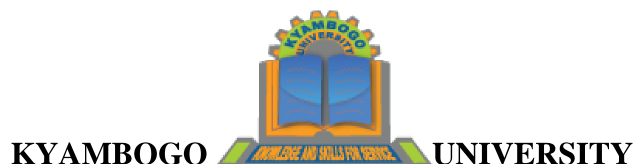
KYAMBOGO  UNIVERSITY

ANALYSIS OF *Kigelia africana* (Lam.) Benth FOR ANTI-MICROBIAL ACTIVITIES

BY

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A DESERTATION SUBMITTED TO THE DIRECTORATE OF RESEARCH AND GRADUATE TRAINING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY OF KYAMBOGO UNIVERSITY

OCTOBER, 2024

DECLARATION

I, **Mary Achiro** (17/U/14267/GMCH/PE)**,** declare that the work contained in this dissertation is original and done by me. It has never been submitted or published to this or any other institution of higher learning for a similar or other award. Where other people's work has been used, this has been properly acknowledged in literature citations.

Signature……………………………….... Date………………………….

APPROVAL

The Dissertation entitled Analysis of *Kigelia africana* for anti-microbial activities by Ms. Mary Achiro has been written and submitted to the Directorate of Research and Graduate Training, Kyambogo University, with our approval as supervisors.

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DEDICATION

To my adorable children and husband, Shane Benedict Tusingwire, Shanei Bernadette Tusingwire and Ambrose Tusingwire. I am grateful for their gracious understanding as I replaced their quality time with my academic pursuits. To my mother, Margret Ayella, God divinely delivers us so many gifts and graciously extends many doors, but the rich gift of my mother is more than I could ask for.

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LIST OF ACRONYMS AND SYMBOLS

CD	:	Circular Dichroism
DCM	:	Dichloromethane
DNA	:	Deoxyribonucleic acid
IC_{50}	:	50% Inhibition Concentration
IR	:	Infrared
Rb	:	Root bark
Sb	:	Stem bark
MeOH	:	Methanol
NMR	:	Nuclear Magnetic Resonance
nM	:	Nanometer
µM	:	Micrometer
WHO	:	World Health Organization
ESI-MS	:	Electron Spray Ionisation Mass Spectrometry
FAD	:	Flavin-adenine dinucleotide
GCGB	:	(gamma-glutamyl-cysteinyl glycinyl)spermidine
HMG-CoA	:	3-Hydroxy-3-methylglutaryl coenzyme A
HMBC	:	Heteronuclear Multiple Bond Correlation
HSQC	:	Heteronuclear Single Quantum Coherence
NOESY	:	Nuclear Overhauser Effect Spectroscopy
QSAR	:	Quantitative Structural Activity Relationship
UV	:	Ultraviolet
MDR	:	Multi-drug Resistance
TB	:	Tuberculosis
NTP	:	National TB control Programme
REF	:	Reference

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ABSTRACT

Fungal infections and bacterial infections, including tuberculosis, are among the illnesses that have plagued humanity for a very long time. Numerous medications have been utilized to treat these infections thus far. However, there has been a challenge of microbial resistance which puts much of the population to be vulnerable to diseases and infections. There is, therefore, a need for research to overcome the existing health challenge. Throughout Africa, traditional medicine practitioners have utilized *Kigelia africana* as a plant remedy for persistent cough and other infectious disorders. In this research, the stem bark of *Kigelia africana* was analyzed for anti-tuberculosis, antibacterial and antifungal activities. 1000 g was extracted from the groundpowder of stembark using dichloromethane: methanol, 1:1 for 24 h at room temperature. The extract was subjected to column chromatographic separations to obtain different fractions which were further purified and four compounds were isolated; Stigmasterol (**1**), a long aliphatic chain ester; 4-Hydroxyphenethyl butyrate (**2**), a coumarin derivative; 6- demethylkigelin (**3**) and Stearic acid (**4**). The structures were established using nuclear magnetic resonance (NMR) Spectroscopic techniques. Compounds **2** is hereby isolated for the first time from the plant whereas compound **3** is hereby reported for the first time from the stem bark of *K. Africana*, but previously isolated from the roots of the tree. Anti-tuberculosis activities for the plant extract and four isolated compounds were expressed as MIC values (μg/mL). Antibacterial and antifungal activities were expressed as mean zones of inhibition (mm). The crude extract was moderately active against *M. tuberculosis* with MIC of 44.2 ±0.5 (μg/mL). Compounds 1-**3** exhibited strong activity against *M. tuberculosis* (33.5±0.4, 31.2±0.4 and 34.5±0.7, respectively) whereas compound **4** was moderately active (43.3±0.1) against *M. tuberculosis*. All the four compounds were weakly active in the antibacterial tests (7.0±0.4, 7.0±0.3, 9.5±0.7 and 7.5±0.4, respectively) and antifungal tests (8.0±0.5, 7.0±0.4, 7.5±0.4 and 7.5±0.7, respectively). There was a noticeable moderate antibacterial activity (10.0±0.3) against *Staphylococcus aureus* and antifungal activity (11.3±0.5) against *Candida albicans* exhibited by the crude extract. The findings show that *K. africana* stem bark has potential for treating bacterial and fungal-related diseases in addition to aiding in the fight against tuberculosis.

CHAPTER ONE INTRODUCTION

1.1 Background

Our communities are seeing increasing rates of sickness and mortality as a result of the global rise in antibiotic resistance. It is imperative to address the issue of resistant bacteria and the environmental propagation of antibiotic resistance. To stop its spread, several tactics have been devised, including as enhanced surveillance and antimicrobial stewardship initiatives. The total extent of antibiotic resistance and the current monitoring, stewardship, and research efforts in Uganda, however, are not well understood (Kivumbi & Standley, 2021).

Antimicrobial resistance (AMR) can significantly reduce treatment efficacy and increase the burden of disease and its repercussions, and Africa continues to be the continent most affected by infectious diseases. According to Tadesse *et al.*'s 2017 systematic analysis, which detailed the state of AMR in Africa, "more than 40% of people in African countries did not have access to recent AMR data." Significant levels of resistance to routinely given antibiotics were found in the nations for which data were available. AMR was reported to be widespread in this sub region by the 2017 systematic review that focused on West Africa. It was more common in hospitalized patients with bloodstream infections (BSI) and urinary tract infections (UTIs), both in outpatient and inpatient settings. Research on antimicrobial resistance (AMR) in East and sub-Saharan Africa also showed that clinical bacterial isolates have a significant incidence of AMR to routinely used antibiotics (Tornimbene *et al*., 2022). The studies also emphasized the limitations of the accessible information and the difficulties low- and lower-middle-income nations had while putting AMR surveillance into practice (Tornimbene *et al*., 2022). The Global Antimicrobial Resistance and Surveillance System (GLASS) were established by the World Health Organization (WHO) in 2015 to help strengthen the body of evidence supporting antimicrobial resistance (AMR). According to the GLASS Report 2020, rather than relying exclusively on laboratory data, GLASS encourages countries to employ monitoring methods relying on systems which integrate population-level, clinical, and epidemiological data. GLASS encourages the development of national AMR surveillance networks in addition to data collecting (Tornimbene *et al*., 2022).

The resistance problem demands that efforts be made to seek/search for antimicrobial agents that may have different modes of action and therefore effective against *M. tuberculosis*,

bacterial and fungi infections. Possible strategies towards this objective include search for natural products from biodiversity especially plants which are used by local communities to treat/manage *Mycobacteria tuberculosis,* bacterial and fungi related infections.

Tuberculosis (TB) manifests itself by affecting the victim's lungs and respiratory system (Heemskerk *et al*., 2015). The symptoms of the illness are an irritating cough (with or without sputum), hemoptysis (coughing blood), tiredness plus poor appetite among others (Luies & du Preez, 2020). Mycobacterium tuberculosis is the microbe that causes tuberculosis (MayoClinic, 2023). Tuberculosis incidence in Uganda stood at 201 per 100,000 persons in 2016 and more than 4 per 100,000 persons have multi-drug resistant TB (Martinez *et al*., 2023; WHO, 2018). Majority of the affected individuals are immune-compromised and immune suppressed people mostly those living with HIV (Bagcchi, 2023). There have been reports of resistance showed by *M. tuberculosis* against a range of standard medicine such as isoniazid, streptomycin, and rifampicin(Wu *et al*., 2019).

Certain endemic regions have reported *M. tuberculosis* exhibiting resistance to up to eight medicines in first line regimens, rendering some tuberculosis patients incurable (Wu *et al*., 2019). Individuals previously inadequately treated for the disease are at great risk of developing MDR tuberculosis. Drug resistance is no longer confined due to re-infections and cross-infections. Antibiotic-resistant infections have been seen to typically quadruple hospital stays and raise TB management expenses (WHO, 2018).

Using an examination of WHO case notifications and tuberculosis death statistics, the global tuberculosis situation in 1990 and its continuing trends are assessed. Basic epidemiological models were used to estimate the number of cases of tuberculosis infection and the incidence of tuberculosis sickness and mortality for the year 1990. About one-third of all people on the planet are infected with Mycobacterium tuberculosis. In the last ten years, 2.5 to 3.2 million cases were reported year globally; population growth has somewhat offset recent declines in notification rates. In 1990, an approximate 8 percent globally, 2.6 to 2.9 million people died from tuberculosis, while millions more contracted the disease. The bulk of these infections and fatalities happened in Asia, and the number of people living with HIV is clearly on the rise, particularly in Africa. Reports on tuberculosis cases reported by WHO Member States should be carefully examined, even though they demonstrate the extent of the problem. Since the number of cases is lower than expected, they highlight the inadequacies of TB control

initiatives. This study highlights the urgent need to rekindle international tuberculosis control efforts and confirms the incredibly large worldwide spread of the disease (Ou *et al*., 2021).

MDR-TB poses a significant threat to global TB control efforts. East Africa, where HIV co-infection is prevalent, has the highest rates of the disease and associated risks. MDR-TB has only recently gained significant attention. Even in high-income settings, MDR-TB treatment successes are below 100%, despite the fact that it is nearly 100% curable. Furthermore, it is one of the primary wellness issues that commonly results in immunocompromised individuals dying. A third of people worldwide are afflicted with TB. The drug-resistant gene present in Mycobacterium tuberculosis is the primary barrier to TB control. MDR TB was present in 3.6% of recently identified TB patients and 18% of TB cases that had previously received treatment (5.6% of total cases). Combining second-line anti-TB medications, the majority of which are less beneficial, more likely to cause side effects, and more costly than first-line medications, is necessary for long-term treatment of MDR-TB. MDR-TB is rarely discussed in East Africa and was not regarded as a serious problem until recently. Although a lot of research has been done on the distribution of tuberculosis that is resistant to multiple drugs (MDR-TB) in the area, insufficient has been learned about the risk factors for MDR-TB in East Africa. According to a few local studies, risk factors for the emergence of tuberculosis that is resistant to multiple drugs (MDR-TB) in the area included Viral co-infection, sex, alcohol use, malnourishment, history of diabetes mellitus, low socioeconomic status, and history of anti-TB medication (Molla *et al*., 2022).

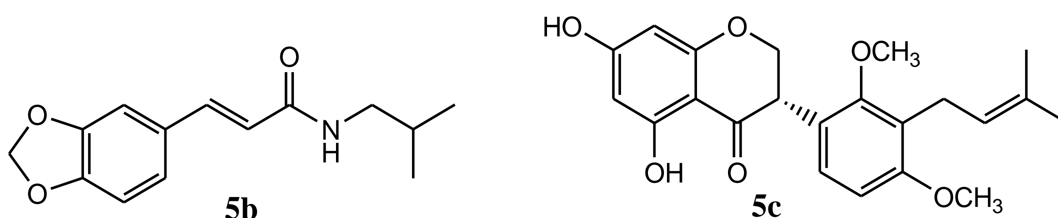
It was estimated that over eight million fresh cases of tuberculosis would have been recorded in 2000. The WHO African Region has one of the greatest levels of tuberculosis, with cases increasing by 6% annually. Nine percent of all newly acquired TB infections in those aged 15 to 49 were caused by HIV infection. However, this number was much higher in the WHO African Region and several wealthy countries, including the US. An estimated 1.8 million TB-related fatalities occurred, with HIV accounting for 12% of those deaths. Of all adult AIDS deaths, 11% were due to tuberculosis. In adults, the frequency of coinfection between M tuberculosis and HIV was 0.36%. In eight African nations, the frequency of coinfection was more than 5%. Two million adults were coinfected in South Africa alone. Management of Mtb worldwide has a significant challenge due to the HIV pandemic. There is an urgent need to prevent HIV and TB, expand WHO DOTS initiatives, and concentrate on controlling HIV-related TB in regions with high HIV prevalence (Mirzayev *et al*., 2021).

In 1993, the disease was declared an international crisis by the World Health Organization (WHO). The illness continues to be an important issue for the community concern in spite of the initiatives that have been put in place by the WHO and some nations. Globally, the prevalence of tuberculosis infection and the burden it carries are unequal, with low-to-middle income nations experiencing higher rates of infection. Despite the various measures put in place by the World Health Organization and numerous nations, one of the biggest global public health concerns is still TB. The TB bacteria infects about one-third of the world's population, yet a higher proportion of people show no symptoms. Approximately 10.4 million people contracted tuberculosis in 2016, and the disease claimed 1.7 million lives.

In this study, the anti-microbial properties of *K. africana*, which traditional medicine practitioners purport to utilize in treating infectious disorders and recurrent cough, were examined. *K. africana* is used to treat measles, cough, diarrhea, and suspected tuberculosis (Tabuti *et al*., 2003; Bunalema *et al*., 2014). Among other things, *K. africana* has been used to treat gynecological issues, cancer, and skin conditions.

(Nabatanzi *et al*., 2020). *K. africana* has been used to develop items that are sold commercially, yet many of them lack complete standardization. The most common uses for fruit extracts are in the management of bacterial and fungal skin diseases (Nabatanzi *et al.*, 2020).

Bacterial and fungal susceptibility assays are crucial in clinical microbiology because they can identify potential therapeutic efficacy or resistance in common infections (Nabatanzi *et al.*, 2020). *Zanthoxylum leprieurii* is another herb used to treat tuberculosis, an infectious condition. Trans-fagaramide (5b), an amido molecule isolated from *Z. leprieurii* stem bark through phytochemical analysis, demonstrated significant in-vitro anti-TB action (MIC = 6µg/ml) (Oloya *et al*., 2021). Similarly, the stem bark of *Platycelphium voënse* (Leguminosae) yielded a prenylated isoflavanone Platyisoflavanone A (**5c**) which exhibited antibacterial activity against *Mycobacterium tuberculosis* in the microplate alamar blue assay (MABA) with MIC = 23.7 mM (Gumula *et al.*, 2012).



1.2 Statement of the Problem

Multi-drug resistant microbial strains are on the increase and spreading around the world, hence, reducing on the number of efficacious antibiotics which may be used in the treatment of bacterial and fungal infections. *Kigelia africana* remains a major medicinal plant that has for a long time been used to manage infectious diseases and other health conditions in Africa. However, information on the phytochemical characterization of the active ingredients in *K. africana* is scanty. This calls for a need to carry out chemical investigations on the tree in the quest for antimicrobial agents. Thus, this study was aimed at identifying and characterizing compounds from the stem-bark of *K. Africana* and testing for their anti-microbial activities.

1.3 Objectives

1.3.1 General Objectives

The general objective of this study was to isolate and characterize the bioactive compounds from *Kigelia africana* stem bark that are responsible for its anti-tuberculosis, antibacterial and antifungal principles.

1.3.2 Specific Objectives

1. To isolate compounds from *Kigelia africana* stem bark.
2. To determine the isolated compounds and crude extract from *Kigelia africana* for anti-tuberculosis, antibacterial and antifungal principles.
3. To elucidate the chemical structures of the pure isolated compounds.

1.3.3 Significance of the study

This study focused on isolating, determining bioactive compounds from *K. Africana stem* bark and elucidating their chemical structures. Since traditional medical practitioners use this plant to manage several disorders like cough "suspected Tb", diarrhea among others, it was thought plausible to do its phytochemical study. Hence promoting sustainable development goal three "Good health and wellbeing".

1.4 Justification of the Study

Since *K. Africana* pant is being used by traditional medicine practitioners to treat recurring cough and infectious disorders, therefore, it was thought plausible that this research will identify molecules with anti-TB, antibacterial and antifungal activities.

CHAPTER TWO

LITERATURE REVIEW

2.1 Kigelia africana

The Worsboom or Sausage Tree is another name for *K. africana* (Bignoniaceae), which gets its name from its enormous, sausage-like fruits. In English, the plant is called the sausage tree; in other languages, it is called *mussa* (Luganda) and *yago* (Acoli). Native to most of Africa, *K. africana* is a species that grows there. It's been around for a while and serves a variety of medicinal functions (Nabatanzi *et al*., 2020; Schultz *et al*., 2020). It is a highly promising and adaptable medicinal herb with several applications. The sausage tree, or *K. africana*, is a lovely and useful tree that is native to continental Africa. Due to its enormous blossoms and peculiar sausage-shaped fruits, tropical and subtropical regions have begun to see it as a decorative wonder (Nabatanzi *et al*., 2020). Pharmacological research has demonstrated the anti-inflammatory, analgesic, and anticancer effects of the plant's extract from its various components. Approximately 150 compounds have been found to be bioactive components in the various plant parts thus far (Ramadan *et al*., 2022). The pre-validated region of "biologically relevant chemical space" is represented by natural products, as evolution has selected molecules that control protein activity. The development of natural ingredients has significantly benefited tuberculosis clinical therapies.

2.1.1 Scientific classification

Kingdom: Plantae

Order : Lamiales

Family : Bignoniaceae

Genus : Kigelia

Species : *K. Africana*

Figure 1: Photo of *K. africana* obtained from Wikipedia

2.1.2 Chemical Constituents of *Kigelia africana*

Chemical investigation has shown the presence of lignans, flavonoids, and important secondary metabolites such as iridoids and naphthoquinoids. Plant secondary metabolites must be identified since they may serve as the foundation for their traditional uses, particularly if they structurally resemble or are identical to compounds with relevant actions from other species. In such cases, one can infer the kind of compounds that are likely to be present based on a taxonomic position. This is demonstrated by *K. pinnata*, a member of the Bignoniaceae family, which is distinguished by the presence of naphthoquinones and iridoids in numerous of its constituent genera (Dhungana *et al*., 2016). The iridoids present in *Kigelia Africana* are consistent with the 9-carbon skeleton type, such as Catalpol (**5**), which is present in other Bignoniaceae members. Significantly, the primary iridoids present in the rootbark and stembark of *K. pinnata* were determined to be the catalpol derivatives esterified with phenylpropanoic acid derivatives at C-6. These were identified as Specioside (**6**), Verminoside (**7**), Minecoside (**8**), and Norviburtinal (**9**), which is generally thought to be a breakdown product of the iridoids (Houghton & Jâger, 2002; Liu & Wen, 2013).

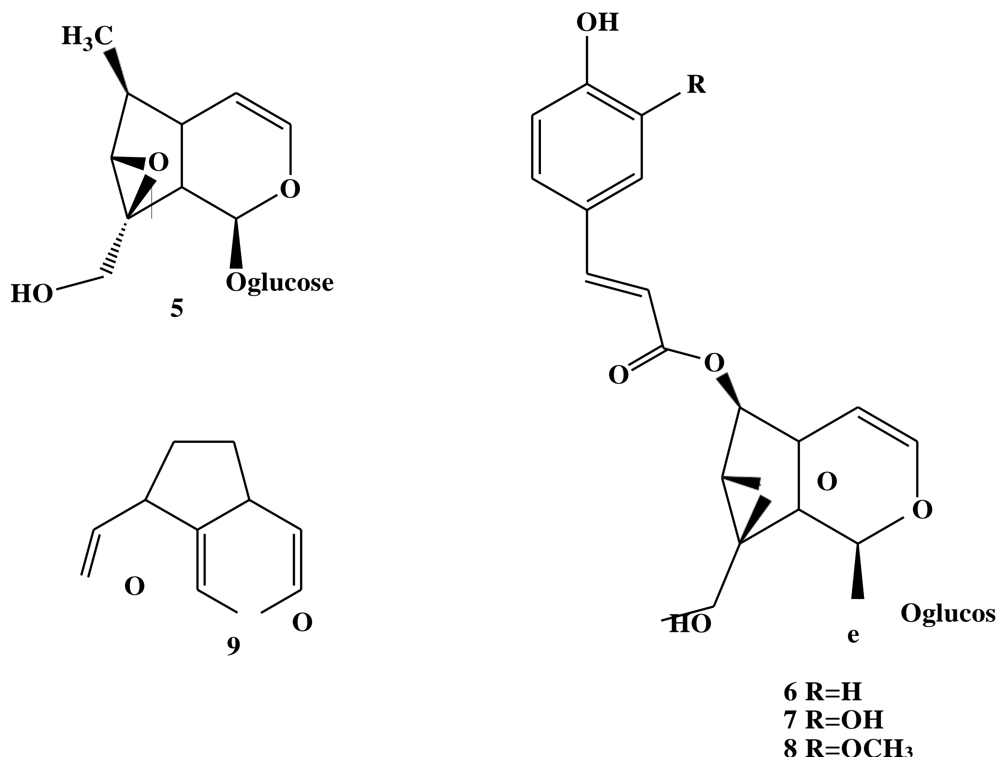


Table 1: Uses of plant parts of *K. africana* in traditional medicine

Plant part	Region	Disease	Method of preparation(s)/ route of administration	Reference
Bark	Kenya	Fungal infestation	The water that has been macerated with bark is used to wash the athlete's feet.	(Kigen *et al.*, 2017)
Bark	Tanzania, Nigeria	Galactogogue	Warm infusion of ground bark used orally followingchildbirth.	(De-Wet & Ngubane, 2014)
Bark	Tanzania, Benin	Malaria, gonorrhea, candida, syphilis, febrile and convulsions	Oral infusion used to reduce headache and body temperature. To cure malaria, mixtures of *K.africana*, Z. scabra, *A. pubescens*, *E. javanica*, and *O. sinuatum* is combined and boiled, and the mixture is administered once aday. The stem bark is cooked and then consumed.	(Hamza *et al.*, 2006; Ugiomoh *et al.*, 2023)
Bark	Uganda,	Anticancer	100 grams of stem bark from *K. africana* and 25 grams of *X. aethiopica* fruits boiled in one liter of water. For two months, three teaspoons of this combination are consumed thrice every	(Gang *et al.*, 2023; Nakaziba *et al.*, 2021)

			day.	
Bark	Tanzania, Beninand Kenya	Rheumatism ,Cough, dysentery	Bark mixture taken orally Pounded bark is mixed with corn powder to make pap. Bark decoction combined with cow's milk. Stem bark decoction boiled with flour and soda.	(Hamza *et al.*, 2006)
Bark	Uganda	Spleen infection	To treat spleen infections, a decoction of *I. gabonensis* leaves and *K. africana* stem bark is employed.	(Nabatanzi *et al.*,2020)
	Uganda	Cough/sterility	The stem bark (or root bark) is decocted and consumed.	(Tabuti *et al.*,2003)
Bark	West and Central Africa	Venereal diseases	Usually applied to kids. managed with a stem bark concoction. Palm wine is made by macerating crushed and dried bark for two to three days. Forat least eight days, 100 Milliliters are taken every day.	(Towns *et al.*, 2014)
Bark + Fruit	Ghana	Rheumatism, wounds and malignant tumors	The mixture is ground into greasy pastes that are applied topically to treat malignant tumors and rheumatoid arthritis.	(Hassan *et al.*, 2018)
Bark +Leaves	Nigeria	Malariafever	Tea made from leaves and stem bark that can be used asa malaria remedy after bathing.	(Evbuomwan *et al.*, 2023)
Fruit	Cote Divore ,South Africa	Penile enlargement	The penis was stroked ordripped with fruit juice.	(Koné *et al.*,2021)
Fruit	Central Africa	Syphilis and rheumatism	A syphilis poultice ismade from the green fruit. The mature fruit powder isapplied topically to relieve rheumatism.	(Tsobou *et al.*, 2016)

Fruit	Africa	Wounds, ulcersand sores	A common method of treating sick body partsis the use of dried fruit.	(Hassan *et al.*, 2015)
Fruit	Uganda	(HIV/AID)	Fruit that has been cooked with milk, chilled, and consumedorally is a cure.	(Lamorde *et al.*,2010)
Fruit +Roots	Central Africa	Parturition haemorrhage .	When boiled together with the fruits and roots of *K. africana*, the stem and tassel of a plantain are used medically to alleviate postpartum hemorrhage.	(Tsobou *et al.*,2016)
Leaves	Uganda	High blood pressure	A tea spoonful of the roasted leaf ashes mixed with honey should be consumed once in the morning and once in the evening.	(Gang *et et al.*, 2020)
Leaves	Benin, Ivory coast and South Africa	Jaundice	For jaundice, stomach infections, stomachaches, anemia during menstruation, vomiting, coughing, and constipation, a decoction of the leaves is used.	(Dossou-Yovo et al., 2022)

Table 2. Extracts of different plant parts with antibacterial activity

Plant	Part	Bacteria	Activity (µg/ml)	Reference
Cytinus hypocistis (L.) L. (*Cytinaceae*)	Whole plant	*S. aureus*	125	(Maisetta *et al*., 2019)
		Staphylococcus epidermidis	250	
		VRE	31.25	
		S. aureus	500	
		S. epidermidis	500	
		VRE	125	
Cytinus ruber (Fourr.) Fritsch (*Cytinaceae*)	Whole plant	*S. aureus*	125	(Maisetta *et al*., 2019)
		S. epidermidis	250	
		VRE	31.25	
		S. aureus	250	
		S. epidermidis	250	
		VRE	125	
Phaseolus vulgaris L. (*Fabaceae*)	Leaves	*E. coli*	256	(Nayim *et al*., 2018)
Punica granatum L. (*Lythraceae*)	Fruit	*S. aureus*	51.67	(Alvarez-Martinez *et al*., 2021)
		MRSA	72.89	
Smilax china L. (*Smilacaceae*)	Stems	*E. coli*	195.31	(Xu *et al*., 2019)
		S. typhimurium	781.25	
		S. aureus	195.31	
		Listeria monocytogenes	781.25	
		Bacillus subtilis	781.25	
Syzygium legatii Burtt Davy & Greenway (*Myrtaceae*)	Leaves	MDR *E. coli*	50	(Famuyide *et al*., 2019)
Theobroma cacao L. (*Malvaceae*)	Beans	*E. coli*	64	(Nayim *et al*., 2018)
		Klebsiella pneumoniae	64	
	Leaves	*Enterobacter aerogenes*	256	
		K. pneumoniae	256	
Triumfetta welwitschia Mast. (*Malvaceae*)	Leaves	*Pseudomonas aeruginosa*	100	(Mombeshora and Mukanganyama, 2019)

2.1.3 Anti-microbial resistance.

Antimicrobial resistance (AMR), which makes it difficult to effectively prevent and treat chronic illnesses, is among the most urgent threats to people's health today. Despite numerous attempts over the past few decades to address this issue, global AMR trends do not appear to be slowing down. It is believed that the overuse and abuse of different antibacterial agents in hospital settings is the main source of antimicrobial resistance. Moreover, bacterial

mutation, horizontal transmission of resistant genes, and spontaneous evolution are the main causes of antimicrobial resistance. Numerous studies have demonstrated the serious financial consequences of AMR, such as the soaring costs of healthcare due to an increase in hospitalizations and medication consumption. Antimicrobial resistance is a multifaceted concern that has led to a number of intricate problems impacting nations worldwide. These effects can be divided into three categories: economic, healthcare, and patient. There isn't any discernible progress to stop the ongoing trends of antimicrobial resistance, despite the fact that there are evident knowledge gaps and areas for improvement on AMR. Public health systems around the world, not just in impoverished countries, are seen to be seriously threatened by AMR (Dadgostar, 2019).

2.2 Tuberculosis

The acid-fast aerobic bacteria *Mycobacterium tuberculosis*, which grow on gram stain as either gram-positive or gram-negative, results into infectious disease known as tuberculosis (TB). There are two types of tuberculosis: extra pulmonary and pulmonary. While extra pulmonary tuberculosis (TB) affects other sites, such as the spinal cord, pulmonary TB affects the lungs (WHO, 2018). Moreover, it can spread to the brain. After HIV/AIDS, these conditions are theprimary cause of infectious disease-related mortality. *Mycobacterium bovis, M. africanum, M.microti, M. pinnipedii, M. caprae, and M. canetti* are the other six species that make up the *Mycobacterium* tuberculosis complex, which includes the bacillus. The species are obligate tuberculosis pathogens, despite having different phenotypic characteristics and host ranges. Genetically, there is close relationship between the *Mycobacterium tuberculosis* complex species: the human and cattle-infecting *M. bovis* genome is closely related to tuberculosis, with a 0.05% genome difference (Dheda *et al*., 2017). *Mycobacteria*, which are characterized by their rod-like form, possess a lipid-rich cell wall that enables them to hold onto carbol fuchsin even when exposed to acidic alcohol. An infection with the *Mycobacterium tuberculosis* bacteria causes the illness known as tuberculosis. The lungs are most frequently impacted, yetit can also affect other bodily parts. Infections come in two flavors: latent and aggressive. Tenpercent of dormant infections reactivate. By speaking, coughing, and sneezing, droplets spread the illness. Before now, the state was referred to as "consumption." Micro bacterial cultures, skin test for tuberculin, and a chest X-ray are utilized in diagnostics (Dheda *et al*., 2017).

TB is most commonly diagnosed by microscopy using Ziehl-Neelsen or Kinyoun stain to detect

acid fast bacilli. To produce a positive smear, a specimen needs to have 104 colony forming units (CFU)/Ml or more. A more straight forward, efficient, and subsequently sensitive alternative for specimen staining in microscopy is the use of a fluorochrome dye, like auramine O. However, microscopic *Mycobacteria* detection cannot differentiate *Mycobacteria tuberculosis* from non-tuberculosis mycobacteria. (Holani *et al*., 2014). *M. tuberculosis* can thrive in the presence of inorganic nitrogen sources and simple carbon. This organism is unique in that it grows slowly in both artificial media and animal tissues. It takes roughly 20 to 24 hours to generate, compared to 20 minutes for organisms like *Escherichia coli* (Xu *et al*., 2022).

2.2.1 Types / Categories of Tuberculosis

There are three categories of tuberculosis: Latent Tb, Active TB, and drug resistant Tb.

a) Latent Tuberculosis

Latent tuberculosis (TB) arises when germs are present in the body but are kept from proliferating by the immune system. It has no symptoms and is not contagious. The infection is still there, though, and it might become active again sometime. If a person has HIV, has had an infection within the last two years or has weakened immune system, they are more likely to undergo re-activation. In this case, the physician will recommend medication to treat active tuberculosis. Latent tuberculosis doesn't show any signs. A blood or skin test can be used to establish whether someone is infected (Carranza *et al*., 2020).

b) Active Tuberculosis

When bacteria multiply and cause illness, active tuberculosis is the result. It is easy for the sickness to spread. In adults, latent TB infections account for 90% of active cases. Signs of active tuberculosis include fever, chills, coughing for longer than three weeks, chest pain, blood in the cough, chronic exhaustion, night sweats, appetite loss, and weight loss (Moule & Cirillo, 2020).

c) Drug-Resistant Tuberculosis

Drug-resistant forms of tuberculosis arise when an antibiotic doesn't totally destroy the microorganisms it targets. The germs that survive grow resistant to the drug in question as well as, most frequently, other antibiotics. Some tuberculosis bacteria have developed resistance todrugs that are often prescribed, such as rifampin and isoniazid. Furthermore, several strains of tuberculosis have developed resistance to less commonly administered drugs, including injectable drugs like amikacin and capreomycin (Capastat) and antibiotics referred to as

fluoroquinolones. Another reason tuberculosis remains a major cause of death is the growth in drug-resistant forms of the bacteria. Since the first antibiotics were used to treat tuberculosis more than 60 years ago, some tuberculosis bacteria have evolved the ability to live in the face of treatment, and this ability is passed on to their descendants (Mirzayev *et al*., 2021).

2.2.2 Tuberculosis Transmission

This happens when someone who has tuberculosis coughs, sneezes, talks or laughs. The germs are released into the air in the form of tiny droplets. These microbes are not surface-growing (Paleckyte *et al*., 2021).

2.2.3 Tuberculosis Test

Three standard tests are utilized to detect tuberculosis:

a) Skin Test

Another name for Mantoux skin test is tuberculin. It includes small injection of fluid under your skin in your lower arm, and after two or three days, we look for any swelling in that arm. You are most likely to have tuberculosis bacteria if your results are positive. However, a false positive can also occur if a person has received the Tb vaccine. In this case, this test could produce a false positive. In the event that a person has a very recent infection, the results could also be false negative. To rule out erroneous results, this test needs to be run multiple times (Sheikh *et al*., 2021).

b) Blood Test

This test, also known as an interferon-gamma release assay (IGRA), assesses how your body reacts to a small amount of TB proteins mixed with your blood. This test does not indicate if your infection is active or latent. The diagnosis of an infection, whether latent or active, will be made by the physician in the event of a positive skin or blood test (Blauenfeldt *et al*., 2020).

c) A Chest X-Ray or CT scan

Primary purpose of this test is to detect alterations in the lungs. The test for tuberculosis (TB) bacteria in sputum, or cough secretions, is called acid-fast bacillus (AFB) (Nijiati *et al*., 2022).

2.2.4 Treatments of Tuberculosis

The FDA has approved anti-tubercular drugs utilization such as pyrazinamide, ethambutol, isoniazid, and rifampin to treat *Mycobacterium tuberculosis* infections. Whether a patient has

an active or latent disease will determine which medications to use for therapy and in what combination and for how long (Alzayer & Al Nasser, 2023). Treatment for tuberculosis can result in multi-drug-resistant tuberculosis (MDR-TB), a dreaded adverse consequence. MDR-TB is unique in that it can resist the first-line medications rifampin and isoniazid. The field of MDR-TB treatment is constantly developing, and so are the recommendations. As second-line therapy for MDR-TB, injections of amikacin, capreomycin, and kanamycin are frequently employed. Second-line medications, also known as sixth agents, include gatifloxacine, moxifloxacine, and levofloxacine. They are used when first-line treatments become ineffective due to medication resistance (Dheda*et al.*, 2017). Pretomanid, when taken with bedaquiline and linezolid, is one of the medicationsfor multi-drug resistant tuberculosis that which has been approved by the FDA. Extensivelymultidrug-resistant tuberculosis is an uncommon and more severe variant of multidrug- resistant tuberculosis (MDR-TB). This infection is distinct due to its resistance to both fluoroquinolones, one second-line aminoglycoside, and the first-line drugs rifampin and isoniazid. Ethambutol, isoniazid, pyrazinamide, and rifampin are examples of first-line medications. Second-line medications include Kanamycin (which is no longer used in the USA), Streptomycin, Capreomycin, Amikacin, Levofloxacin, Moxifloxacin, and Gatifloxacin(Unissa *et al.*, 2016).

Treatment plan is defendant on infection type. When a patient is found to have latent tuberculosis, the doctor will suggest treatment to get rid of the germs and stop the infection from coming back. Isoniazid, rifapentine or rifampin may be administered to the affected individual concurrently or separately. These drugs can be taken for a maximum of nine months. Active TB is treated with a mix of drugs (Menzies *et al.*, 2009). The most widely used ones include rifampin, pyrazinamide, isoniazid, and ethambutol. In situations of drug-resistant tuberculosis, the doctor may also prescribe one or more alternative drugs, which are typically taken for six to twelve months. These drugs have potential negative effects and can be taken for up to thirty months (Padda & Reddy, 2022). Because tuberculosis patients have distinct bacterial populations, anti-tuberculosis medications are always used in combination. A single therapy would lead to drug resistance developing, treatment failure, and inadequate control of bacillary treatment. To eradicate slowly dividing semi-dormant bacilli that were not sufficiently sterilized and prevent relapse, anti-tuberculosis therapy is given for several months. Treatment consists of a continuation phase intended to eradicate any remaining bacilli after an initial intense phase intended to destroy actively growing and semi-dormant bacilli (Menzies *et al.*, 2009).

The WHO advises both newly diagnosed and previously treated patients to follow standard treatment regimens that include daily administration of rifampicin, isoniazid, pyrazinamide, and ethambutol under the close supervision of a medical professional to guarantee complete adherence to medication dosages (WHO, 2018). In addition, the WHO advises against six-month isoniazid-ethambutol regimens in favor of a four-month rifampicin and isoniazid medication combination. Clinical trials have demonstrated that, as long as the bacterially susceptible medications are taken regularly and in the recommended dosages, this is the most effective treatment plan with the lowest risk of relapse (WHO, 2018). Although fixed dose combinations lessen the number of pills taken, increase patient adherence, and lower the risk of drug resistance, the patients still have to deal with this. Second line medications are the preferred option in cases of multidrug-resistant tuberculosis; the first course of treatment lasts six months, with additional extensions of eighteen months following culture conversion and twenty-four months for more advanced disease (McIvor *et al*., 2017). Compared to the first line drug-sensitive TB regimen, the second line regimens are weaker, more toxic, and more costly. In patients with AIDS, successful treatment completion does not ensure safety because it can lead to recurrent tuberculosis (Jang & Chung, 2020). As a result, more involved re- treatment plans are required, which adds to the burden of managing TB. Recurrent tuberculosisthat develops several months after treatment ends is caused by disease reactivation, or the inability to eradicate all semi-dormant bacilli. Recurrent tuberculosis that develops later is morelikely to be the consequence of contracting a new infection (McIvor *et al*., 2017).

a) Active Tuberculosis

The initial phase and the subsequent phase are the two stages of treatment for this ailment. Two months of treatment with ethambutol, pyrazinamide, isoniazid, and rifampin comprise this initial phase. Within 60 days, 56 doses of this regimen are swallowed once day. The continuation phase entails taking isoniazid and rifampin for more 120 days, after completion this regimen is swallowed once daily for eighteen weeks, for a total of 126 doses. Patients who are intolerant to ethambutol may be switched to streptomycin. The weight-based adjustments for these dosages are made. Fluoroquinolones like levofloxacin, gatifloxacin, and moxifloxacin are given orally, while second-line medications like kanamycin, capreomycin, and amikacin are given intravenously. When these drugs become resistant, first-line therapy are options (Babalik *et al*., 2011).

b) Latent Tuberculosis

One of the most well-liked and effective treatments for latent tuberculosis is isoniazid therapy, which lasts nine months. For nine months, this regimen is taken once a day, for a total of 270 doses. Furthermore, three months of isoniazid plus rifampin therapy or four months of rifampin mono therapy are alternatives. Over 90% of latent disease can be affected by isoniazid mono therapy if it is used for the whole nine months. It is advised to take pyridoxine, or vitaminB6, in addition to isoniazid therapy since the lack of vitamin B6 caused by isoniazid used alone might produce peripheral neuropathy (Padda & Reddy, 2022).

2.2.5 Tuberculosis Medication Side Effects

Drug outcomes/ consequences are prevalent after receiving TB treatments. Isoniazid outcomes include weakness, nausea, vomiting, numbness and tingling in the hands and feet, and decreased appetite. The outcomes of ethambutol are headache, nausea, chills, edema or hurting joints, decreased appetite and confusion. Pyrazinamide outcomes are fatigue, nausea, vomiting, decreased appetite and painful muscles or joints. Diarrhea, pancreatitis, poor appetite, nausea, throwing up, upset stomach and skin rash are common outcomes of rifampin (Dasopang *et al*., 2020).

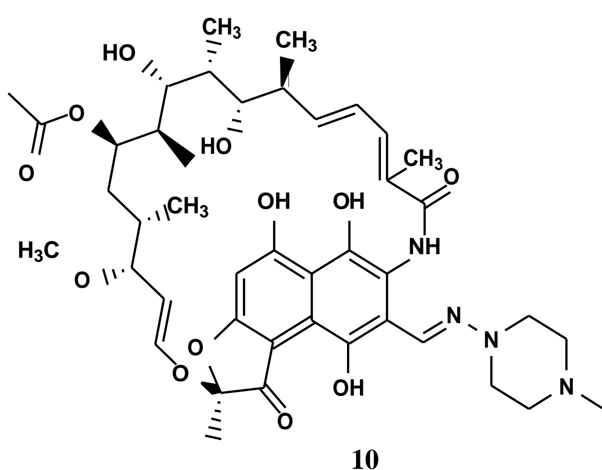
2.2.6 Tuberculosis Prevention

To prevent a latent infection from becoming active and spread, it is important to take all recommended medication. When a person has active tuberculosis, they should minimize their social interactions. A victim should cover the mouth when coughing, sneezing, or laughing. Throughout the initial few weeks of treatment, wear a surgical mask whenever you are in public. Children in TB-endemic nations are frequently immunized with BCG. It isn't frequently used in the US and isn't always effective at preventing infection. Only children who live with an active TB infection involving a highly drug-resistant strain or who are incapable of taking antibiotics are advised to do so by doctors (Borham *et al*., 2022).

2.2.7 Mechanism of Action

Rifampin (10) inhibits transcription and the subsequent synthesis of bacterial proteins by reversibly inhibiting DNA-dependent RNA polymerase. Prodrug isoniazid (**11**) is catalase-peroxidase-transformed into its active form metabolite, which is then used by the body to produce further biosynthesis. It is yet uncertain how precisely pyrazinamide (**12**) works.

Pyrazamide inhibits trans-translation and perhaps coenzyme, an important synthesis for the survival of the bacterium, after converting to pyrazinoic acid. Ethambutol (**13**) inhibits the arabinosyl transferases enzyme and halts the development of the mycobacterial cell wall. Streptomycin (**14**) and amikacin (**15**) are examples of aminoglycosides that function by attaching tothe 30S ribosomal subunit and stopping *Mycobacteria* from making proteins (S. & C., 2012). Quinolones, including **16**-doxifloxacin further inhibit DNA synthesis in the bacterium. These fluoroquinolones also block DNA gyrase and topoisomerase IV (Padda & Reddy, 2022).



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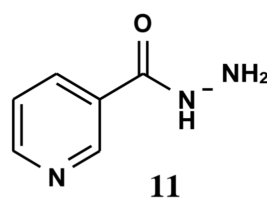

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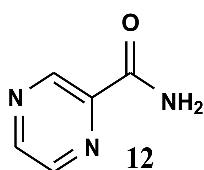

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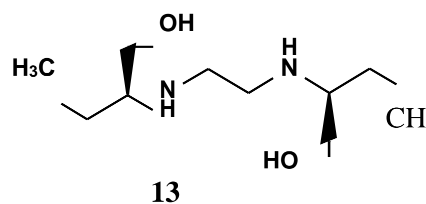

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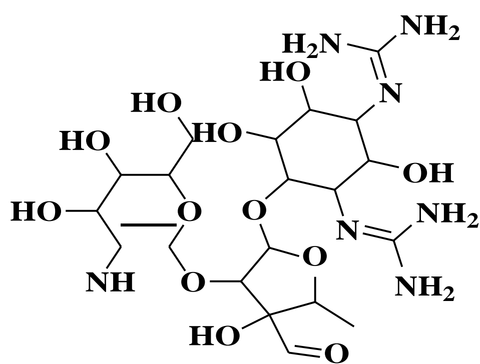

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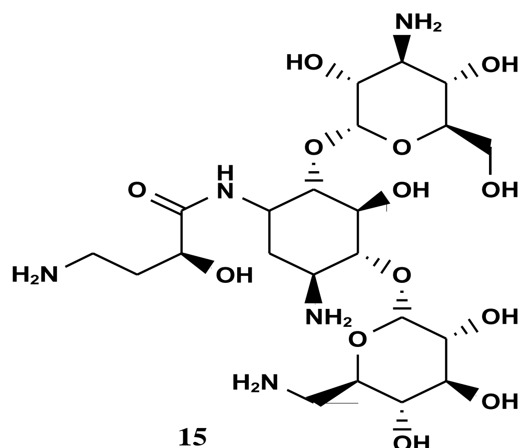

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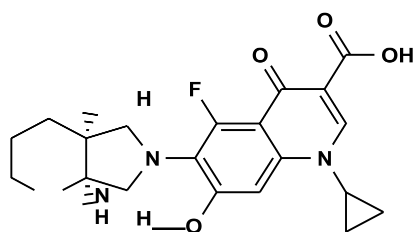

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2.2.8 Toxicity

Hepatotoxic effects are possible with all first-line anti-tubercular medication, including rifampin (10), isoniazid (11), pyrazinamide (12), and ethambutol (13). If liver function test results keep increasing, treatment should be discontinued. When the medication is stopped, nephrotoxicity caused by aminoglycosides can be reversed. Renal toxicity is contingent upon the presence of any underlying renal disease in the patient as well as the medication dose being taken. For most patients, renal insufficiency is preventable (Swain *et al*., 2020).

2.2.9 Extraction

Since chemicals that regulate protein activity have been selected for evolution, natural products represent a pre-validated portion of "biologically relevant chemical space". Natural product introductions have played a major role in improving tuberculosis clinical therapies (Ponphaiboon *et al*., 2023). The most common variables affecting extraction procedures include temperature, extraction duration, solvents employed, and the matrix properties of the plant portion. Bioactive chemicals can only be obtained once the extraction process has been properly completed and subjected to further identification, characterization and separation processes.

Bioactive chemicals can be extracted from plant materials using a variety of conventional

extraction methods. The majority of these techniques rely on the extraction, heating, and/or mixing strengths of the solvents. Three popular traditional processes—soxhlet extraction, maceration, and hydro distillation—gave an extract which was concentrated with a rotating extractor (Altemimi *et al*., 2017; Ponphaiboon *et al*., 2023). In column chromatography, a vertical glass column filled with a solid adsorbent stationary phase is topped with a liquid mobile phase. Gravity causes the liquid mobile phase to flow down the column. Traditionally, column chromatography was employed as a purification method to separate out particular molecules from a mixt (Solomon, 2019). The top of the column was filled with crude extract that would be purified via column chromatography. Gravity caused the liquid solvent, sometimes referred to as the eluent, to flow through the column. The eluting solvent passing through the column and the solute adsorbed on the adsorbent were in balance. A separation happened as a result of the various components of the mixture reacting differently with the stationary and mobile phases, resulting in varied degrees of their transportation along with the mobile phase. The different components, or elutants were collected as the solvent seeped out of the bottom of the column (Hasnat *et al*.,2023). In column chromatography, two common adsorbents are silica gel (SiO_2) and alumina (Al_2O_3). The different mesh sizes in which these adsorbents are sold are indicated by a number on the bottle label. The polarity of the solvent that was poured through the column affected the relative rates at which the compounds went through it (Ponphaiboon *et al*., 2023).

In order to better solvate the polar elements, polar solvents outcompeted polar molecules in a mixture for the polar sites on the adsorbent surface. Thus, even highly polar molecules will pass through the column rapidly in a highly polar solvent. The components of the mixture will be moved too much and separated very little or not at all by an overly polar solvent. A solventwon't elute any compounds from the column if it isn't polar enough.

Because of this, choosing the appropriate eluting solvent is crucial to using column chromatography as a separation method effectively. It is usual practice to use a series of increasingly polar solvent systems to elute a column. The less polar molecules are first eluted using a non-polar solvent. After the less polar molecule was taken out of the column, a more polar solvent was introduced to elute the more polar molecules (Altemimi *et al*., 2017; Ponphaiboon *et al*., 2023).

2.2.10 Thin Layer Chromatography

To determine whether column chromatography fractions include a lot of different components and whether mixing them won't affect their purity, thin layer chromatography, or TLC, is frequently utilized. The relative affinities of the compounds for the stationary and mobile phases dictate the TLC separation process. The compounds travel across the surface of the stationary phase while being influenced by the capillary-driven mobile phase. Compounds with a lower affinity for the stationary phase move faster during this movement, while compounds with a higher affinity move more slowly. The components of the combination are so scattered. Iodine vapor staining makes each component visible as dots on the plate upon separation (Kowalska & Sajewicz,2022).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Kigelia africana stem bark samples were collected from Kaliro District using a plant taxonomist's assistance. The Makerere University Herbarium received the sample voucher specimens for identification and confirmation. In the shade, the finely chopped plant matter was left to dry. A summary of the main analytical methods employed in natural product research demonstrates how important method choice is for both qualitative and quantitative studies of bioactive compounds derived from plants. This section includes some of the methods commonly used in natural product research.

3.1.2 Chemicals, Apparatus and Organisms

The chemical reagents included Sodium hydroxide, ferric chloride, ethyl acetate, ether, ethanol, butanol, chloroform all analytical grade manufactured by Rankem, ammonia solution analytical grade manufactured by SRL chemicals, Hydrochloric acid analytical grade manufactured by Mercklife science Pvt limited. The above chemicals were all purchased from ChemiQuip Uganda. Disposable gloves, autoclave, laminar flow hood, incubators including carbon dioxide, incubator for *mycobacterium tuberculosis*, micro titer plates ,90 mm-diameter with quadrants, platinum wire loop, 6.35mm discs supplied in cartridge sortubes under anhydrous conditions, 0.2µm disposables syringe filter for cold sterilization, disposable syringes, auto clavable bottles, micro pipette tips, vortex, bio-hazard bags, sterile distilled water, dimethyl sulphoxide, middlebrook7H9broth, Middlebrook7H10 agar, Lowenstein Jensen, isoniazid ,rifampicin and susceptible strains for testing *Mycobacterium tuberculosis* and rifampicin for quality control were also used.

3.2 Methods

3.2.1 Extraction Process

The dried stem bark plant material was ground using a blender, 1000 grams of a brownish-red powder was obtained, and soaked in Dichloromethane and methanol in a ratio of 1:1 for 24 hours and a brownish-red crude extract was obtained. The soaking was repeated twice, and exhaustive extraction was achieved (Salih *et al*., 2020; Sarker *et al*., 2006). This was filtered and then dried on a rotary evaporator at low pressure which led to 150 g of the extract being

obtained. The crude extract was divided into two portions: one was used for the bioassay tests and the other was used for chromatographic fractionation and isolation. The extracts were preserved until additional analysis was performed by being stored in a refrigerator at a temperature of -15 ^{0}C.

3.2.2 Fractionation and Compound Isolation

According to (Saulnier, n.d.), A prepackaged 200 g of silica gel (70–230 mesh ASTM) was used for column chromatography on the crude extract (55 x 5 cm). As shown in figures 2, 3, and 4, the crude extract (1g) was put through chromatographic separations, the column was eluted using n-hexane as the mobile phase. In order to boost the polarity, more ethyl acetate was gradually added to the solvent. TLC was used to assess each fraction's purity after the resultant fractions were dried on a rotary evaporator, as shown in figures 5, 6, 7, 8, and 9. Fifteen simplified fractions were obtained by pooling the fractions with comparable Retention factor values.

Figure 2

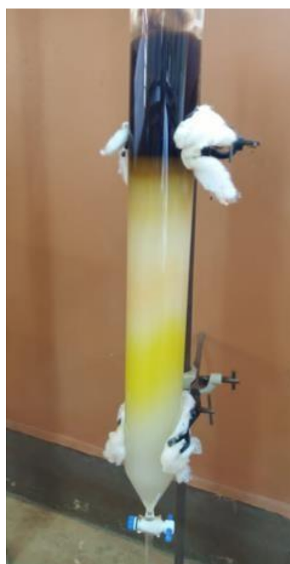


Figure 3

Figure 4

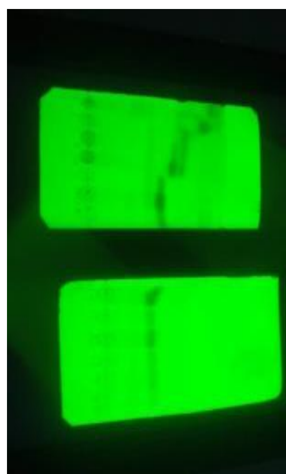


Figure 7

Figure 6

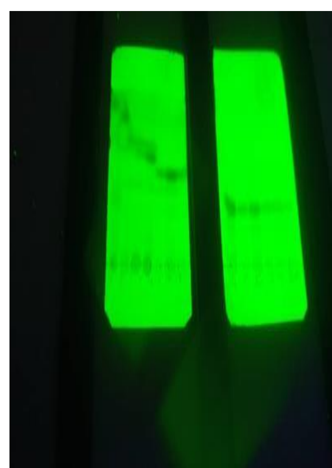


Figure 5

Figure 9

Figure 8

Under a lower vacuum, each of these fractions was evaporated on a rotary evaporator until it was dry. Fraction I (that eluted with 2% ethyl acetate in n-hexane) gave an amorphous solid of compound (**3**) (35 mg). Fraction II (that eluted with 3% ethyl acetate in n-hexane) consisted of two compounds which were separated by further purification over Sephadex LH-20 (eluent; dichloromethane-methanol, 1:1) to give unidentified compound FIIA (6 mg), and unidentified compound FIIB (3 mg). Fraction III (that eluted with 5% ethyl acetate in n-hexane) consisted of five compounds which were separated by further purification over Sephadex LH-20 (eluent; dichloromethane-methanol, 1:1) to give a colorless, oily liquid with no odour of compound (**2**) (25 mg), and other four unidentified compounds (3 mg, 5 mg, 3 mg, 3 mg). Fraction IV (that eluted with 10% ethyl acetate in n-hexane yielded a white amorphous solid of compound (**1**) (47 mg). Fraction V (that eluted with 15% ethyl acetate in n-hexane) yielded a white solid of compound (**4**) (29 mg). On performing thin layer chromatography and viewing under uv lamp, fraction II showed two single spots which necessitated further purification, Fraction III gave single spots and further purification was carried out over sephadex (eluent;

dichloromethane-methanol 1:1) with consequent TLC processes and thus compound **2** was isolated. The fractions which showed minimal spotting on viewing under ultra violet light were not considered for further purification.

3.2.3Anti-bacterial, anti-fungal and anti-tuberculosis activities

3.2.4 Sample pretreatment

The crude extract together with four pure isolated components which were separated for antibacterial, antifungal, and anti-TB properties underwent bioassay studies. The serial dilution method was used to determine the lowest inhibitory concentration of the crude extract and isolated compounds against bacterial, fungal, and Mycobacterium tuberculosis activities, respectively. These tests were conducted at Kyambogo University's biology lab (anti-bacterial and anti-fungal activities) and the national tuberculosis reference laboratory (anti-Tb activity).

3.2.5 Preparation of the crude and isolated compounds for antibacterial, antifungal and anti TB tests

Dimethyl sulfoxide (DMSO) was used to dissolve 0.5g of dried crude and 0.5g of isolated compounds, yielding a concentration of 50 mg/ml of stock solutions. Two-micron single-use filters were used to sterilize both the separated chemicals and the crude.

3.2.6 Selection and collection of the bacterial, fungal and mycobacterial strains

The isolates of bacterial and fungal were obtained from the Biology Laboratory, Department of Biological Sciences, Kyambogo University. Bacterial and fungal strains isolated were *Staphylococcus aureus* and *Candida albicans*.

The Rifampicin resistant (TMC331) strain, isoniazid and susceptible strains of *mycobacteria* were obtained from Butabika Tuberculosis Reference Laboratory in Kampala, Uganda, where*mycobacteria* bioassay was done.

3.2.7 Preparation of growth media and Inoculation for antibacterial, antifungal and anti-Tuberculosis preparation Bioassay tests

Eloff's approach of serial dilution was applied (Eloff, 2019). In 1.5 ml micro centrifuge tubes (Eppendorff), stock solutions of the crude extract and the isolated compound were made by dissolving the extract in dimethyl sulphoxide (DMSO) to a final concentration of 64 mg/ml. 96-well microplates were used to create serial dilutions of the stock solution, which ranged from 32 mg/mL to 0.25 mg/mL, using Mueller-Hinton broth (Becton Dickinson, Sparks,

MD,USA).

The bacterial suspension was made from a 24-hour culture plate and contained about 5 × 105 colony-forming units/mL. 100 µl of this slurry was added to each well for inoculation. For the strain, a sterility control well and a growth control well were also investigated. Since yeasts need more time to grow, the microtiter plates were incubated at 37 °C for 24 hours for bacteria and 48 hours for yeasts. As a measure of microbial development, 40µl of an INT solution containing 0.4 mg/ml was added to each well following incubation at 37 °C. 30 minutes were spent incubating the plates for bacteria and 24 hours for yeast at 37°C. The MIC values were ascertained using the eyes. Extract's lowest concentration, 0.30 uL, was the minimal inhibitory concentration since it showed no signs of growth. The concentration of the extract that inhibited bacterial/yeast growth completely (the first clear well) was 0.6uL and taken as the MIC value, 0.35 uL of compounds **1** and **2** inhibited bacterial growth and were recorded asMIC values. These were determined in triplicates (Manyawi *et al*., 2023).

A sterile 41-well round-bottom microplate was rimmed with 200 µL of sterile deionized water for each well. The remaining wells received 50 µL of 7 H9 broth, whereas the wells in the drug-free column received 98 µL of soup. 2 µL of INH from the 2.5 µg/mL working stock concentration was added to the well designated for the control and 2 µL of the test compounds from the 20 mg/mL stock concentration was added to the wells chosen for test compounds. Using 2 µL from each well, created the same serial dilutions (1:2). The extra medium was removed from each well until only 20 µL of volume remained in each well. As a control, a compound free well effectively reconstituted to 2×10^7 CFU/mL of *M. tuberculosis* H37Rv on ice after thawing the frozen stock cryovial. To calculate the CFU/mL, one vial was taken from each freezer box, and the boxes from which the vials were obtained were noted. Serial dilutionswere then plated out. This action was taken to prevent discrepancies in CFU/mL between cryovials that were filled at different times. In order to reduce the amount of medium evaporation in the test wells during incubation, water was added to the wells around the outsideof the microplate. This was thoroughly mixed after adding 100 µL of thawed stock to 10 mL of fresh 7 H9 medium. 50 µL of this mixture was added to wells that contained the drug and the compounds separately. The goal was to reach a final concentration in each well of 1 × 105 CFU/mL. The final concentrations for the test chemicals and INH varied from 200 µg/mL to 0.78 µg/mL and 0.25 µg/mL to 0.0009 µg/mL, respectively. In the negative control wells, only the solvent and the bacterial culture were added. After using Parafilm to

seal the plate, it was incubated for 5 days at 37°C. After five days, 10 μL of Alamar blue reagent was put into a compound well. The plate was re-incubated at 37°C for a full day. All the other wells in the microplate received the Alamar blue reagent mixture after the well-turned pink. After resealingthe plate, it was incubated for a further twenty-four hours at 37°C (Tuyiringire *et al*., 2022).

This was done following the method of Quality Control of Individual Components Used in Middlebrook 7H10 Medium for *Mycobacterial* Susceptibility Testing (Guthertz *et al*., 1988). In this method to revive and culture the *Mycobacteria*, Middlebrook7H10 agar mixed with oleic acid-albumin-catalase (OAAC) was utilized. To prepare them, 19.0 g of dehydrated medium were added to 900 ml of filtered water that contained 15.0 ml of glycerol. After giving the mixture a good swirl to dissolve it, it was autoclaved for 10 minutes at 121°C. After chilling to45°C, 100 ml of oleic acid-albumin catalase was aseptically added to the medium. There were no P^h corrections performed because most bacteria are neutrophiles and grow at near neutral P^h (Fang *et al*., 2013).

This was done according to the method of Formulation of Microbial Inoculants by Encapsulation in Natural Polysaccharides: Focus on Beneficial Properties of Carrier Additives and Derivatives (Vassilev *et al*., 2020). Before anti-TB susceptibility testing, preserved strains of multi-drug resistant *Mycobacteria tuberculosis* were resurrected on Lowenstein-Jensen. Three-week-old, freshly developing colonies on Lowenstein-Jensenslants were scraped of theirbacterial cells, which were then added to one milliliter of middle Brook 7H9broth.After givingthe tubes a violent 30-second vortex in a plastic tube filled with glass beads, the particles wereleft to settle for around ten minutes (Tuyiringire *et al*., 2022). The supernatant was collected and adjusted to a turbidity of opacity equivalent to McFarland standard of 0.51. The inoculumwas then ready for use.

3.2.8 Determination of Minimum Inhibitory Concentration (MIC) for the bacterial, fungal and mycobacteria strains

With positive control experiments, the sensitivity of the microorganism was ascertained by starting concentrations of 0.10 mg/mL of ampicillin (Sigma-Aldrich) for bacterial strains. 0.10 mg/mL of fluconazole for anti-fungal strains in DMSO and water (where 1.00 mg/mL was prepared in DMSO and diluted to 0.1 mg/mL in sterile water thereafter). For these trials, the ultimate concentrations varied between 25.0 μg/mL and 0.19 μg/mL. The only substance used in the negative control experiment was DMSO. Mueller-Hinton broth (Becton Dickinson,

Sparks, MD, USA) was employed to create serial dilutions of the separated compounds' stock solution in 96-well microplates, with concentrations starting from (5.0 µg/mL to 3.90 µg/mL) (Manyawi *et al*., 2023). The microbroth dilution approach was used to determine the lowest inhibitory concentration, or MIC, of the Mycobacterial tuberculosis strain (Getahun *et al*., 2022). The medium used was Middle Brook7H9. The isolated compounds and crude extract were serially diluted to check the lowest Inhibitory Concentration of the extract together with isolated compounds on the strain resistant to rifampicin (TMC331). The use of resazulin allowed for the confirmation of either *mycobacterial* growth or lack thereof. Separate serial dilutions were prepared and then mixed in various micro-Titer plates. A turbidity standard and McFarl were created (Mpeirwe *et al*., 2023; Tuyiringire *et al*., 2022). The typical medication, rifampicin, had MIC values of 0.06 and 1.8 M in MABA, indicating a notable cytotoxicity (Mpeirwe *et al*., 2023; Tuyiringire *et al*., 2022).

3.3 Determination of chemical structure and Structural Elucidation for the isolated compounds

1D and 2D nuclear magnetic resonance (NMR) spectroscopic methods were among the spectroscopic and spectrometric methods used to determine the chemical structures of the isolated substances (Přichystal *et al*., 2016). The analysis was carried out at the Institute of Chemistry, Potsdam University, Germany.

NMR spectra were run using a Bruker Avance Neo spectrometer operating at 500 MHz for the ^{1}H nuclei and 125 MHz for ^{13}C nuclei with test samples dissolved in deuterated chloroform solvent. The spectra were processed using MestReNova software version 10. Structural elucidation and NMR assignments were based on both one-dimensional (^{1}H, and ^{13}C NMR) and two-dimensional (^{1}H-^{1}H COSY, HSQC, HMBC) experiments using residual solvent peaks as internal references.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Extraction and Isolation

The crude extract from the stem bark of *K. Africana* weighed 1000g and was analyzed using thin layer chromatography. This revealed the presence of phytochemicals as visualized under Ultraviolet light (254 and 366 nm) and with the aid of iodine vapour. The phytochemical constituents of the extracts were isolated using a combination of chromatographic techniques such as column chromatography. Solvents used for extraction were methanol and dichloromethane both analytical grades. Four pure compounds were isolated and elucidated. Bioassays were then carried out for anti-TB, anti-bacterial and anti-fungal tests. The results obtained are discussed in the sub-sections that follow.

4.2 Characterization of compounds isolated from the stem bark of *K. africana*

Compounds from the air-dried ground stem bark of *K. africana* were extracted with dichloromethane/methanol (1:1) by cold percolation at room temperature which resulted into isolation of a stigmasterol (**1**), 4-Hydroxyphenethyl butyrate (**2**), 6-demethylkigelin (**3**) and Stearic acid (**4**).

4.2.1 Stigmasterol (1)

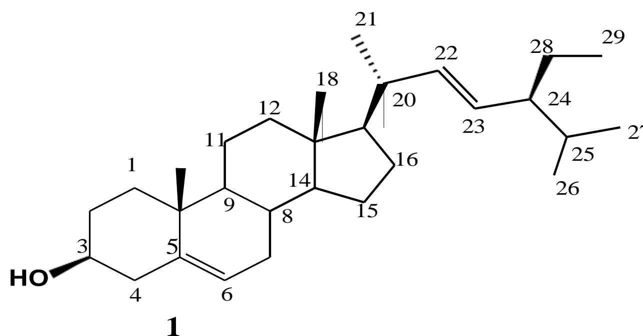
Compound **1** was isolated as a white amorphous solid. It was active against iodine vapour which implied presence of unsaturation. Its ^{13}C NMR spectrum (Table **4.2**) showed twenty-nine signals and this is consistent with a steroid skeleton (Tsobou *et al*., 2016). Four of these signals at δ_C 141.5, 138.9, 130.0 and 122.0 ppm were for olefinic carbon atoms, which are consistent with two carbon-carbon double bonds (Manyawi *et al*., 2023). The other signals included the six methyl carbons δ_C 12.2, 12.3, 19.8, 21.6, 22.7 and 23.05, one of which at δ_C 72.2 ppm was for an oxygenated C-3 of steroids (Manyawi *et al.*, 2023). ^1H NMR spectra of Compound 1 showed the presence of two methyl singlets at δ 0.71, and 1.01; three methyl doublets that appeared at δ 0.80, 0.82, and 0.91; and a methyl triplet at δ 0.83.

The compound also showed protons at δ 3.53, 5.15, and 5.35 suggesting the presence of three protons corresponding to that of a tri-substituted and a di-substituted olefinic bond. The proton corresponding to the H-3 of a sterol moiety appeared as a triplet of doublet of doublets at δ 3.52. The above spectral data supported the presence of sterol skeleton having a hydroxyl group at C-3 position with two double bonds at C-5/C-6 and C- 22/C-23 with six methyl groups which was supported by the key COSY and HMBC correlations. The ^{1}H and ^{13}C NMR values for all the protons andcarbons (**Table 4.2**) were assigned on the basis of COSY, HMQC and HMBC correlations and in comparison, with data in the literature (Dube *et al*., 2023). By direct comparison of its NMR spectral data in Table **4.2** with literature, compound **1** was identified as stigma sterol (Tornimbene *et al*., 2022). Thus, the structure of compound **1** was assigned as the known compound stigmasterol.

In a study done by (Manyawi *et al*.,2023) and it was showed that the roots of *Rhoicissus tridentata* afforded good yield of stigmasterol. The compound has been reported to show pharmacological activities like as anti- immunomodulatory, antitumor, antimutagenic and neuroprotective outcomes among others (Manyawi *et al*., 2023). Furthermore, some sterols have been associated with the management of skin afflictions such as fungal infections and skin cancer (Akhtar *et al*., 2015), and β-sitosterol has exhibited anti-cancer activities and can further be developed for the chemo prevention and chemotherapy of the different cancer types (Awad *et al*., 2000; Komakech *et al*., 2017). The stem bark of *K. africana* is considered to be an excellent source of medicines for skin fungal infections (Nabatanzi *et al*., 2020). Therefore, the isolation stigmasterol is a strong support for the traditional uses of the plant in treating skin problems (Grace *et al*., 2003; Nabatanzi *et al*., 2020).

Table 4.2: ^{1}H ($CDCl_3$ 500 MHz) and ^{13}C ($CDCl_3$ 125MHz) NMR spectral data for Compound 1

Position	δ_H Experimental data	δ_C Experimental data	δ_C literature data (Luhata & Munkombwe, 2015)
1	1.84	36.8	36.72
2	1.88	30.2	29.71
3	3.52	72.2	71.97
4	2.31	42.9	42.35
5	-	141.5	140.94
6	5.35	122.0	121.32
7	1.50	32.5	31.71
8	1.45	30.5	29.24
9	0.94	50.7	50.03
10	-	36.7	36.16
11	1.06,1.56	24.4	24.32
12	1.18	39.9	39.82
13	-	40.1	40.45
14	1.00	57.4	56.90
15	1.54	24.5	24.32
16	1.84	29.5	28.90
17	1.12	56.6	56.03
18	0.71	12.2	12.06
19	0.96	19.8	19.06
20	1.36	40.2	39.82
21	0.92	23.1	23.12
22	5.01	138.9	138.40
23	5.15	130.0	129.34
24	0.95	51.3	51.26
25	1.68	33.9	34.01
26	0.85	21.6	21.12
27	1.26	22.7	22.82
28	0.99	25.1	25.32
29	1.01	12.3	12.06



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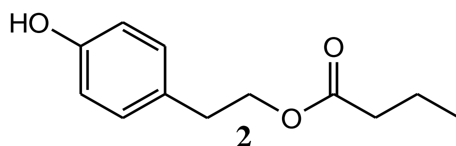
4.2.2 4-Hydroxyphenethyl butyrate (2)

Compound **2** was isolated as a colorless and odourless, oily liquid with a molecular ion peak in the EI-MS at *m/z* 208.2 (appendix 2E), corresponding to $C_{12}H_{16}O_3$. It was identified to be an ester based on its ^{13}C NMR spectrum that displayed a carbonyl peak of δ_C 174.0 (Aljubiri *et al.,* 2021). The ^{1}H NMR spectrum had a characteristic AA′XX′ spin system [δ_H 7.08 (m, 2H, H-2/6) and 6.76 2H, (H-3/5)] which is attributable to a *para*-substituted phenyl ring. Presence of an ethenyloxy group was deduced from the ^{1}H NMR spectrum which showed corresponding signals of an A_2X_2 spin system at δ_H 2.86 and 4.23. This revelation was further supported by the ^{13}C NMR and the HSQC spectra which exhibited carbon peaks at δ_C 34.4 (C-1′) and 65.1 (C-2′). The ^{1}H NMR spectrum further displayed features of an alkanoate with a terminal methyl group [δ_H 0.90, (t, J = 6.9 Hz, H-4″). This was further supported by the HMBC correlation observed between methylene protons at δ_H 2.27 (H-2″) and the carbonyl carbon at δ_C 174.0 (C-1″). Complete assignment was achieved on the basis of COSY, HSQC and HMBC correlations (**Appendices: 2B, 2C and 2D, respectively**) and are given in **Table 4.3**. The compound was, thus, identified as 4-Hydroxyphenethyl butylate (**2**), isolated for the first time from *Kigelia africana*. Hydroxytyrosyl derivatives such as compound 2 are reported to exhibit antioxidant properties (Mentos *et al*., 2008).

Table 4.3: ^{1}H ($CDCl_3$ 500 MHz) NMR spectral data for compound 2

	Experimental data		**Literature data** (Mariana *et al*.,2006)	
Position	**δ_c /ppm**	**δ_c /ppm (mult., *J* in Hz)**	**δ_H/ppm**	**δ_c /ppm (mult., *J* in Hz)**
1	130.2	-	129.7	-
2/6	130.2	7.08 (m)	127.7	6.62 (dd)
3/5	115.6	6.76 (m)	116.1	6.44 (dd)
4	154.3	-	145.0	-
1′	34.4	2.86 (t, 7.1)	33.5	2.67 (t,6.9)
2′	65.1	4.23 (t, 7.1)	64.1	4.10 (t,7.0)
1″	174.0	-	172.7	-
2″	34.5	2.27 (t, 7.5)	33.5	2.27 (t,7.3)
3″	22.8	1.30 (m)	24.4	1.25 (m)
4″	14.3	0.90 (t, 6.9)	13.8	0.84 (t,7.0)



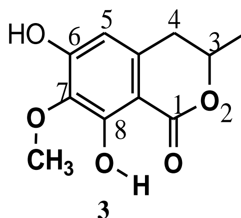
4.2.3 6-demethylkigelin (3)

Compound **3** was isolated as a white amorphous solid with a molecular ion peak, in the EI-MS, at m/z 224 (**Appendix 3F**) corresponding to a molecular formula of $C_{11}H_{12}O_5$. The ^{13}C NMR spectrum of the compound (**Appendix: 3E**) showed eleven signals including peaks due to an aromatic ring system (δ_C 102.3, 105.4, 133.1, 135.6, 155.0 and 155.6); an aliphatic cyclic system (δ_C 20.8, 34.6, 76.0) with oxygen as a heteroatom; and a diortho-substituted aromatic methoxyl peak (as collaborated with HSQC data) at 61.0 ppm (7-OCH_3). The presence of a signal at δ_C 170.2, in the ^{13}C NMR spectrum, was suggestive of a lactone moiety. The ^{1}H NMR spectrum exhibited broad singlet at δ_H 11.38 which was attributed to an intra-molecularly hydroxyl group (8-OH) chelated to the oxygen atom of the lactonyl carbon (C-1).

Table 4.3: ^{1}H ($CDCl_3$ 500 MHz) and ^{13}C (125 MHz) NMR spectral data for compound 3

	Experimental data		**Literature data (**Atsumi *et al.*,2020)	
Position	**δ_C/ppm**	**δ_H/ppm (mult., *J* in Hz)**	**δ_C/ppm**	**δ_H/ppm (mult., *J* in Hz)**
1	170.2	-	170.1	-
3	76.0	4.66 (m)	75.9	4.66 (m)
4a 4b	34.6	2.83 (m) 2.84 (m)	34.4	2.82 (m) 2.85 (m)
5	105.4	6.32 (s)	105.2	6.33 (s)
6	155.0	-	154.9	-
7	133.1	-	133.6	-
8	155.6	-	155.4	-
8a	102.3	-	102.2	-
9	20.8	1.51 (d, 6.1)	20.6	1.50 (d,6.4)
7-OMe	61.0	3.98 (s)	60.9	3.98 (s)
8-OH	-	11.38 (*br* s)	-	11.38 (s)

Both the ^{1}H NMR and the ^{1}H-^{1}H COSY spectra revealed the presence of 2-hydroxy-propyl moiety of a typical cyclic ester [δ_H 1.51, d (J = 6.1 Hz, 3H, H-9), 2.83-2.84, m (2H, H-4a & H-4b), 4.66, m (1H, H-3)] whose attachment at C-5a of the aromatic ring was based on the HMBC correlation between H-4a, 4b with carbons resonating at δ_C 102.3 (C-8a), 105.6 (C-5) and 135.6 (C-5a).From the above data, compared with literature, compound **3** was deduced to be a dihydroisocoumarin, 6-demethylkigelin previously obtained from the root bark of *K. africana* (Atawodi & Olowoniyi, 2015; Govindachari *et al.,* 1971), but hereby reported for the first time from the stem bark of the plant.



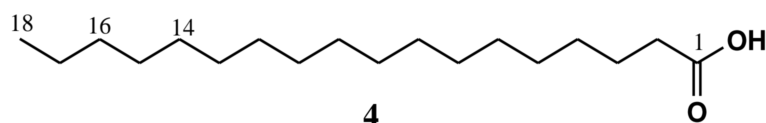
Coumarin derivatives are known for their anticoagulant properties and are responsible for maintaining the fluidity of blood (Jain and Joshi, 2012). The isolation of compound **3** supports the use of the plant in the management of cardiovascular diseases in Africa (Nabatanzi *et al*., 2020). Some of the bioactivities of 6-demethylkigelin include anti-trypanosomal and antiprotozoal properties (Atawodi & Olowoniyi, 2015).

4.2.4 Stearic Acid (4)

Compound **4** was isolated as white amorphous solid whose EI-mass spectrum (**Appendix 4G**) gave a molecular ion peak at m/z 284.2715 corresponding to $C_{18}H_{36}O_2$. The ^{1}H NMR spectrum of compound **4** displayed the terminal methyl protons at δ_H 0.88 (t, J = 6.9 Hz, H-18). The presence of a carboxylic acid was deduced from ^{13}C NMR spectrum (**Appendix 4F**) which exhibited a signal at δ_C 180.5 (C-1). Furthermore, the ^{1}H NMR spectrum together with the HSQC revealed fourteen CH_2 groups at δ_H 1.17 - 1.40 (m, 28H, CH_2-4 to CH_2-17) characteristic of an aliphatic chain. The signals at δ_H 2.34, (t, J = 7.5 Hz) and 1.63 (quintet, J = 7.5 Hz), ^{1}H NMR spectrum, exhibited two ethyl protons adjacent to a carboxyl group assignable to H-2 and H-3, respectively.

Table 5: ^{1}H ($CDCl_3$, 500 MHz) NMR spectral data for compound **4**

	Experimental data		**Literatre data** (Abdurrahman *et al.*,2020)	
Position	**δ_C/ppm**	**δ_H/ppm (mult., *J* in Hz)**	**δ_C/ppm**	**δ_H/ppm (mult., *J* in Hz)**
1	180.5	-	178.74	-
2	34.0	2.36 (t, 7.5)	33.81	2.34 (t, 7.5)
3	24.7	1.63 (quintet, 7.5)	31.91	1.63 (m)
4-15	29.2 - 29.9	1.17 - 1.40 (m)	29.7-30.00	29.7-30.0 (m)
16	32.0	1.28 (m)	29.7-30.00	1.28 (m)
17	22.8	1.28 - 1.30 (m)	22.68	1.28 - 1.30 (m)
18	14.3	0.88	4.44	0.88



HMBC correlations observed between the carbonyl carbon at δ_C 180.5 (C-1) with both protons resonating at δ_H 2.34 and 1.63 further supported the assignment of H-2 and H-3. On this basis, together with the information in the ^{1}H-^{1}H COSY, HSQC and HMBC (**Appendices:4B, 4C** and **4D**), the structure of compound **4** was established to be that of stearic acid.

4.3 Bioassay Tests

Bacterial, fungal and tuberculosis tests were performed on the crude extracts and the separated compounds. These testing included an anti-TB test as well as antifungal and antibacterial tests.

Table 4.6: Anti-TB activity of plant extract and four isolated compounds expressed as MIC values (µg/mL)

Sample	**Ant-TB test (µg/mL) (Rifampicin Resistant strain)**
Stigmasterol (**1**)	33.5 ±0.4
4-Hydroxyphenethyl butyrate (**2**)	31.2±0.4
6-demethylkigelin (**3**)	34.5±0.7
Stearic acid (**4**)	43.3±0.1
Crude extract	44.2±0.5
Rifampicin as (Positive control)	30.2±0.1
Negative control	0

Table 4.7: Showing the mean zones of inhibition (mm) of isolated compounds 1, 2, 3, 4 and the crude extract against *Staphylococcus aureus*

Sample	**Zone of inhibition (mm)**
Stigmasterol (**1**)	7.0 ± 0.4
4-Hydroxyphenethyl butyrate (**2**)	7.0 ± 0.3
6-demethylkigelin (**3**)	9.5 ± 0.7
Stearic A cid(**4**)	7.5 ± 0.4
Crude extract	10.0 ± 0.3
Ampicillin (positive control)	14.0±0.2
Negative control	0

Table 4.8: Showing the mean zones of inhibition (mm) of isolated compounds 1, 2, 3, 4 and the crude extract against *Candida albicans*

Sample	**Zone of inhibition (mm)**
Stigma sterol (**1**)	8.0±0.5
4-Hydroxyphenethyl butyrate (**2**)	7.0±0.4
6-demethylkigelin (**3**)	7.5±0.4
Stearic Acid (**4**)	7.5±0.7
Crude extract	11.3±0.5
Fluconazole (positive control)	13.0±0.3
Negative control	0

By using the lowest concentration of the extract at which no appreciable growth was seen, the minimal inhibitory concentration was ascertained. By measuring the concentration that completely inhibited the growth of mycobacteria, yeast and bacteria (the first clear well), minimum inhibitory concentration (MIC) was found. These values were determined in triplicate to confirm activity. The crude extract was moderately active (7.0 -8.5 µg/ml) according to Anywar (2022) against the TB strain with an MIC value of 44.2±0.5 µg/ml Compounds **1**-**3** exhibited strong activity against *M. tuberculosis* (33.5±0.4, 31.2±0.4 and 34.5±0.7, respectively) whereas compound **4** was moderately active (43.3±0.1) against *M. tuberculosis*. All the four compounds were weakly active with MIC values in the range (9.5-10.5µg/ml) according to Anywar (2022) in the antibacterial tests (7.0±0.4, 7.0±0.3, 9.5±0.7 and 7.5±0.4, respectively) and moderately active in antifungal tests (8.0±0.5, 7.0±0.4, 7.5±0.4 and 7.5±0.7) with MIC values in the range (7.0 -8.5 µg/ml). There was a noticeable moderate antibacterial activity (7.0±0.3) against *Staphylococcus aureus* and strong antifungal activity (MIC <5µg/ml) according to Anywar (2022) against *Candida albicans* exhibited by the crude extract. The results of the anti-mycobacterial assays showed that compounds **1** - **3** do contribute, significantly, towards the anti-mycobacterial activity of the extracts from the plant. These results further support the claimed use of the crude extract in the treatment of TB related ailments such as cough (Tabuti *et al.,* 2003). Furthermore, the moderate antifungal activity exhibited by the crude extract as well as stigmasterol is a validation for the use of herbal preparations from the sausage tree aimed at treating skin infections (Murakami *et al*., 2003).

Apart from the activity exhibited by compound **4** (IZ: 14–20.5 mm) on C. *albicans*, the preceding samples showed equal or greater antifungal activity compared to fluconazole on the two tested fungi which has previously proven resistant to several antibiotics and many other plant extracts which inhibit the growth However, the zones of inhibition were relatively small, indicating that growth inhibition was not particularly strong for the extract against any of the fungi.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

- Anti-microbial phytochemical studies were carried out on the stem bark of *K. africana* and atotal of four compounds were isolated and elucidated from the plant material.
- The Four compounds isolated and characterized from the stem bark of *K. africana* were elucidated as follows: stigmasterol (**1**), 4-Hydroxyphenethyl butyrate (**2**), 6-demethylkigelin (**3**) and stearic acid (**4**) and have been reported.
- The isolated compounds and crude extract showed significant antimicrobial activity. The results revealed the presence of medicinally important constituents in *Kigelia africana* since many evidences from earlier studies confirmed the identified phytochemicals above as medicinal.

5.2 Recommendations

1. Further phytochemical analysis of *K. africana* stem bark is needed to establish the toxicity/safety limits of the crude and its isolated compounds.
2. The crude extract and the isolated compounds of *K. africana* should be tested against other microorganisms as well as other forms of biological activities such as anti-hypertensive activity.
3. Other plant parts of *K. africana* should be phytochemically studied as well.
4. The isolated compounds should be synthesized so as to have their quantities scaled up for comprehensive and indepth studies.
5. I recommended that this plant be included as protected species in communities wherethey are found and established as immediate source of the identified active ingredientsfor the clinical trials.

7. It would be beneficial to synthesize the active ingredients in large quantities for testing in higher animals like monkeys and chimpanzees prior to big -scale clinical trials in humans before their deployment as therapeutic agents in humans, given the pharmacological and medicinal properties. Efficacies of numerous phytochemicals isolated from various parts of *Kigelia africana* in experimental models.

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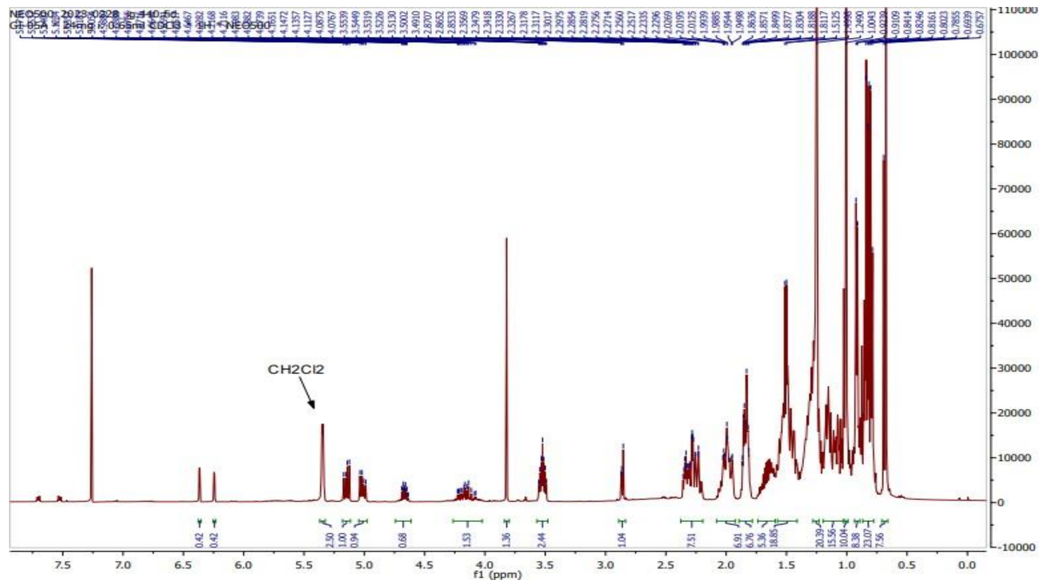
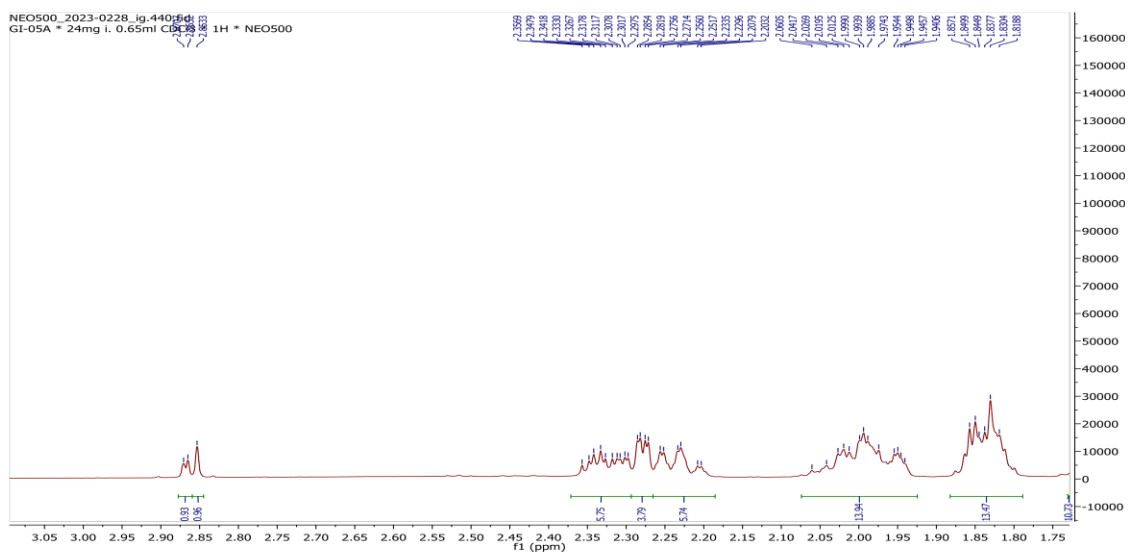
APPENDICES

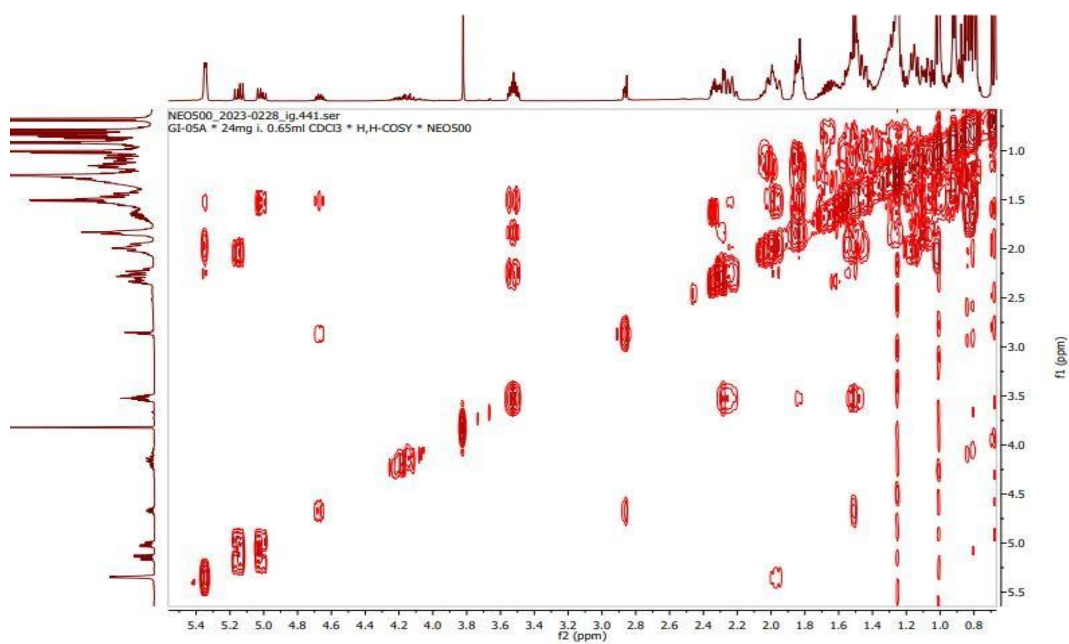
Appendix 1A: ^{1}H ($CDCl_3$, 500 MHz) NMR Spectrum of Stigmasterol (1)



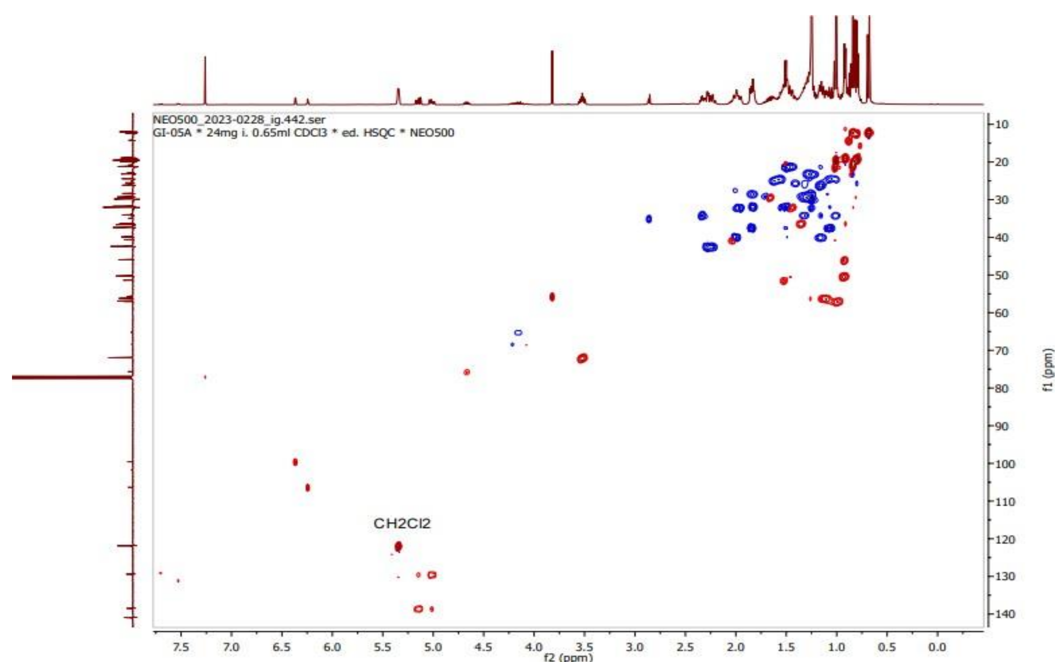
Appendix 1B: Expanded ^1H ($CDCl_3$, 500 MHz) NMR Spectrum of Stigmasterol (1)



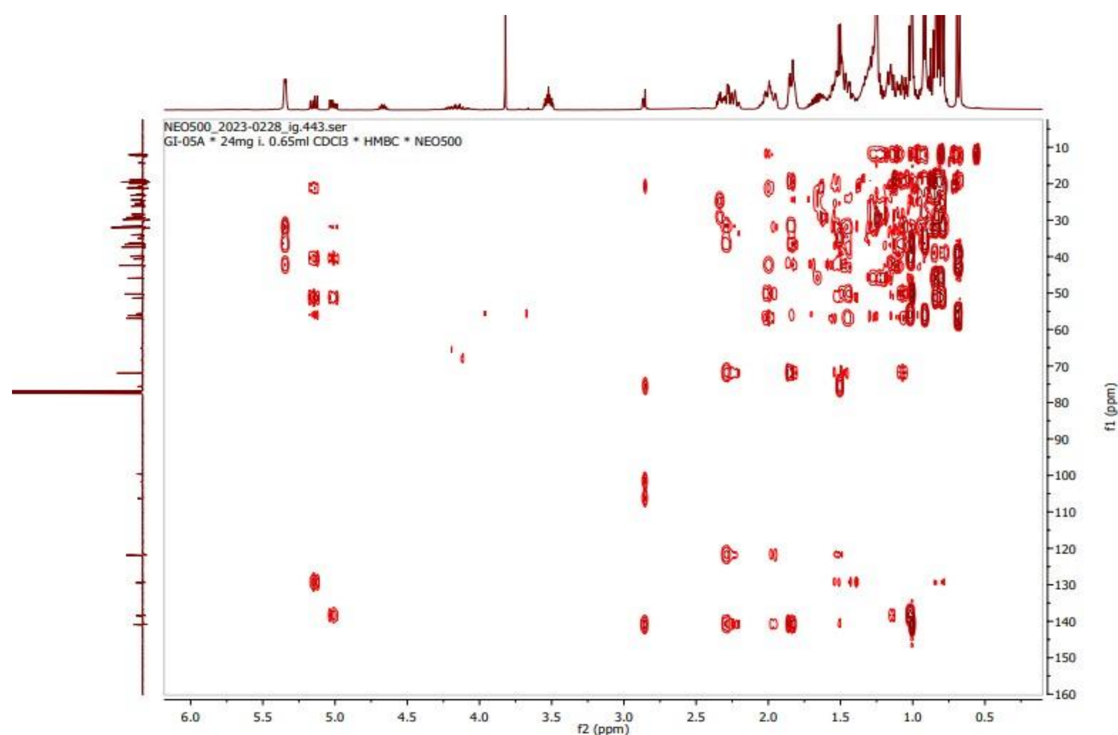
Appendix 1C: ^1H-^1H COSY ($CDCl_3$, 500 MHz) Spectrum of Stigmasterol (1)



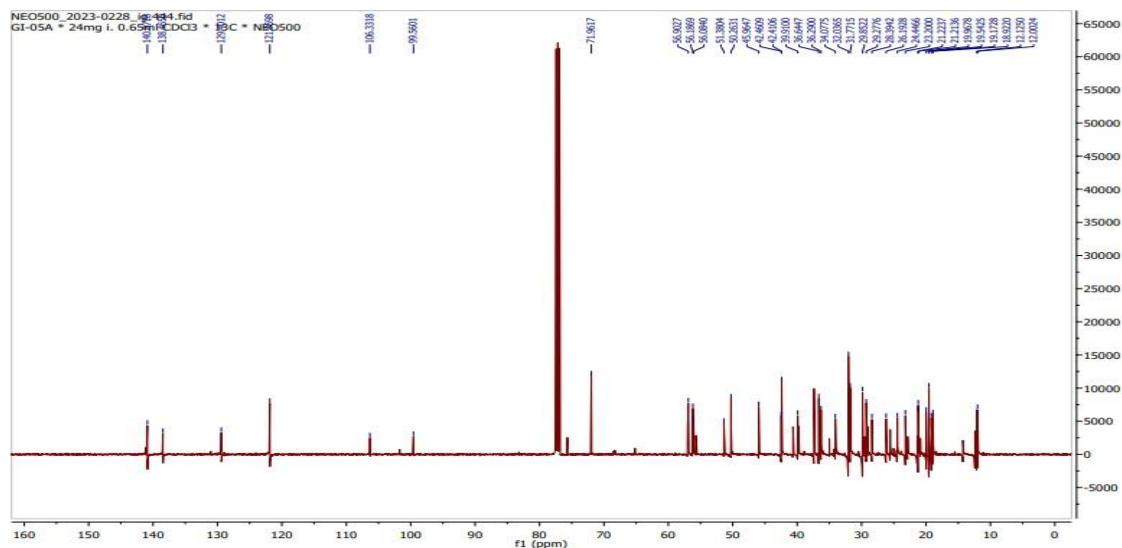
Appendix 1D: HSQC ($CDCl_3$, 500 MHz) Spectrum of Stigmasterol (1)



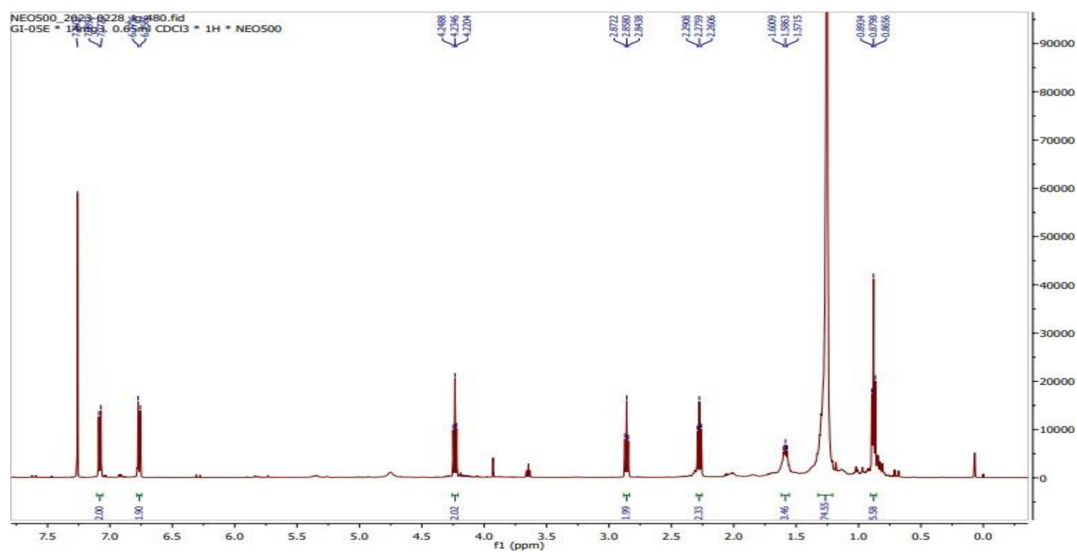
Appendix 1E: HMBC ($CDCl_3$, 500 MHz) spectrum of Stigma sterol (1)



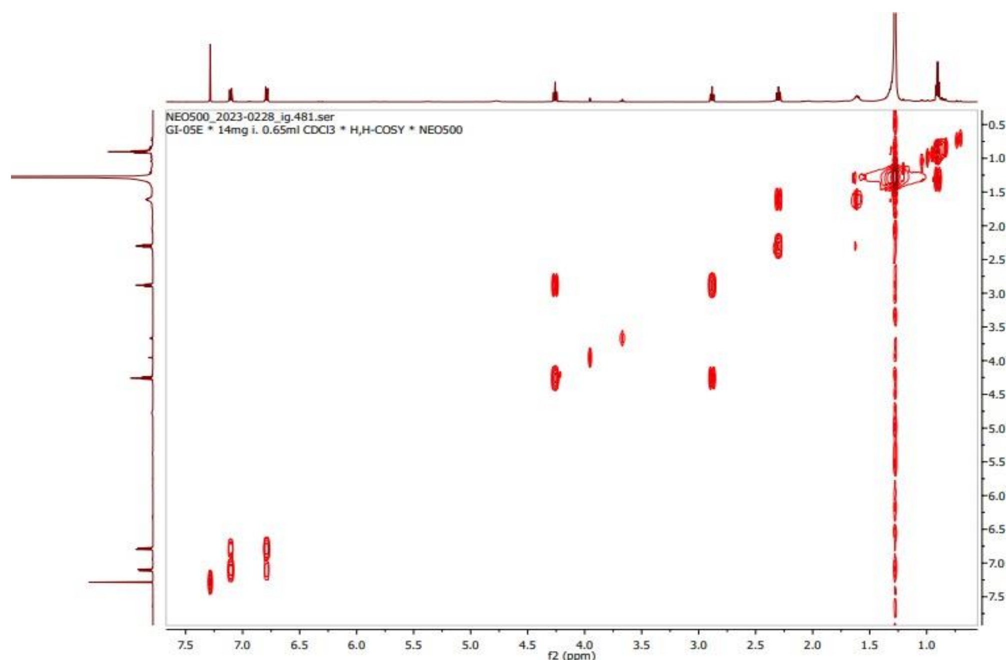
Appendix 1F: ^{13}C ($CDCl_3$, 125 MHz) spectrum of Stigma sterol (1)



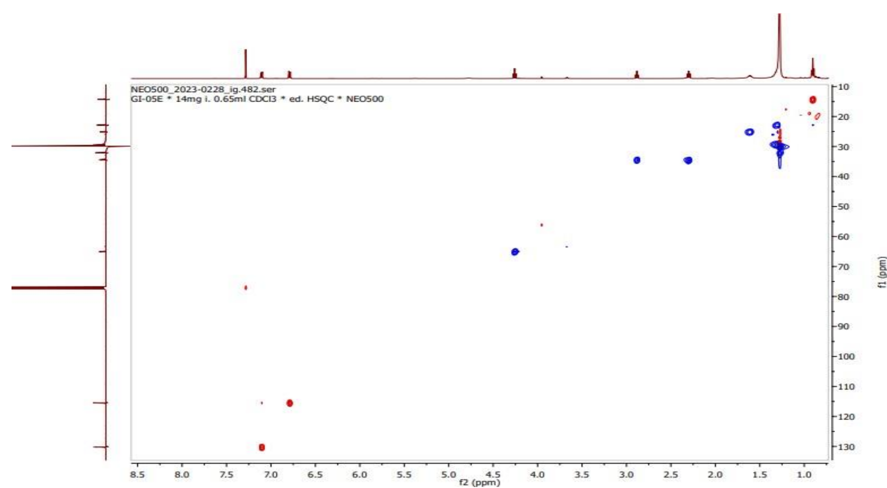
Appendix 2A: ^{1}H ($CDCl_3$, 500 MHz) NMR Spectrum of 4-Hydroxyphenethyl butyrate (2)



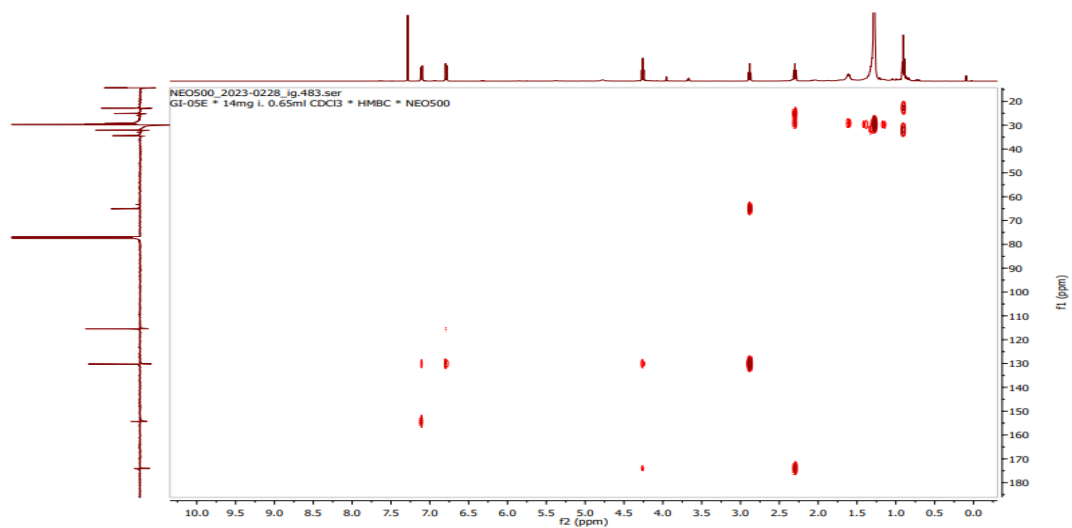
Appendix 2B: ^{1}H-^{1}H COSY ($CDCl_3$, 500 MHz) NMR Spectrum of 4-Hydroxyphenethyl butyrate (2)



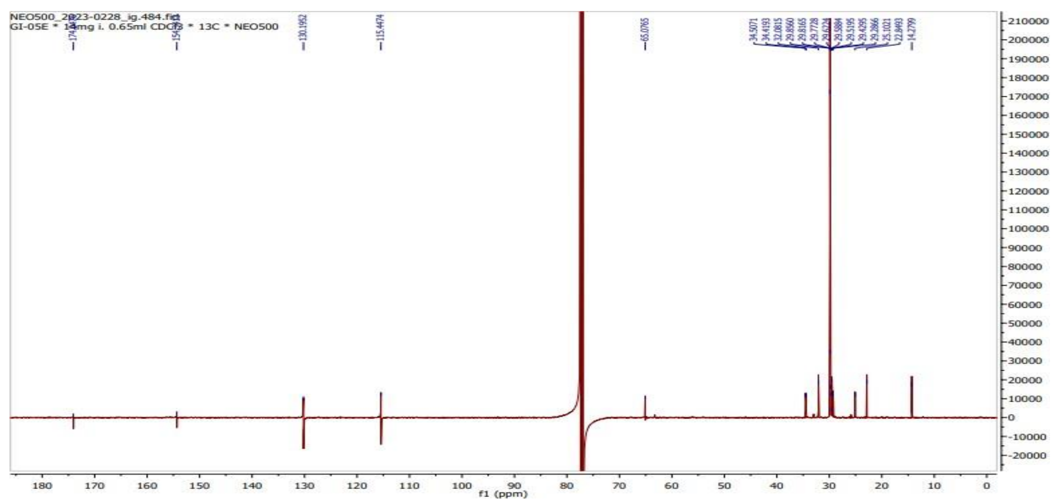
Appendix 2C: HSQC ($CDCl_3$, 500 MHz) NMR Spectrum of 4-Hydroxyphenethyl butyrate (2)



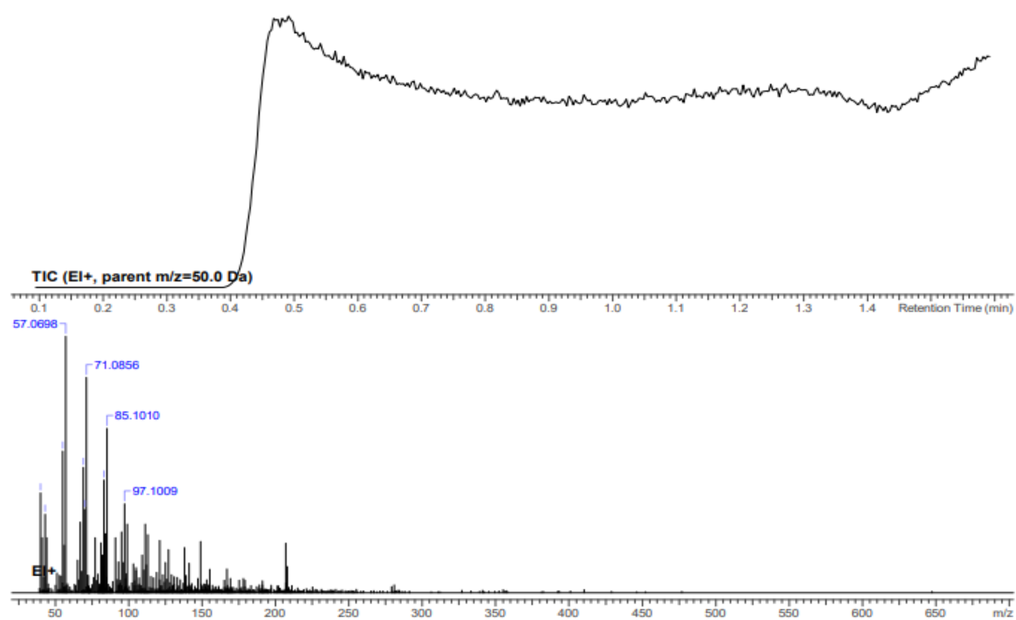
Appendix 2D: HMBC ($CDCl_3$, 500 MHz) NMR Spectrum of 4-Hydroxyphenethyl butyrate (2)



Appendix 2E: ^{13}C ($CDCl_3$, 125 MHz) NMR Spectrum of 4-Hydroxyphenethyl butyrate (2)



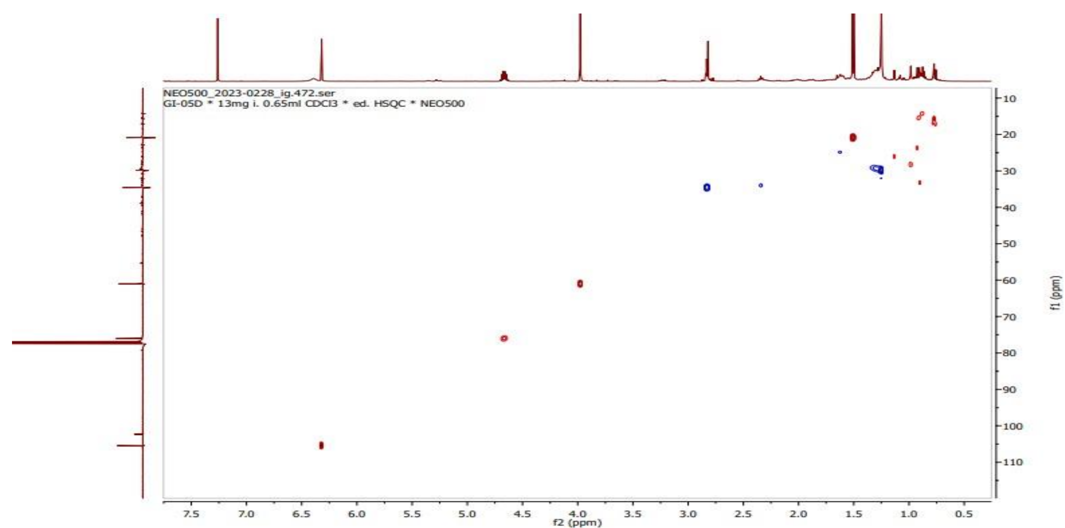
Appendix 2F: HREI-Mass Spectrum of 4-Hydroxyphenethyl butyrate(2)



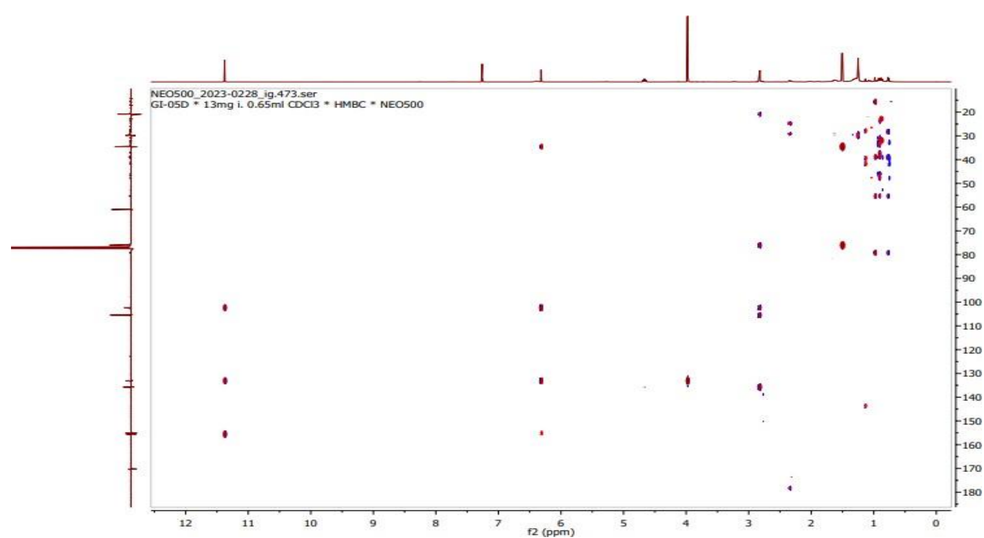
Appendix 3A: ^{1}H ($CDCl_3$, 500 MHz) NMR Spectrum of 6-demethylkigelin (3)

Appendix 3B: ^{1}H-^{1}H COSY ($CDCl_3$, 500 MHz) NMR Spectrum of 6-demethylkigelin (3)

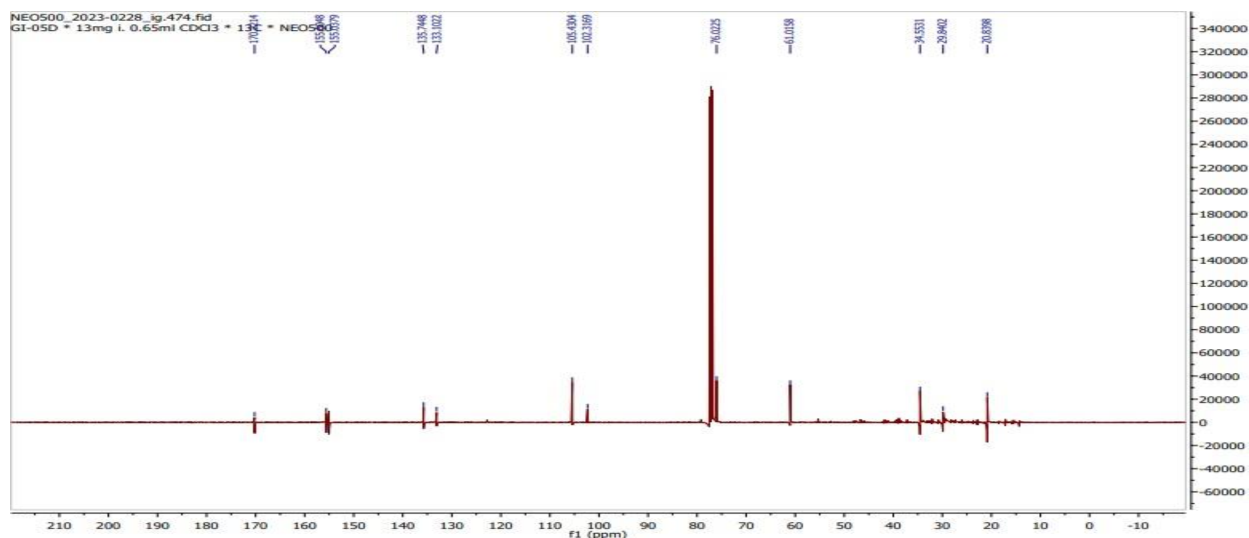
Appendix 3C: HSQC ($CDCl_3$, 500 MHz) NMR Spectrum of 6-demethylkigelin (3)



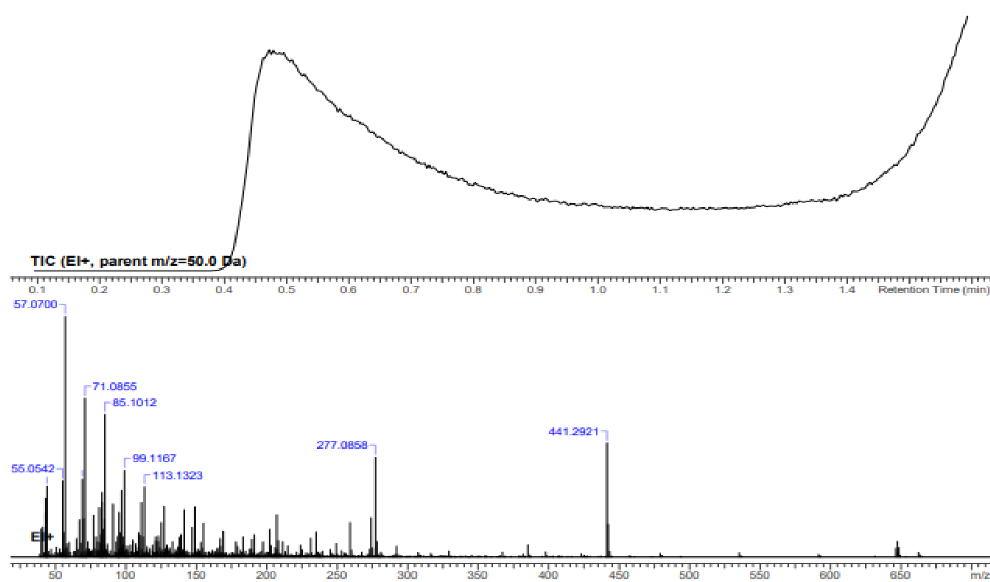
Appendix 3D: HMBC ($CDCl_3$, 500 MHz) NMR Spectrum of 6-demethylkigelin (3)



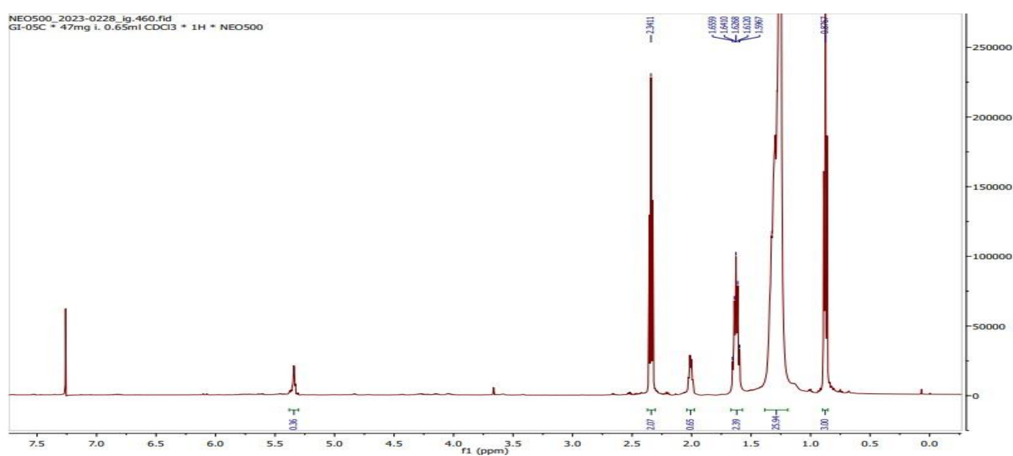
Appendix 3E: ^{13}C ($CDCl_3$, 125 MHz) NMR Spectrum of 6-demethylkigelin (3)



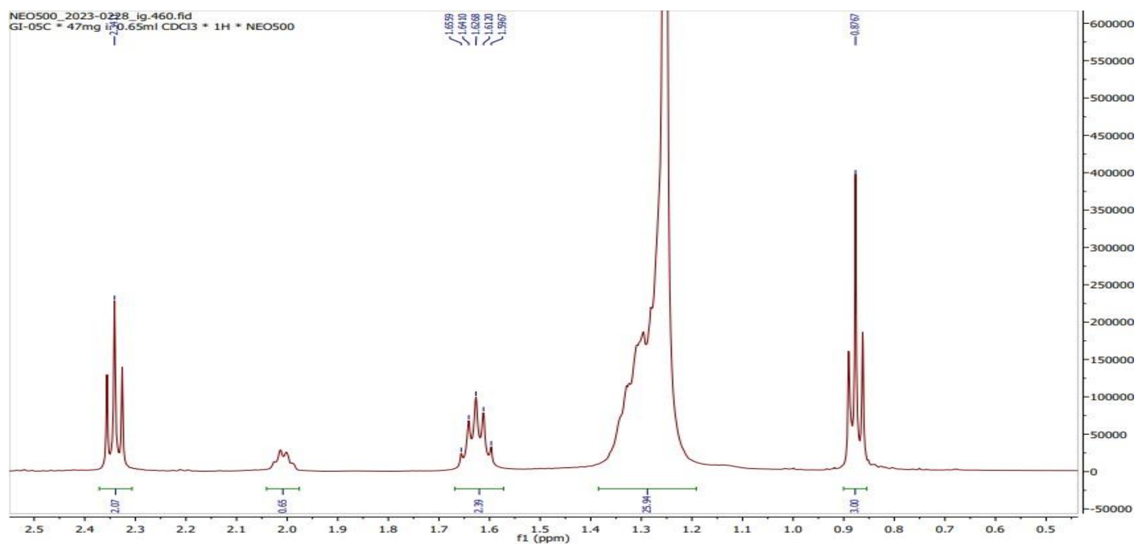
Appendix 3F: HREI-Mass Spectrum of 6-demethylkigelin (3)



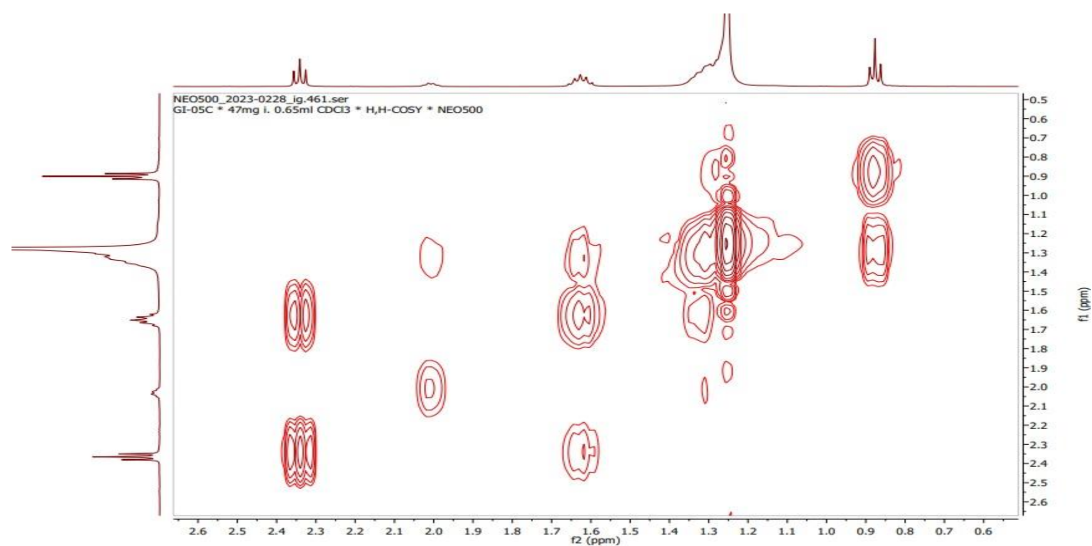
Appendix 4A: ^{1}H ($CDCl_3$, 500 MHz) NMR Spectrum of Stearic Acid (4)



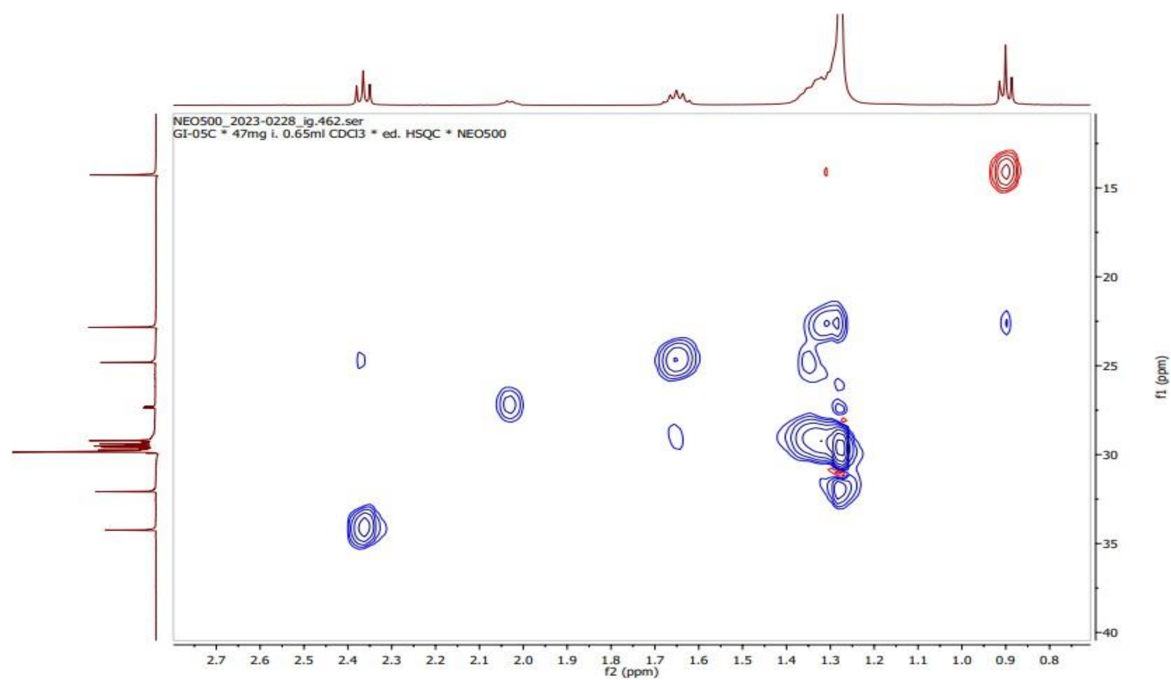
Appendix 4B: Expanded ^{1}H ($CDCl_3$, 500 MHz) NMR Spectrum of Stearic Acid (4)



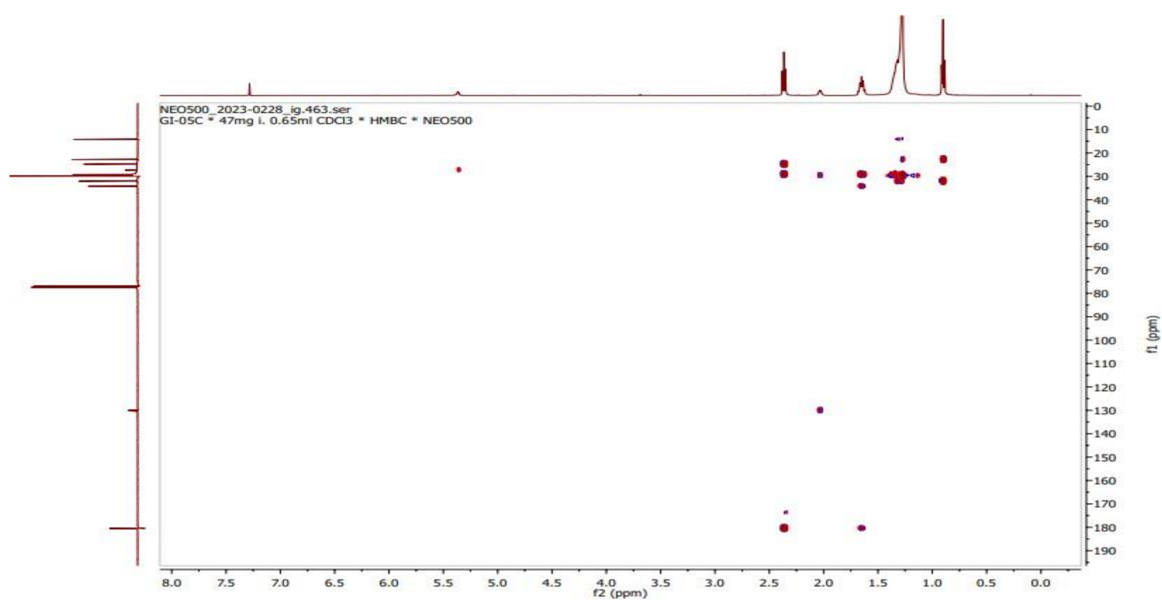
Appendix 4C: ^{1}H-^{1}H COSY ($CDCl_3$, 500 MHz) NMR Spectrum of Stearic Acid (4)



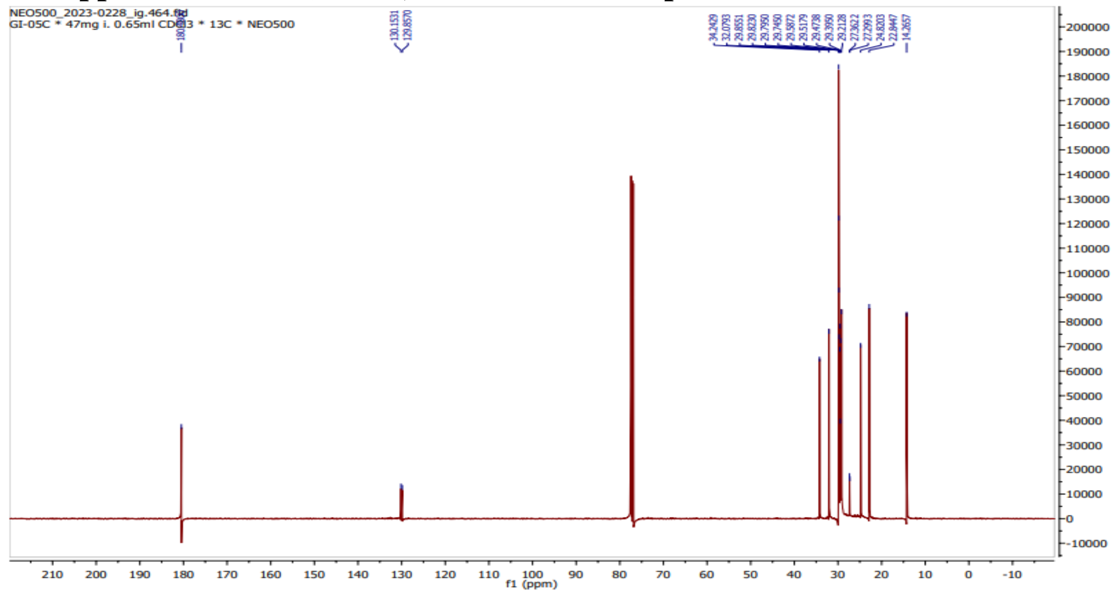
Appendix 4D: HSQC ($CDCl_3$, 500 MHz) NMR Spectrum of Stearic Acid (4)



Appendix 4E: HMBC ($CDCl_3$, 500 MHz) NMR Spectrum of Stearic Acid (4)



Appendix 4F: ^{13}C ($CDCl_3$, 125 MHz) NMR Spectrum of Stearic Acid (4)



Appendix 4G: HREI-Mass Spectrum of Stearic Acid (4)