˚C.

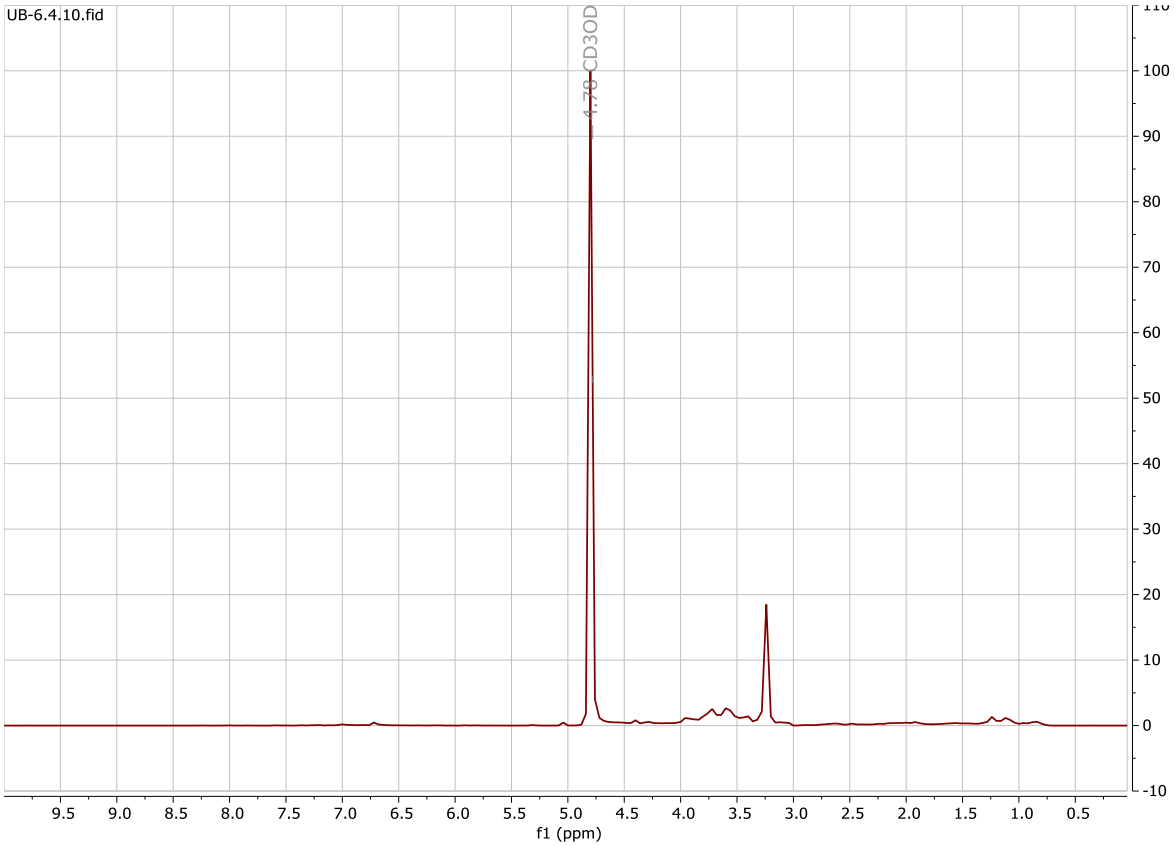


Figure A.51: ^{1}H NMR spectrum of the polyherbal formulation AC, which was stored over a period of 14 days at 4˚C.

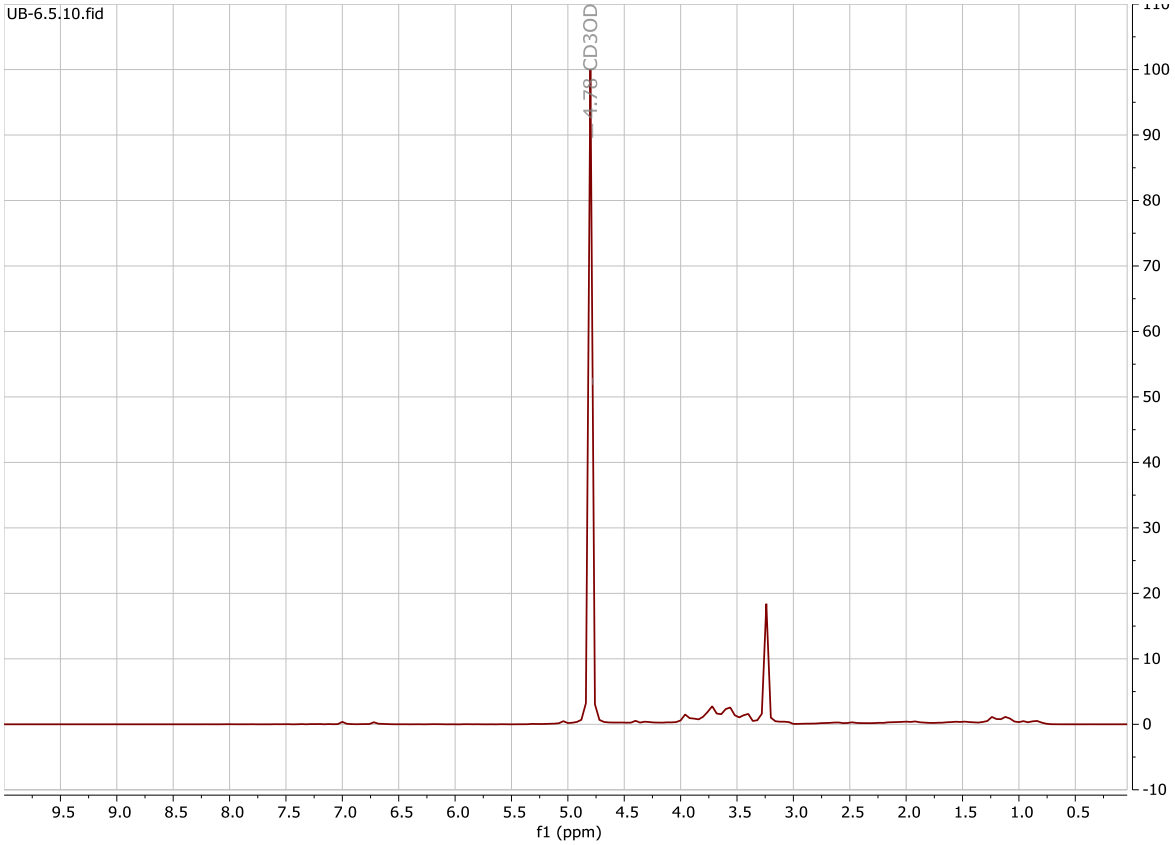


Figure A.52: ^{1}H NMR spectrum of the polyherbal formulation AC, which was stored over a period of 14 days at 25˚C.

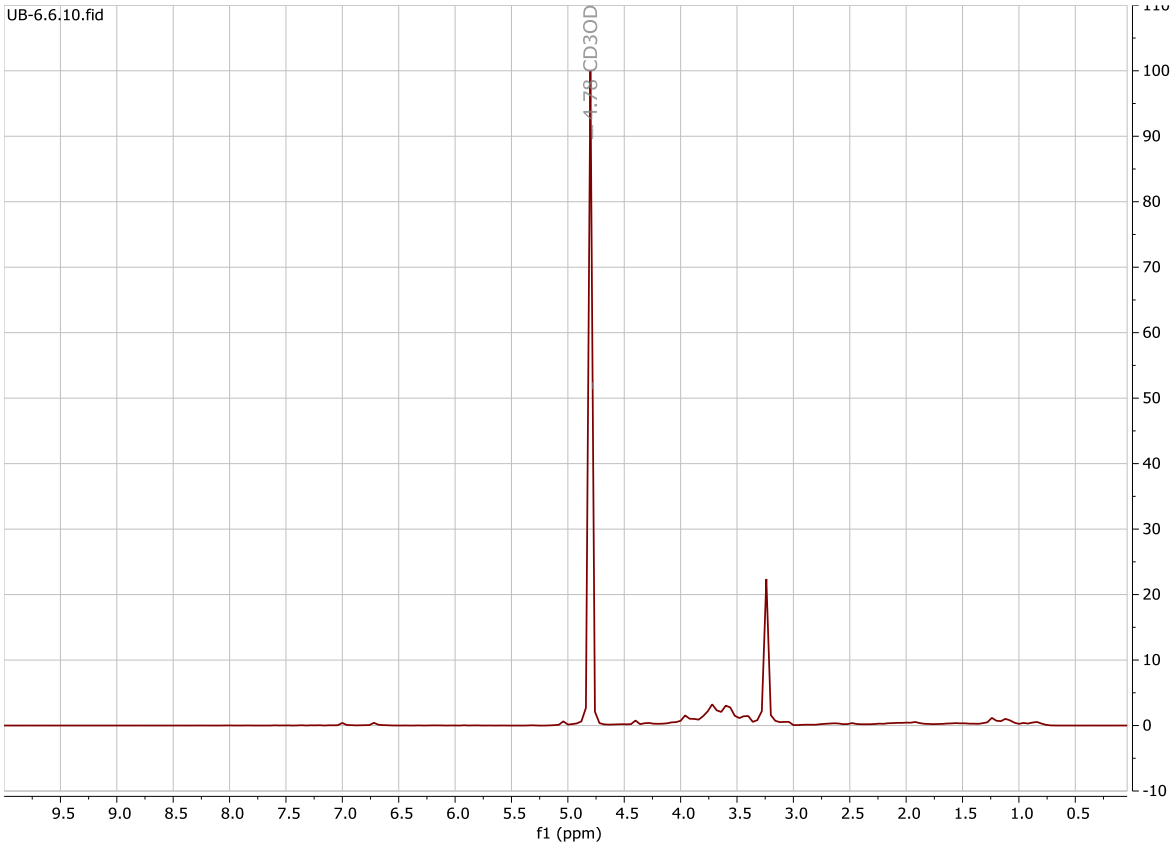


Figure A.53: ^{1}H NMR spectrum of the polyherbal formulation AC, which was stored over a period of 14 days at 37˚C.

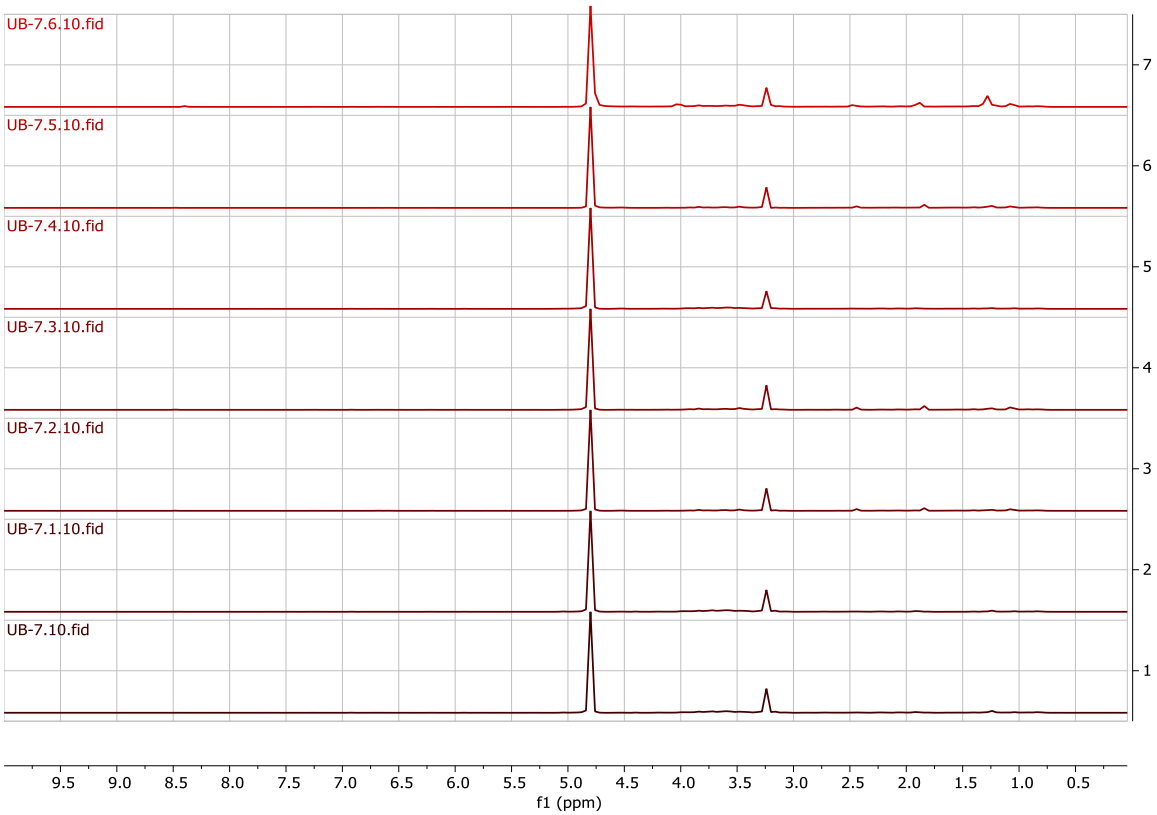


Figure A.54: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants AD.

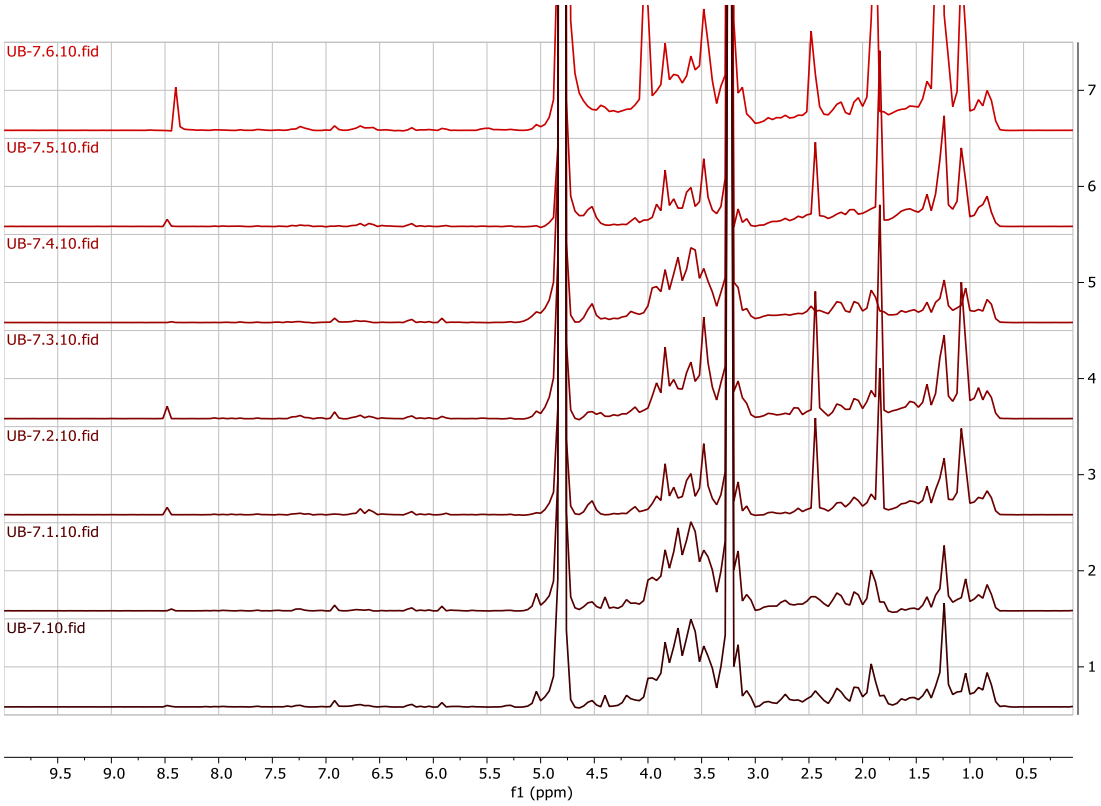


Figure A.55: Zoomed in stacked ^{1}H NMR spectra of the polyherbal formulation containing plants AD, containing combinations that were stored at various temperatures over two different storage periods.

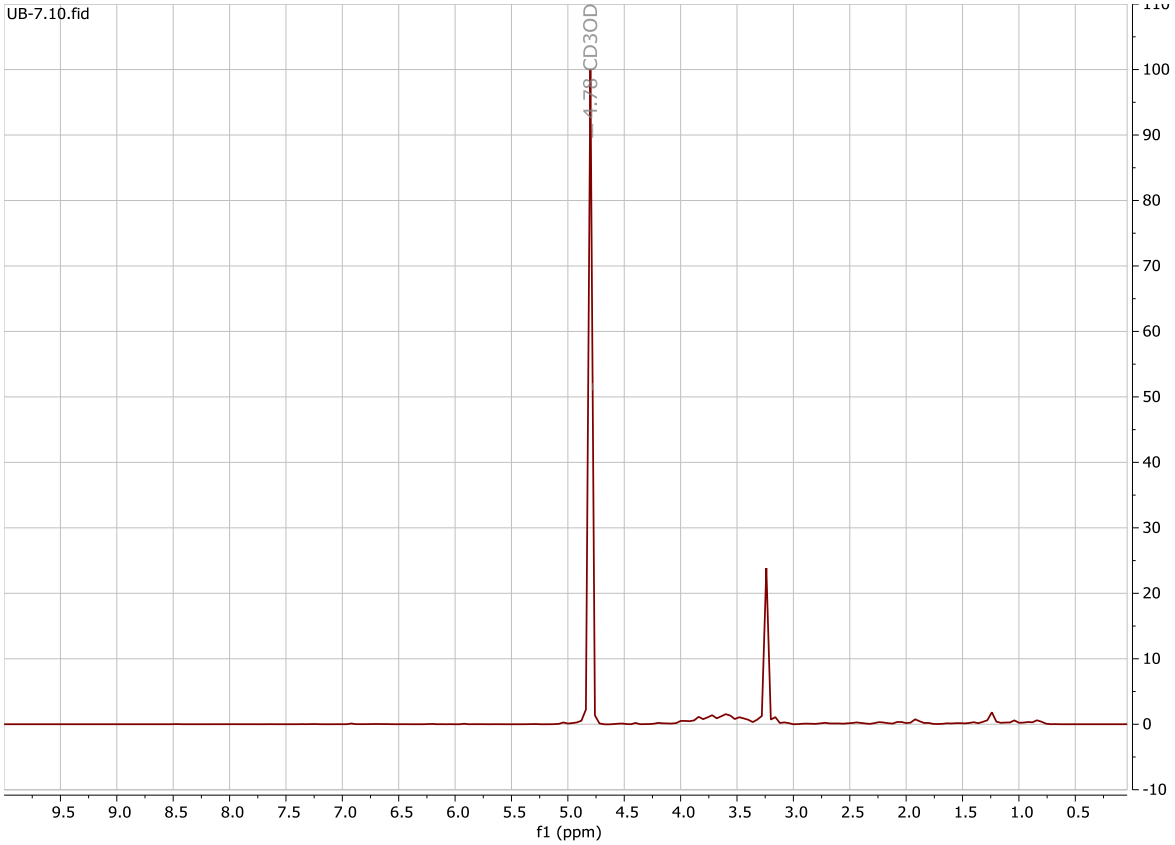


Figure A.56: ^{1}H NMR spectrum of the polyherbal formulation AD, which was not stored over a period.

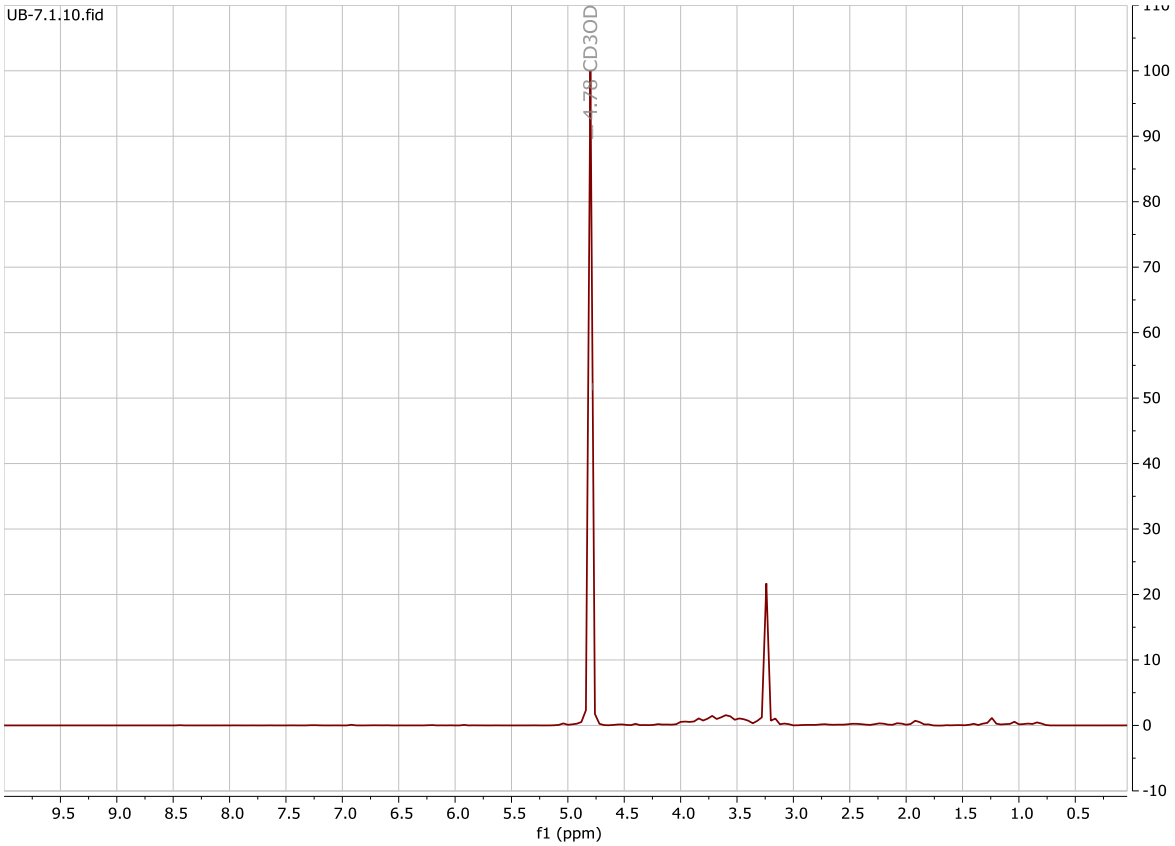


Figure A.57: ^{1}H NMR spectrum of the polyherbal formulation AD, which was stored over a period of 7 days at 4˚C.

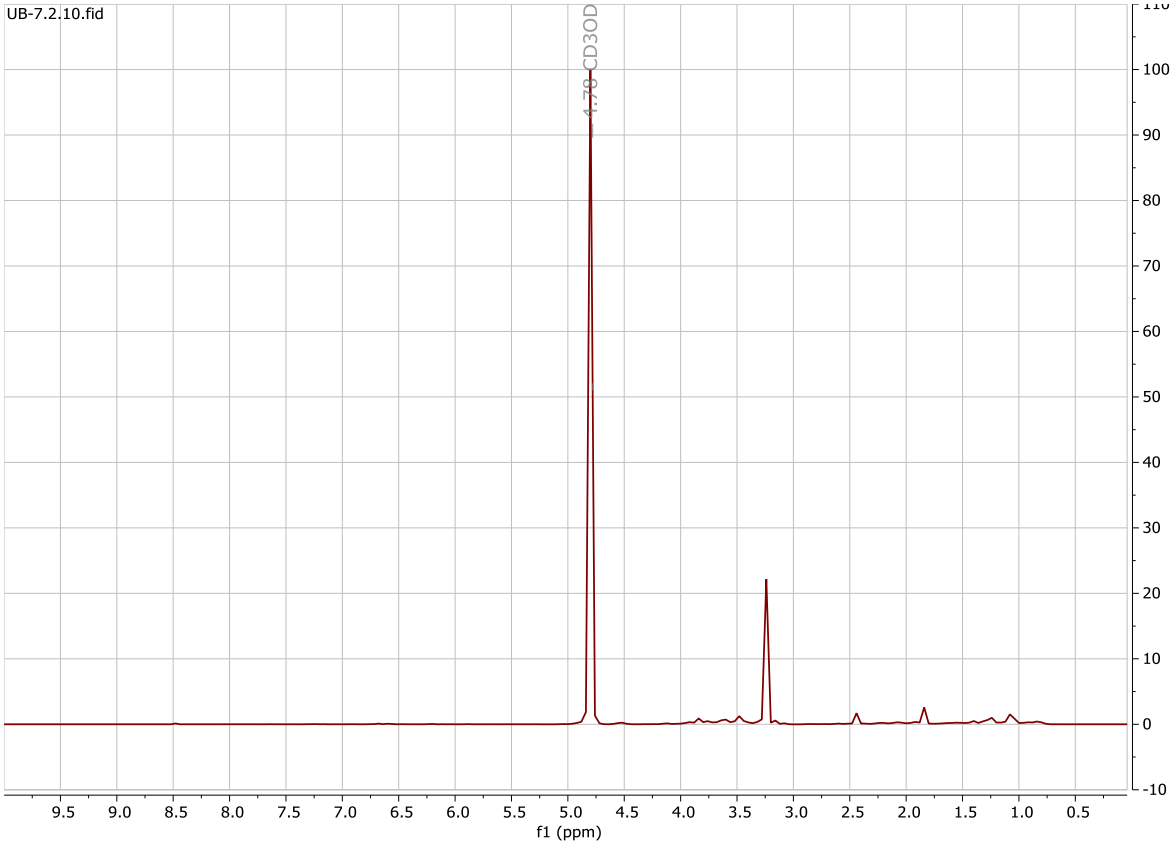


Figure A.58: ^{1}H NMR spectrum of the polyherbal formulation AD, which was stored over a period of 7 days at 25˚C.

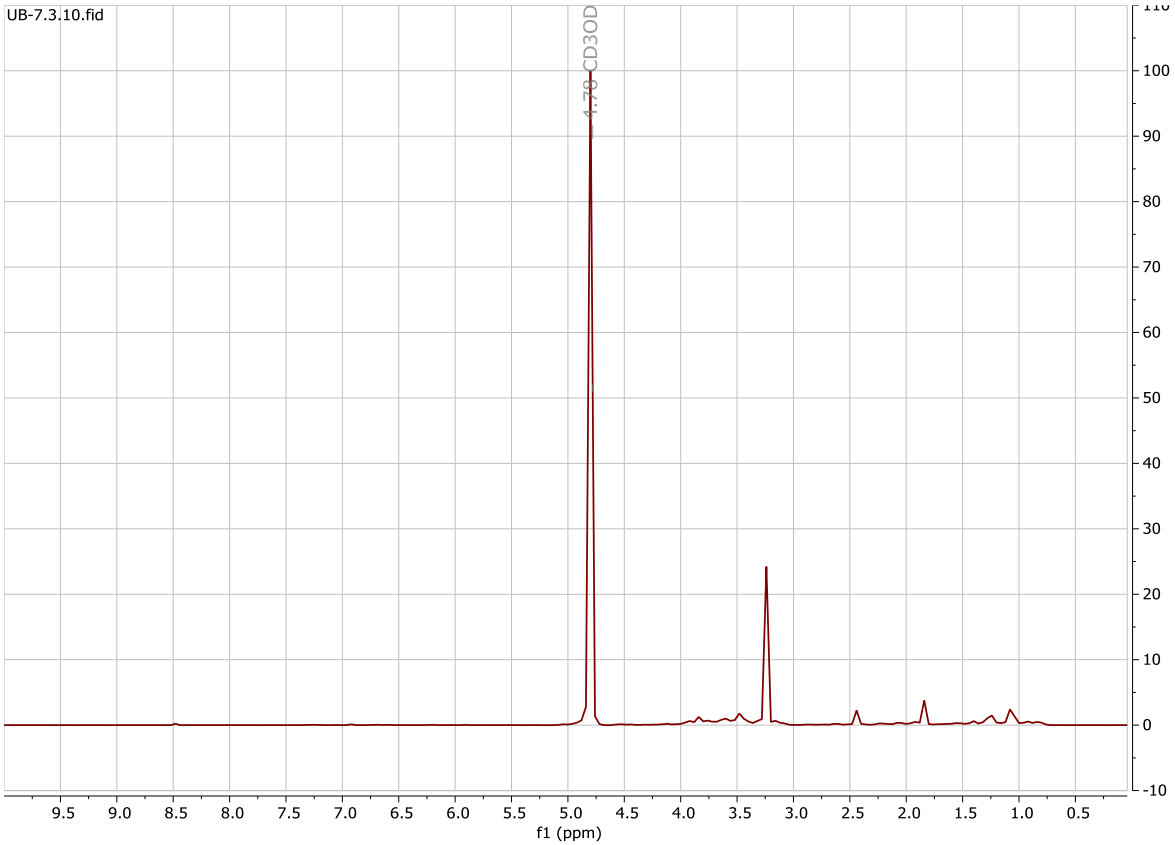


Figure A.59: ^{1}H NMR spectrum of the polyherbal formulation AD, which was stored over a period of 7 days at 37˚C.

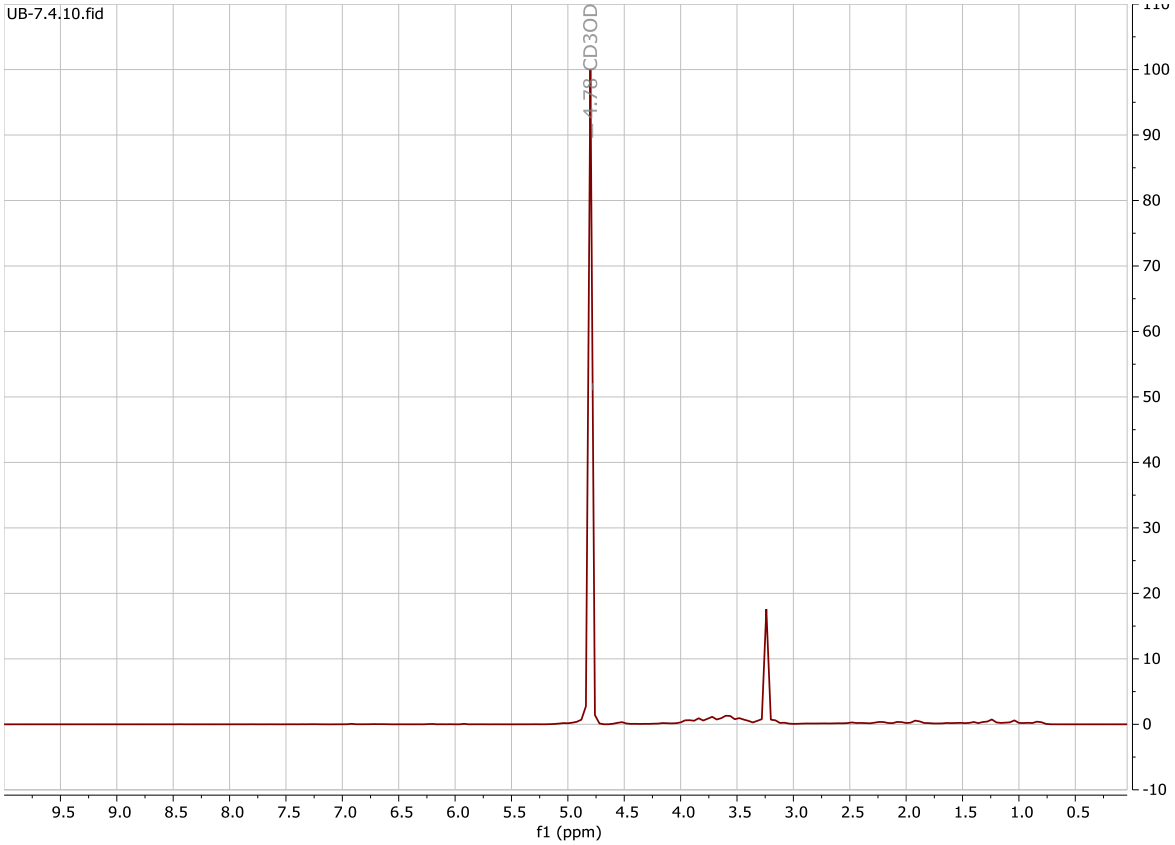


Figure A.60: ^{1}H NMR spectrum of the polyherbal formulation AD, which was stored over a period of 14 days at 4˚C.

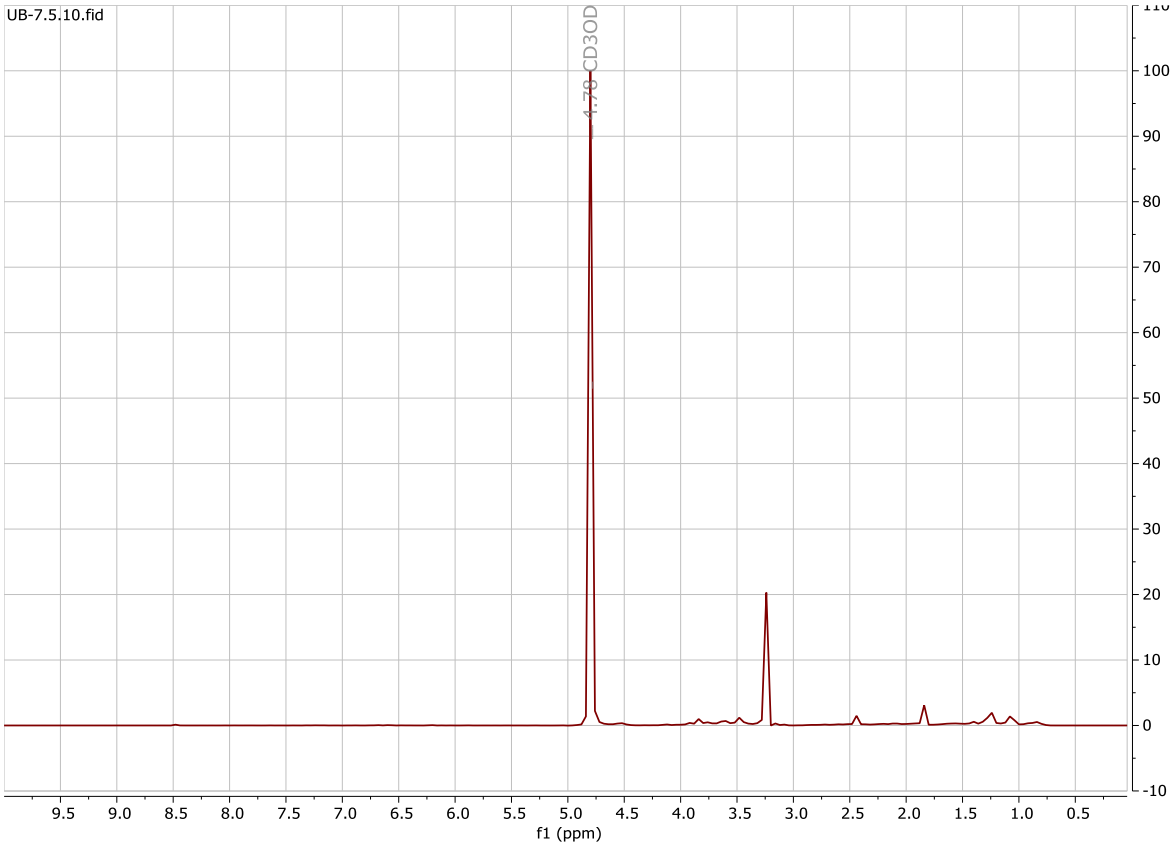


Figure A.61: ^{1}H NMR spectrum of the polyherbal formulation AD, which was stored over a period of 14 days at 25˚C.

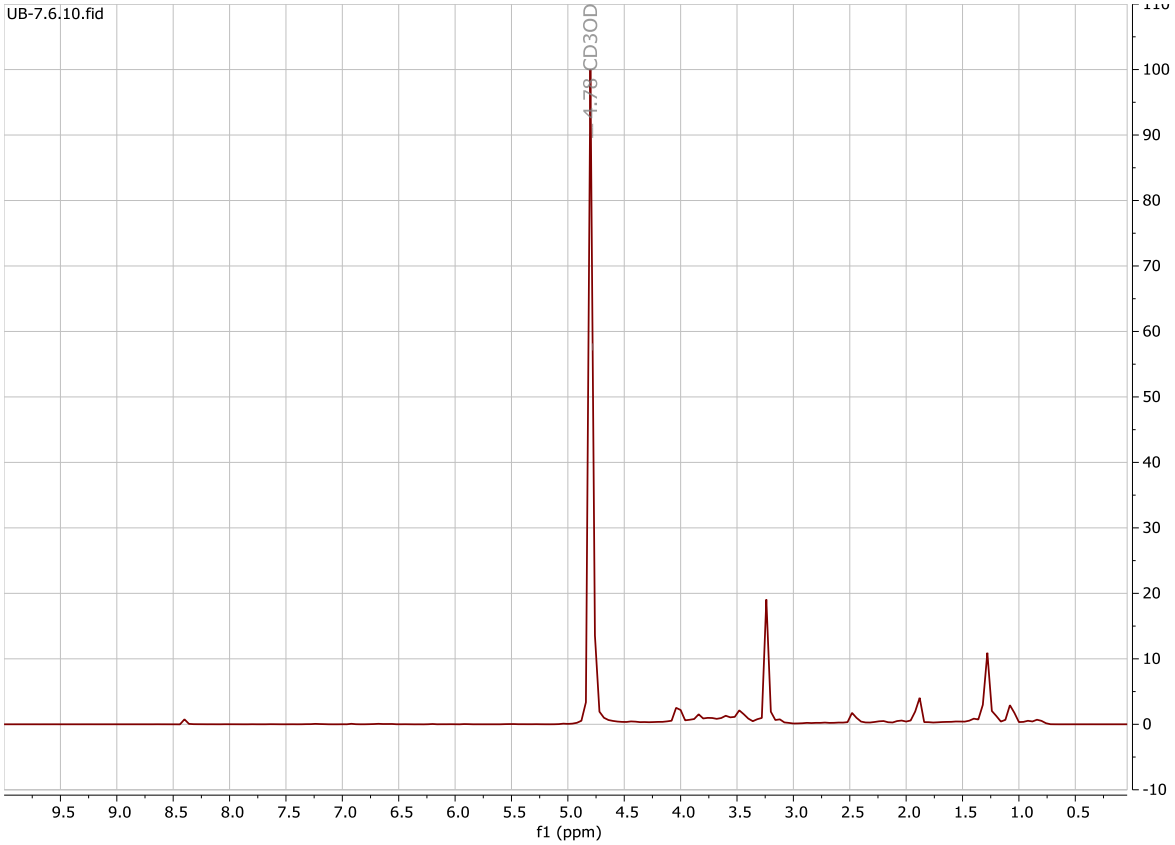


Figure A.62: ^{1}H NMR spectrum of the polyherbal formulation AD, which was stored over a period of 14 days at 37˚C.

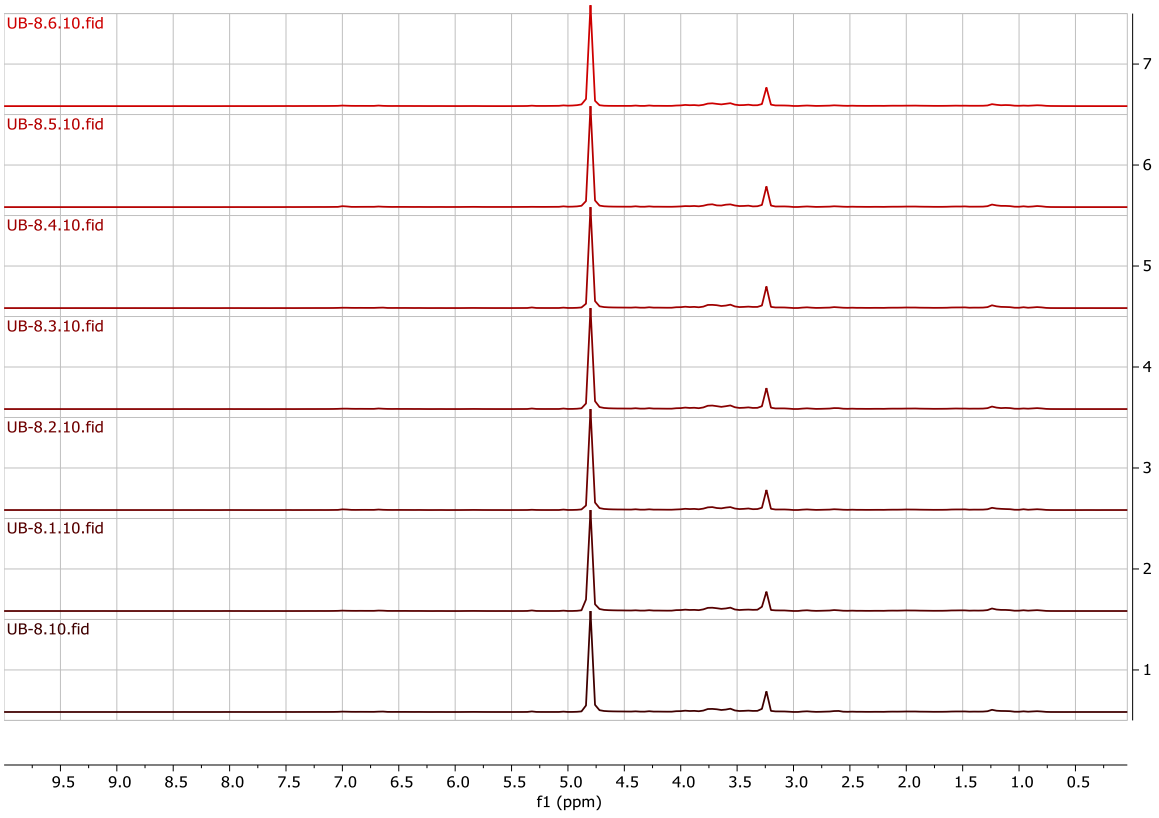


Figure A.63: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants BC.

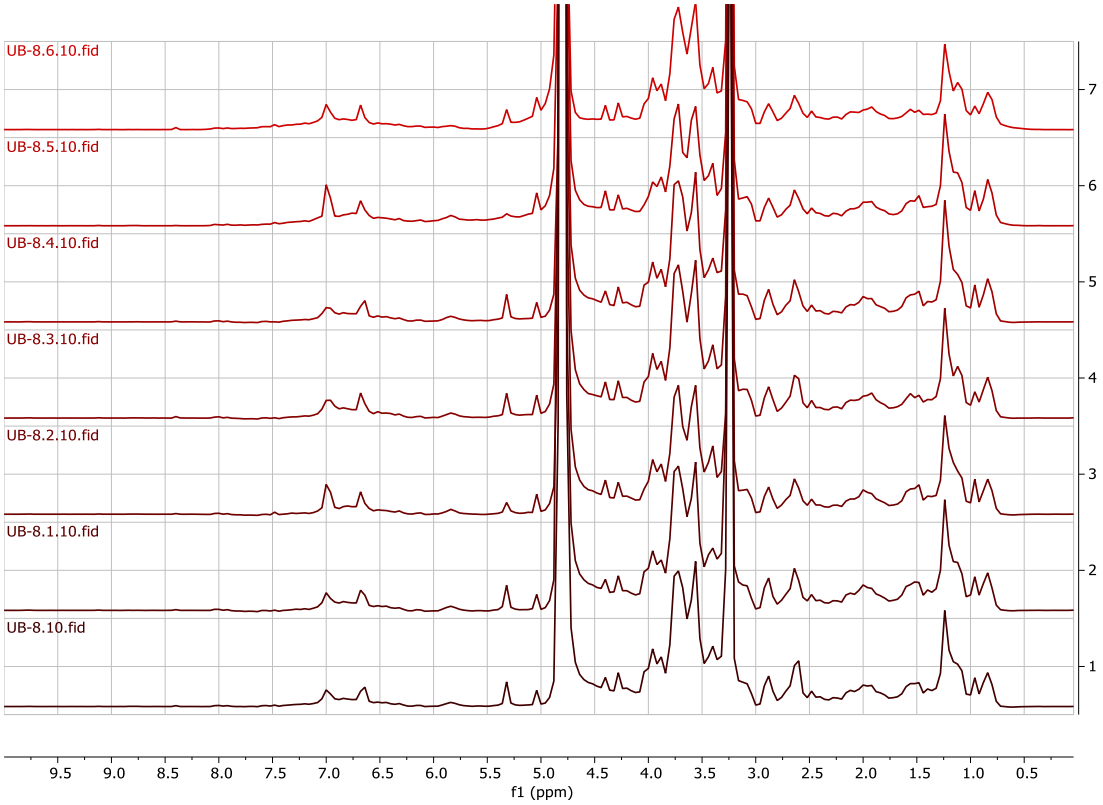


Figure A.64: Zoomed Stacked ^{1}H NMR spectra of the polyherbal formulation containing plants BC, containing combinations that were stored at various temperatures over two different storage periods.

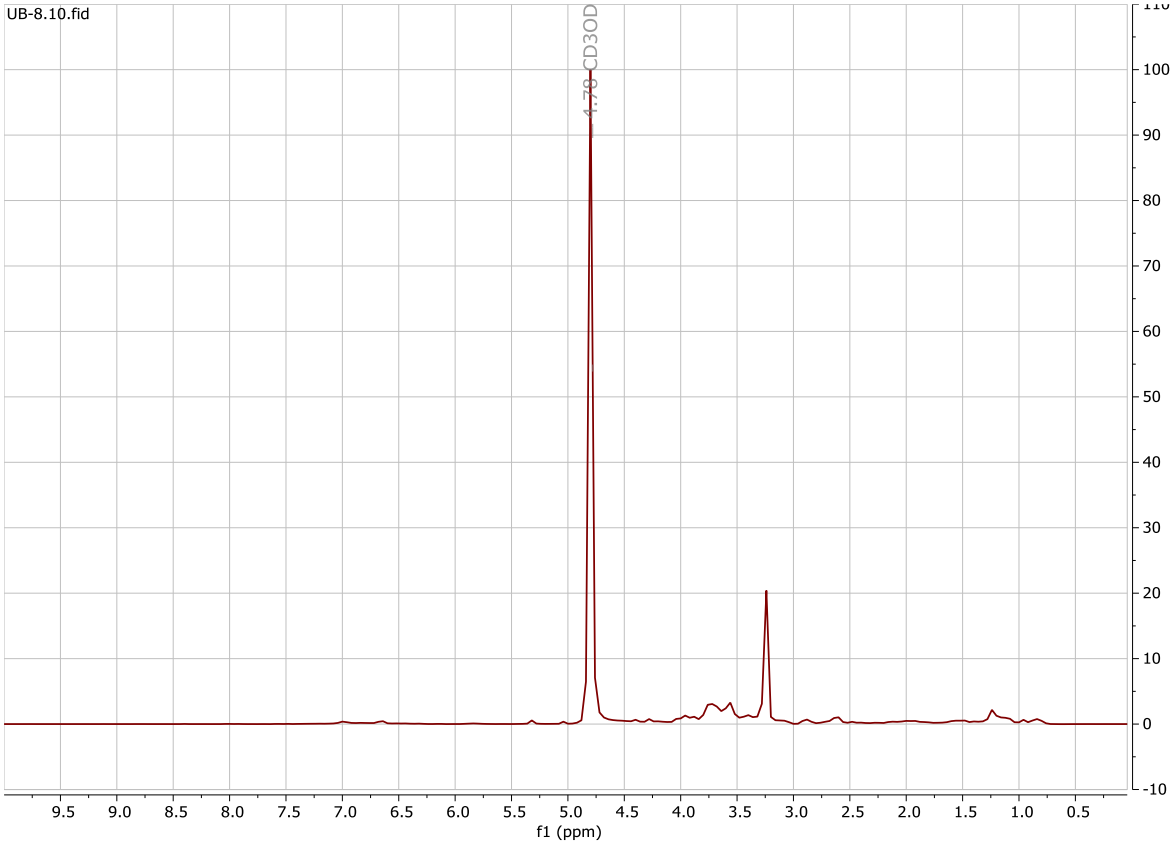


Figure A.65: ^{1}H NMR spectrum of the polyherbal formulation BC, which was not stored over a period.

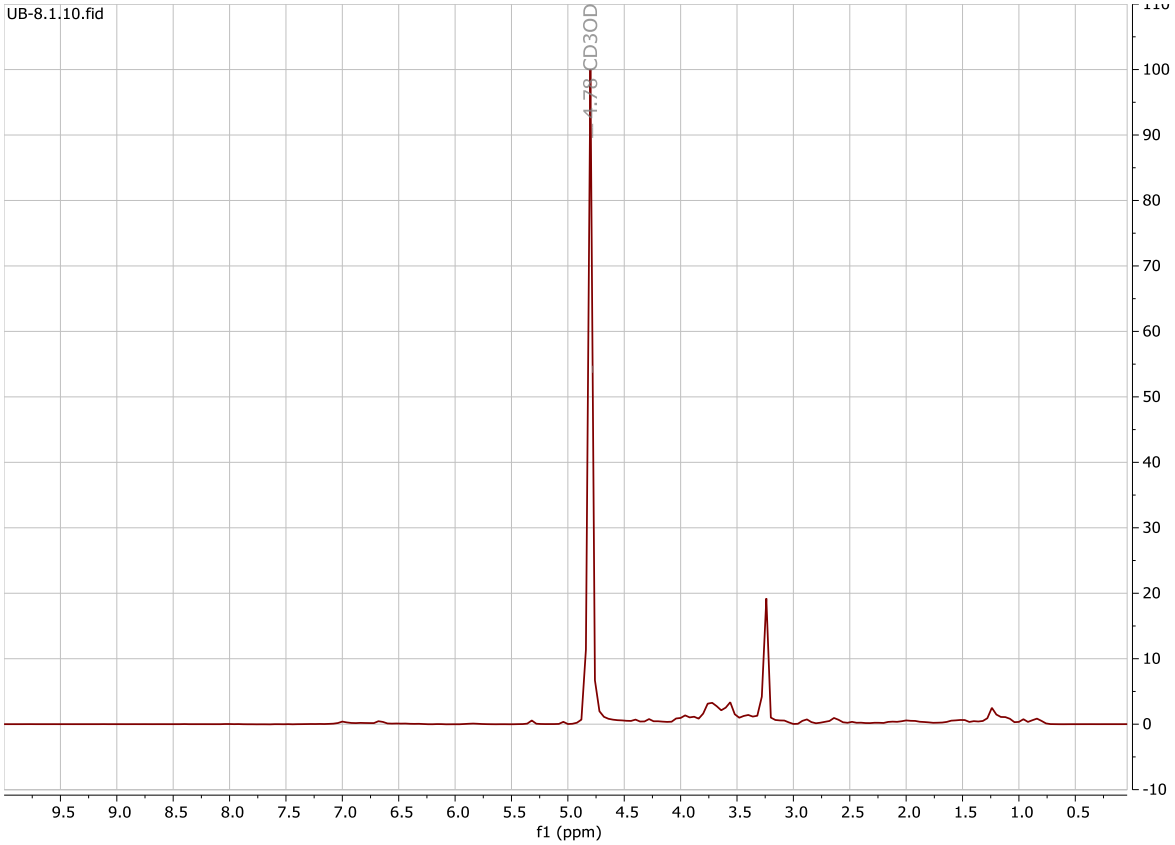


Figure A.66: ^{1}H NMR spectrum of the polyherbal formulation BC, which was stored over a period of 7 days at 4˚C.

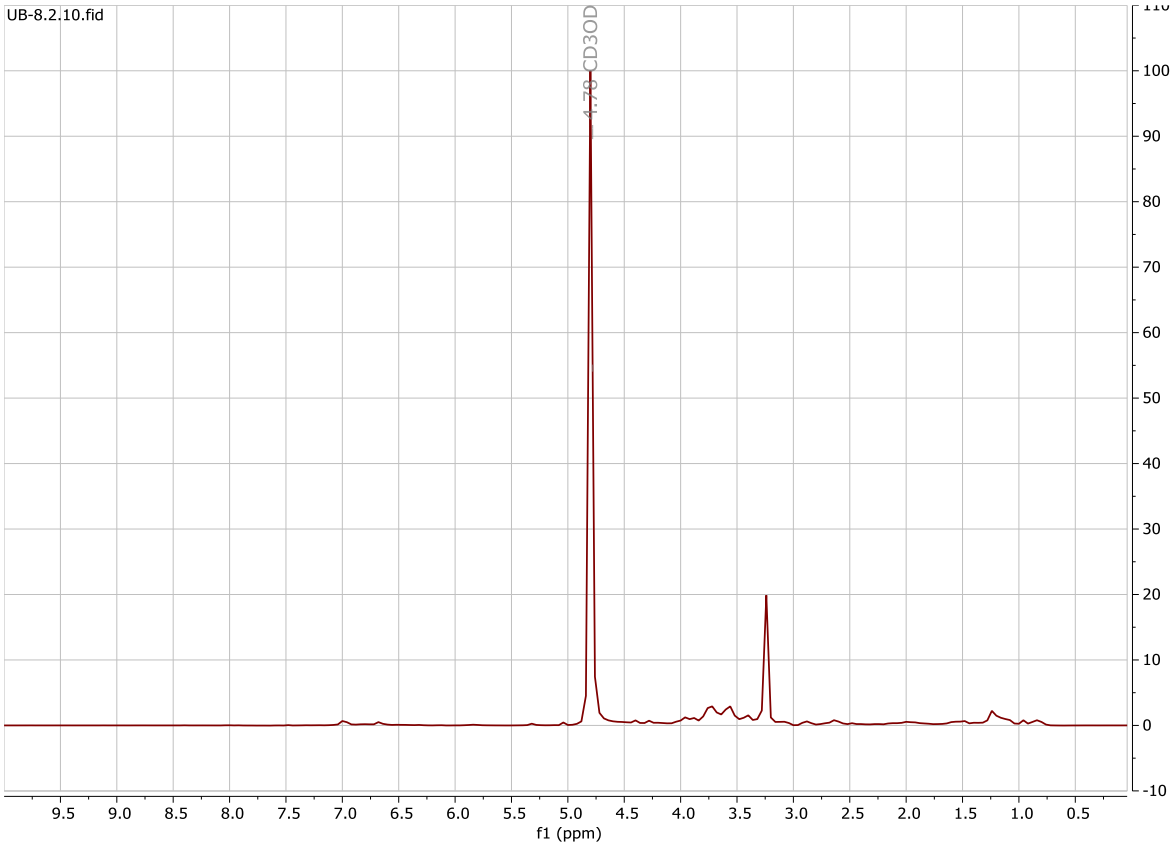


Figure A.67:^{1}H NMR spectrum of the polyherbal formulation BC, which was stored over a period of 7 days at 25˚C.

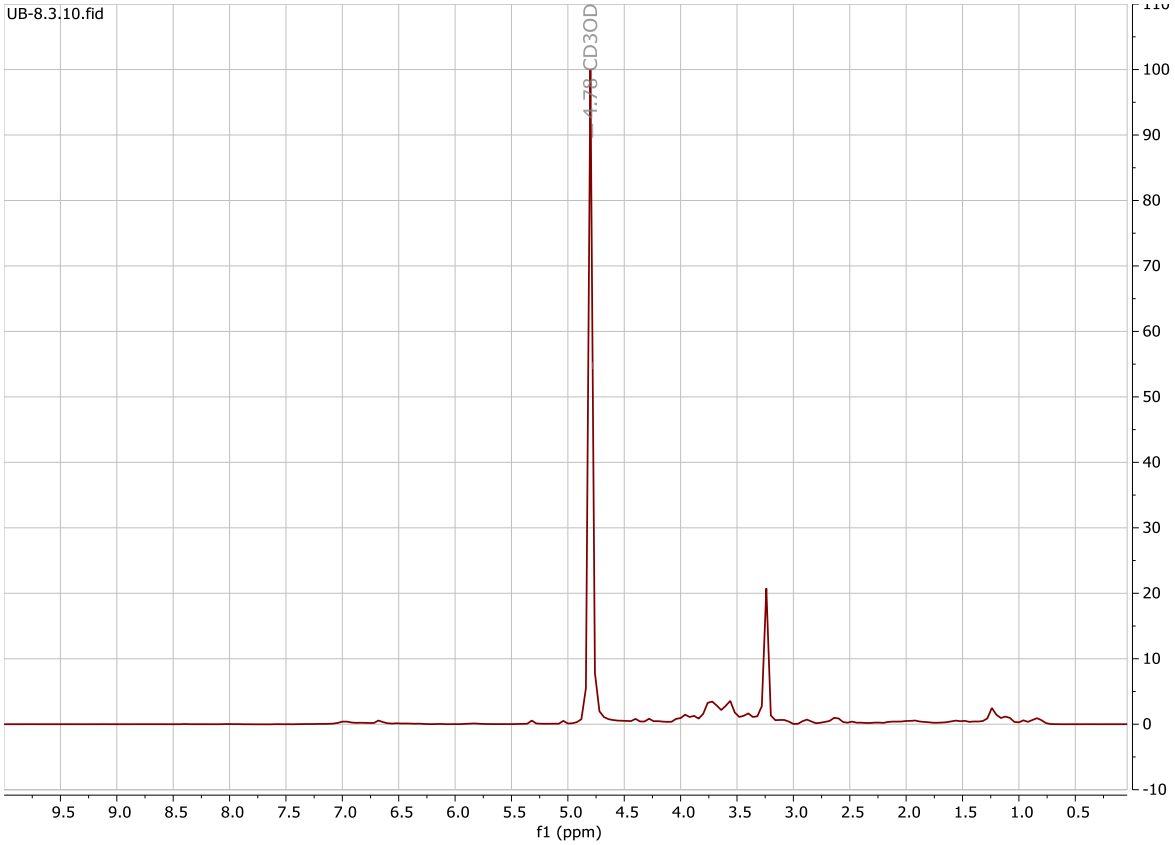


Figure A.68: ^{1}H NMR spectrum of the polyherbal formulation BC, which was stored over a period of 7 days at 37˚C.

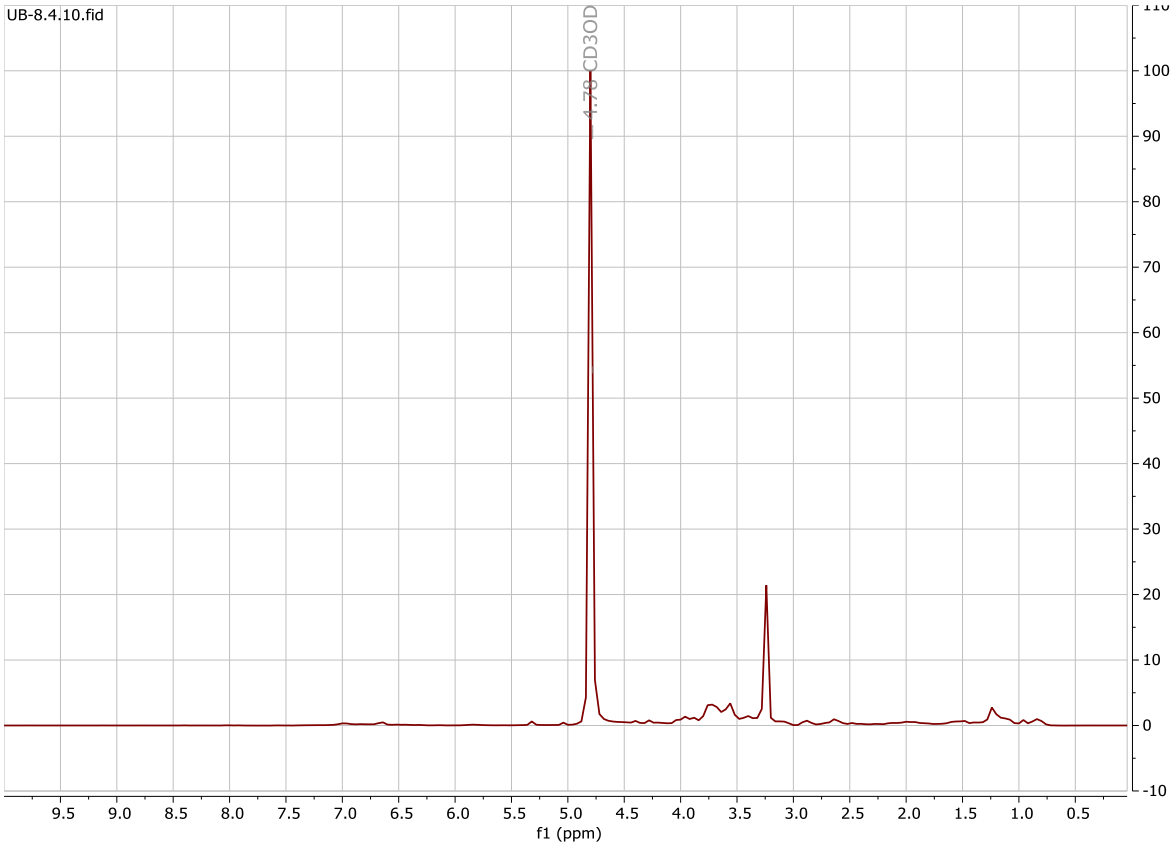


Figure A.69: ^{1}H NMR spectrum of the polyherbal formulation BC, which was stored over a period of 14 days at 4˚C.

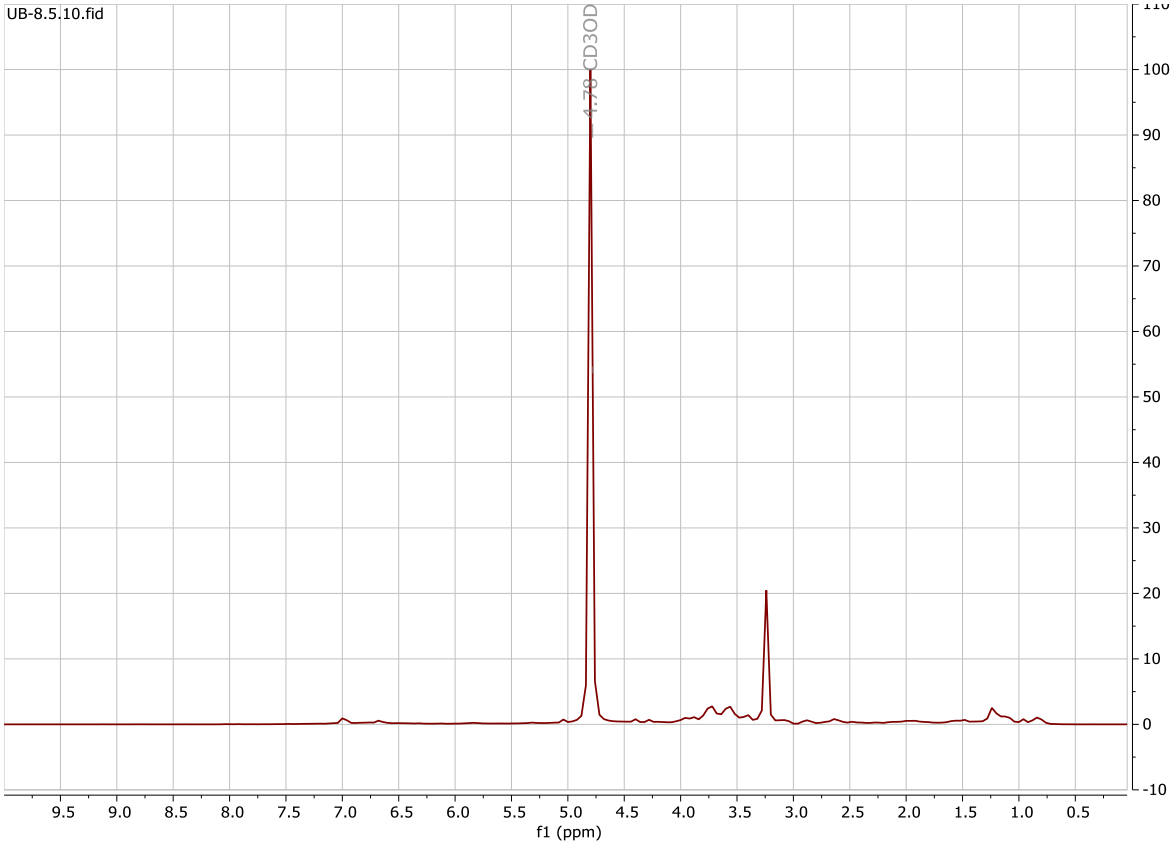


Figure A.70: ^{1}H NMR spectrum of the polyherbal formulation BC, which was stored over a period of 14 days at 25˚C.

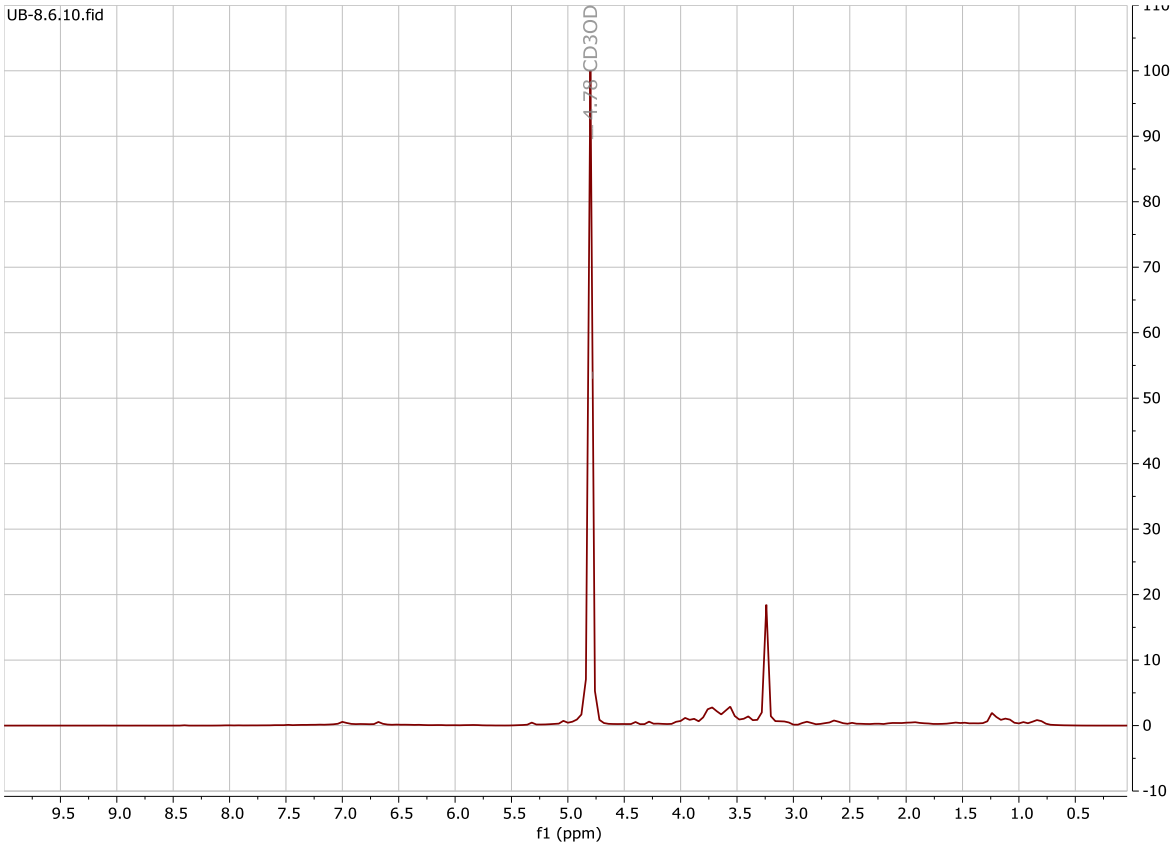


Figure A.71: ^{1}H NMR spectrum of the polyherbal formulation BC, which was stored over a period of 14 days at 37˚C.

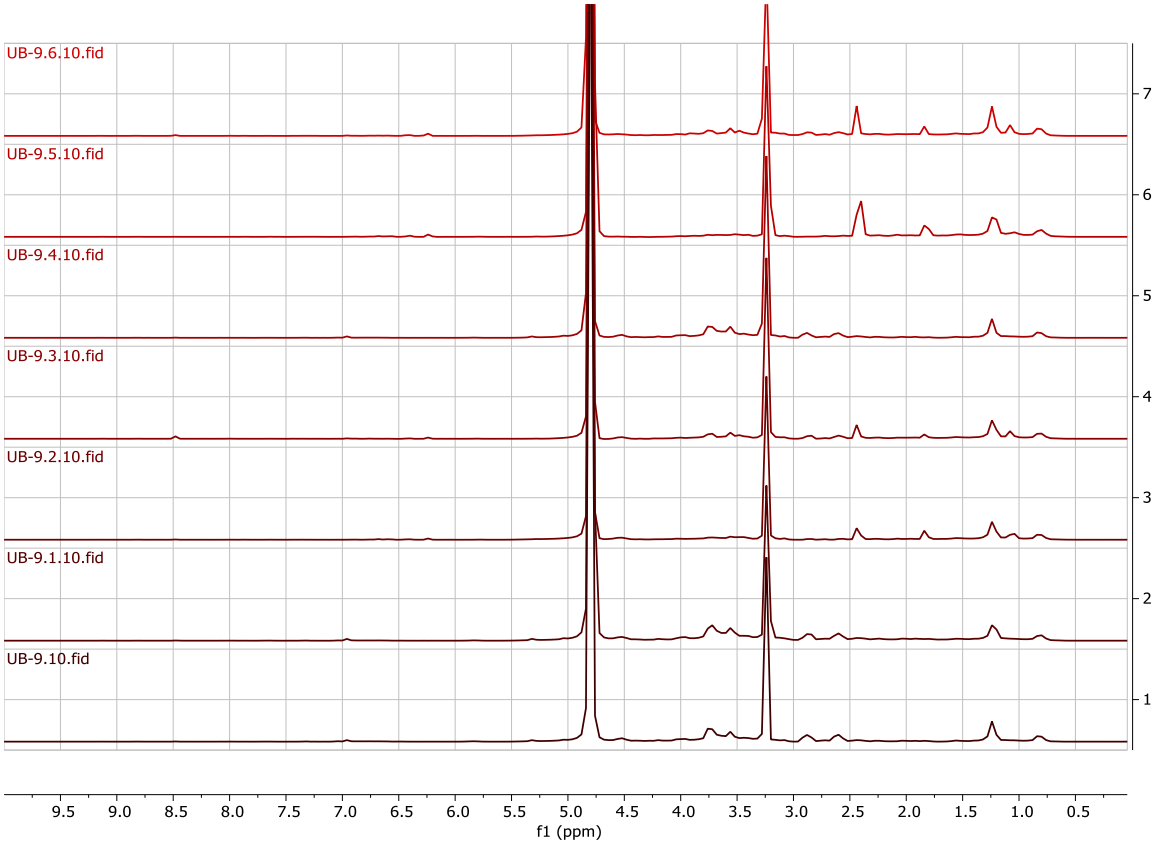


Figure A.72: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants BD

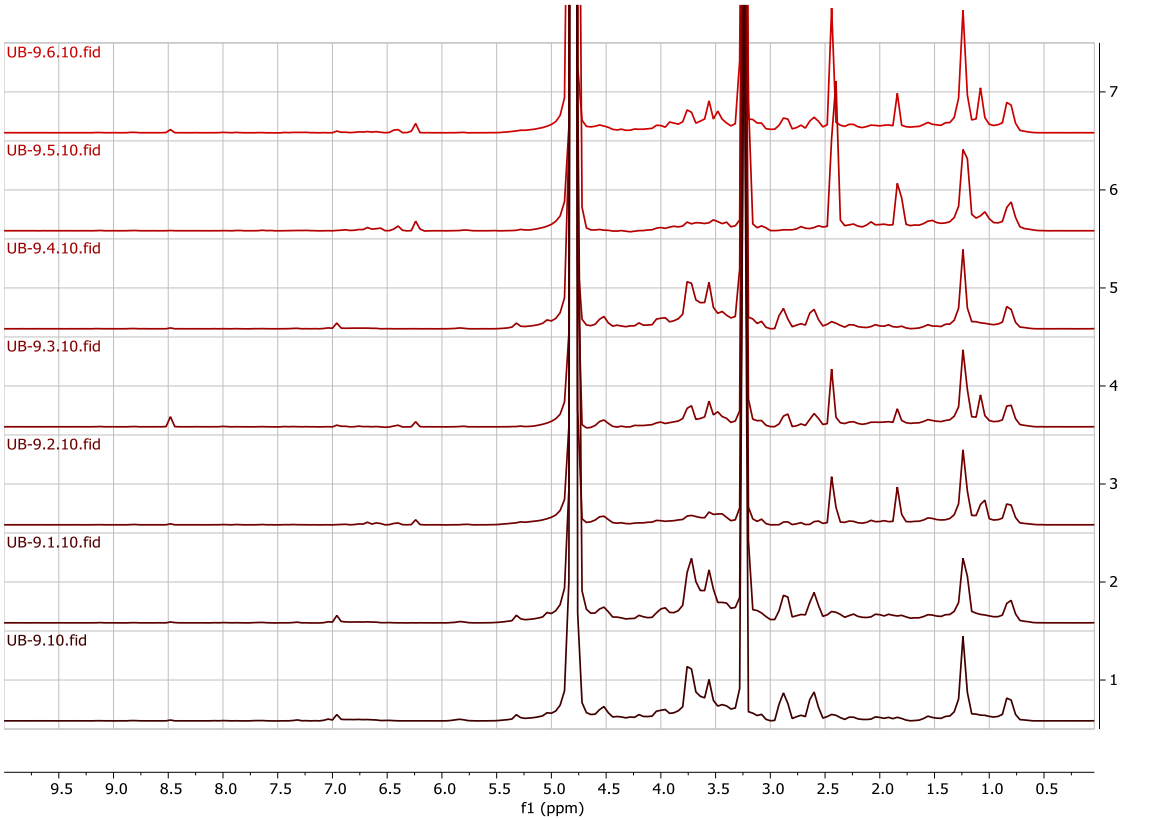


Figure A.73: Zoomed in stacked ^{1}H NMR spectra of the polyherbal formulation containing plants BD, containing combinations that were stored at various temperatures over two different storage periods

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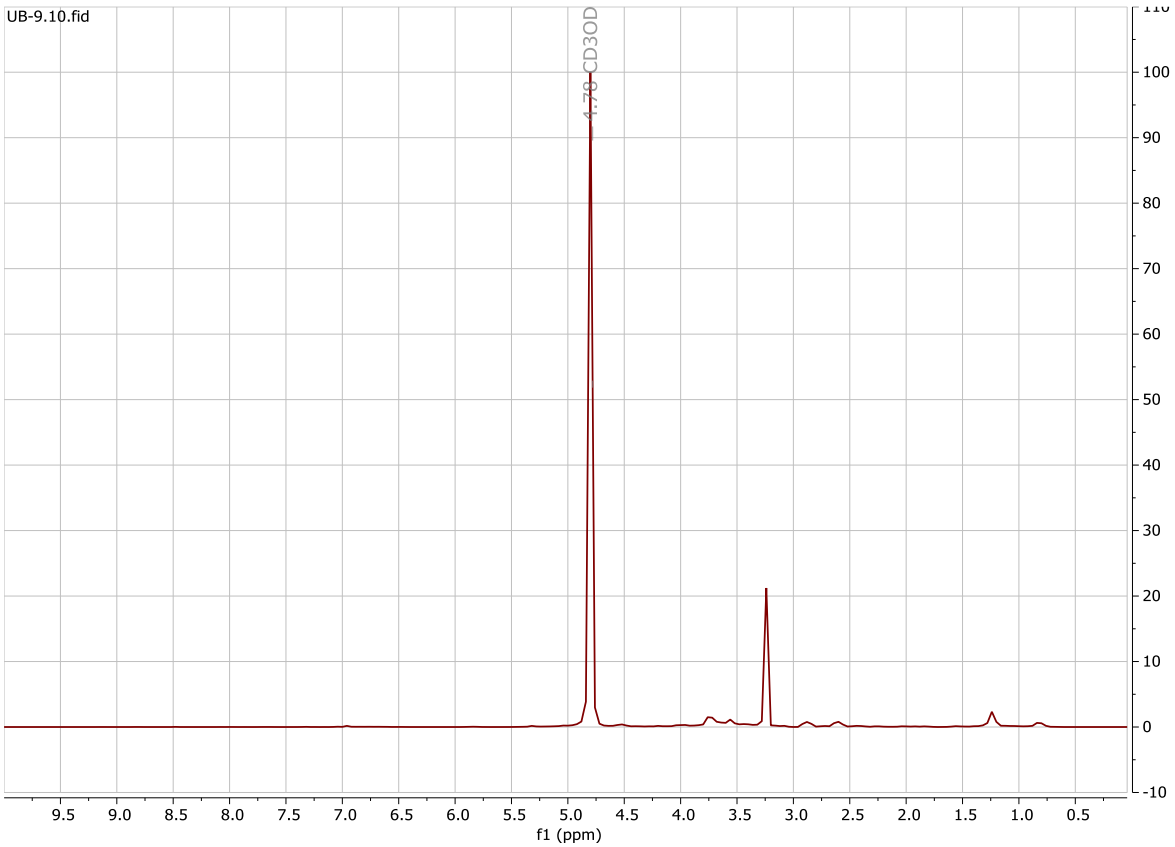

Figure A.74: ^{1}H NMR spectrum of the polyherbal formulation BD, which was not stored over a period.

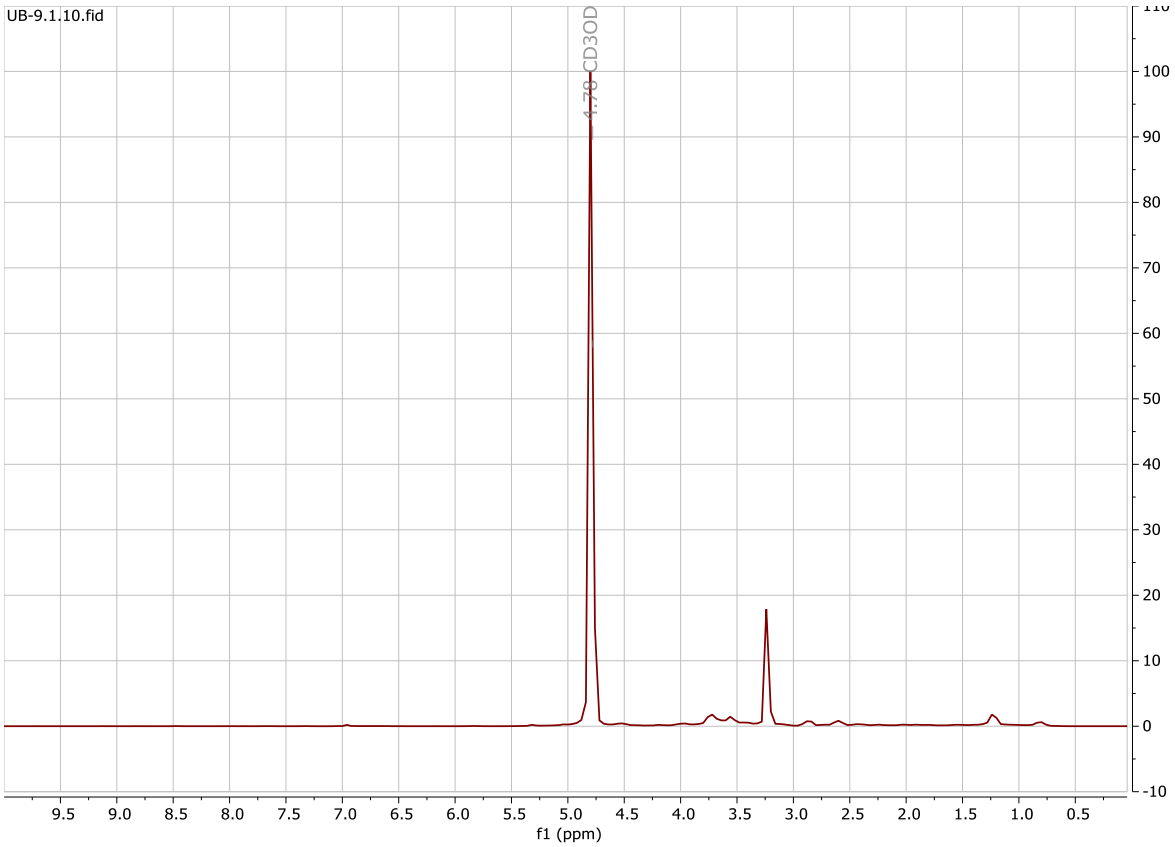

Figure A.75: ^{1}H NMR spectrum of the polyherbal formulation BD, which was stored over a period of 7 days at 4˚C.

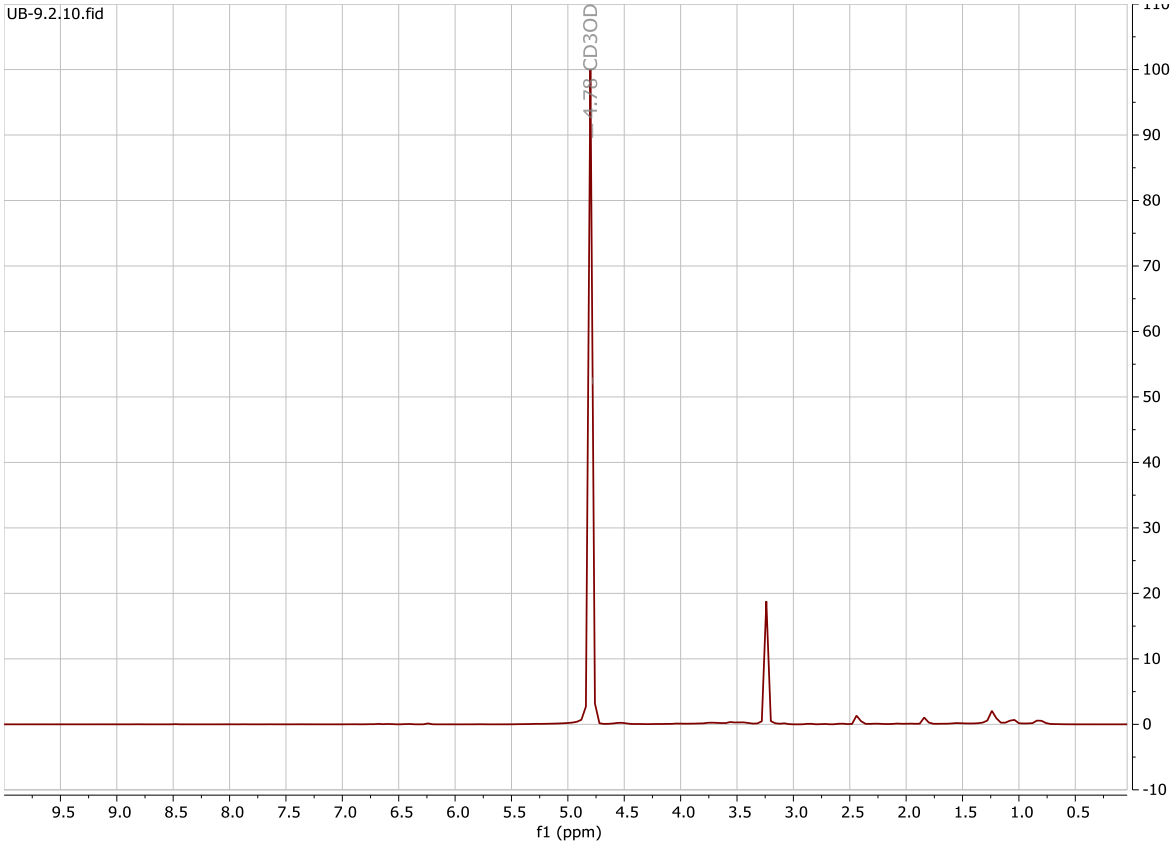

Figure A.76: ^{1}H NMR spectrum of the polyherbal formulation BD, which was stored over a period of 7 days at 25˚C.

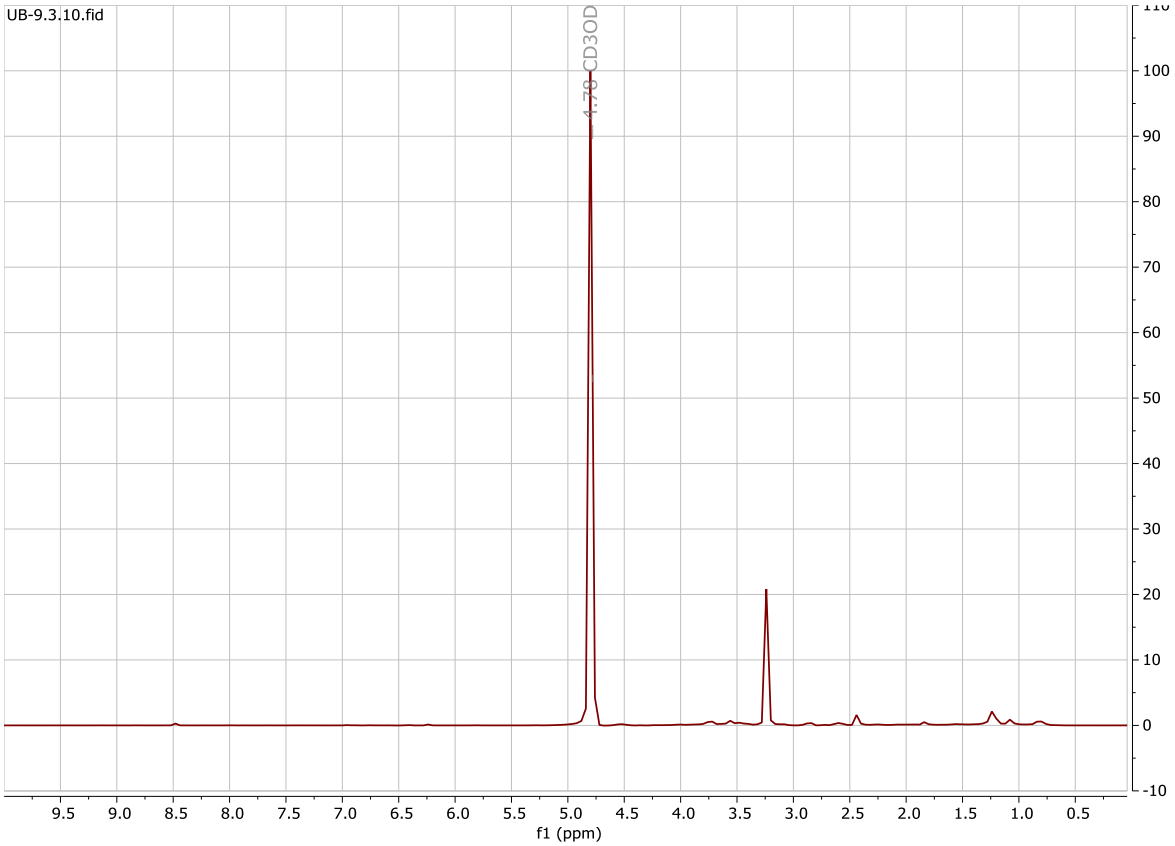

Figure A.77: ^{1}H NMR spectrum of the polyherbal formulation BD, which was stored over a period of 7 days at 37˚C.

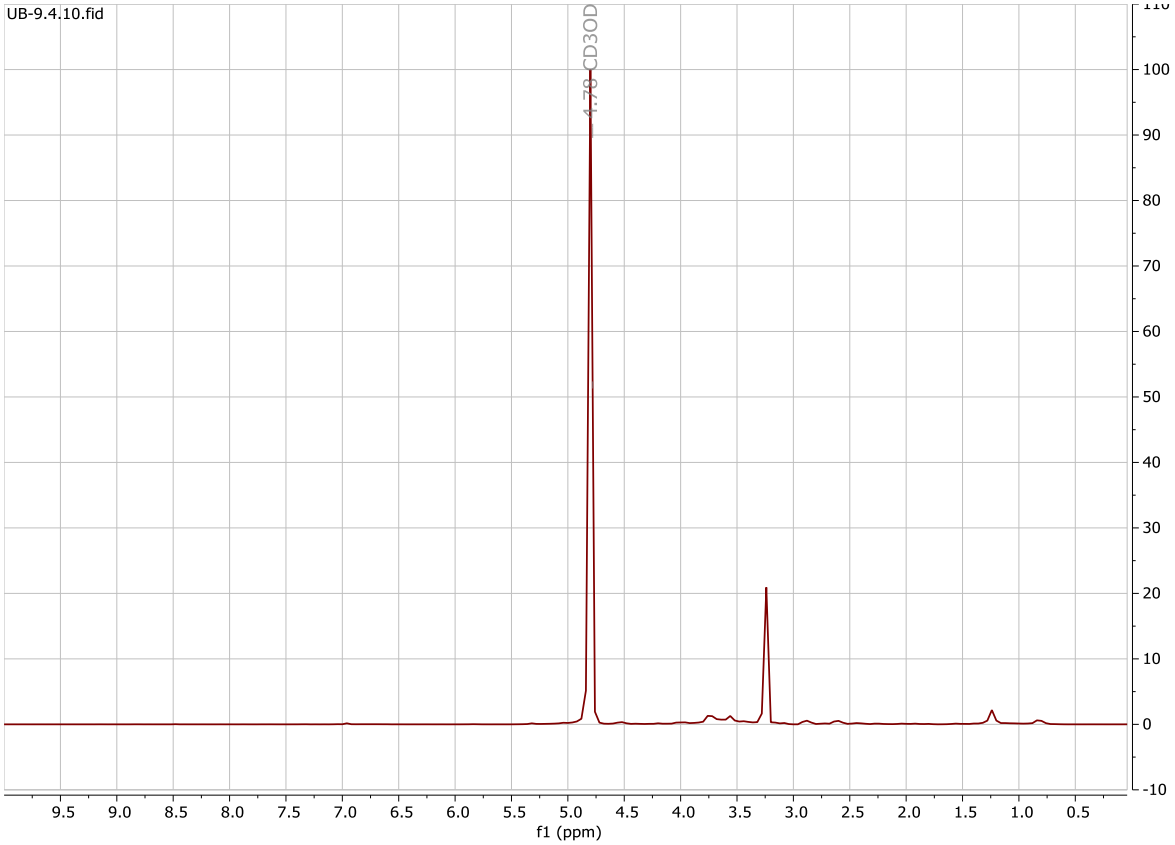

Figure A.78: ^{1}H NMR spectrum of the polyherbal formulation BD, which was stored over a period of 14 days at 4˚C.

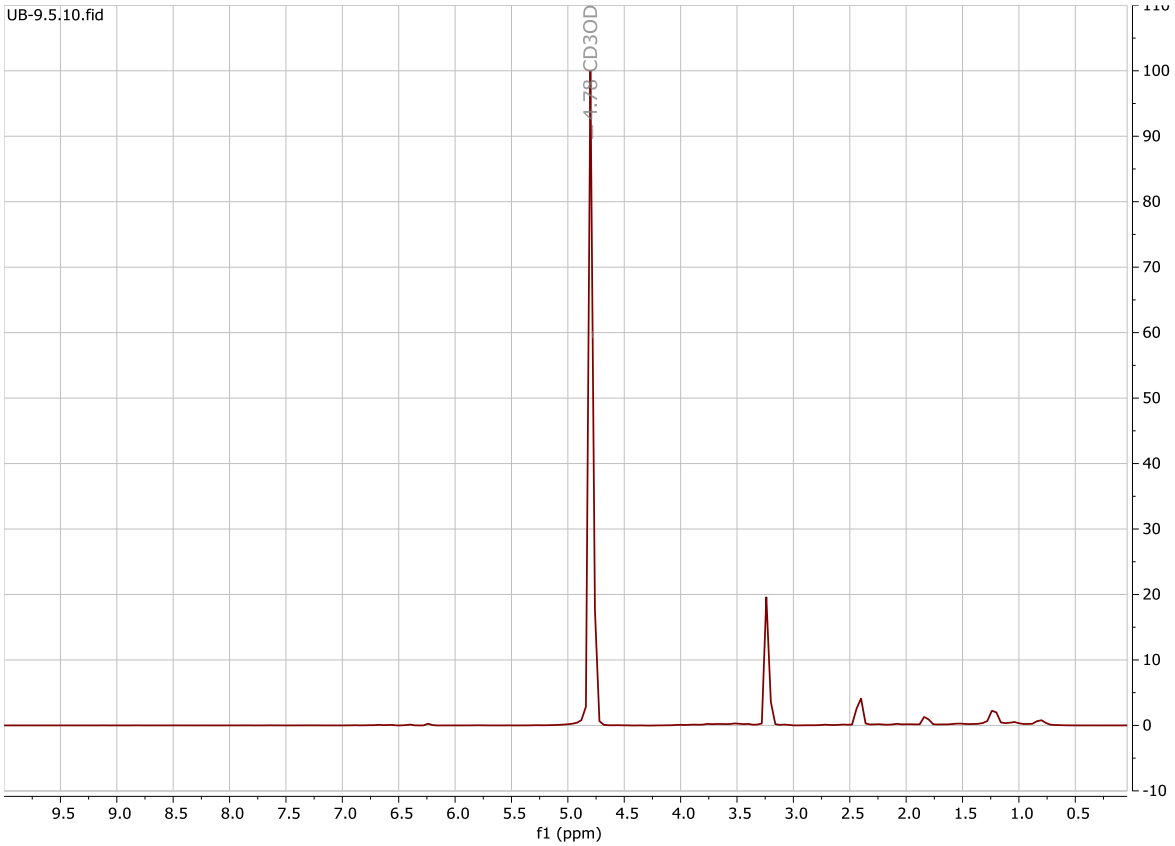

Figure A.79: ^{1}H NMR spectrum of the polyherbal formulation BD, which was stored over a period of 14 days at 25˚C.

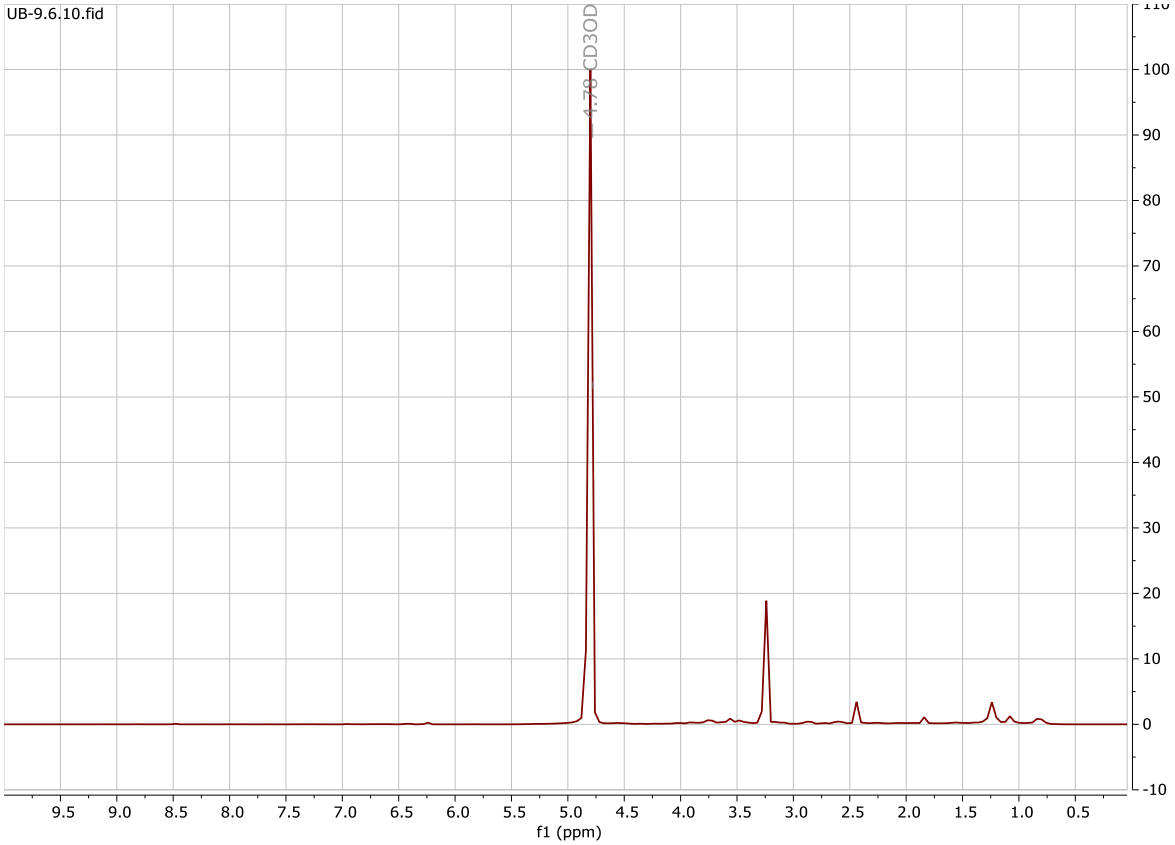

Figure A.80: ^{1}H NMR spectrum of the polyherbal formulation BD, which was stored over a period of 14 days at 37˚C.

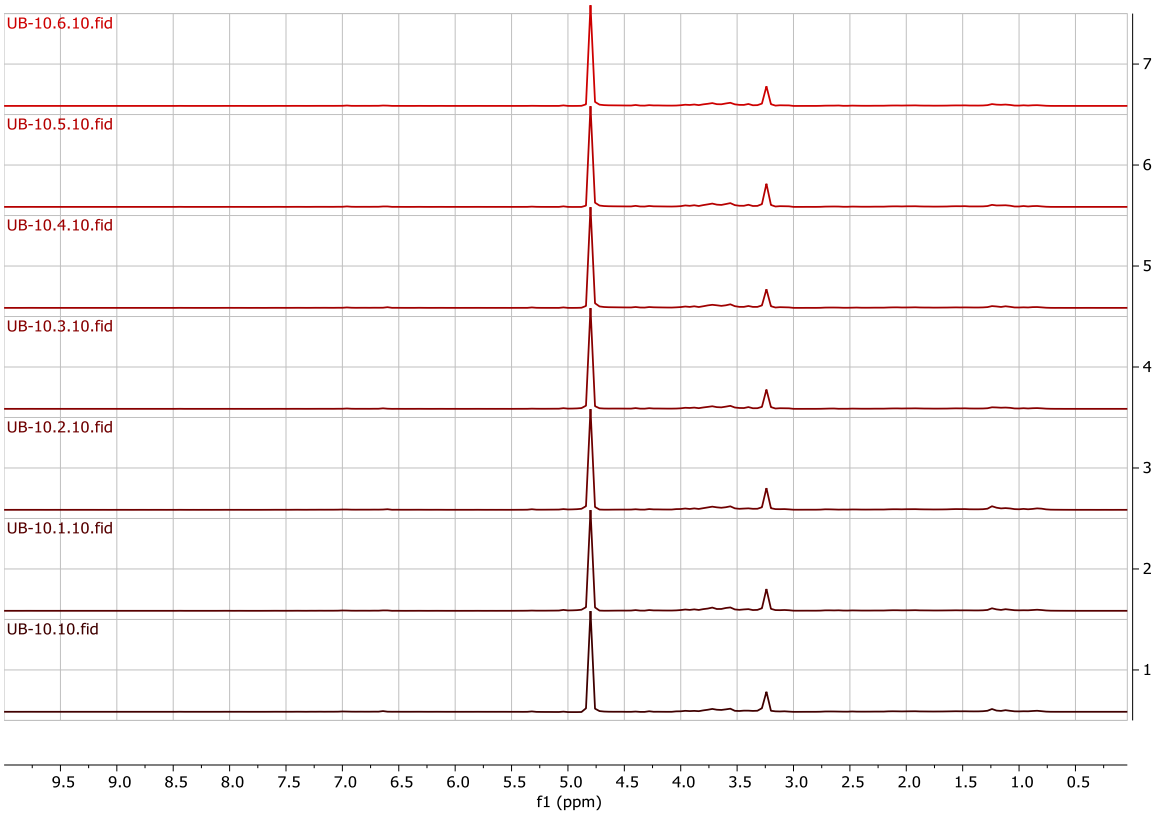

Figure A.81: Stacked ^{1}H NMR spectra of polyherbal formulation containing medicinal plants CD.

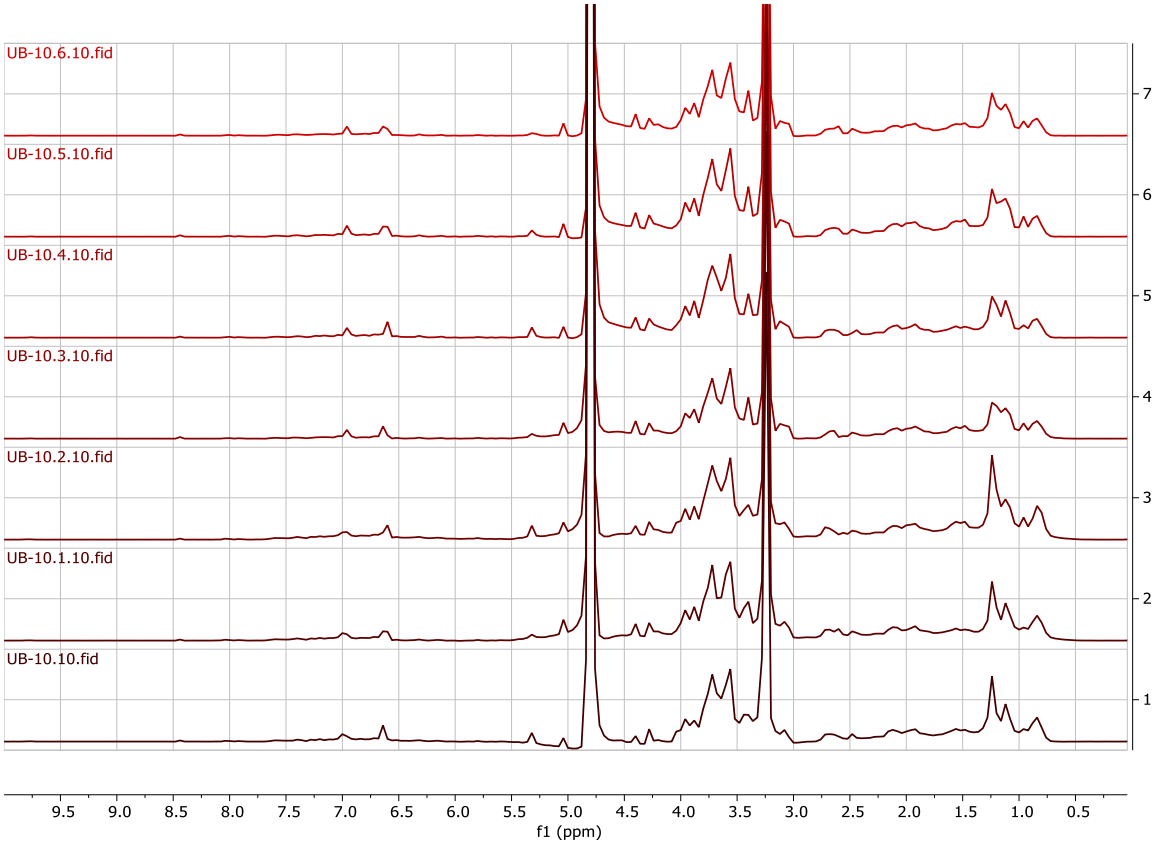

Figure A.82: Zoomed in stacked ^{1}H NMR spectra of the polyherbal formulation containing plants CD, containing combinations that were stored at various temperatures over two different storage periods.

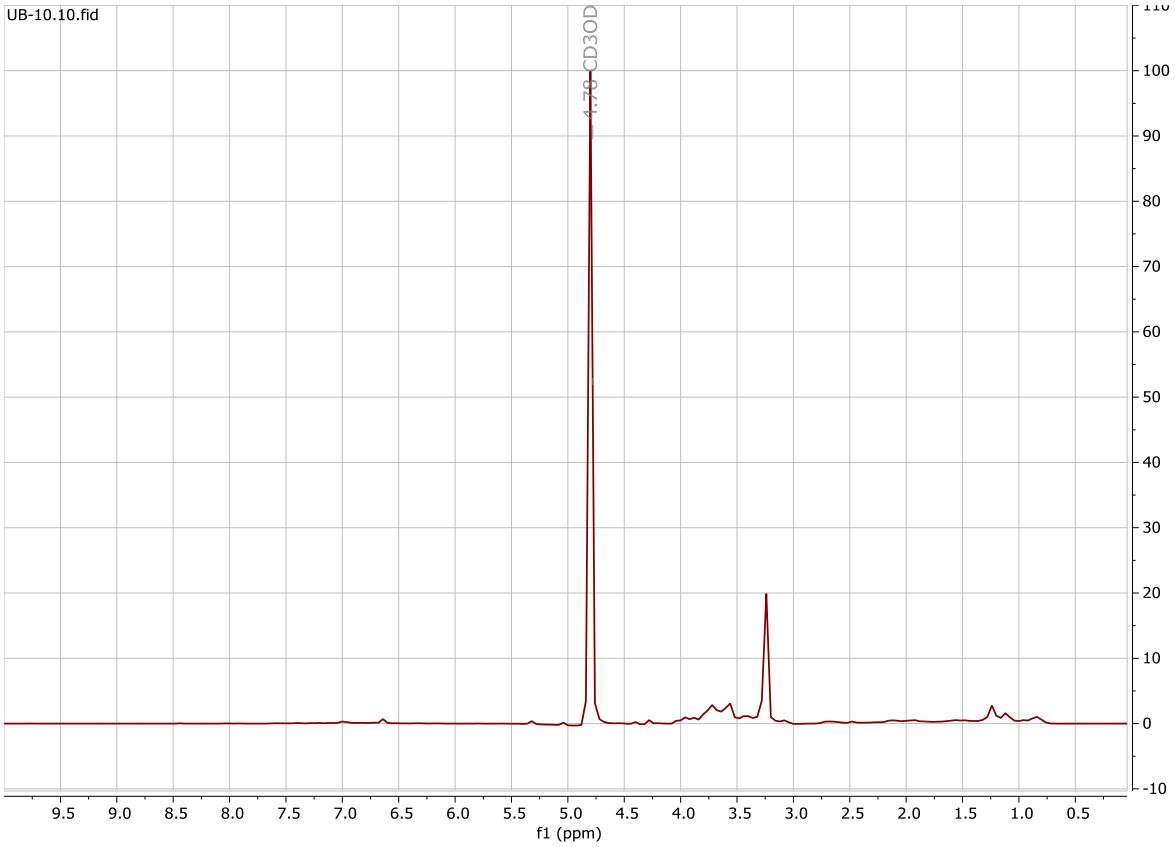

Figure A.83: ^{1}H NMR spectrum of the polyherbal formulation CD, which was not stored over a period.

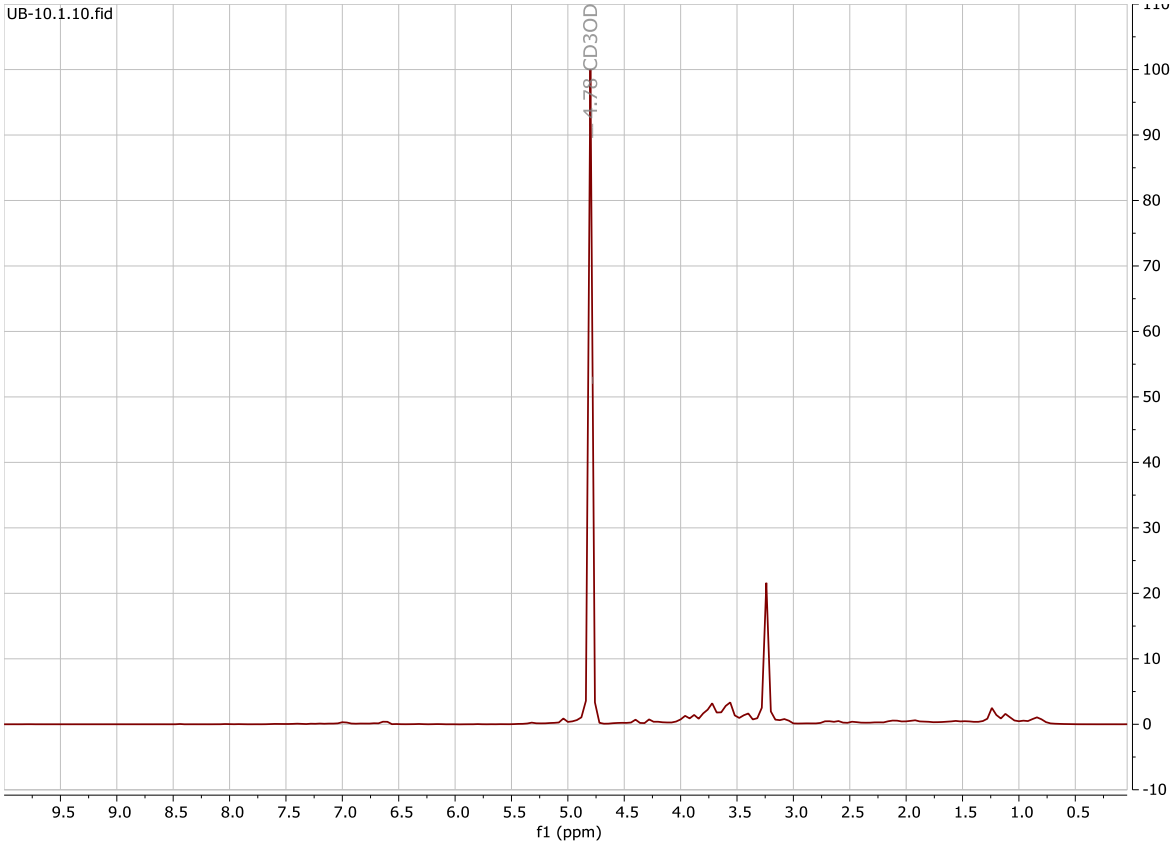

Figure A.84: ^{1}H NMR spectrum of the polyherbal formulation CD, which was stored over a period of 7 days at 4˚C.

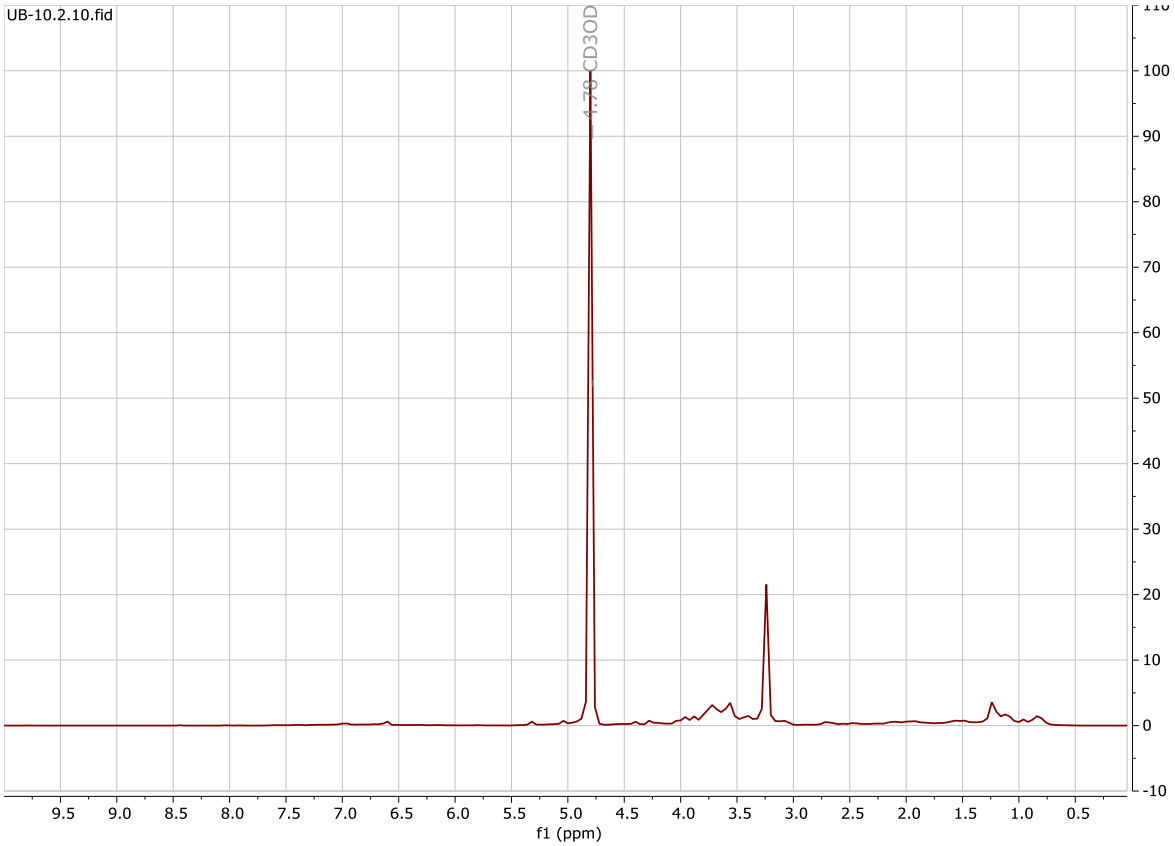

Figure A.85: ^{1}H NMR spectrum of the polyherbal formulation CD, which was stored over a period of 7 days at 25˚C.

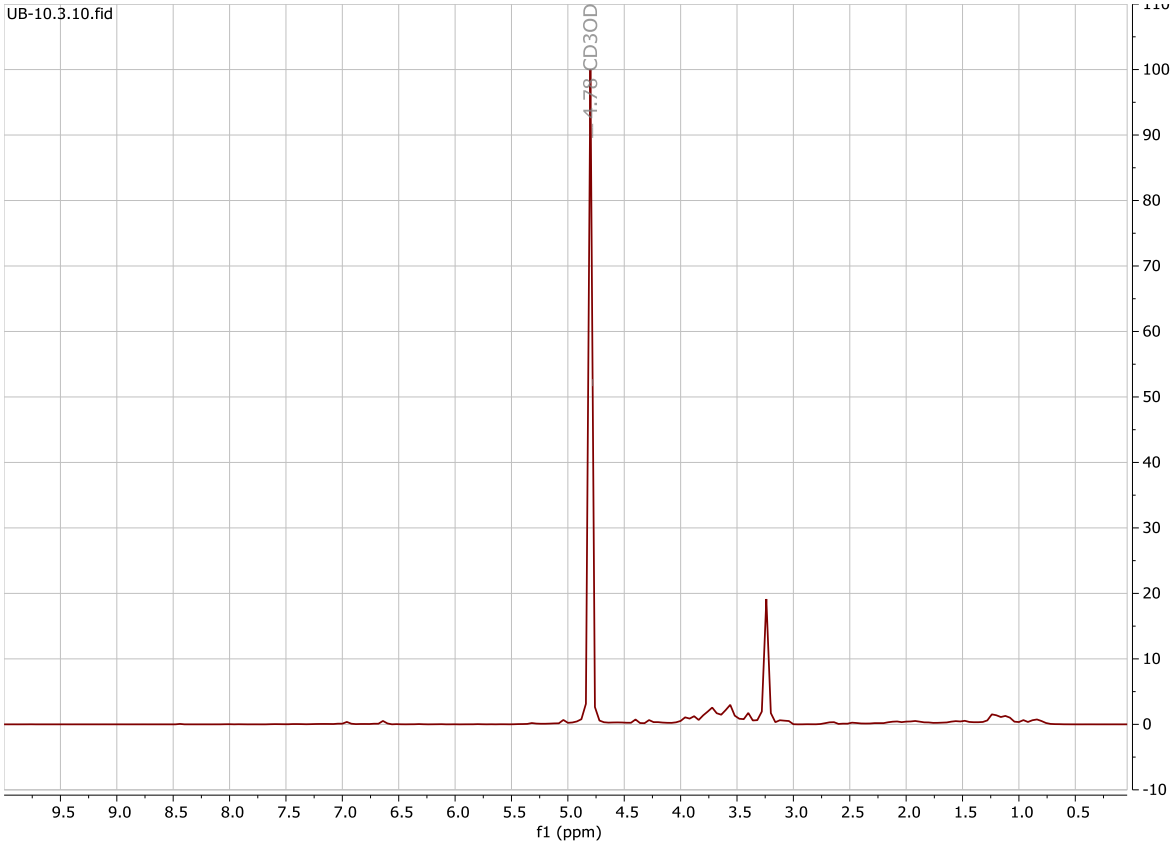

Figure A.86: ^{1}H NMR spectrum of the polyherbal formulation CD, which was stored over a period of 7 days at 37˚C.

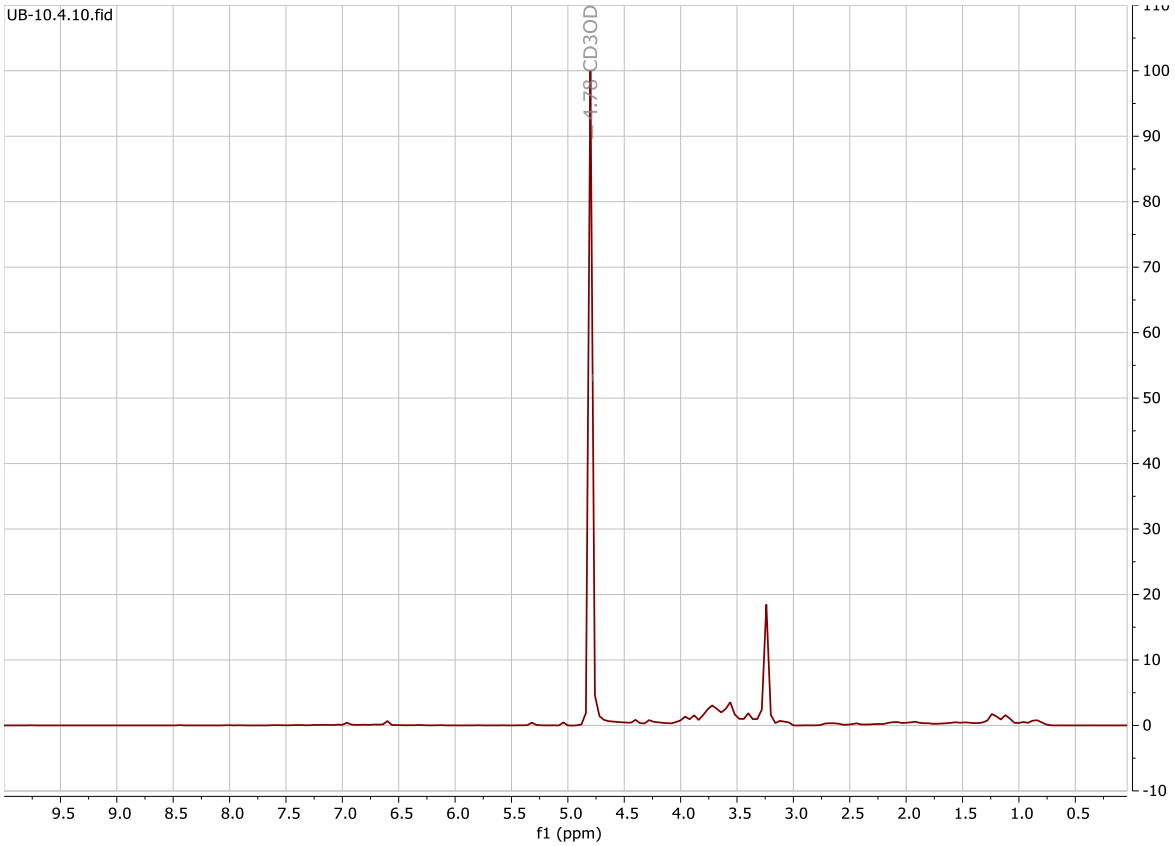

Figure A.87: ^{1}H NMR spectrum of the polyherbal formulation CD, which was stored over a period of 14 days at 4˚C.

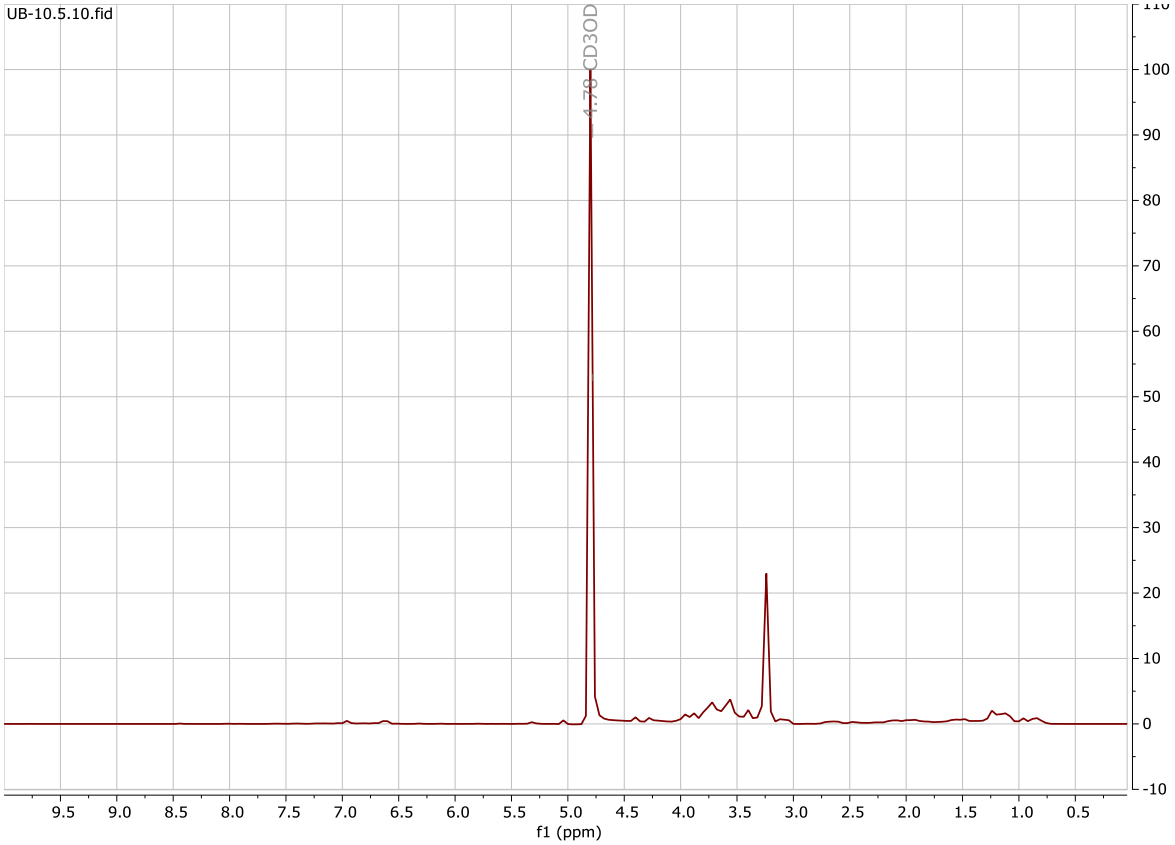

Figure A.88: ^{1}H NMR spectrum of the polyherbal formulation CD, which was stored over a period of 14 days at 25˚C.

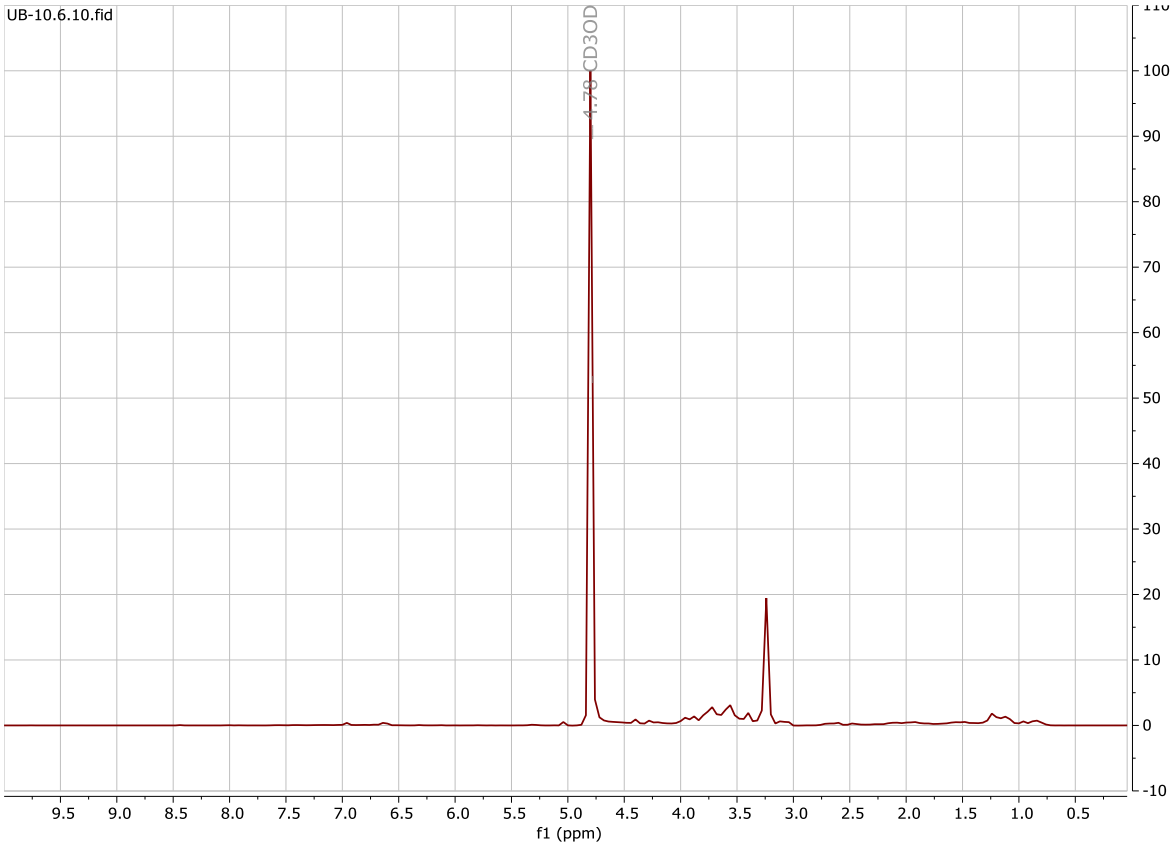

Figure A.89: ^{1}H NMR spectrum of the polyherbal formulation CD, which was stored over a period of 14 days at 37˚C.

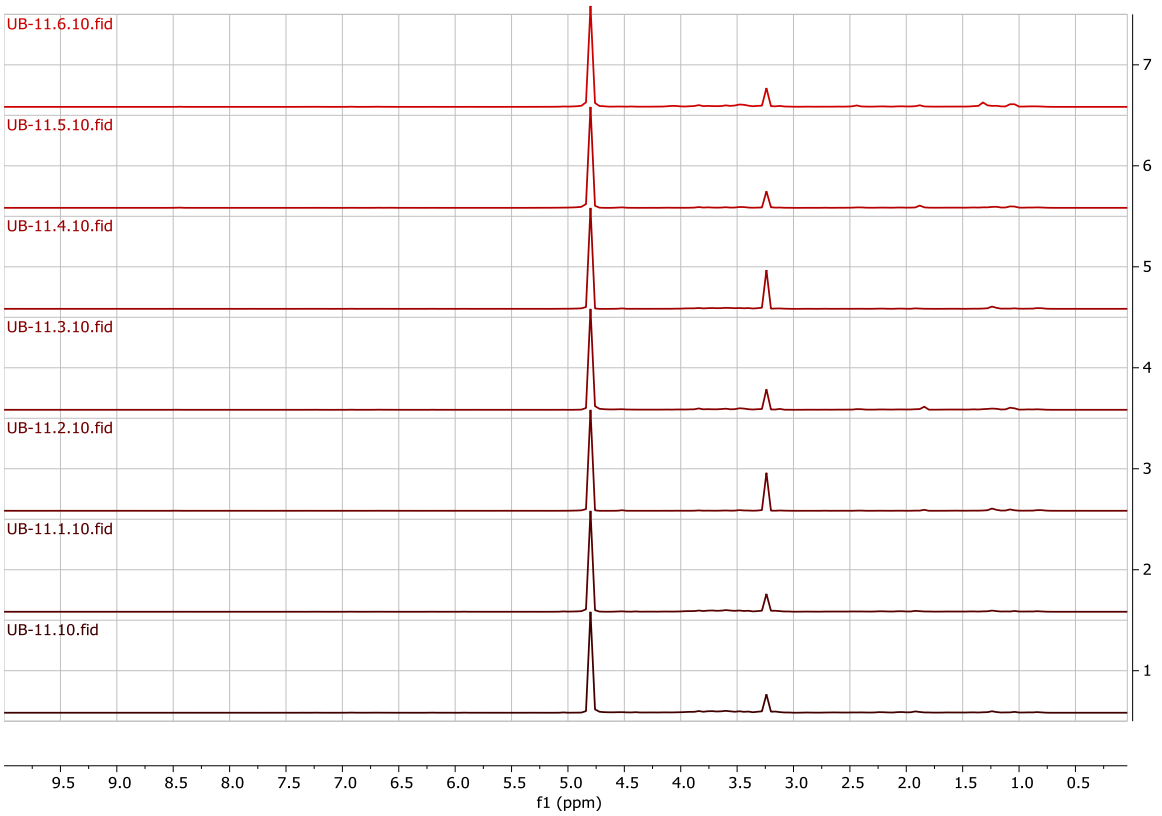

Figure A.90: Stacked ^{1}H NMR spectra of *Dicoma anomala Sond. subsp anomala,* stored under various conditions.

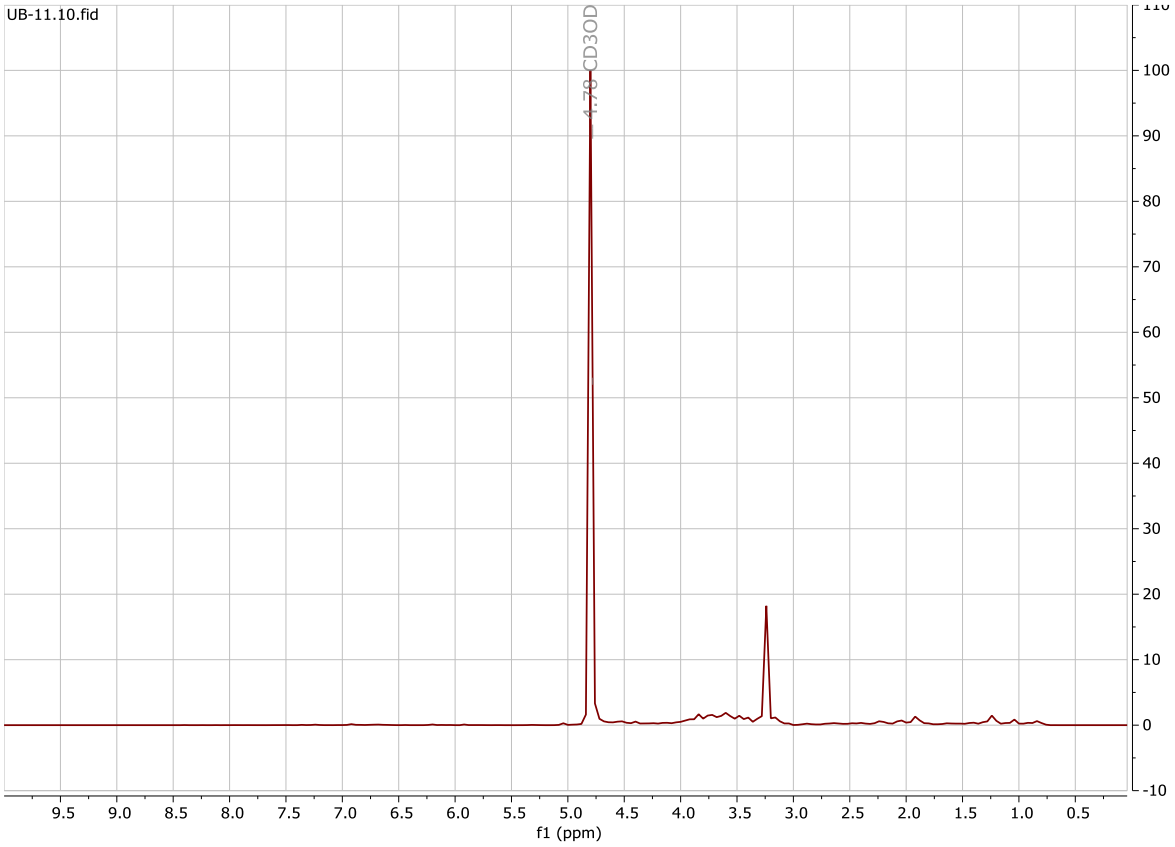

Figure A.91: ^{1}H NMR spectrum of *Dicoma anomala Sond. subsp anomala,* which was not stored.

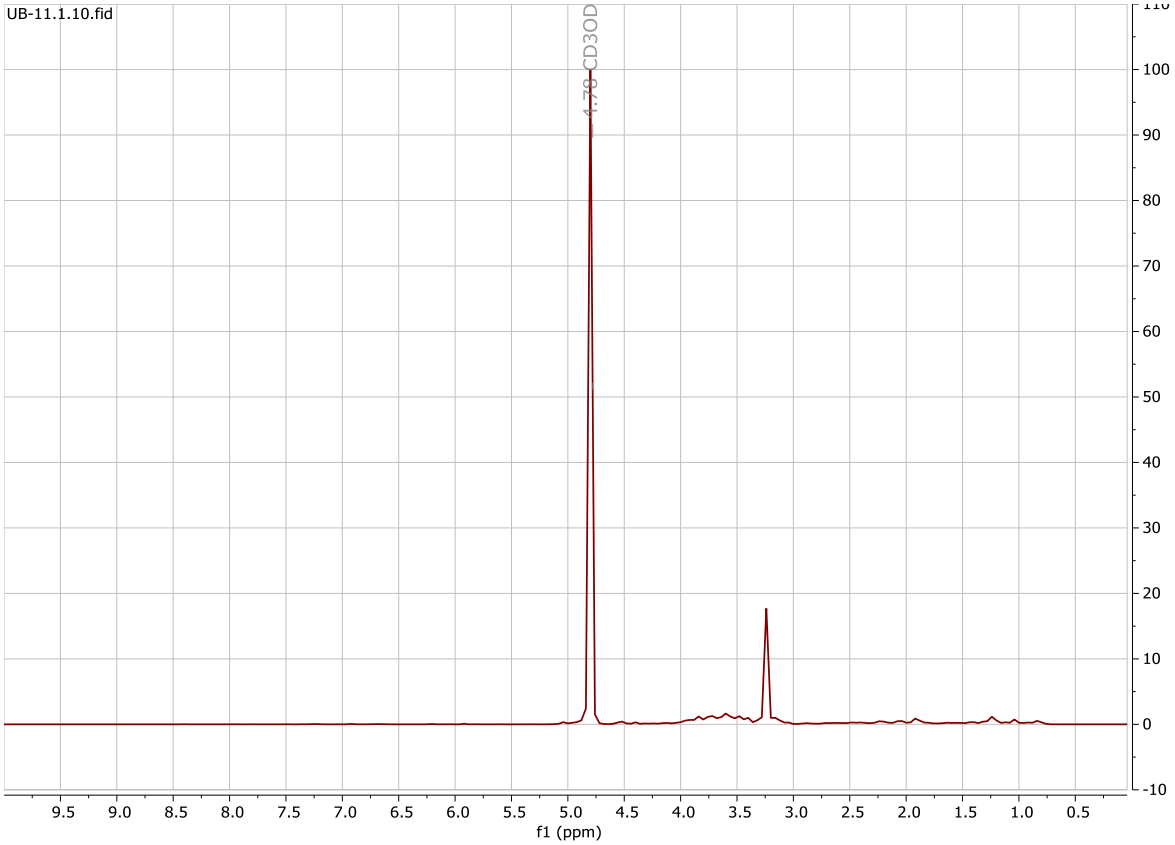

Figure A.92: ^{1}H NMR spectrum of *Dicoma anomala Sond. subsp anomala,* which was stored for 7 days at 4˚C.

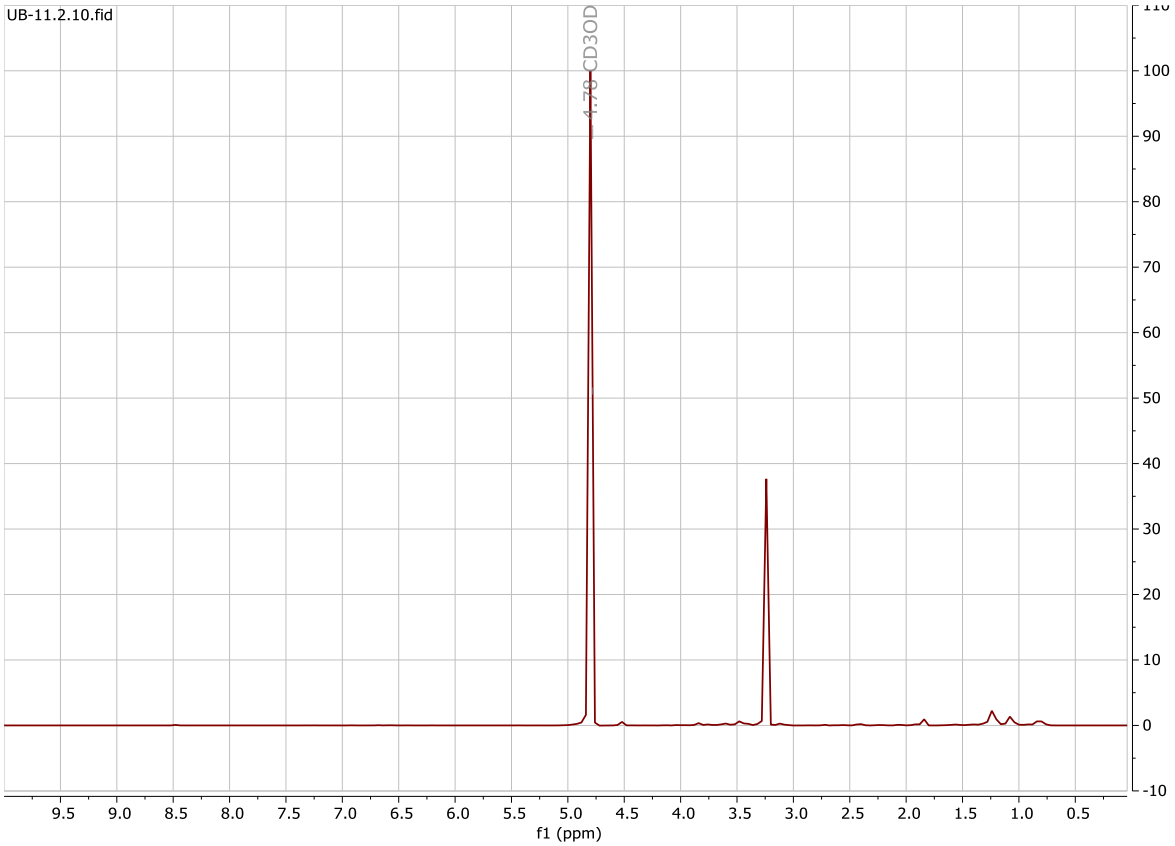

Figure A.93: ^{1}H NMR spectrum of *Dicoma anomala Sond. subsp anomala,* which was stored for 7 days at 25˚C.

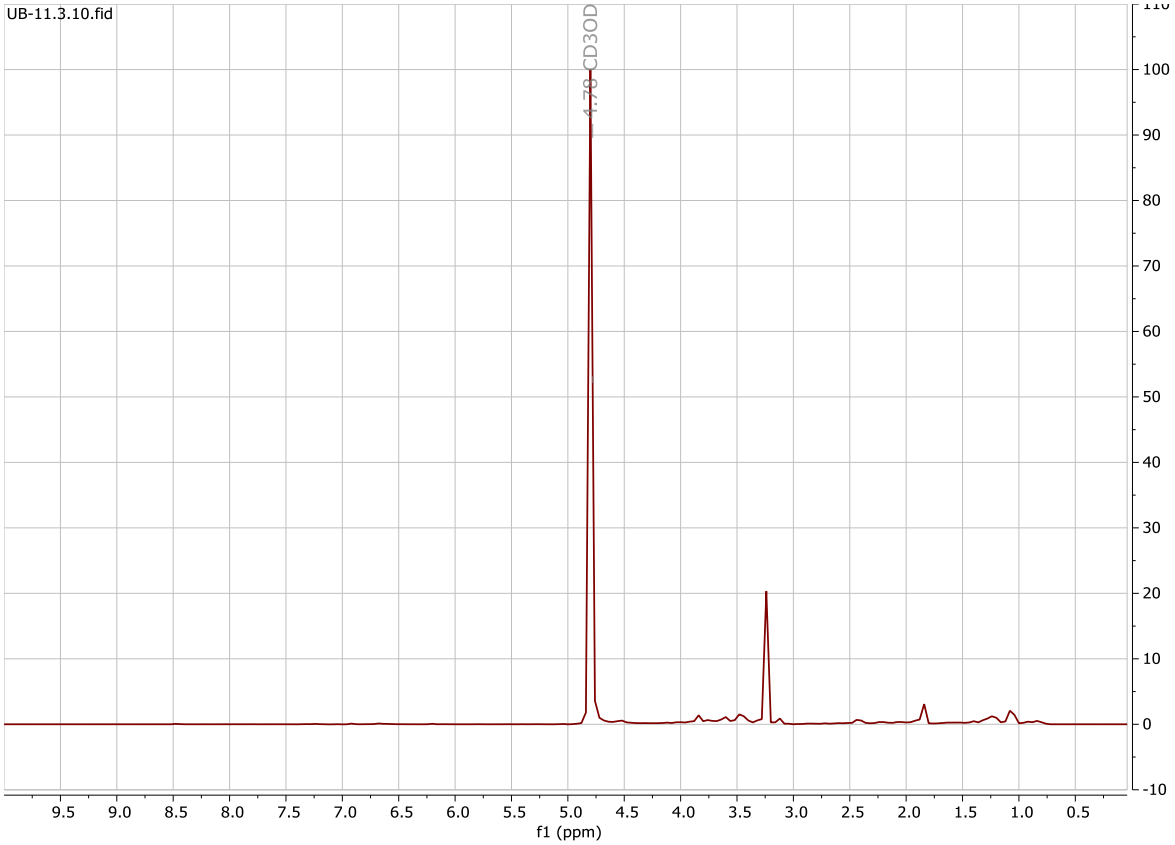

Figure A.94: ^{1}H NMR spectrum of *Dicoma anomala Sond. subsp anomala,* which was stored for 7 days at 37˚C.

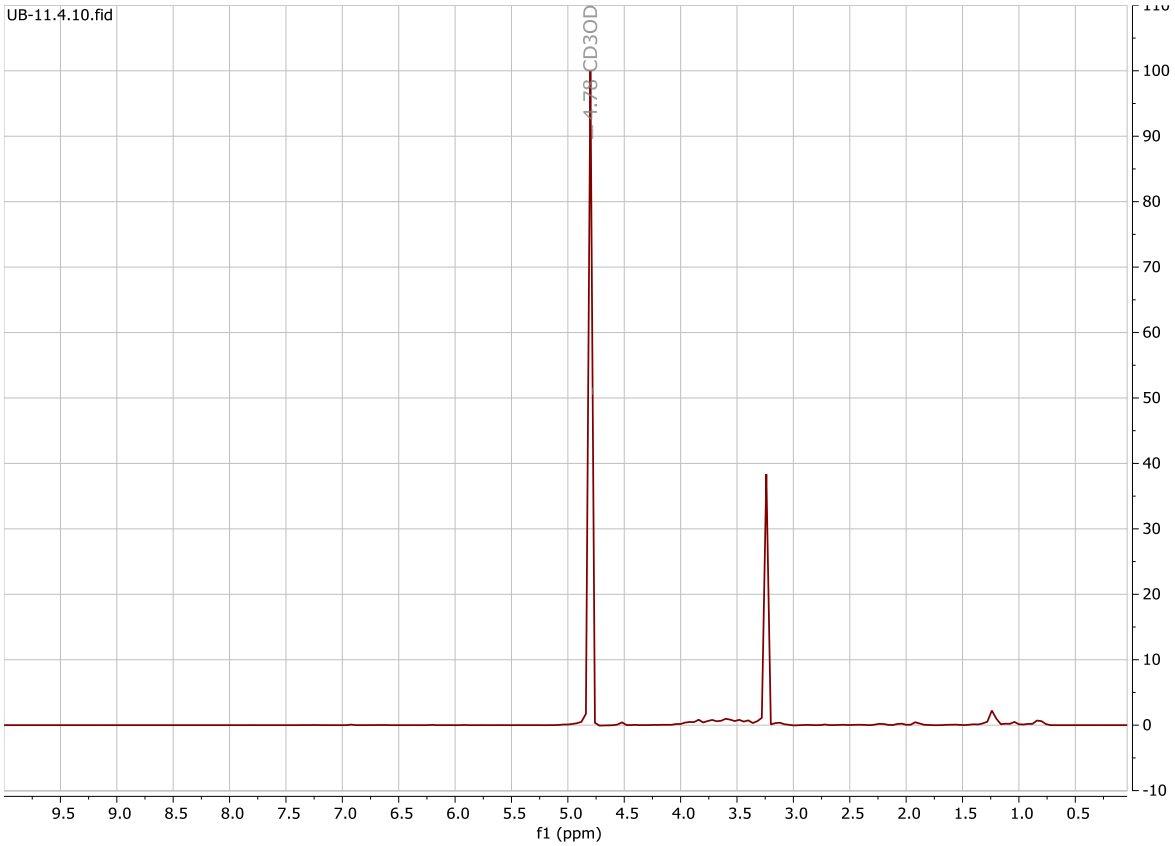

Figure A.95: ^{1}H NMR spectrum of *Dicoma anomala Sond. subsp anomala*, which was stored for 14 days at 25˚C.

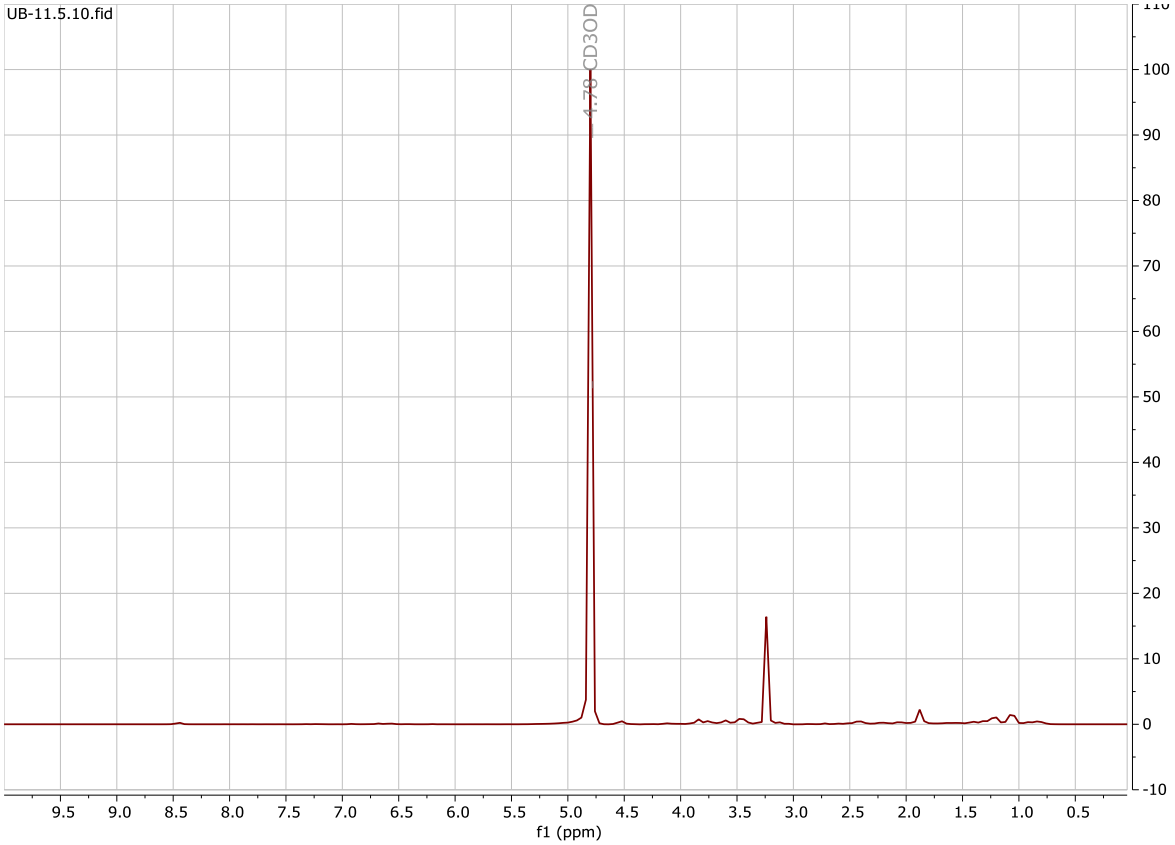

Figure A.96: ^{1}H NMR spectrum of *Dicoma anomala Sond. subsp anomala,* which was stored for 14 days at 25˚C.

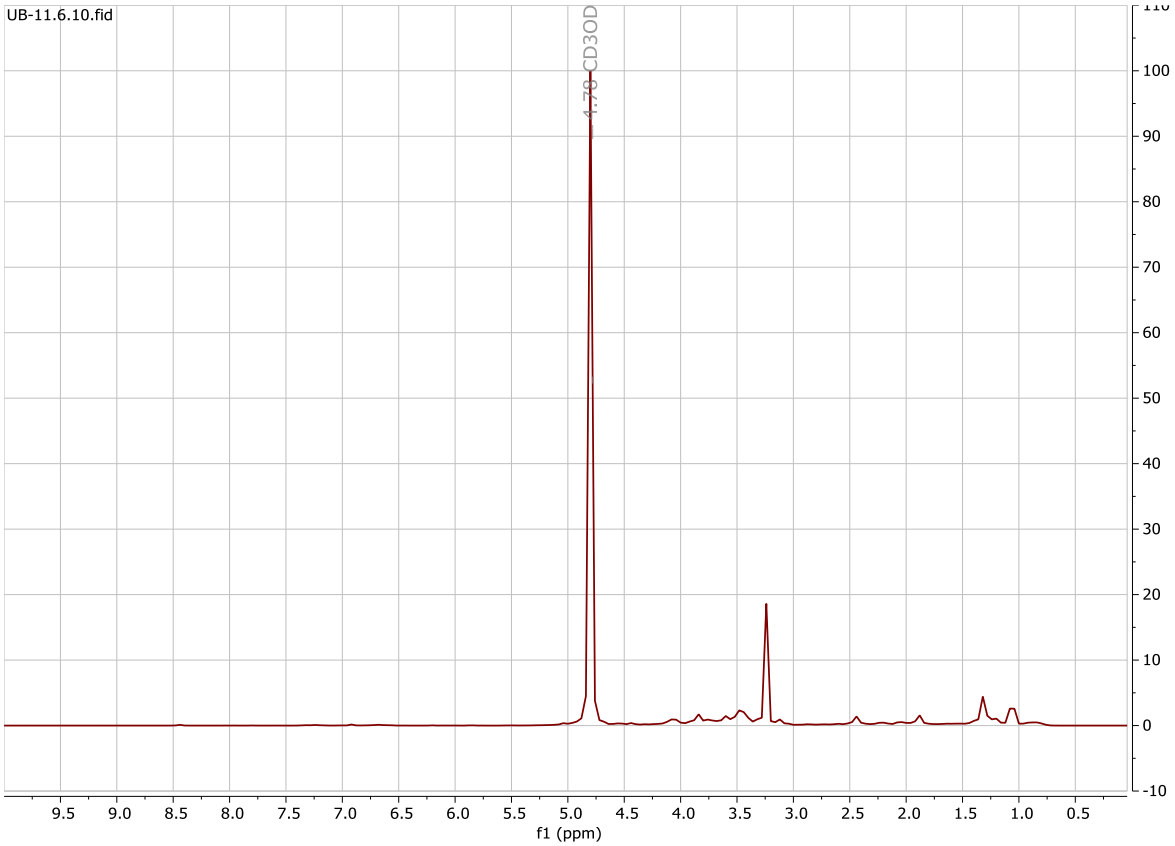

Figure A.97: ^{1}H NMR spectrum of *Dicoma anomala Sond. subsp anomala,* which was stored for 14 days at 25˚C.

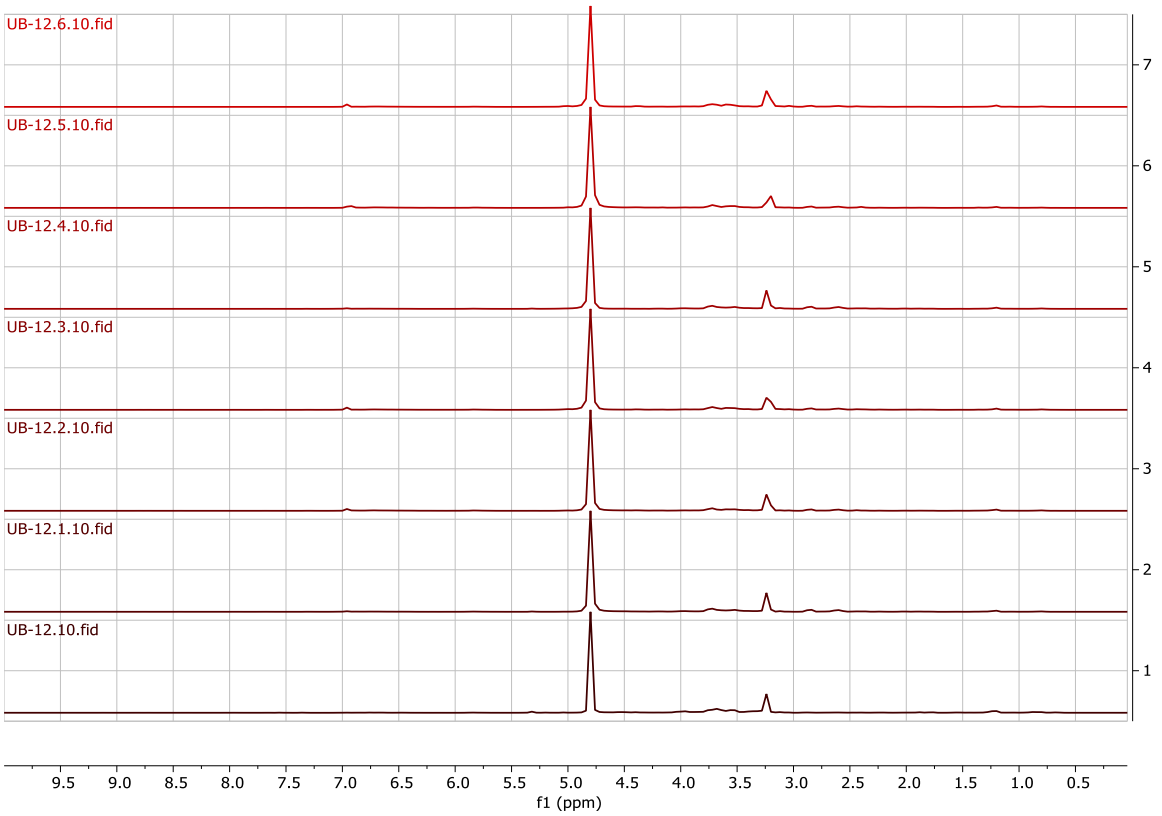

Figure A.98: Stacked ^{1}H NMR spectra of *Elephantorrhiza elephantina,* stored under various conditions.

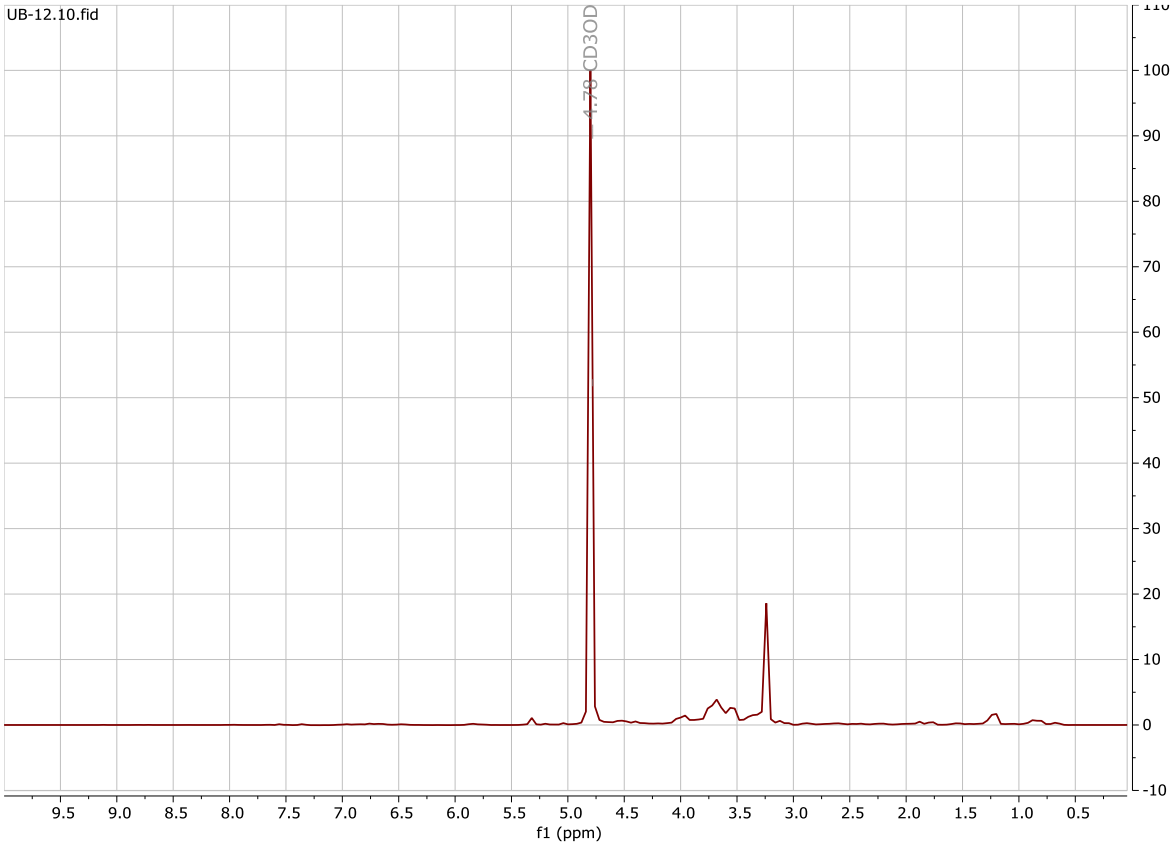

Figure A.99: ^{1}H NMR spectrum of *Elephantorrhiza elephantina*, which was not stored.

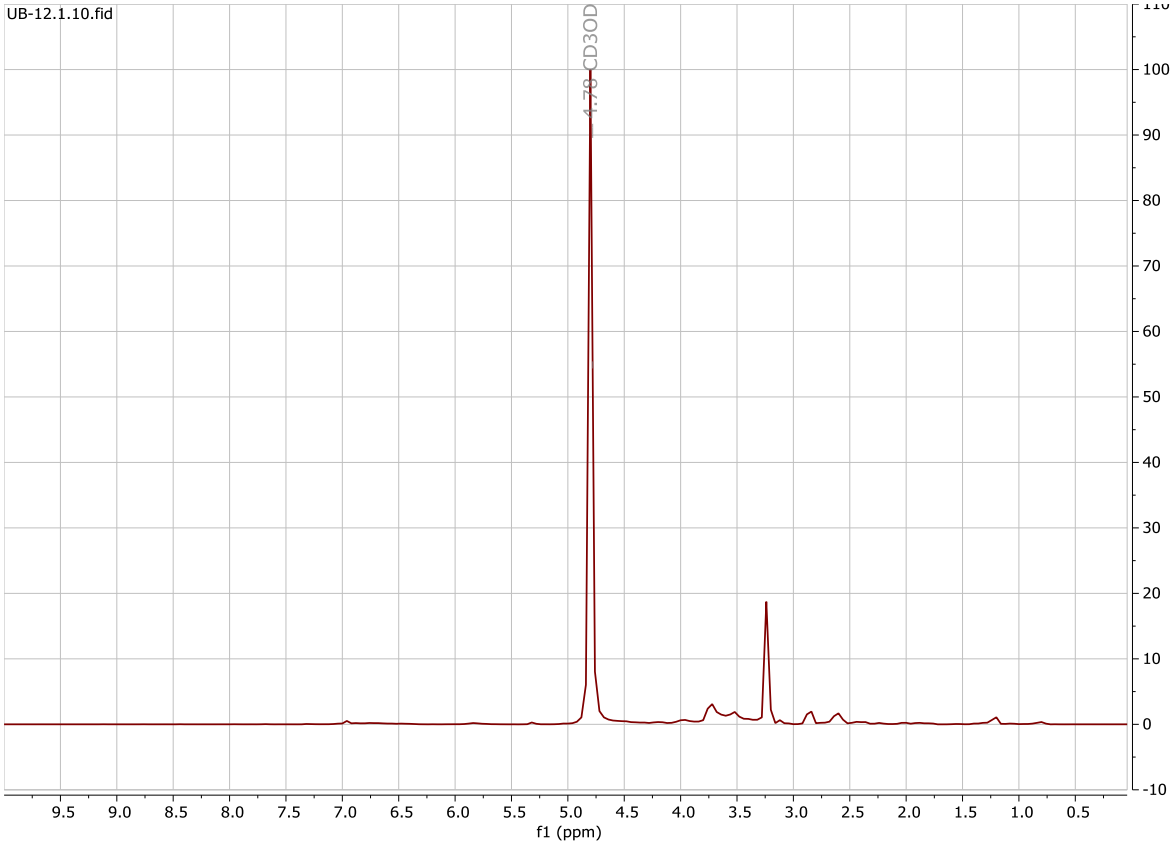

Figure A.100: ^{1}H NMR spectrum of *Elephantorrhiza elephantina,* which was stored for 7 days at 4˚C.

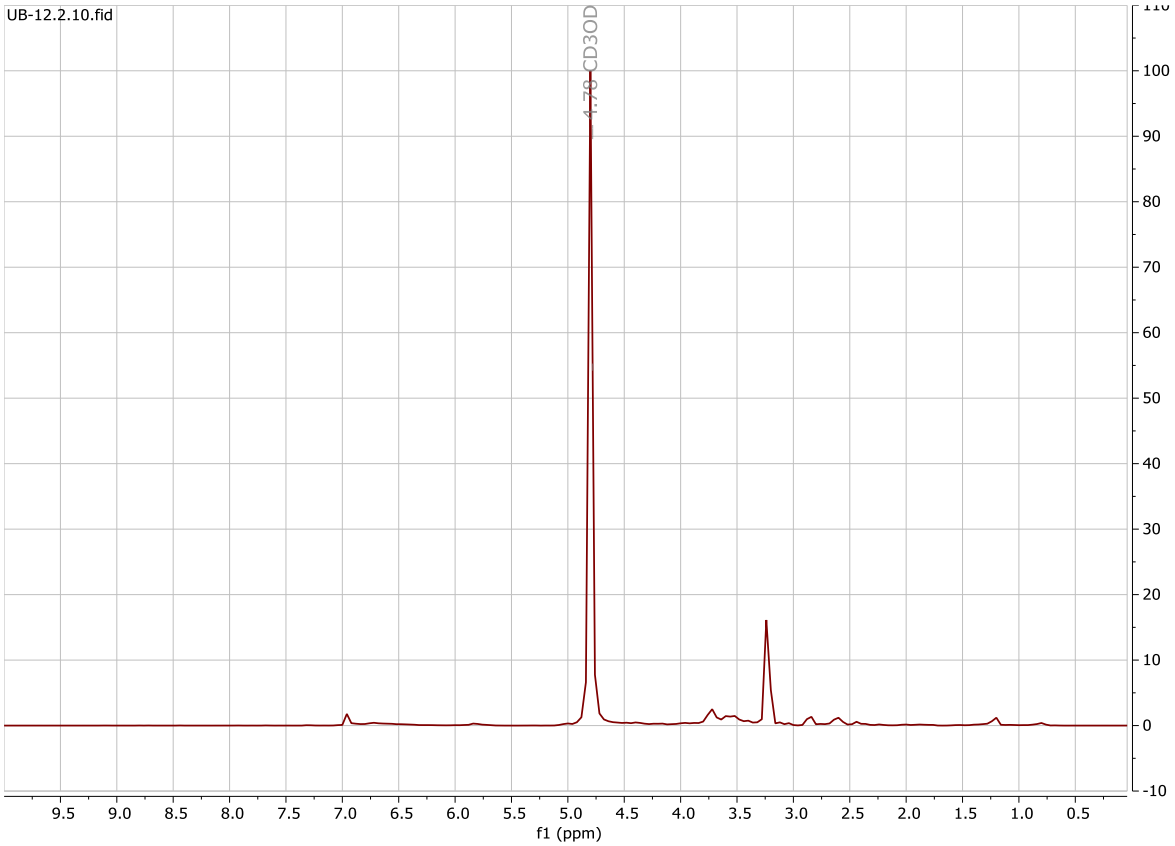

Figure A.101*:* ^{1}H NMR spectrum of *Elephantorrhiza elephantina,* which was stored for 7 days at 25˚C.

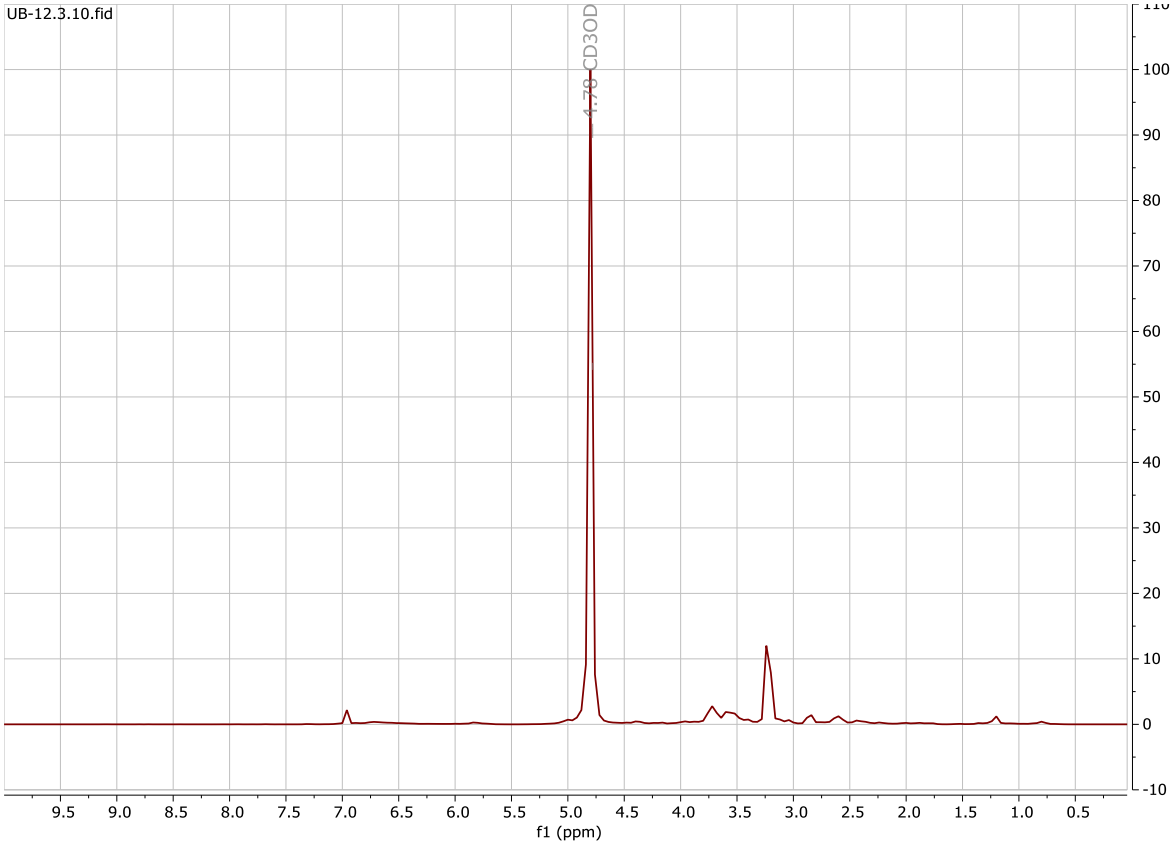

Figure A.102*:* ^{1}H NMR spectrum of *Elephantorrhiza elephantina,* which was stored for 7 days at 37˚C.

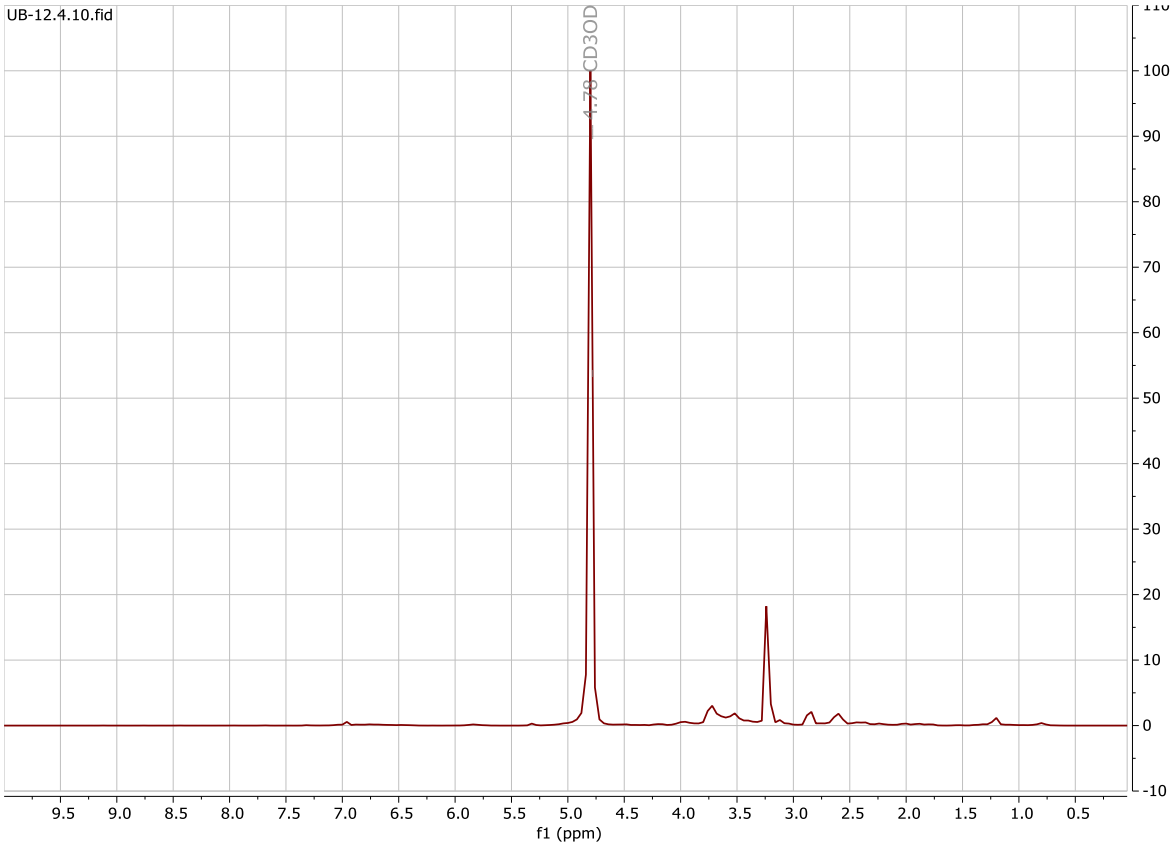

Figure A.103*:* ^{1}H NMR spectrum of *Elephantorrhiza elephantina,* which was stored for 14 days at 4˚C.

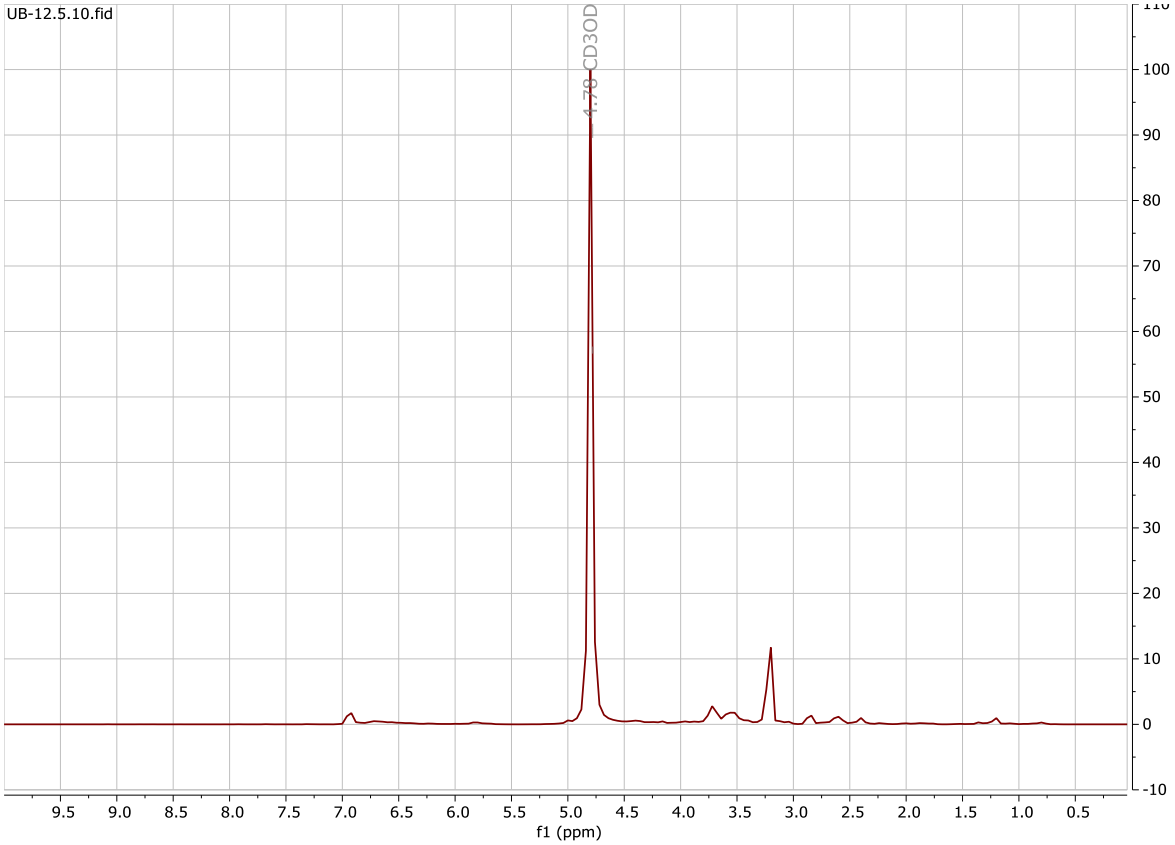

Figure A.104*:* ^{1}H NMR spectrum of *Elephantorrhiza elephantina,* which was stored for 14 days at 25˚C.

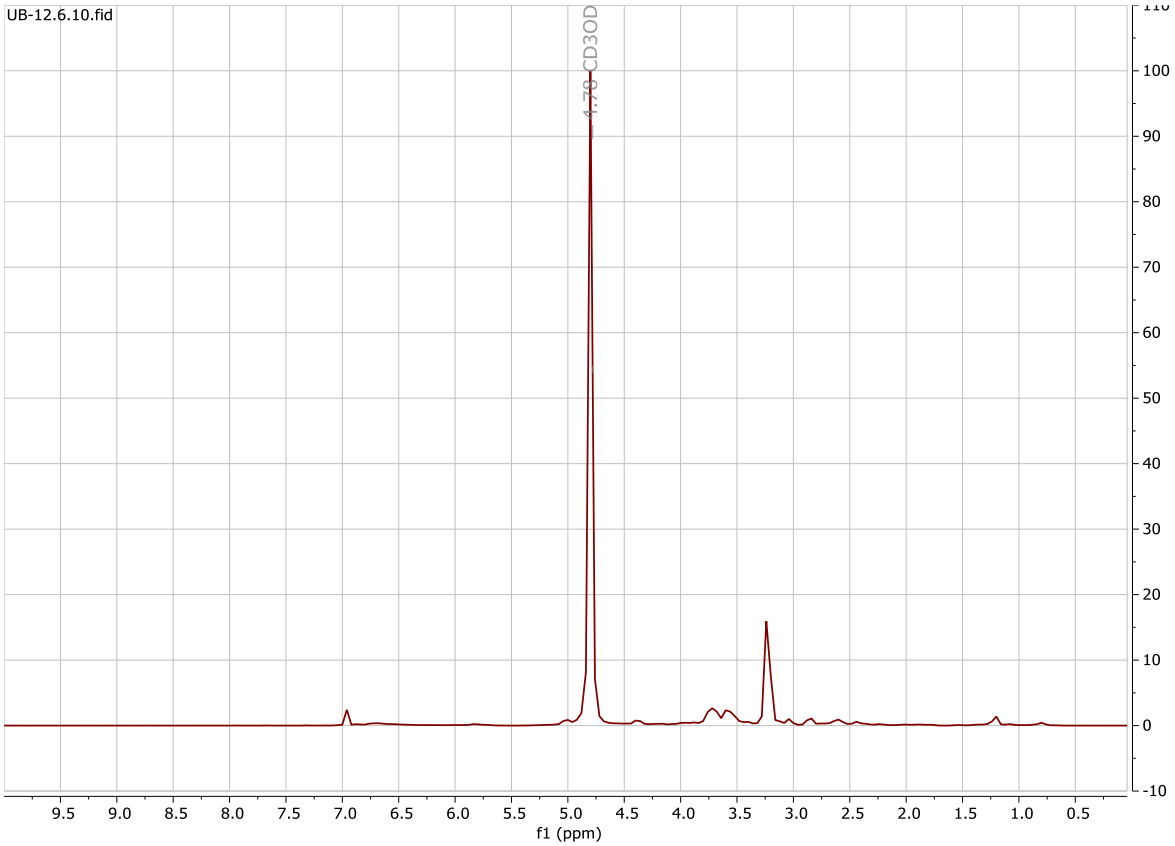

Figure A.105*:* ^{1}H NMR spectrum of *Elephantorrhiza elephantina,* which was stored for 14 days at 37˚C.

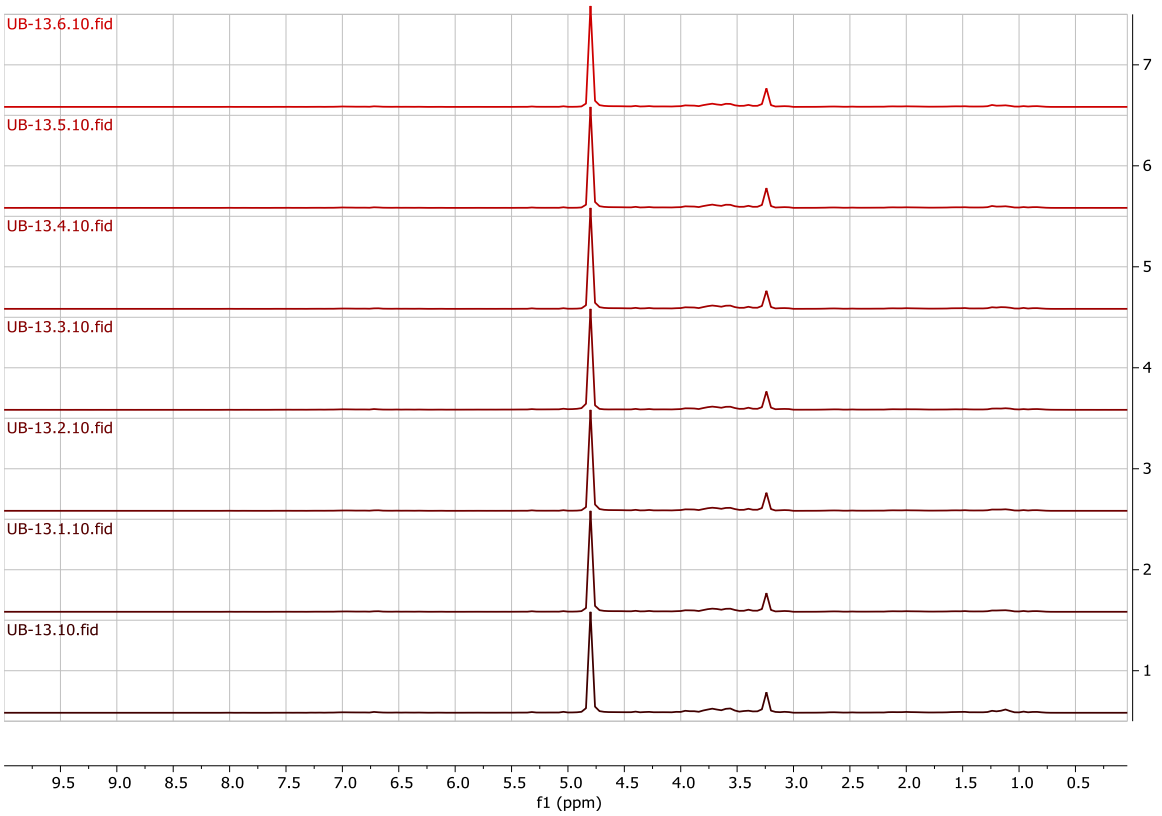

Figure A.106*:* Stacked ^{1}H NMR spectra of *Eucalyptus cinerea*, stored under various conditions.

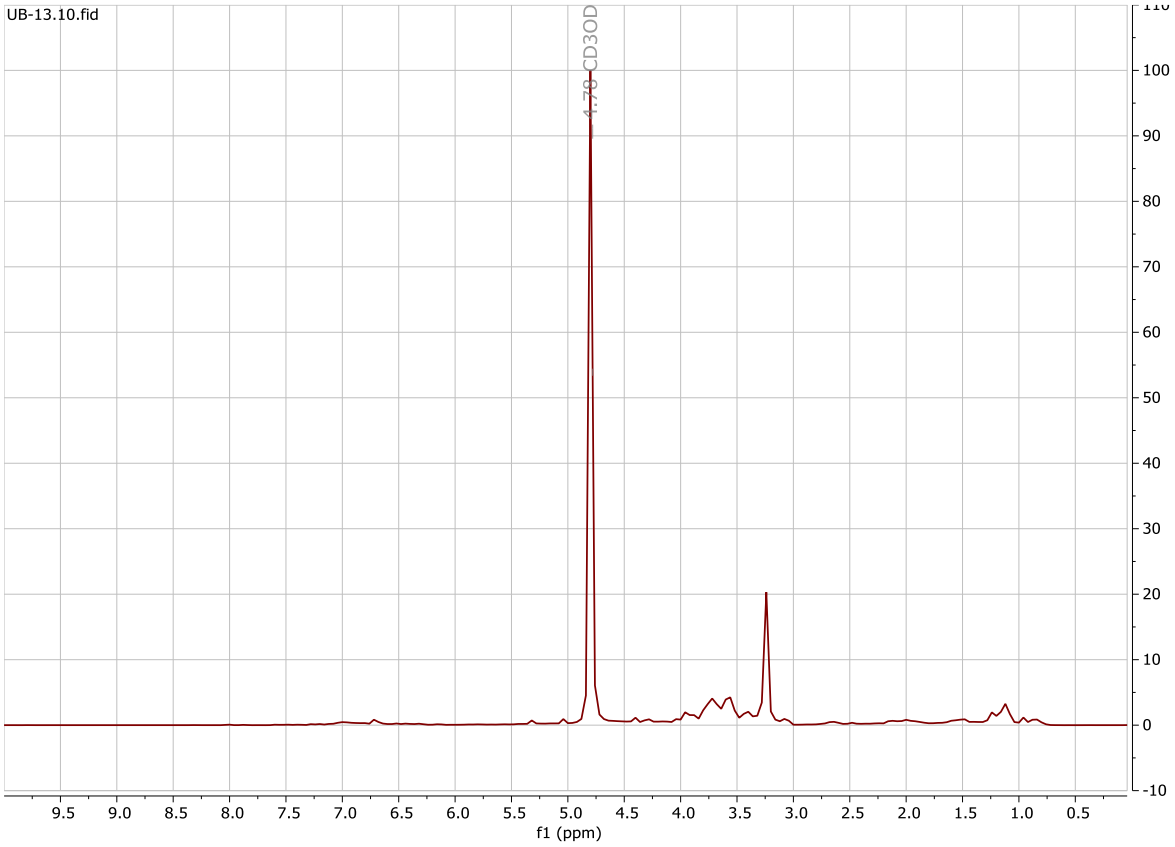

Figure A.107: ^{1}H NMR spectrum of *Eucalyptus cinerea,* which was not stored.

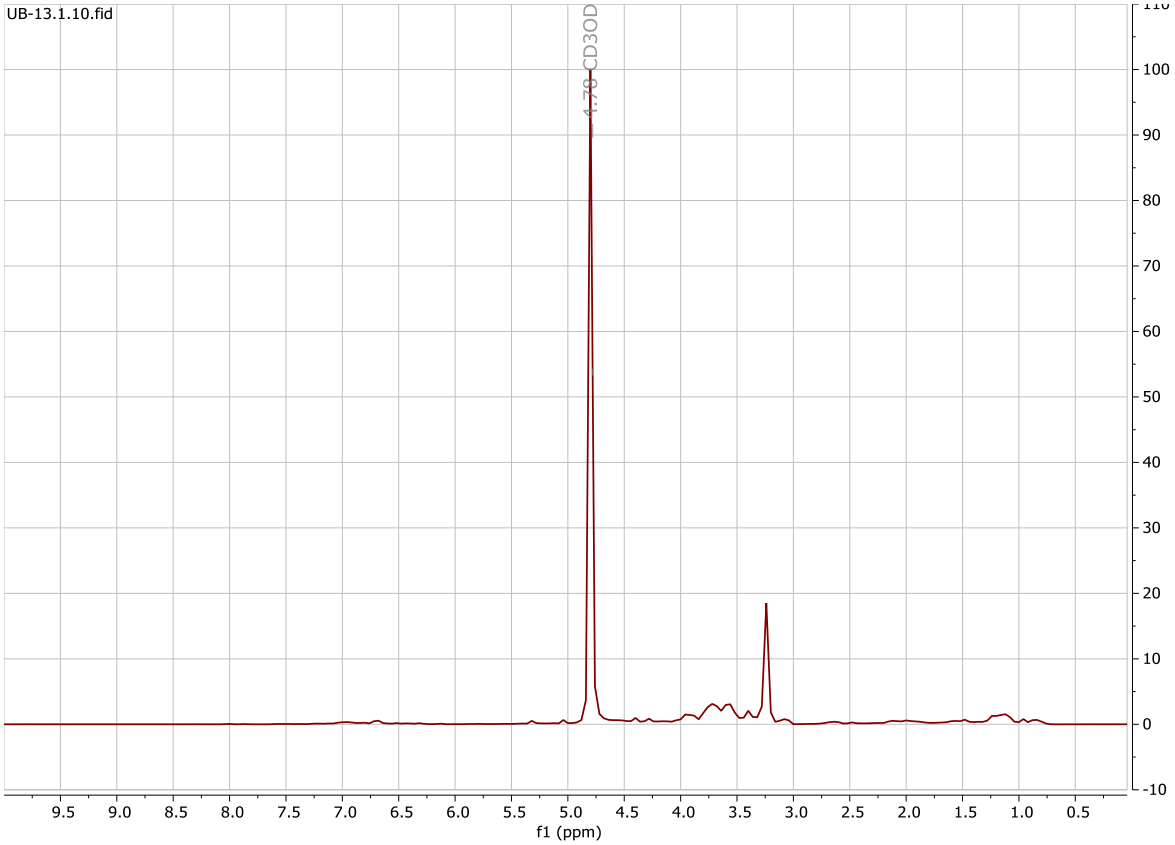

Figure A.108: ^{1}H NMR spectrum of *Eucalyptus cinerea,* which was stored for 7 days at 4˚C.

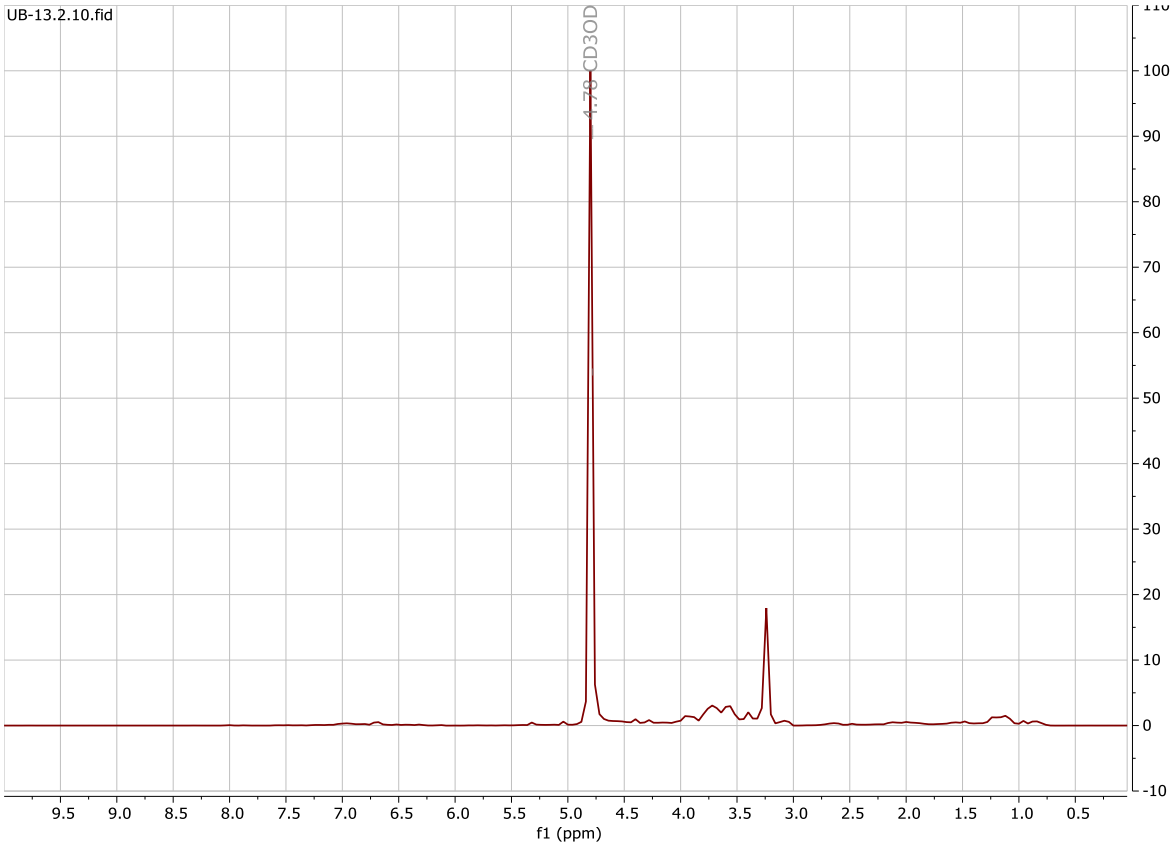

Figure A.109: ^{1}H NMR spectrum of *Eucalyptus cinerea*, which was stored for 7 days at 25˚C.

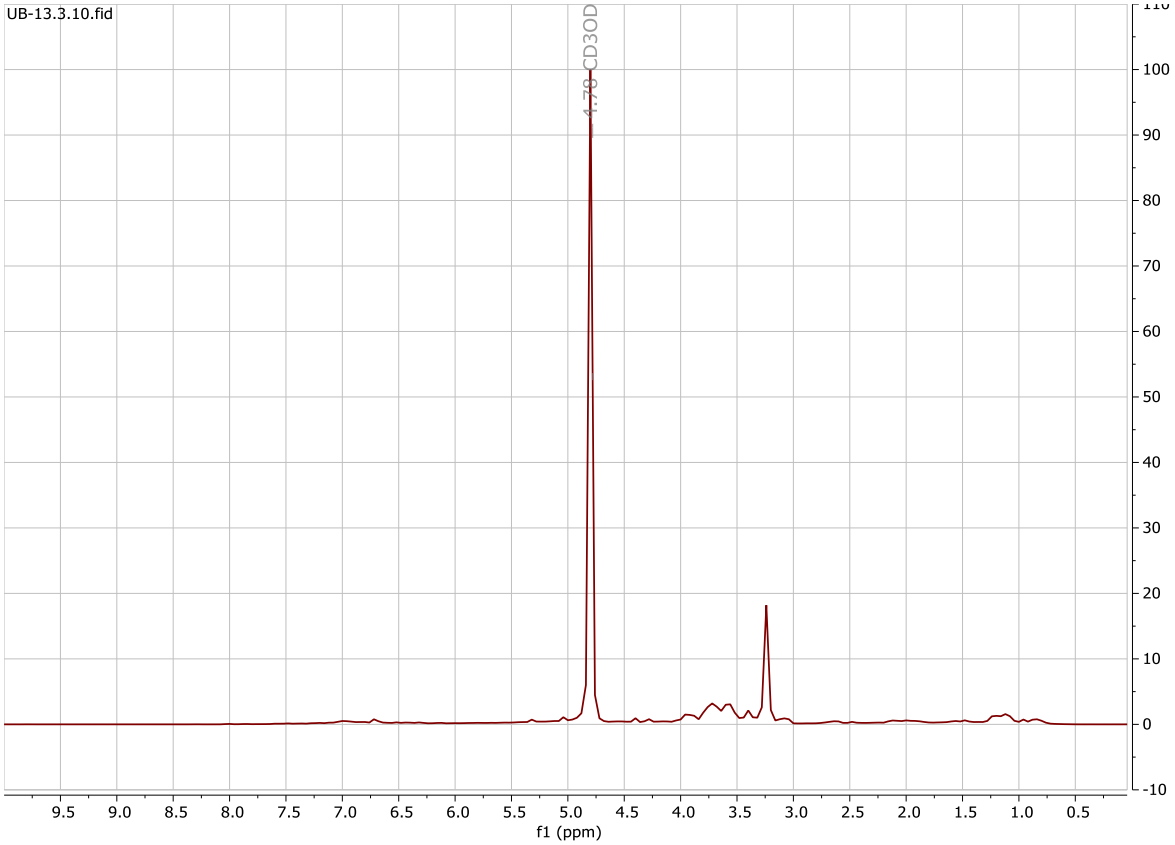

Figure A.110: ^{1}H NMR spectrum of *Eucalyptus cinerea,* which was stored for 7 days at 37˚C.

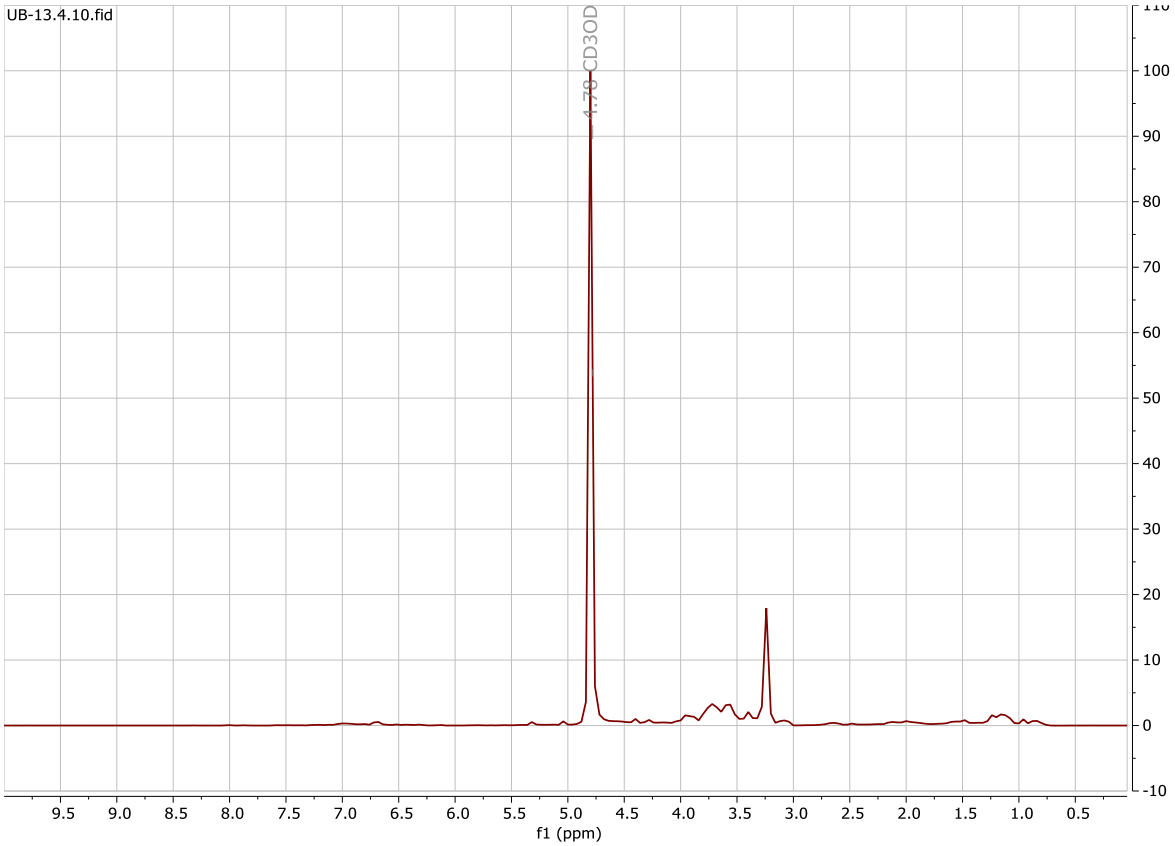

Figure A.111*:* ^{1}H NMR spectrum of *Eucalyptus cinerea,* which was stored for 14 days at 4˚C.

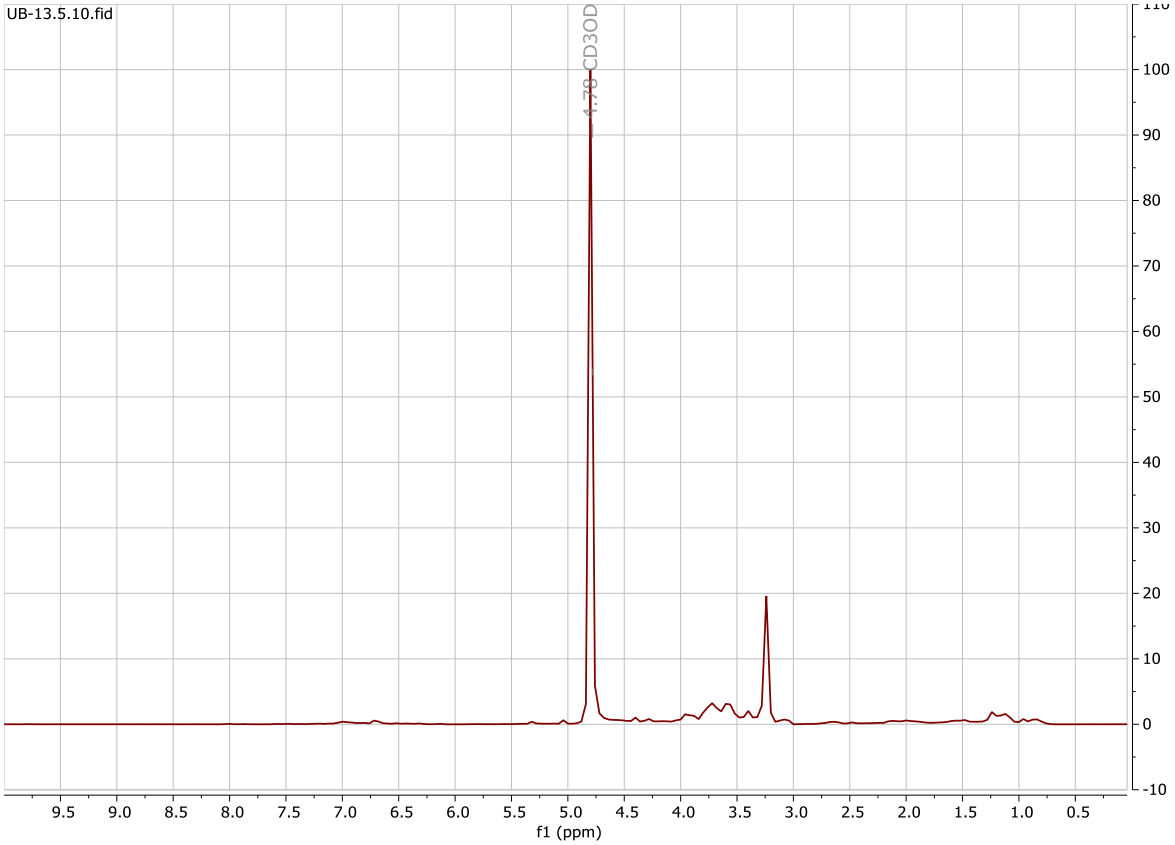

Figure A.112: ^{1}H NMR spectrum of *Eucalyptus cinerea*, which was stored for 14 days at 25˚C.

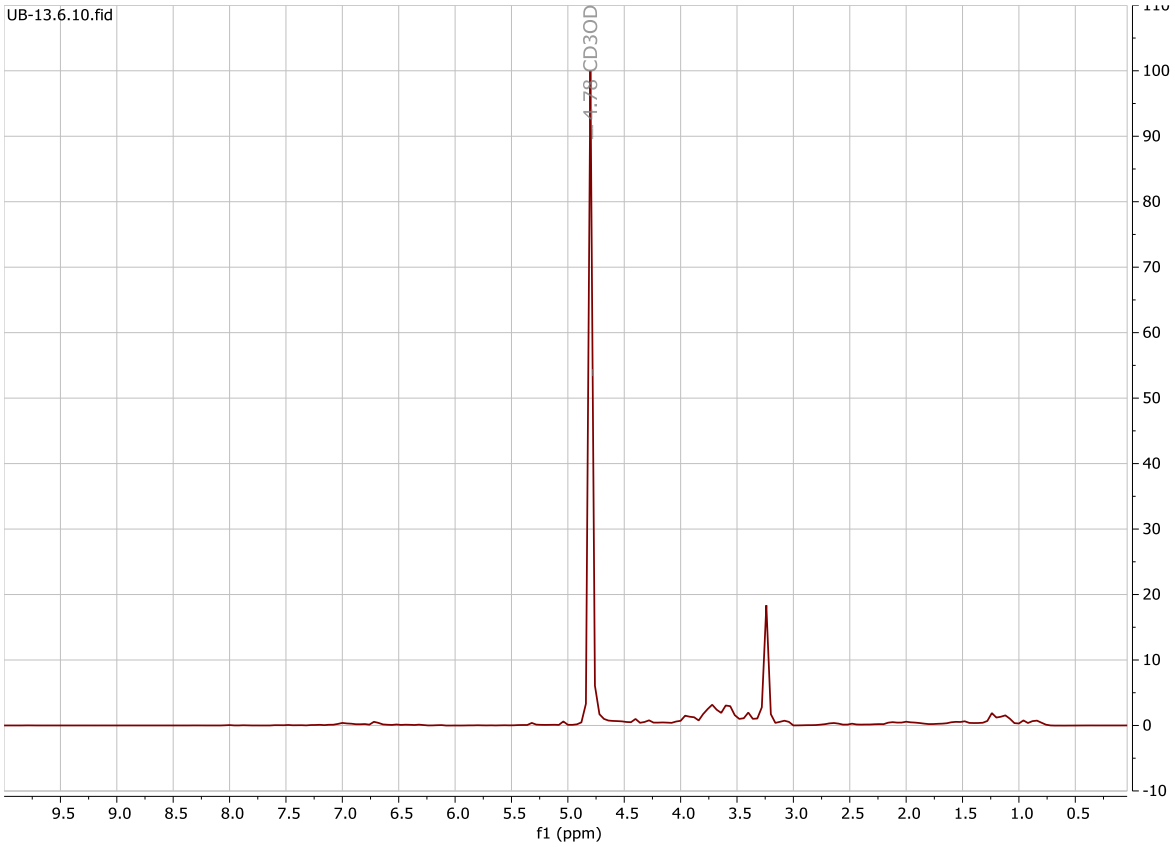

Figure A.113*:* ^{1}H NMR spectrum of *Eucalyptus cinerea,* which was stored for 14 days at 37˚C.

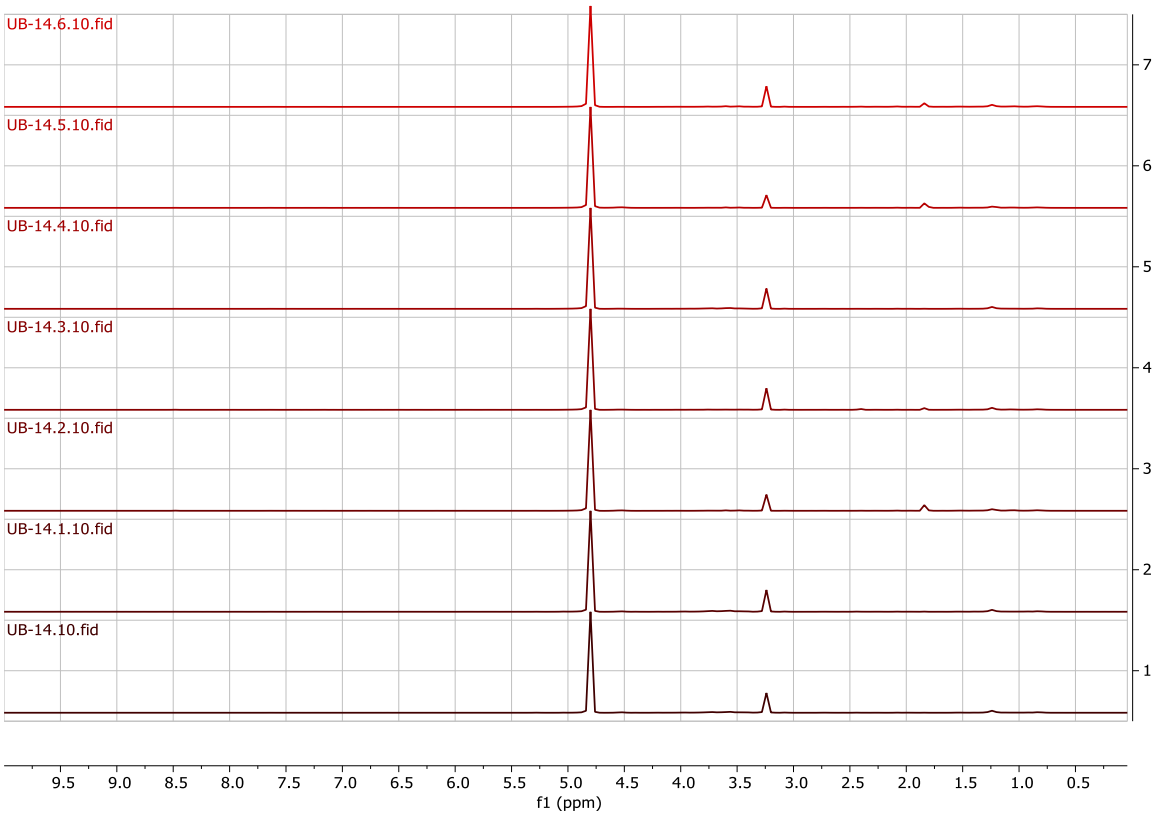

Figure A.114*:* Stacked ^{1}H NMR spectra of *Kigelia africana*, stored under various conditions.

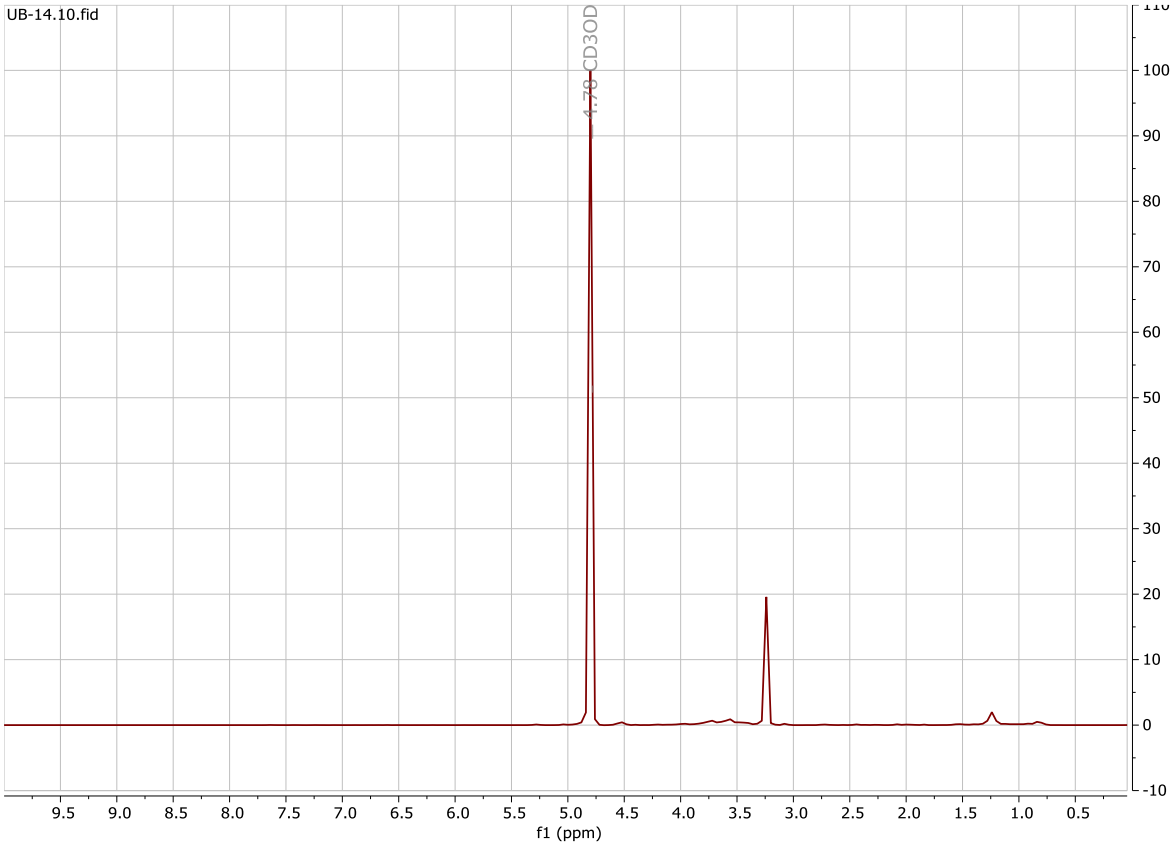

Figure A.115*:* ^{1}H NMR spectrum of *Kigelia africana,* which was not stored.

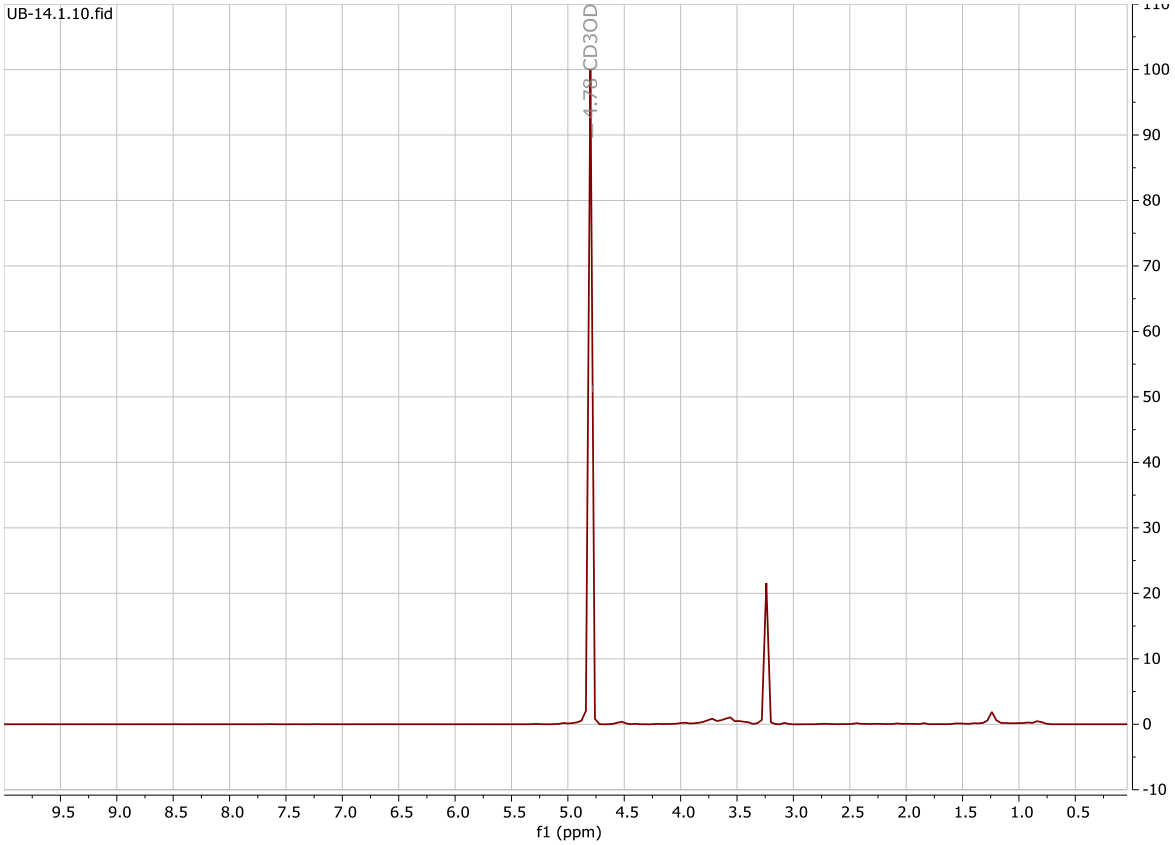

Figure A.116: ^{1}H NMR spectrum of *Kigelia africana,* which was stored for 7 days at 4˚C.

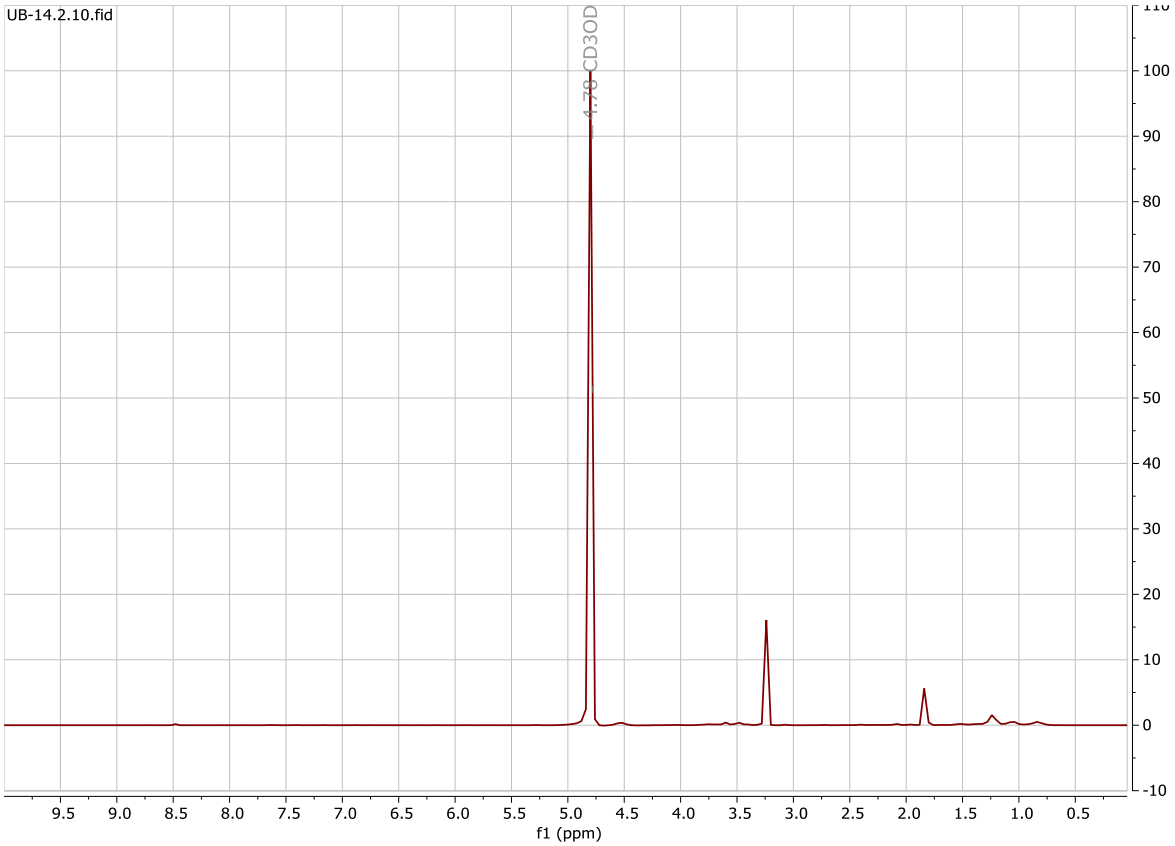

Figure A.117: ^{1}H NMR spectrum of *Kigelia africana,* which was stored for 7 days at 25˚C.

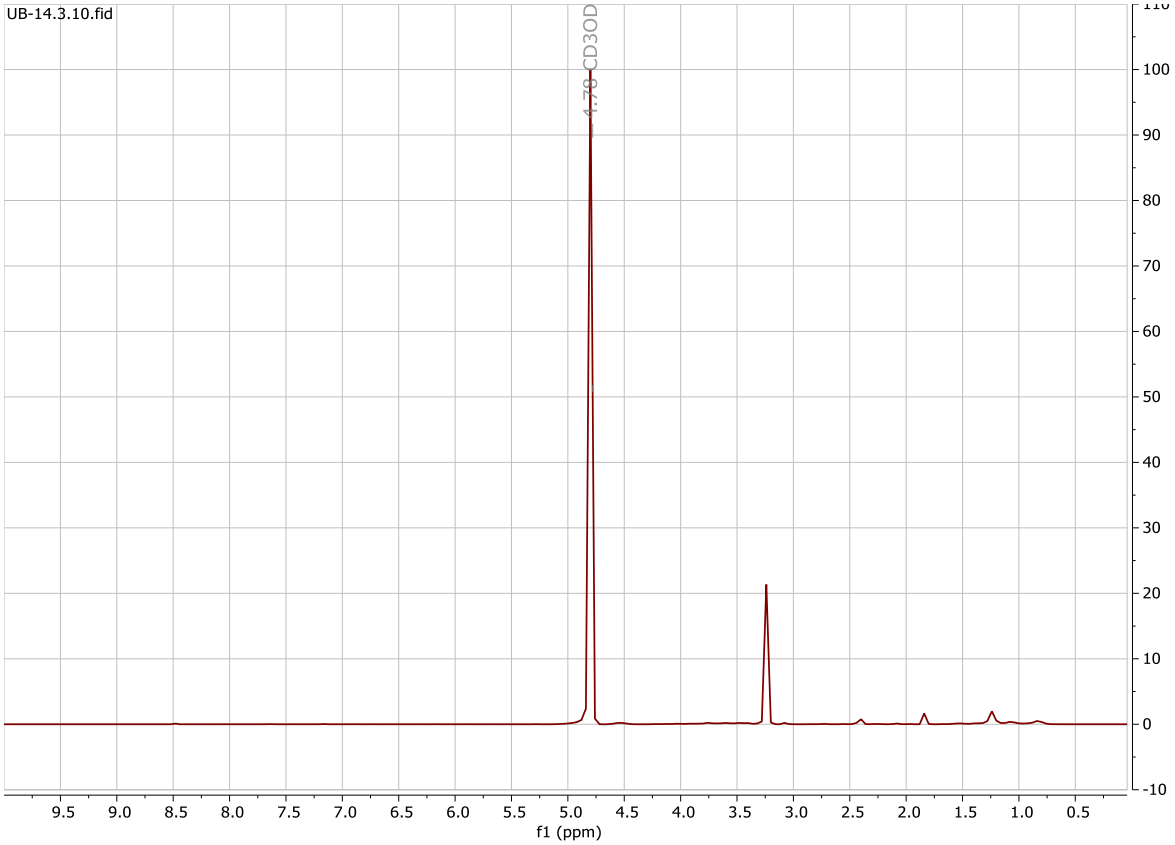

Figure A.118*:* ^{1}H NMR spectrum of *Kigelia africana,* which was stored for 7 days at 37˚C.

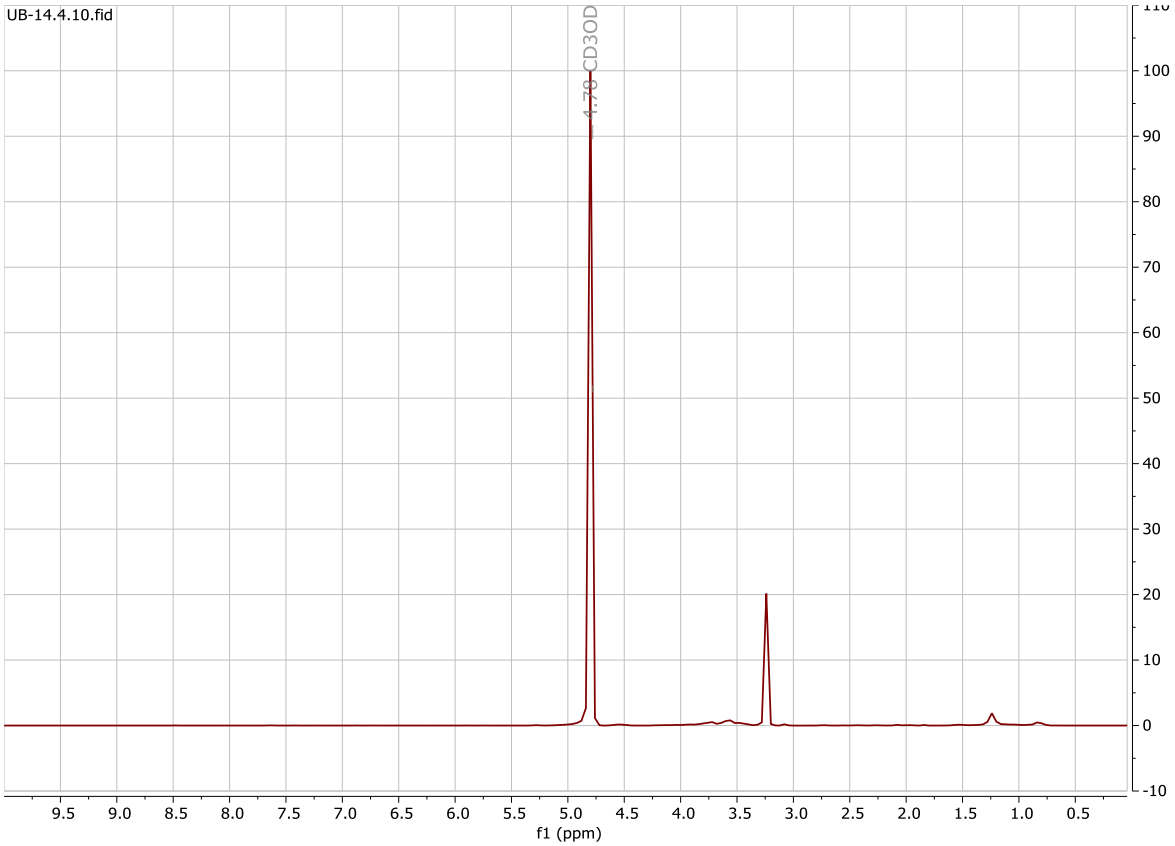

Figure A.119*:* ^{1}H NMR spectrum of *Kigelia africana,* which was stored for 14 days at 4˚C.

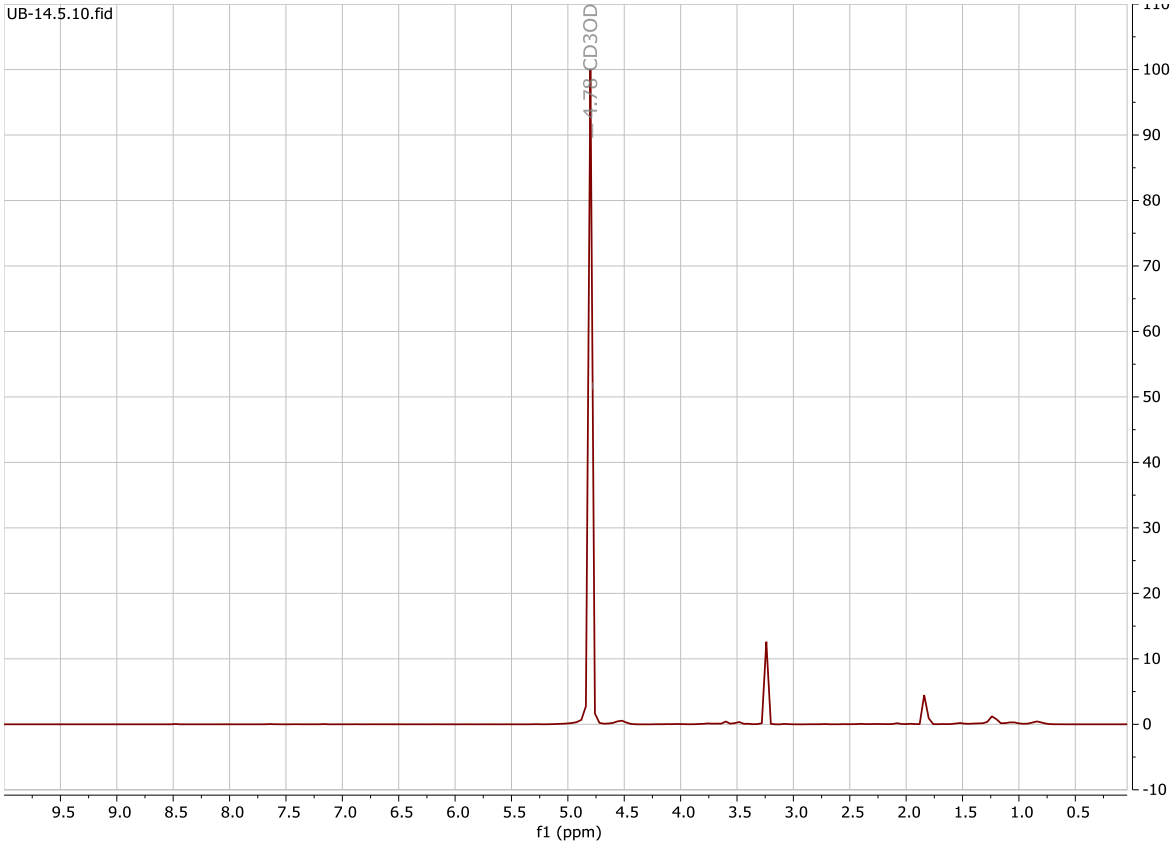

Figure A.120: ^{1}H NMR spectrum of *Kigelia africana,* which was stored for 14 days at 25˚C.

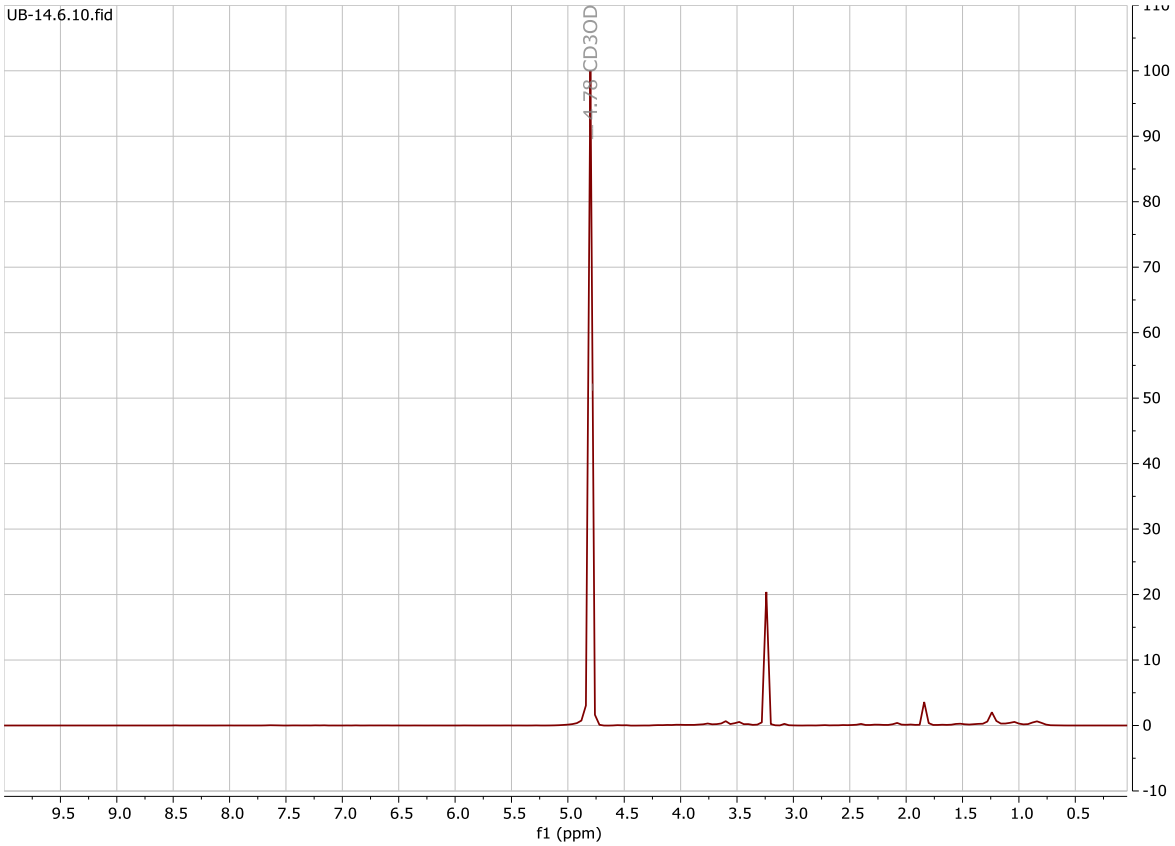

Figure A.121: ^{1}H NMR spectrum of *Kigelia africana,* which was stored for 14 days at 37˚C.

Negative and positive spectrum of the blank solvent used in the High-performance liquid chromatography (HPLC)

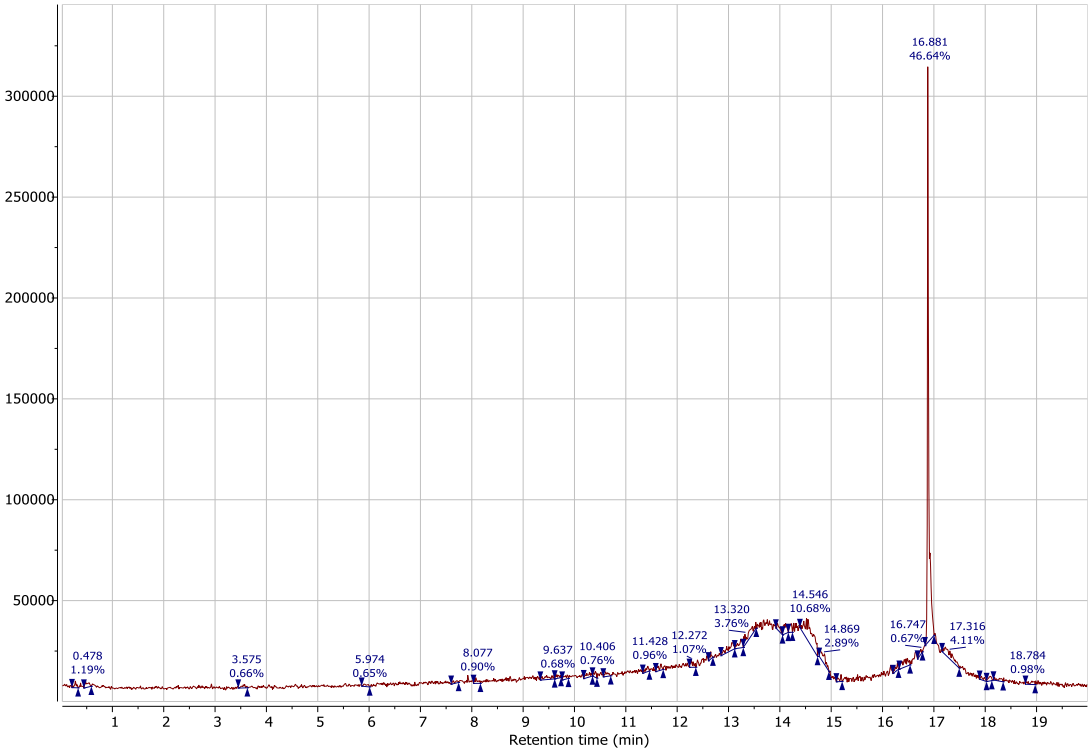

Figure A.122: Spectrum of the blank solvent (negative) used to analysis high performance liquid chromatography analysis.

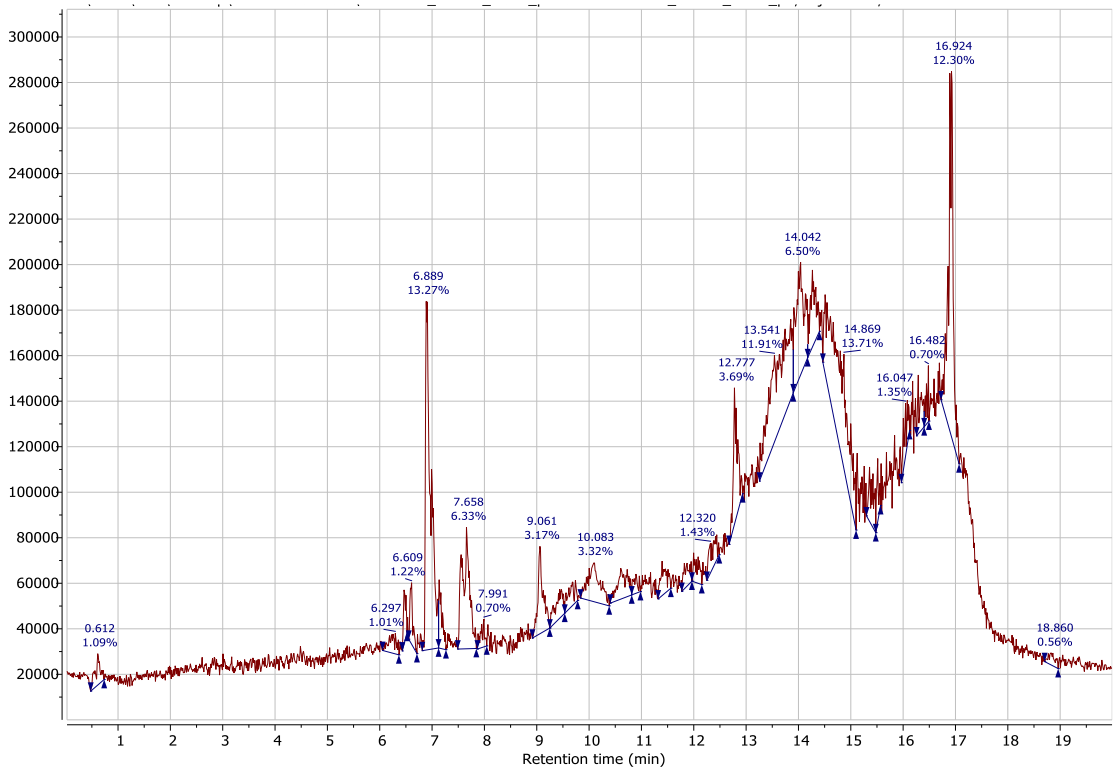

Figure A.123: Spectrum of the blank solvent (positive) used to analysis high performance liquid chromatography analysis.

Appendix B

Chapter 6: A mycotoxic analysis of the most active polyherbal combinations of selected medicinal plants used in the treatment against *Niessseria gonorrhoea* and *Candida albicans*

Descriptive method of High-Performance Liquid Chromatography (HPLC)

The method provided was amended for the extraction of the following mycotoxins; Deoxynivalenol (DON), Nivalenol (NIV), Zearalenone (ZEA), Ochratoxin A, Aflatoxin B1, Fumonisin B1, B2 and B3. Weighed plant sampled (1 g) was placed into a 50 mL tube and 20 mL of solvent extraction mixture containing 50 % MillQ® water: 25% Methanol: 25% Acetonitrile, which is sonicated for an hour. Aiquot 1 mL of the supernatant into a 2 mL Eppendorf tube and add 1 mL of solvent mixture 75% MillQ® water:25% Methanol and the was then centrifuged for 5 minutes at 13 000 rpm. Aliquot 1 mL of sam

Compound separation and detection was performed using a Waters® Acquity Ultra Performance Liquid Chromatography (UPLC) was coupled to a Xevo triple quadrupole tandem mass spectrometer (MS/MS) (Waters Inc., Milford, Massachusetts,USA) and was used for a high-resolution UPLC-MS/MS analysis. Multi mycotoxins were separated in multiple reaction monitoring (MRM) using electrospray ionization in positive mode. The mass spectrometry operating parameters used were; capillary voltage; 3.5 V; cone voltage range 15-50V; collision energy range, 10-40 eV; source temperature, 140˚C desolvation temperature, 400 ˚C; desolvation gas, 800 L/h and cone gas, 50 L/h. Separation was achieved with an Aquity BEH C18 (2.1 x 100 mm; 1.7u particle size) column at 50 ˚C and a flow rate of 0.35 mL/min. An injection volume of 2 µL was used and the mobile phase consisted of water acidified with 0.1% formic acid (A) and acetonitrile acidified with 0.1% formic acid (B). The gradient used for the initial conditions was 98% A, which was held for 0-0.5 min followed by a gradual gradient change of solvent A to 60% from 0.5-7 min. After a 60% gradient it was further reduced to 30% from 7-10 min change of solvent A, followed by a drastic change of 5% from 10-11 min. From 11-12 min the gradient change of solvent A is 0%. Thereafter the gradient quickly changed to initial condition of 98% solvent A from 12-12.1 min, and further kept from 12.1-14 min.

Chromatograms of mycotoxin standards used in mycotoxin studies.

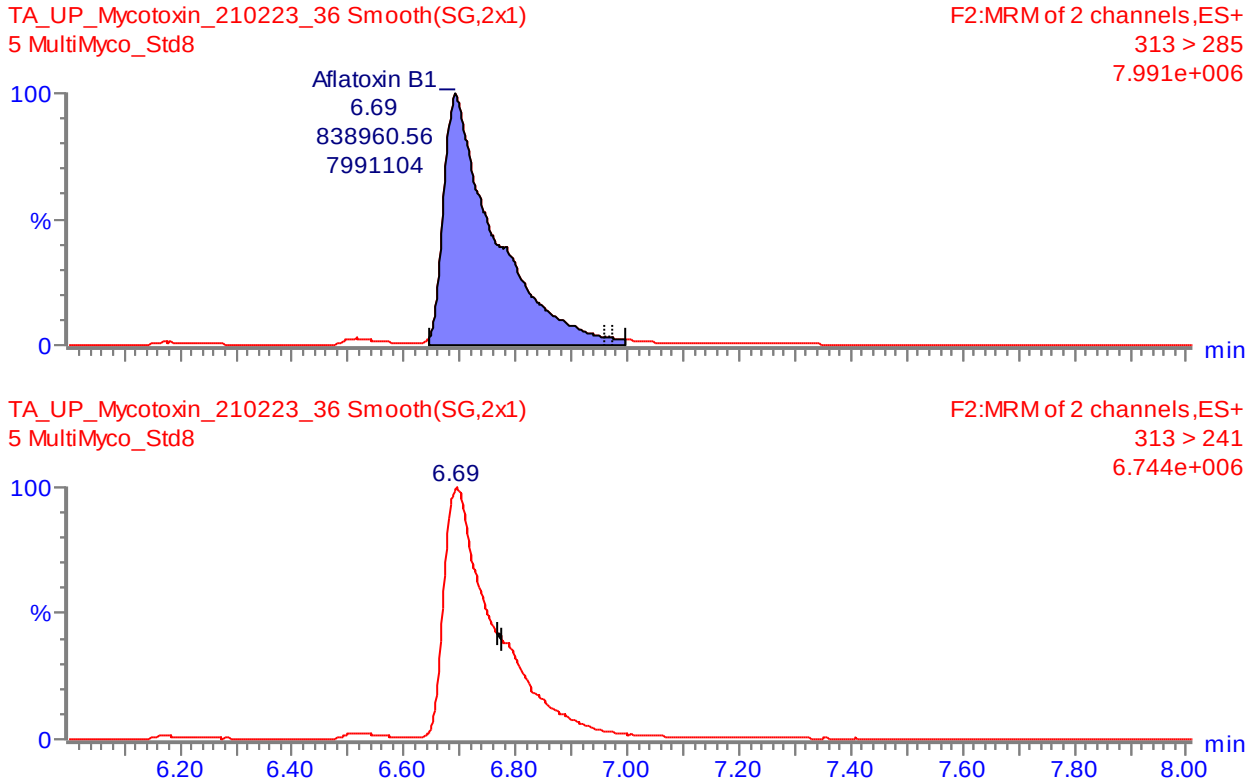

Figure A.124: Chromatogram of the standard, mycotoxin Alfatoxin B_1 at a retention time of 6.69 minutes.

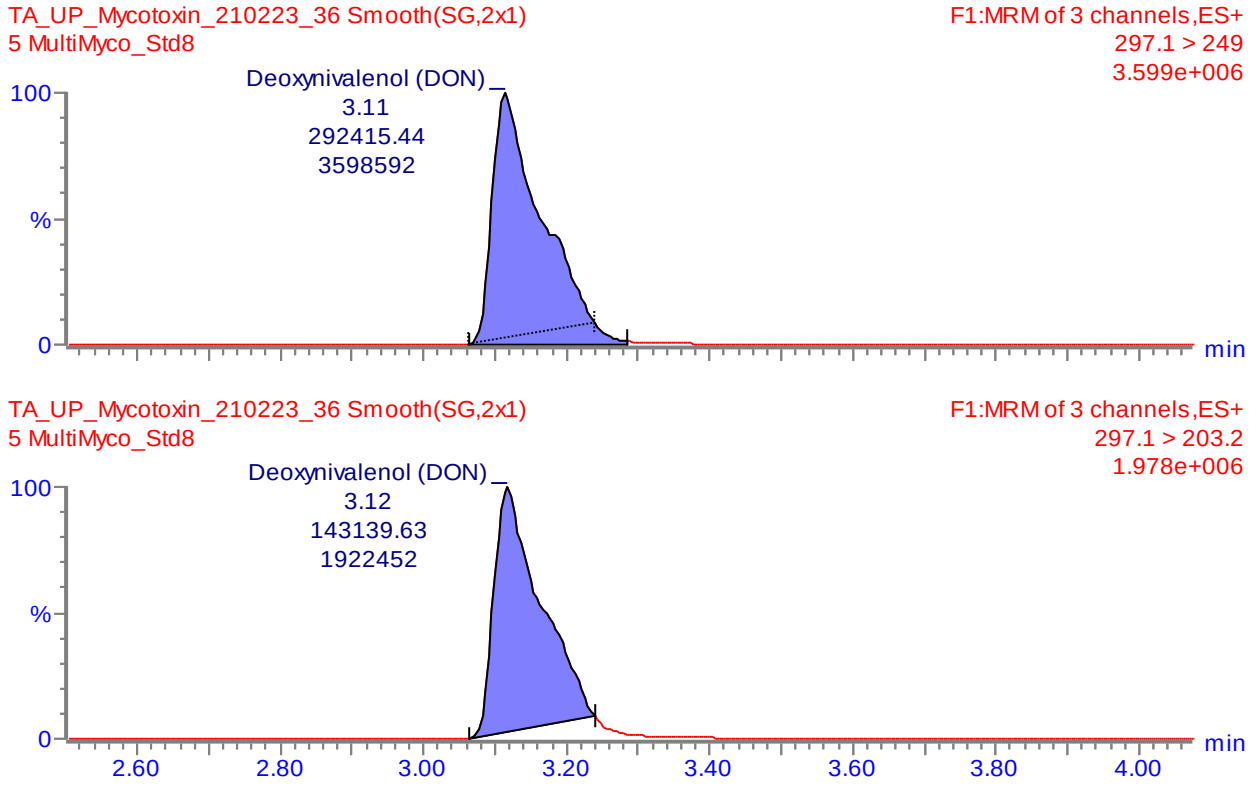

Figure A.125: Chromatogram of the standard, mycotoxin Deoxynivalenol at a retention time of 3.12 minutes.

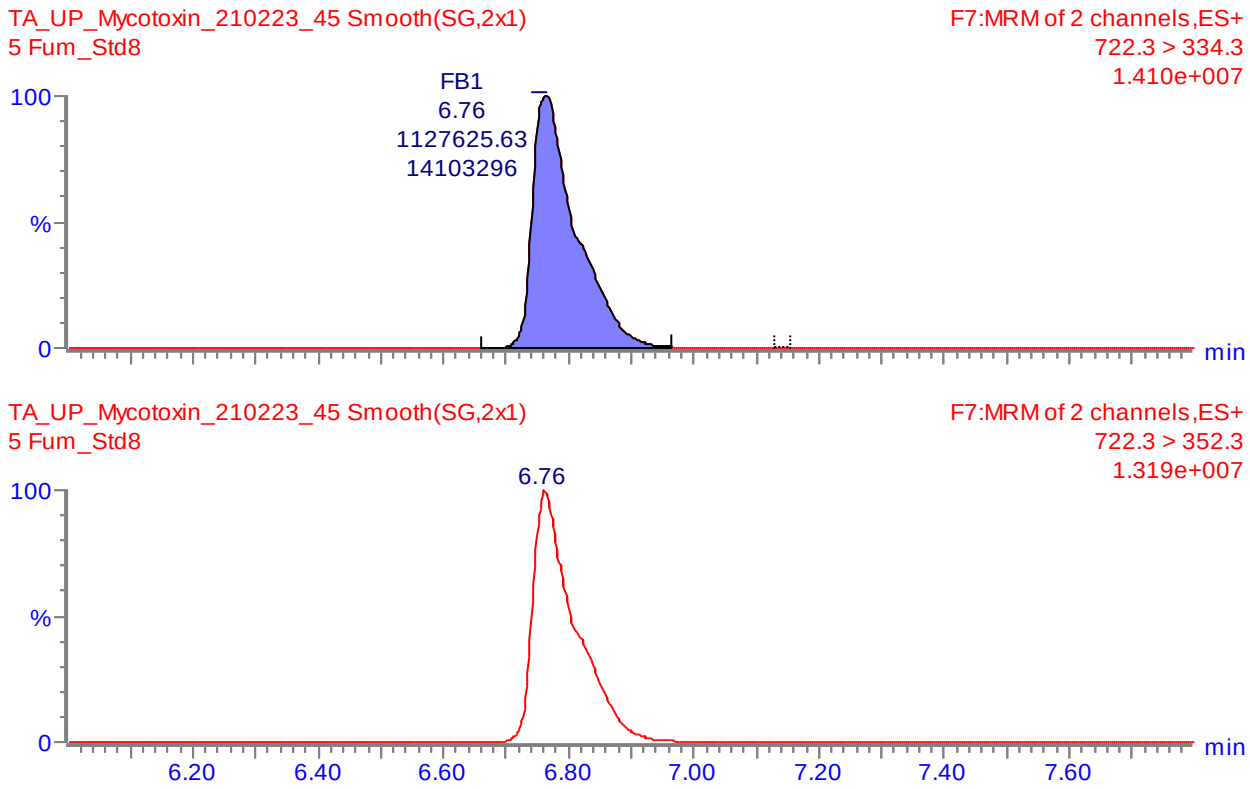

Figure A.126: Chromatogram of the standard, mycotoxin Fumonisin B_1 at a retention time of 6.76 minutes.

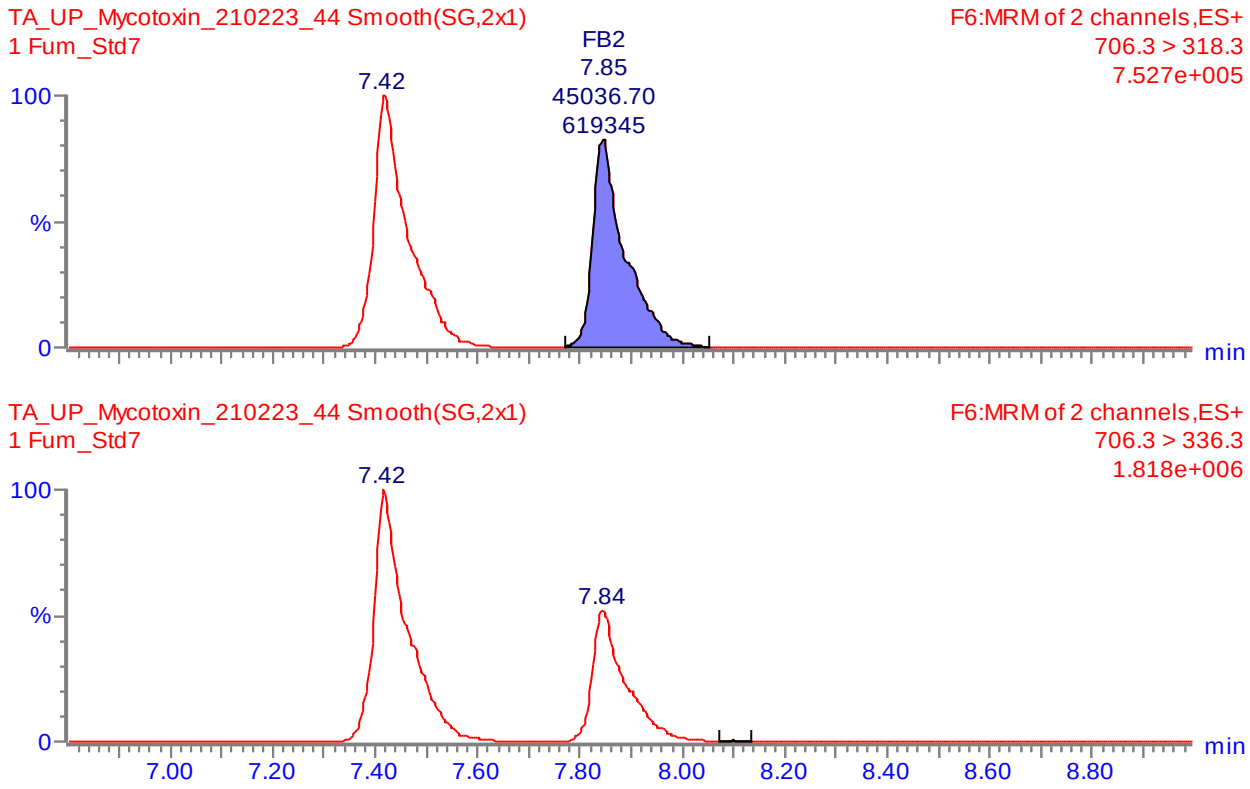

Figure A.127: Chromatogram of the standard, mycotoxin Fumonisin B_2 at a retention time of 7.42 minutes.

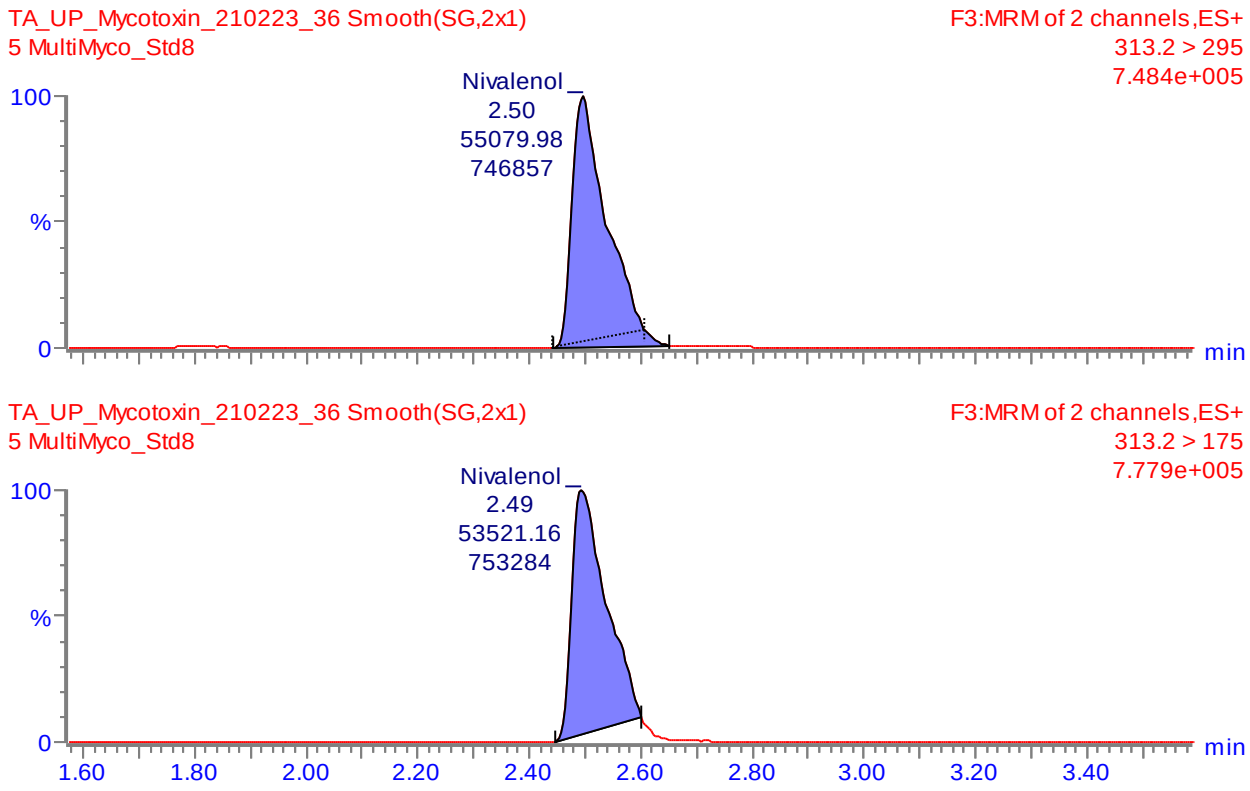

Figure A.128: Chromatogram of the standard, mycotoxin Nivalenol at a retention time of 2.50 minutes.

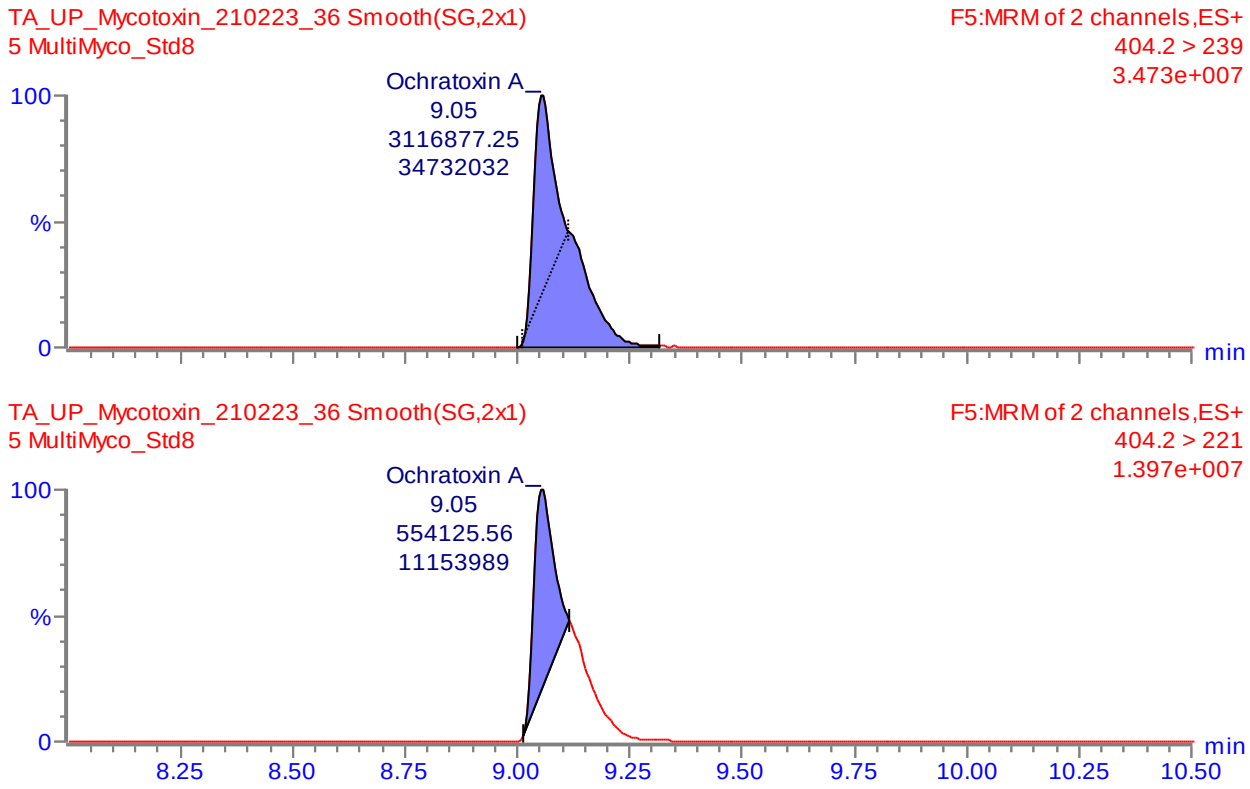

Figure A.129: Chromatogram of the standard, mycotoxin Ochratoxin A at a retention time of 9.05 minutes.

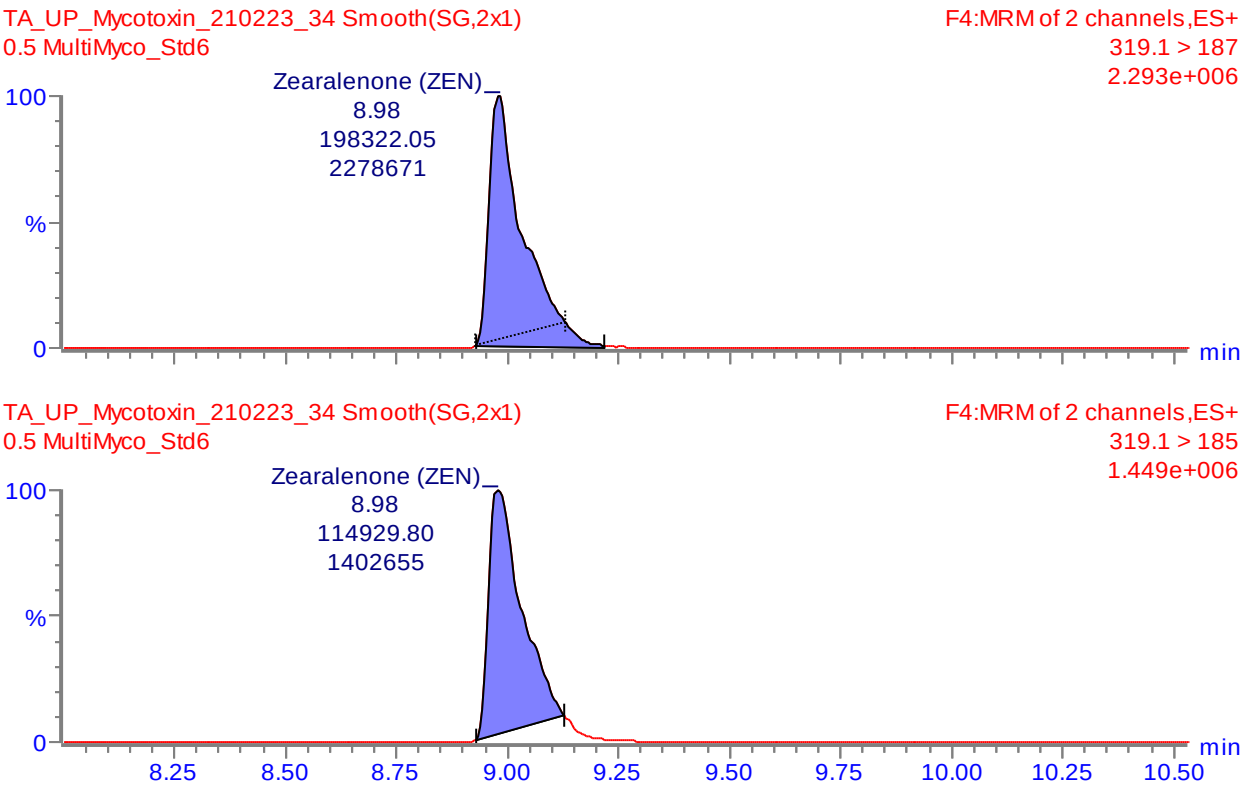

Figure A.130: Chromatogram of the standard, mycotoxin Zearalenone at a retention time of 8.98 minutes.