KYAMBOGO UNIVERSITY

PHYTOCHEMICAL INVESTIGATION OF *Alstonia Boonei* FOR ANTIMICROBIAL ACTIVITIES

BY

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A DISSERTATION SUBMITTED TO THE DIRECTORATE OF RESEARCH AND GRADUATE TRAINING IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF MASTERS OF SCIENCE IN CHEMISTRY OF KYAMBOGO UNIVERSITY

JULY, 2023

DECLARATION

I, **George Busulwa**, declare that the work carried out and reported in this dissertation is my own work, never before submitted to another source for any academic award and where other people's works have been used, this has been properly cited in literature.

Sign: …… Date: ……/……./……

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APPROVAL

We confirm and approve the work done by the candidate under our supervision;

Signature: …… Date: ……./ ……/ ……...

Dr Ivan Gumula

Signature: ……. Date: ……./ ……/ ……...

Dr Sarah Nanyonga Kiwanuka

ACKNOWLEDGEMENT

First, I thank Almighty God for the gift of life and good health He gave me throughout the period of study and writing of this research Dissertation. I am fully honored to thank and appreciate my supervisors, Dr. Ivan Gumula, and Dr. Sarah Nanyonga for their guidance, advice and patience they continued to offer to me in the preparation of this research dissertation.

I am appreciative to my dear wife and children for the support they gave me and for accepting to sacrifice part of family time and resources to my studies.

DEDICATION

This dissertation is dedicated to my entire family especially my mother Mrs. Christine Oliver Nambi, my dear wife Mrs. Clemency Busulwa, all my brothers and sisters for their encouragement, support, and unconditional love.

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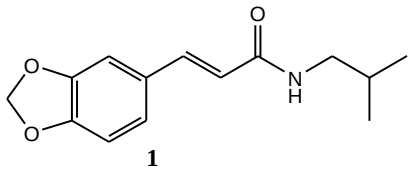
ABSTRACT

Alstonia boonei was investigated with the aim of identifying bioactive compounds present in its leaves. The plant was chosen because it is one of the most widely used plants in traditional herbal medicine. The leaves of *Alstonia boonei* were collected from Nakawuka village, Wakiso District, Central Uganda, in November 2017. Extraction of the crude was done by soaking the dry powdered leaves in a mixture of dichloromethane and methanol (1:1, v/v) followed by preliminary phytochemical screening. Isolation and purification of compounds was carried out using Column Chromatography over silica gel and the extent of separation was monitored using Thin Layer Chromatography (TLC). Bioactivity of the crude extract, as well as one isolated compound, was determined against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhi* Preliminary phytochemical analysis revealed the presence of tannins, flavonoids, saponins, terpenoids, cardiac glycosides and alkaloids. The isolated compounds were identified with the help of a combination of both 1D and 2D NMR spectroscopic techniques. Two compounds were isolated and identified; *viz*: an amide derivative, *trans*-fagaramide (**1**) and a triterpene, lupeol (**2**). Compound **1** is hereby reported for the first time from the genus *Alstonia.* The crude extract exhibited strong antibacterial activity against *E. coli* in the disc diffusion assay with a diameter zone of inhibition of 20 mm and moderately active against *P. aeruginosa* and *S. aureus* (diameter zone of inhibition of 10 and 9 mm, respectively), but it was inactive against *S. typhi*. *Trans*-fagaramide showed moderate antibacterial activities in the disc diffusion assay with zones of inhibition (diameter) of 13, 12, 10 and 10 mm against *S. typhi*, *P. aeruginosa*, *S. aureus* and *E. coli*, respectively. The minimum inhibitory concentration of *trans*-fagaramide was 125 mg/mL against *P. aeruginosa* and 250 mg/mL against the rest of the test bacterial strains. The results in this study justify the use of *Alstonia boonei* in the management of infectious diseases by traditional healers.



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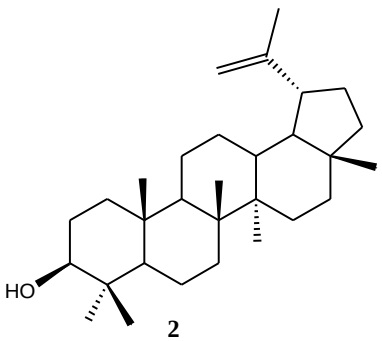

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CHAPTER ONE
INTRODUCTION

1.1 Background

Majority of people especially in developing countries use traditional medicine to treat very many diseases caused by microorganism (Ahmad de Beg., 2001, Akinmoladun et al., 2007). This has been due to two main factors; inaccessibility of modern drugs to many people in the rural areas and the economic factor. It is reported that at least 40% of the prescription drugs used in United States of America (USA) and Canada were derived from or modeled after natural products (Eskilsson & Bjorklund., 2000). In recent decades, several antitumor drugs have been derived from plants, including paclitaxel (from *Taxus brevifolia*), Aspirin, Morphine and camptothecin, using fractionation techniques based on bioactivity.

Medicinal plants are natural products which provide numerous essential services in the ecosystem (Tabuti et al, 2007). A medicinal plant is defined as any plant with one or more of its organs containing substance which is used for therapeutic purpose or which can be used as precursors for the synthesis of antimicrobial drugs (Adotey et al, 2012). Plants are presently, the sources of medicines for many people of different age in many countries of the world, where diseases are treated primarily with traditional medicines obtained from plants. The modern pharmaceutical industry itself still relies largely on the diversity of secondary metabolites in plants and secondary metabolites of which at least 12,000 have been isolated; a number estimated to be less than 10% of the total (Ganza, 2014).

Alstonia boonei, a large evergreen tree belonging to the family Apocynaceae is one of the widely used medicinal plants in Africa and beyond. The important plants of genus *Alstonia* includes *Alstonia scholaris, Alstonia boonei, Alstonia congensis* and *Alstonia macrophylla* which have proved to be useful in various diseases (Opuku & Akoto, 2015). In Africa, only one species is known to occur distributed throughout the tropics and the rain forest of West and Central Africa (Kumar, 2012). It is known by different names in different cultures and tribal settings. It is not edible as food but possess roots, stems, barks, leaves fruits, seeds, flowers, and latex which are claimed to have medicinal properties in some cultures (Shang et al, 2010).

Antioxidants and antibiotics have gained significant attention due to the confirmed role they play in preventing and treating/managing chronic and degenerative diseases such as arthritis, cancer,

cataract and cardiovascular diseases. However, antibiotics have been at times linked to the adverse effects on the host which include hypersensitivity, depletion of beneficial gut and mucosal microorganism's immune suppression and allergic reactions (Erhenhi & Obadoni, 2015). Therefore, the need for development of alternative antibacterial and antifungal agents for treatment of infectious diseases has risen. *Alstonia boonei* (Apocynaceae), is a deciduous plant largely found in rain forest regions of Senegal, Western Cameroon extending across Africa to Egypt, Sudan, Uganda and Zaire. It is referred to by different local names including Awun (Nigeria), Sinupo (Ghana), Mubajangalabi (Uganda), Botuk (Cameroon), Emien (Ivory Coast), and Kaini (Sierra Leone) (Ogwu et al, 2017).

In traditional African medicine, *Alstonia boonei* is used to treat chronic diarrhea and dysentery, fever, pain, intestinal disorders as well as an antidote for strophanthus poison. Plant extracts are still widely used in the treatment of malaria and other ailments, and up to 80% of the African population rely on traditional medicines for primary health care (Eskilsson & Bjorklund, 2000). However, little scientific information to validate antimicrobial properties of *Alstonia boonei* is available, thus, it is paramount that it's claimed antimicrobial properties are investigated, in order to establish their efficacy and determine their potential as sources of new antimicrobial drugs. This dissertation provides details in extraction, isolation and characterization, antimicrobial assay of two compounds from *Alstonia boonie* leaves extract with the use of chromatographic techniques, such as TLC, Nuclear Magnetic Resonance (NMR) and GC - MS.

1.2 Problem statement

Alstonia boonei is a herbal medicinal plant whose leaf, stem and root bark is traditionally used in the treatment of chronic diarrhea and dysentery, fever, pain, intestinal disorders in Uganda. However, the compounds responsible for the biological activities of this traditional remedy are not yet known. It is on this basis that this study aimed at isolating, characterizing and identifying antimicrobial compounds from leaves of the plant, was carried out. Antimicrobial infections are being resistant to the current/available antimicrobial drugs whereas Alstonia boonei is being used traditionally, its compounds and properties need to be determined.

1.3 Objectives

1.3.1 General objective

The general objective of this study was to assess the antimicrobial compounds from the leaves of *Alstonia boonei.*

1.3.2 Specific objectives

The specific objectives of the study were;

1. To carry out preliminary phytochemical screening of the *A. boonei* extract.
2. To isolate and characterize chemical compounds from the leaves of *A. boonei*
3. To test for antimicrobial activities of isolated compounds and the crude extract from *A. boonei.*

1.4 Hypotheses

1. The classes of compounds in the leaves of *A. boonei* can be identified by phytochemical screening.
2. The compounds in the leaves of *A. boonei* can be isolated and their structures established.
3. The extracts from the leaves of *A. boonei* and the compounds therein have antimicrobial activities.

1.5 Significance of the study

This study would yield important lead molecular structures for possible antimicrobial drug development especially in the production of synthetically improved antimicrobial agents. The identified compounds may as well be used as markers for the standardization of herbal formulations from *Alstonia boonei*.

Furthermore, the antimicrobial activity of the crude extract was tested against selected organisms. The bioactivity results of *Alstonia boonei* provided preliminary scientific justification for the traditional medicinal uses of this ethno remedy, an important step towards its acceptance and development as alternative antimicrobial agent.

The indiscriminate use of antimicrobial drugs has led to the increase in emergence of resistant microbes to major classes of drugs in recent years. Therefore, this has caused various clinical problems associated with infectious diseases. These antibiotics are sometimes associated with side effects like hypersensitivity in the host and this call for the search of new anti-microbial agents. Since *Alstonia bonnie* is used traditionally for treatment of a number of illnesses, it could be a source of alternative antimicrobial agents.

CHAPTER TWO
LITERATURE REVIEW

2.1 Introduction

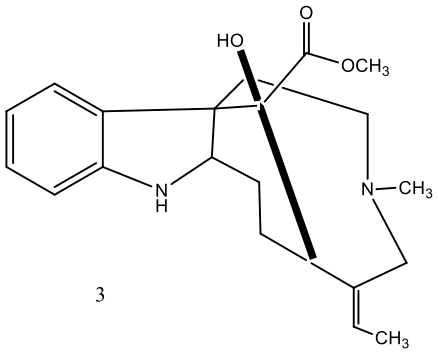
Microbes such as bacteria, fungi, protozoa or viruses are known to cause microbial infections (Kumar, 2012) especially the West Nile virus which has been previously associated with Africa and the Middle East as well as the United States (Ogwu et al, 2017). Thus, microbes play a significant role in most of the diseases occurring on earth (Sasidharan et al., 2011). Microbes are living organisms which occur in different shapes and sizes and are found everywhere. *Alstonia* is made up of almost 40 species widely distributed in the tropics of Africa, South Asia (Adotey et al., 2012), Austria and Central America (Oghenesuvwe et al., 2015). *Alstonia boonei* belongs to the family Apocynaceae (Adotey et al., 2012). The plant consists of tropical trees, shrubs and vines (Ahmad & Beg, 2001), and has more than 300 genera and 13000 species (Oghenesuvwe *et al*., 2015). Based on the variety of medicinal values of *A. boonei*, it is mostly likely that inhibition of oxidative stress and free radicals might contribute to its medicinal effects.

Alstonia boonei can also be drunk or used during bathing as a cure for dizziness, and given after childbirth to help in placenta recovery (Ogwu et al., 2017). Its leaves can be pounded to a mash and can be applied topically to reduce oedema, and leaf sap is used to cleanse sores (Ogwu et al., 2017). Various ethnopharmacological studies done on *Alstonia boonei* products indicate that the extracts possess antimalarial, antipyretic, analgesic and anti-inflammatory properties (Okwu, 2004; Olajide et al., 2000) anthelmintic, diuretic, spasmolytic and hypotensive properties, immunostimulant property, antipsychotic and anxiolytic effect and reversible antifertility effect (Oghenesuvwe et al, 2015).

The stem bark is anti-venom for snake bites and also used in traditional medicine to treat painful urination, insomnia and chronic diarrhea (Sati and Joshi, 2011). A mixture of its roots with stem bark can also be used in the treatment of asthma, while stem bark and leaves can be used to treat impotence (Opoku and Akoto, 2015). Therapeutically, the bark possesss antimicrobial and antibiotic properties (Ogwu et al., 2017). A decoction can be mixed with pure honey for daily use as an effective painkiller for conditions like: Painful menstruation (dysmenorrhoea), when associated with uterine fibroid or ovarian cysts in women; lower abdominal and pelvic congestion associated with gynaecological problems such as pelvic inflammatory diseases; and to relieve the painful urethritis common with gonococcus or other microbial infections in men (Adotey et al., 2012). Its mixture can also be taken

for the purpose of treating round worms, threadworms and other intestinal parasites in children (Eskilsson et al, 2000).

The bark decoction of *Alstonia boonei* is used with other preparations in the treatment of fractures or dislocation and its latex is taken as a purgative (Adotey et al., 2012). Various chemical compounds including; alkaloids, tannins, iridoids, and triterpenoids have been isolated from *A. boonei*, (Akinmoladun et al., 2007). The alkaloids isolated from the plant include echitamine and other alkaloids, and the triterpenes b-amyrin, lupenol, and ursolic acid have all been isolated from leaves and stem bark (Adotey et al., 2012). Echitamine has anticancer activities (Adotey et al., 2012; Oigiangbe et al., 2010), (Z)-9- Octadecenoic acid was found to have the most volatile oil in the leaf and stem bark, while methyl (7 E)- 7-octadecenoate was the most abundant in the roots (Moronkola and Kunle, 2012).



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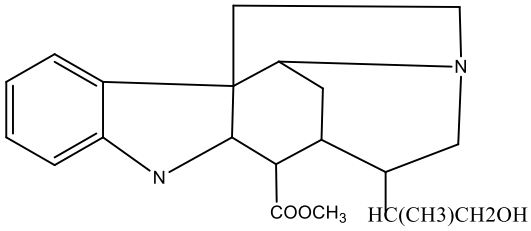

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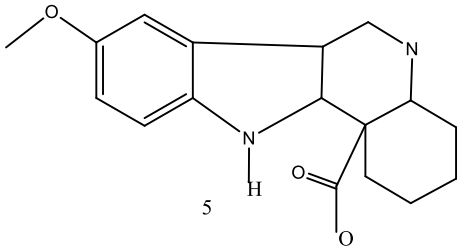

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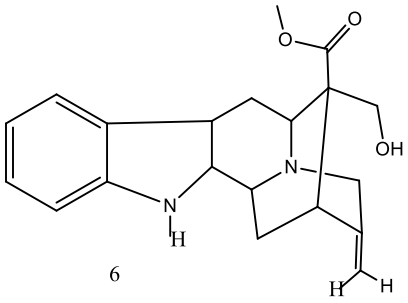

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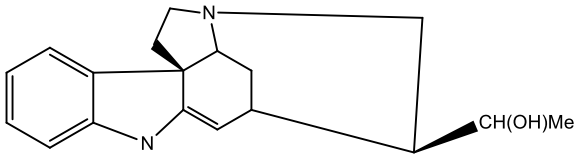

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2.2 Classification, Cultivation, and Ethnobotanical uses of *Alstonia Boonei*

The species are found all over the world of which *Alstonia boonei*, *Alstonia congensis* and *Alstonia scholaris* are indigenous in Africa. *Alstonia* grows into big trees in most of the evergreen rain forests of tropical West Africa. The plant can grow very well in damp riverbanks and it is also used by all the traditional healers practicing along the west coast of Africa. *Alstonia boonei* is a deciduous tree up to 35 meters high and it buttresses deep-fluted high (Shang et al, 2010). Its white latexes are copious. The leaves are shaped in whorls at nodes, with rounded apex rounded to acuminate, lateral vein prominent almost at right angle to midrib. The plant produces white flowers with lax terminal cymes and the fruits are paired with a slender follicle up to 16cm long with brown floss at each end (Sasidharan et al, 2011).

Figure 1: Showing clockwise order from top left; the leaves, stem, branches, and whole plant of *Alstonia boonei* species.

Popularly known as God's tree or "Onyame dua" *Alstonia boonei De Wild* is a herbal medicinal plant of West African origin, ". Within West Africa in some forest communities, the plant is considered as sacred consequently the plant parts are not eaten (Opoku & Akoto, 2014). The plant parts are traditionally used for its antimalarial, aphrodisiac, antidiabetic, antimicrobial, and antipyretic activities, and this been proved scientifically. The plant parts are rich in various bioactive compounds such as echitamidine, Nα-formylechitamidine, boonein, loganin, lupeol, ursolic acid, and β-amyrin among which the alkaloids and triterpenoids form a major portion (Okwu, 2004).

Alstonia has about 40 species of which about twelve species of the genus *Alstonia and Alstonia boonei* De Wild belongs to the family Apocynaceae. Also, *Alstonia* is called; Australian fever bush, Australian quinine, Devil tree, Dita bark, fever bark, or palimara (Savova et al, 2004). In uganda, it is called Mubajjangalabi.

Alstonia boonei is found in dry, peripheral, semi-evergreen transitional rainforest. It also occurs in similar habitats and in swamp and riverside forests. *A. boonei* requires large amounts of light and consumes large amounts of space in forests. It has plenty of natural regeneration in young secondary forest. According to Shai et al (2008), in Nigeria, *A. boonei* occurs in moist lowland forest but may extend into drier types, including gentle to steep, rocky hill sites. It can survive in different areas that is; from rocky hillsides to seasonal swamps but mostly prefers damp situations and can grow satisfactorily on well-drained slopes.

2.3 Uses of *Alstonia boonei*

Plants need minerals for healthy growth and to build up the active nutrients to enrich their pharmacological properties and antioxidant activity and these minerals can only be obtained from soil. Likewise, animals also need to regularly feed on minerals and vitamins for their good health and boost their productivity, thus they largely depend on plants through the food nutrients they take. In cases where the food lacks sufficient minerals and vitamins, deficiency diseases have always come into existence (Akinmoladun et al, 2007). This medicinal value has led to their growing demand owing largely to the discovery that extracts from plants contain not only minerals and primary metabolites as well as antioxidant potential. Antioxidant substances block the action of free radicals and this has been implicated in the pathogenesis of many diseases including atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease, Parkinson's disease and in the aging process (Tabuti et al., 2007).

Alstonia boonei stem bark has been found to possess anti-inflammatory, analgesic and antipyretic activities (Olajide et al., 2000). The stem bark is mostly used in the treatment of malaria, and is listed in the African Pharmacopoeia as an antimalaria drug. An infusion of the bark is used as antivenom for snake bites and can also used to treat painful micturation and rheumatic conditions (Okwu et al, 2004). A solution of roots and stem bark can be applied to manage asthma and its stem bark and leaves liquid is drunk to treat impotence.

A. boonei extracts have macro elements which play significant roles in the metabolism of living organisms including man. Ca salts provide rigidity to the skeleton and calcium ion plays a role in many if not most, metabolic processes. Many neuromuscular and other cellular functions depend on the maintenance of the ionized calcium concentration in the extracellular fluid. Calcium fluxes are important mediators of hormonal effects on target organs through several intracellular signaling pathways (Ogwu et al, 2017). Phosphorous is also important in bone formation and many essential metabolic activities in the body for example phosphorylation reactions.

The bark of *Alstonia* tree is one of the most effective analgesic herbs in nature. All the parts of the plant are very useful but the thick bark cut from the matured tree is the part that is most commonly used for therapeutic purposes. The bark of the tree is highly effective when it is used in its fresh form; however, the dried one could equally be used. Therapeutically, the bark has been found to possess antirheumatic, anti-inflammatory, analgesic/pain-killing, antimalaria/antipyretic, antidiabetic (mild hypoglycaemic), antihelminthic, antimicrobial and antibiotic properties (Shai et al, 2008).

Alstonia solution also helps in relieving the aches and pains associated with malaria fever and painful menstruation (dysmenorrhoea). *Alstonia* can also be taken for conditions that exhibit antipyrexia and anti-malaria effects, to combat rheumatic and arthritic pains. The solution of *Alstonia* bark is also an effective pain-killing agent (Moronkola & Kumle, 2012). A cold solution made from the fresh or dried bark of *Alstonia* taken orally two to three times daily exerts a mild hypoglycaemic effect on diabetic patients, used to cure round worms, threadworms and other intestinal parasites in children (Erhenhi & Obadoni, 2015).

The fresh bark of *Alstonia* is also useful as an effective antidote against snake, rat, or scorpion poison, it could also be useful in expelling retained products of conception and afterbirth when given to women. Parts of *Trema orientalis* and the bark of *Alstonia boonei can be* mixed with the roots and

bark of cola and fruits of *Xylopia parviflora* with hard potash and used to treat Asthma (Ganza, 2014). It is used with other preparations in the treatment of fractures or dislocation, jaundice, and for inducing breast milk. Its latex is taken as a purgative. The hardened latex is used for the treatment of yaws and it also has potential anti-HIV indicators (Olajide et al., 2000).

Several plants and vegetables that are used in traditional medicine are attributed to their antioxidant compounds. Antioxidants are also used to preserve food quality mainly because they arrest oxidative deterioration of lipids. Plant-based antioxidants are now preferred to the synthetic ones because of safety concerns (Okoye et al, 2014; Kumar, 2012; Ganza, 2014; & Sati et al, 2011). This has led to extensive screening of plants for possible medicinal and antioxidant properties, the isolation and characterization of diverse phytochemicals and the development and utilization of antioxidants of natural origin (Sasidharan et al., 2011; Savova et al., 2012). *Alstonia boonei* (Apocynaceae) as a medicinal plant is widely used across Africa for managing various ailments.

The following are the traditional uses of *Alstonia boonei*; its latex gives an inferior resinous coagulate which has been used to adulterate better rubbers. A solution of stem bark and cold water is drunk as a cure for venereal diseases, worms, and snakebite and rheumatic pains and to relax muscles (Okoye et al., 2014). Additionally, a mixture of the root and stem bark is also taken as a treatment for asthma. A liquid made from the stem bark and leaves is drunk to treat impotence. In Ghana (helping in toothache and, after child delivery, to aid in expelling the placenta), in Cote d'Ivoire and Burkina Faso (used to reduce oedema and to clear suppurant sores and exposed fractures), in Nigeria (used for ulcers), and in Cameroon and Liberia (for snake bite and arrow poison as well as dizziness) (Erhenhi et al., 2015). An infusion of root and stem back and fruit is drunk as a remedy for asthma (Okwu et al, 2017). It is used from Cote d'voire through to Burkina Faso as a decoction to cleanse suppurating sores and exposed fractures (Erhenhi et al., 2015). In Nigeria for sores and ulcers, and in Cameroon and Liberia for snakebit and arrow poison. The bark has widespread use in Ghana to assuage toothache; in Seira Leone it is used as an anthlmintic (Ooku & Akoto, 2015).

2.4. A review of some analytical methods used in natural products research

The qualitative and quantitative studies of bioactive compounds from plant materials mostly rely on the selection of proper methods. In this section, some of the commonly used methods in natural products research are discussed.

2.4.1. Extraction

Extraction is the first step of any medicinal plant study and largely determines the final outcome of the study. Extraction methods are at times called "sample preparation techniques". The development of modern chromatographic and spectrometric techniques has made bioactive compounds analysis easier than before though the success still depends on the extraction methods, input parameters and the exact nature of plant parts (Sasidharan et al, 2011). The most common factors affecting extraction processes include; the matrix properties of the plant part, the solvents used, temperature and extraction time. And further separation can be conducted only if, identification, and characterization of bioactive compounds and the extraction process has been appropriately done. Bioactive compounds from plant materials can be extracted by use of various classical extraction techniques including; soxhlet extraction, maceration and hydro distillation to obtain a crude extract which is then concentrated using a rotary evaporator. These techniques are based on the extracting power of different solvents used and the application of heat and/or mixing (Ogwu et al, 2017).

2.4.2. Column chromatography

In column chromatography, the stationary phase (a solid adsorbent) is placed in a vertical glass column and the mobile phase (a liquid) is added to the top and flows down through the column (by either gravity or external pressure). Column chromatography is commonly used as a purification technique to separate desired compounds from a mixture (Siddique & Saleem, 2011).

The extract to be purified by column chromatography is applied at the top of the column. The liquid solvent (the eluent) is passed through the column by gravity or by the application of air pressure. Equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase to varying degrees and a separation will be achieved. The individual components, or elutants, are collected as the solvent drips from the bottom of the column (Moronkola & Kunle, 2012).

Silica gel (SiO_2) and alumina (Al_2O_3) are the two adsorbents commonly used for column chromatography, they are sold in different mesh sizes, indicated by a number on the bottle label. The polarity of the solvent which is passed through the column affects the relative rates at which compounds move through the column (Ogwu et al, 2017). Polar solvents can compete more effectively with the polar molecules of a mixture for the polar sites on the adsorbent surface and will

also solvate the polar constituents better. Consequently, a highly polar solvent will move even highly polar molecules rapidly through the column. A solvent which is too polar allows rapid movement with little or no separation of the components of a mixture in the end and when a solvent is not polar enough, no compounds will elute from the column. Therefore, proper choice of an eluting solvent is very crucial for the successful application of column chromatography as a separation technique. Often a series of increasingly polar solvent systems are used to elute a column. A non-polar solvent is first used to elute the less-polar compounds. Once the less-polar compound is off the column, a more-polar solvent is added to the column to elute the more-polar compounds (Okwu et al, 2010).

2.4.3. Thin layer chromatography

Thin layer chromatography (TLC) is often used to analyze the fractions obtained from column chromatography to determine if the fraction contains more than one component and if fractions can be combined without affecting their purity (Adotey et al, 2012). The separation by TLC depends on the relative affinity of compounds towards stationary and mobile phase. The compounds under the influence of mobile phase (driven by capillary action) travel over the surface of the stationary phase. During this movement, the compounds with higher affinity to the stationary phase travel slowly while those with less affinity to the stationary phase travel faster and this facilitates the separation of components in the mixture. Once separation has been achieved, the individual components are visualized as spots on the plate after staining with iodine vapour (Okwu et al, 2010).

2.4.4. High performance liquid chromatography

This is basically a highly improved form of column chromatography where a solvent is forced through under high pressures of up to 400 atmospheres instead of being allowed to drip through a column under gravity which makes it move faster. It also allows the use of a smaller particle size for the column packing material which gives it a greater surface area for interactions between the stationary phase and the molecules flowing through it, thus allowing better separation of the components (Sati et al, 2011). Preparative high-performance liquid chromatography (HPLC) has become a favorite method of natural product isolation and purification and the different modes available like; normal-phase, reversed-phase, size exclusion, and ion-exchange can be used to purify most classes of natural products. Although preparative HPLC is very similar to analytical HPLC, instead of injecting a small amount of sample to maximize the resolution, the amount of feed is very high in order to maximize the purification productivity and minimize the amount of solvent used (Kumar, 2012).

2.4.5. Mass spectrometry (MS)

Mass spectrometry allows the determination of the molecular mass and the molecular formula of a compound, as well as certain structural features. When a small amount of the compound is vaporized and then ionized, it produces a molecular ion (a radical cation). Many of the molecular ions break apart into cations, radicals, neutral molecules, and other radical cations (Okoye et al, 2014). The weakest bonds are most likely to break and those that result in the formation of the most stable products. These fragments of the molecules are detected individually on the basis of their mass-to-charge ratios (Sati & Joshi, 2011). The details of exactly how these positively charged fragments are separated and detected differ according to the specific design of the mass analyzer portion of the instrument. In any case, the information acquired and displayed by the data system (the so-called mass spectrum) allows the analyst to reconstruct the original molecule and thereby identify it. Besides the significant applicability to molecular compound identification, mass spectrometry also finds application in elemental analysis, such as to determine what isotopes of an element might be present in a sample (Shai et al, 2008). Here, various Mass Spectrometry Ionization Methods including Atmospheric Pressure Chemical Ionization, Atmospheric Pressure Photoionization (APPI), Electrospray ionization (ESI) and Matrix-Assisted Laser Desorption Ionization (MALDI) were used.

2.4.6. Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance spectroscopy depends on the absorption of energy when the nucleus of an atom is excited from its lowest energy spin state to the next higher one. Many elements are difficult to study by NMR, and some cannot be studied at all. Fortunately though, the two elements (carbon and hydrogen) are the most common in organic molecules and have isotopes (^{1}H and ^{13}C) capable of giving NMR spectra that are rich in structural information. A proton nuclear magnetic resonance (^{1}H NMR) spectrum tells us about the environments of the various hydrogen atoms in a molecule; a carbon-13 nuclear magnetic resonance (^{13}C NMR) spectrum does the same for the carbon atoms (Erhenhi et al, 2015). Together, ^{1}H and ^{13}C NMR is used in determining a substance's molecular structure. It is mostly used with other spectrometric techniques such as FTIR, mass spectrometry and 2DNMR.

In 2DNMR, there is HMBC spectroscopy, correlates ^{1}H and ^{13}C nuclei through two, three, or sometimes four bonds, HSQC determines the correlations between two different types of nuclei

(commonly ^{1}H with ^{13}C), which are separated by one bond and H-HCOSY, ^{1}H and ^{1}H which determines a correlation between a proton and a proton (Erhenhi et al, 2015).

2.4.7 Phytochemical properties of *Alstonia boonei*

Different chemical compounds including alkaloids, tannins, iridoids, and triterpenoids have been isolated from *Alstonia boonei*. Chromatography of bark extracts of *Alstonia boonei* on silica gel plates with the solvent system AcOEt-MeOH-H_2O (150 : 26 : 19) produced 6 separate spots with alkaloid reactions and the alkaloids isolated from the plant included echitamine (3) , echitamidine (4), voacangine (5) , ekuammidine (6) and Nα-formylechitamidine (7) (Opoku & Akoto, 2014). Echitamine is also isolated from the bark of *Alstonia scholaris*. There is absence of alkaloids and terpenoids in aqueous extracts and presence of tannins (14.75%), alkaloids (8.79%) and terpenoids (7.19%) in methanolic extract of leaves (Wemambu et al., 2018)

The medicinal effects of plants are often attributed to the antioxidant activity of the phytochemical constituents, mainly the phenolics (Scholichin, 1980). The antioxidant activity of phenolics is due to their redox properties which allow them to act as reducing agents, metal chelators and free radical quenchers (Siddique & Saleem, 2011). Plants having significant medicinal values are always rich in phenolics and to have high antioxidant potentials. It was expected that *A. boonei*, which has many medicinal uses, could have a larger amount of phenolics and possess a high antioxidant potential. However, Siddique & Saleem (2011) findings confirmed that the opposite was the case. The antioxidant indices evaluated showed low values for the plant. This presupposes that other classes of phytochemicals, like the alkaloids, could be major players in the medicinal and therapeutic value of *A. boonei*. A synergistic relationship amongst phytochemicals has been confirmed to be responsible for the overall beneficial effect that can be achieved from plants (Savova et al, 2004), though tests have not yet been carried out to measure this synergy. The synergy of phytochemicals may make-up for the apparent low values for individual classes of phytochemicals. The important minerals and vitamin found in the plant might also be major contributors to the medicinal value of the plant (Shai et al, 2008). Mineral elements may have more roles to play, than presently acknowledged, in the synergy of phytochemicals for the health benefit of man. The phytochemicals present in the methanolic and aqueous extracts were identical. In traditional usage, decoction or infusions of herbs are usually made with either alcohol or water as the solvent. At times, differences exist between the phytochemical profile of alcoholic and aqueous extracts (Wemambu et al, 2018). In the case of *A.*

boonei, the aqueous extract is recommended because no vital phytochemical seems to be left out and also because of probable unwanted effects that alcohol.

Flavonoids are potential water-soluble antioxidants and are free radical scavengers which prevent oxidative cell damage and have strong anticancer activity (Salah et al., 1995; Okwu, 2004). They also minimize the risk of heart diseases. Additionally, Saponins have the capability to neutralize some enzymes in the intestines that can become harmful, building the immune system and promoting wound healing. Alkaloids also possess analgesic, antispasmodic and bactericidal effects with tannins being confirmed to hasten the healing of wounds and inflamed mucous membrane (Okwu and Okwu, 2004). Cardiac steroids are widely used to treat congestive heart failure since they help in increasing the force of contraction of the heart (positive inotropic activity) in heart failure patients and their presence supports the medicinal use of *A. boonei* (Shang et al, 2008).

According to Wemambu et al (2018), sequential extraction of leaves, stem and root bark samples was done with the solvents of increasing polarity and these extracts were assayed for total phenolic content and *in vitro* antioxidant and antidiabetic activities using different experimental models. There is ability of these extracts to inhibit α-glucosidase and α-amylase was also examined *in vitro*. The ethanol extract of leaves had high total phenolic content when compared with other extracts and had free radical scavenging abilities in different experimental models (Kumar, 2012). The ethanol and aqueous extracts significantly inhibit the activities of key enzymes linked to type 2 diabetes. These compounds have a high binding affinity with α-glucosidase and α-amylase enzymes under molecular docking studies. Phytochemical screening on the presence of phytochemicals responsible for the antimicrobial potential of roots of *Alstonia boonei* revealed the presence of alkaloids, cyanogenetic glycosides, flavonoids, terpenoids and steroids and saponins. Thus research has been conducted using dichloromethane and methanol (1:1 v/v) extracts of the stem bark of *Alstonia boonei (*Apocynaceae), for antimicrobial activities against *Candida* species, *Pseudomonas aeruginosa, Escherichia coli, Streptococcus pyogenes, Bacillussubtilis* and *Staphylococcus aureus* using the agar diffusion method.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant collection and Identification

The leaves of *Alstonia boonei* were collected from Wakiso District (Nakawuka village). Identification of the plant was done at Makerere University Herbarium and Vourcher number wasBG-2017/001.

3.2 Extraction

The leaves were air dried at room temperature for 21 days. The dry leaves were ground into fine powder using an electric grinder. The powdered plant material (1 Kg) was sequentially extracted three times with 5 litres of a mixture of dichloromethane and methanol (1:1 v/v) at room temperature for 2 days (48 hours). The extract was filtered using cotton wool and Whatman No. 1 filter paper and concentrated with a rotary evaporator at 40 ^{0}C to dryness. The dried extract was transferred to a sample bottle which was put in a dessicator containing anhydrous sodium sulphate to remove any content of water that could have remained. The dried extract was then put in tightly closed containers which were kept in a refrigerator for further analysis.

The dichloromethane and methanol extract was then subjected to preliminary phytochemical analysis to identify the presence of phytochemical constituents as indicated in the following sections here below;

3.3 Phytochemical screening assay

Phytochemicals are substances got from plants and refer to the large number of secondary metabolic compounds found in plants. After obtaining the crude extract from the plant material, phytochemical screening was done with the appropriate tests in order to identify the type of phytochemicals existing in the avde extract mixture (Opoku & Akoto, 2015) as shown in Table 1 below.

Table 1: Phytochemical screening methods used

Metabolite	Test	Procedure	observation
Alkaloids	Wagner test	2 ml of extract was mixed with1% HCl .Then to 1ml of this solution 6 drops of Wagner's reagent.	Yellow precipitate
Cardiac glycosides	Kellar – Kiliani Test	2ml of extract was mixed with 1 ml of glacial acetic acid, 1 ml of ferric chloride and 1ml of concentrated sulphuric acid.	Green-blue colouration
Anthraquinone	Borntrager's test	50 mg of extract was heated with 1 ml 10% ferric chloride solution and 1 ml of concentrated hydrochloric acid. The extract was then cooled and filtered. The filtrate was then shaken with an equal amount of diethyl ether. The ether extract was further extracted with strong ammonia.	No pink red colour in ammoniac (lower layer)
Flavonoid	NaOH test	The extract was treated with dilute NaOH, followed by addition of dilute HCl.	Intense yellow colour
Saponin	Frothing test / Foam test	0.5 ml of extract was dissolved in 5 ml of distilled water and shaken well.	Foam persists
Tannin	Braemer's test	10% alcoholic ferric chloride was added to 2 ml of the extract.	Greenish grey colouration

Terpenoid	Salkowski test	5 ml extract was mixed with 2 ml of chloroform and 3 ml of concentrated sulphuric acid.	Reddish brown colour of interface

3.4 Isolation of compounds

Column Chromatography (CC) was used to carry out fractionation and isolation. The level of separation/purification was monitored using Thin Layer Chromatography (TLC) viewed under ultra-violet (UV) light (254 nm and 366 nm wave lengths).

3.4.1 Column Chromatography

The crude extract was mixed with silica gel (70-230 mesh) Column Chromatography. Then silica gel was mixed with n-hexane and formed a homogenous suspension which was stirred using a glass-stirring rod to remove bubbles. The silica gel slurry was then poured into a glass column of internal diameter 5cm. The sample to be l7oaded on the column was first prepared by dissolving 15 g of the extract in 50 ml of dichloromethane. To the solution, 30 g of silica was added and mixed by stirring with a glass rod so as to adsorb the crude extract onto the silica gel. The mixture was dried using a rotary evaporator at 45-50 ^{0}C and the adsorbed silica gel was layered on the column layer bed. The column was first eluted with n-hexane as the mobile phase with the polarity increasing by 5 % increments of ethyl acetate and after getting to 100 % ethyl acetate, the polarity was further increased by 5 % increments of methanol. For each eluent system, two litre volumes were used and 250 ml fractions collected in 250 ml glass beakers. The collected fractions were dried using a rotary evaporator at 40^{0}C and the fractions were further divided by small Column Chromatography with dichloromethane and methanol (1:1) as the eluent system to obtain the pure compounds.

3.4.2 Thin layer chromatography

The concentrated fractions collected from the column chromatography were subjected to Thin Layer Chromatography (TLC). The fraction viles were dissolved in little dichloromethane and briefly a spot of each fraction was carefully applied onto a thin layer chromatographic plate (coated with silica) and left to dry. After about five minutes, the plate was dipped in a suitable solvent which allowed the compounds in the spot to move upwards by capillary attraction. The plate was then removed from the solvent and left to dry. The positions of different compounds were observed under UV-light (254 nm and 366 nm) followed by exposure to iodine vapour. Fractions with similar TLC profiles were combined for further separation.

3.5 Structure determination of the bioactive compounds

The structure of each of the compound isolated was then determined from NMR data obtained on a Bruker AV-500 spectrometer (MC-Murry) to generate 1D (^{1}H and ^{13}C) as well as 2D (H-H CoSY, HSQC and HMBC) spectra . Here 10mg of the sample was dissolved in 0.6ml of deuterated chloroform and then filtered through a Pasteur pipette equipped with glass wool plug that discharged into a 5mm NMR tube (Aldrich Z412848) which was labeled clearly with concentric label. This was carried out in German. The spectra in FID format were processed using MestReNova version 8.1.1. and the structure of a compound in the given test sample was determined by interpretation of both the 1D (^{1}H, ^{13}C) and 2D (H-H CoSY, HSQC and HMBC) NMR results with residual chloroform peaks used as the references. The results were compared with published data in the literature.

The pure extract was 29.1 mg of a white powder.

3.6 Bioassay of the crude and pure compounds

The crude extract and pure compound were tested against four types of bacterial strains: *Pseudomonas aeruginosa, Stapylococcus aureus, Salmonella typhi* and *Escherichia coli* all of which were obtained from the Department of Biological Sciences Kyambogo University , Kampala.

3.7 Modified Agar disc diffusion assay for bacterial screening

Each test microorganism was aseptically inoculated (approx. 1.0×10^{8} colony forming units/ml) on sterile Mueller Hinton agar by surface spreading to make a uniform microbial inoculum. Using sterile glass cork borers (6 mm in diameter), two wells both containing the test extract were carefully made on the agar plate without distorting the media and a separate agar plate was used to test the control drug; Tetracycline (0.5 mg/ml) against the bacteria. The second plate containing dimethyl sulfoxide (DMSO) was used as a negative control. Fifty microlitres (50 µl) of the extract and the controls were carefully dispensed into the respective wells and the plates left on the bench for 60 minutes to allow the system stabilize as the inoculated microorganisms get acclimatized to the new environment. The culture plates were then incubated at 37 ^{0}C for 24 hours and the diameter of the zone of inhibition was measured using a metric ruler (Oigaiangbe et al, 2010).

3.8 Determination of Minimum Inhibitory Concentration (MIC) using Broth dilution method

MIC was performed on organisms that exhibited highest sensitivity, to the extract and pure compounds, of diameter above 12 mm upon screening. Five hundred microlitres of the test extract

was serially diluted from 2-fold to 4-fold dilution in sterile Mueller Hinton broth. Five hundred microlitres (500 µl) of the test organism was aseptically inoculated in each of the four tubes containing the extract in order of increasing dilution (500, 250, 125 and 62.5 mg/ml). Thereafter, the tubes were incubated at $37\,^{0}C$ for 24 hours (Wemambu et al, 2018). After incubation, the tube next to the one showing no microorganism turbidity was considered as containing the MIC of the extract in question.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Phytochemical screening results of *Alstonia boonei*

Table 4.1.1 Phytochemical screening results of *Alstonia boonei*

Phytochemicals	Reagent	Observation	Results
Saponin test	Foam Test	Produced foam that persisted	+
Alkaloid test	Wagner's Reagent	Formation of yellow precipitate	+
Tannins test	Braemer's test	Greenish grey coloration of the solution	+
Cardiac glycosides	Kellar- Kiliani test	Green-blue coloration of Solution	+
Flavonoids test	Alkaline Reagent Test	intense yellow colour obtained	+
Terpenoid	Salkowski test	Reddish brown color obtained	+
Anthraquinone	Borntrager's test	No pink-red color in the ammoniacal (lower) layer	-

The phytochemical screening of the Methanol/DCM samples of *Alstonia boonei* gave an indication of bioactive constituents which include: saponins, flavonoids, alkaloids, tannins, cardiac glycosides, terpenoids. These biologically active constituents of the extract are indicators of its antimicrobial activity.

4.2 Characterization of chemical compounds from the extract

Two compounds were isolated from the samples of the leaves of *Alstonia boonei* by a combination of chromatographic techniques. The structures of these isolated compounds were identified as *trans*-fagaramide(**1**) and Lupeol (**2**). This is the first time compound 1 is being reported from this species.

4.2.1 Compund 1

Compound 1 produced a white powder soluble in Dichloromethane.

^{1}H NMR analysis in 500 MHz spectrometer with a solvent system of $CDCl_3$

Table 4.2.2: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data together with the HMBC correlations of compound 1 in $CDCl_3$.

Connectivity in the molecule

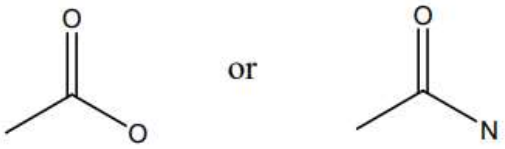
Position	δ_C	δ_H [ppm, matter, (J in Hz)	COSY	HMBC (H→C)
1	148.9	-- -- --	---	-----
2	148.2	-- -- --	---	------
3	106.4	6.98 d (1.7)	H–5	C-1, 2,5,7
4	129.4	-- -- --	---	--------
5	123.7	6.95, dd (8.0, 1.7)	H–6, 3	C-1,3,7)
6	108.5	6.75 d	H–5	C–2, 4.
7	140.1	7.54, d (15.6)	H–8	C–3, 5, 9
8	119.2	6.32 d (15.6)	H–7	C–4, C–9
9	166.3	-----	------	-----
10	-----	-----	----	-----
11	47.2	3.21 m	H–12, N–H	C-9, C-13/14, C–12
12	28.8	1.85 m	H -11, 13/14	C-13/14, 11
13/14	20.3	0.95 d (6.7, 1.7)	H–12	C-11 12, 14/15
1-$OCH_2$0-2	101.4	5.69 d (1.7)	---	C–1, 2

N – H	----	6.21 LS	H – 11	----

Partial Structure 1

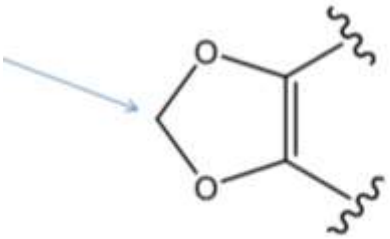
The compound was isolated leading to formation of a white amorphous solid

The ^{13}C NMR 125 MHz showed 13 peaks including that of an ester (or amide) carbonyl (δ 166.3)



Partial Structure 2

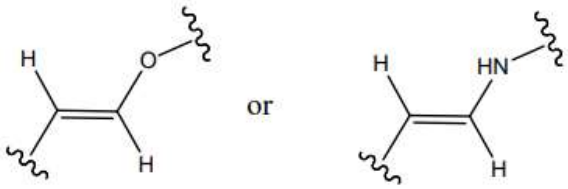
The HSQC showed a dioxymethylene group with a cross peak coordinate at δ_H 5.69 (2H), (δ_C 101.4)



Partial Structure 3

From ^{1}H NMR and ^{1}H-^{1}H COSY, showed a trans-double bond which was deduced [δ_H 7.54 (1H, d, J = 15.6 Hz); δ_H 6.32 (1H, d, J = 15.6 Hz). Furthermore, the HSQC spectra revealed a cross peak at δ_H 7.54, δ_C 140.1, implying that the double bond was adjacent to an electron-withdrawing atom:

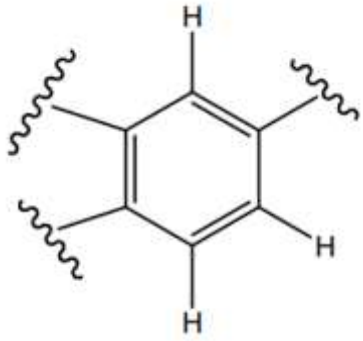
Hence;



Partial Structure 3

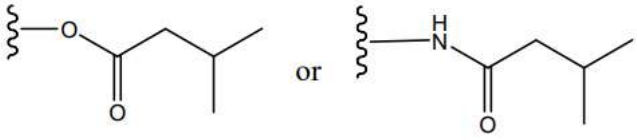
From ^{1}H NMR and ^{1}H-^{1}H COSY spectra, it was also deduced that 3 aromatic protons were present [d_H 6.98(brs, 1H); d_H 6.94(d, 1H, J = 8.0) and d_H 6.76 (dd, 1H, J = 8.0, 1.7 Hz), two of which were *ortho* to each other.

Hence:



The ^{1}H NMR and ^{1}H-^{1}H COSY spectra also indicated an isobutyl moiety [δ_H 3.21 (2 Hz, multiplet H – 11, H – 13/14); δ_H 1.85 (1H, a septet of doublets, J = 6.5. 1.7Hz) and δ_H 0.95 (6H,d d, J = 6.7, 1.7. Hz)

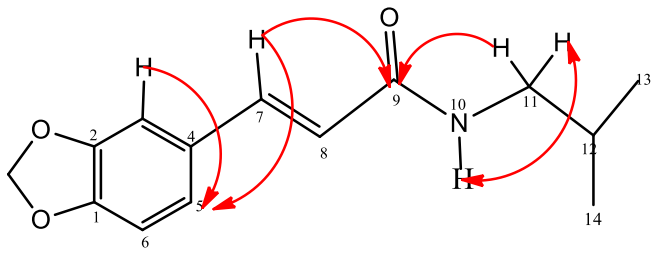
In addition the HMBC exhibited a correlation between the methylene proton at δ_H 3.21 H – 12 and a carbonyl carbon at δ_C 166.3, (C – 9) thus suggesting a connection with the ester (or amide) group.



Final Structure

The linkage between the trans-double bond with aromatic ring system at position 4 was deduced from the HMBC interaction between a proton at δ7.54 (H – 7) with aromatic carbons at δc 106.4 (C – 3) and at δc 123.7 (C – 5).

From the above data and complete analysis of HMBC, the partial structures were joined to give the final structure as (*E*))-3-(benzo[d] [1,3] dioxol-6-yl) - N-isobutyl acrylamide also known as trans-fagaramide shown below



4.3 Compound 2

Compound 2 produced a brown amorphous solid, having been eluted with ethylacetate hexane 3.7 from the silica gel column.

Table 4.3.1: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data together with the HMBC correlations of compound 2 in $CDCl_3$.

C-NO	δ_C	δ^1H [ppm] (J [HZ])	H-H COSY	HMBC (H→C)
1	38.8 (CH_2)	0.89 (m)	1.65	C-5,25
2	27.5 (CH_2)	1.71 (m) 1.59 (m)	1.59, 1.71	
3	79.1 (CH)	3.18 (m)	1.57	C-23,24
4	38.9 (q)	-		
5	55.4 (CH)	0.67 (m)	1.38	C-1,25
6	18.4 (CH_2)	1.39 (m) 1.52 (m)	0.67,1.92,2. 37,1.67,0.98	
7	34.4 (CH_2)	1.39 (m)		
8	40.8 (q)	-		
9	50.4 (CH)	1.29 (m)		
10	37.3 (q)	-		
11	21.1 (CH_2)	1.41 (m)	1.22	C-19
12	25.2 (CH_2)	1.68 (m)	1.07	
13	38.1 (CH)	1.66 (m)		
14	42.9(q)	-		
15	27.4 (CH_2)	1.57 (m) 1.03 (m)	1.03, 1.57	
16	35.6(CH_2)	1.49 (m) 1.37 (m)	1.37 1.49	
17 18	43.1 (q) 48.4 (CH)	- 1.36 (m)		
19	48.1 (CH)	2.38 (m)		C-13,18,20,21 ,29, 30

20	150.6 (q)	-		
21	29.9 (CH_2)	1.33 (m)	1.92	C-13,17, 19,20
22	40.1 (CH_2)	1.38 (m) 1.20 (m)	1.20, 1.38	
23	28.1 (CH_3)	0.97 (s)		C-3,4, 5, 24
24	15.4 (CH_3)	0.76 (s)		
25	16.0 (CH_3)	1.03 (s)		C-1,5,
26	16.2 (CH_3)	0.83 (s)		
27	14.6 (CH_3)	0.94 (s)		13,14,15 ,
28	18.1 (CH_3)	0.79 (s)		
29	109.5 (CH_2)	4.69 (m)	1.68, 4.57 1.68, 4.68	C-19,30
30	19.4 (CH_3)	1.68 (s)	4.57, 4.69	C-19,20,29

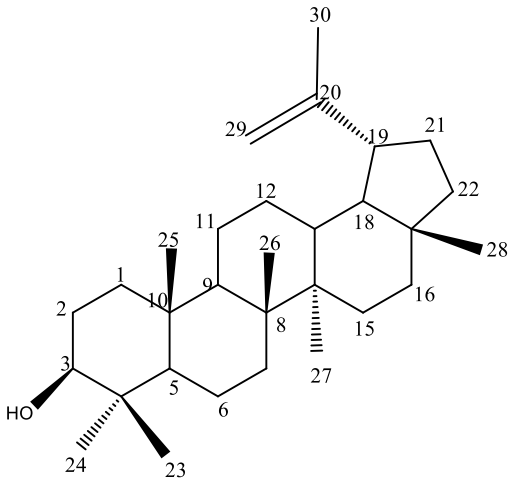
The ^{1}H NMR spectrum produced seven methyl protons at δ 0.76, 0.79, 0.83, 0.94, 0.97, 1.03 and 1.68 (integrated for 3H-each). A sextet of one proton at δ 2.38 ascribable to H-19 is a feature of lupeol. The H-3 proton showed a multiplet at δ 3.2 while a pair of broad singlets at δ 4.57 and δ 4.69 (IH, each) was indicated olefinic protons at (H-29 a & b).

The structure was further substantiated by the ^{13}C NMR experiments which showed seven methyl groups at δ 28.1 (C-23), δ 18.1 (C-28), δ 16.2 (C-25), δ 16.0 (C-26), δ 15.4 (C- 24), δ 14.6 (C-27) and δ 19.4 (C-30)]; there is an exomethylene group at δ 109.5 (C-29) and δ 150.6 (C-20)]. A signal at δ_C 79.1 was attributed to C-3.

The confirmation of the structure of compound 2 was accomplished through the 2D NMR experiments (COSY, HSQC and HMBC).The H-H COSY spectrum of the compound exhibited some cross peaks such as between δ 2.38, H-19 and one methylene proton δ1.33, H-21 and between methine proton δ3.18, H-3 and methylene (δ 1.57, H-2).

In the HMBC spectrum, the methine proton at δ3.18 (H-3) showed cross peaks with a methyl carbon at δ28.1, C-23) by J_2 correlation and a methyl carbon at δ 18.4, C-6) by J_3 correlation. The methine proton at δ 2.38 (H-19) showed cross peaks with two methylene carbon atoms at δ29.9 (C-21) and δ109.5 (C-29), a methine carbon at δ48.4 (C-18), a methyl carbon at δ 19.4 (C-30)] and a quaternary carbon at δ 150.6 (C-20). The pair of broad singlets of protons at δ 4.57 and 4.69 showed cross peaks with a methylene carbon at δ 48.1(C-19), δ150.6 (C-20) and δ 109.5 (C-29)] by J_3 correlation.

In conclusion, the forgoing spectral analysis and comparison with reported data (Jain and Bari, 2010), led to the proposition of the structure as lupeol, a pentacylic tri-terpenoid, as shown below.



4.5 In vitro activity of crude extract against selected bacteria

The activity of the crude extract was tested against four bacterial strains namely: *Pseudomonas aeruginosa* and *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* the extract's zone of inhibition diameter was 9 mm, 20 mm, 0 mm and 10 mm respectively as presented below;

Table 4.5.1: Bacteria strain and zone of inhibition by crude extract

		Zone of inhibition(mm)	
Bacterial strain	**Sensitivity**	**Crude extract**	**Tetracycline (positive control)**
Escherichia coli	Reactive	20	50
Staphylococcus aureus	Reactive	10	50
Pseudomonas aeruginosa	Reactive	9	50
Salmonella typhi	Reactive	0	60

Escherichia coli was found to be susceptible to the crude extract as the zone of inhibition of diameter (20mm) was with in the range for standard antibiotics such as tetracycline zone of (inhibition diameter 17-25) and ampicillin (inhibition diameter 16-22) as required by Clinical and Laboratory Standard Institute CLSI(2007)

When the *Trans* faragamide was tested against five bacterial strains namely: *Pseudomonas aeruginosa* and *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* the extract's zone of inhibition diameter was 12 mm, 10 mm, 13mm and 10mm respectively as presented below;

Table 4.5.2: Bacteria strain and zone of inhibition by *Trans* faragamide.

		Zone of inhibition(mm)	
Bacterial strain	**Sensitivity**	***Trans* Fagaramide**	**Tetracycline (positive control)**
Escherichia coli	Reactive	10	38
Staphylococcus aureus	Reactive	10	40
Pseudomonas aeruginosa	Reactive	12	40
Salmonella typhi	Reactive	13	30

The *trans*-fagaramide showed largest zone of inhibition on *Salmonella typhi* followed by *Pseudomonas aeruginosa* then *Escherichia coli* and *Staphylococcus aureus* although its zone of inhibition was below the range for standard antibiotics as required by Clinical and Laboratory Standard Institute.

The Minimum Inhibitory Concentration (MIC) against each of the bacterial results are presented below.

Table 4.5.3: Bacteria strain and Minimum Inhibitory Concentration of the *Trans* faragamide

Bacteria	Minimum Inhibitory Concentration (mg/ml)
Pseudomonas aeruginosa	125
Salmonella typhi	250
Escherichia coli	250
Staphylococcus aureus	250

The *trans*-fagaramide showed a Minimum Inhibitory Concentration of 125mg/ml on *Pseudomonas aeruginosa,* and *250mg/ml* on *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus.* This

showed that the fagaramide was more effective on *Pseudomonas aeruginosa than the other* bacteria. This high MIC value call for a high dose for effective treatment. This result support high dosage of (2-3 cups per day) used by traditional healers on adults. This is the first time this compound is reported from leaves of this plant.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The study found out that the crude extract obtained from *Alstonia boonei* leaves was strongly active antibacterial solution against *Escherichia coli* in disc diffusion agar assay, moderately active against

Pseudomonas aeruginosa and *Staphylococcus aureus* but it was inactive against *Salmonella typhi.* The Preliminary phytochemical analysis of the extract revealed the presence of tannins, flavonoids, saponins, terpenoids, cardiac glycosides and alkaloids. Separation of the crude extract yielded two compounds of; *trans*-fagaramide and lupeol. The *trans*- fagaramide showed strong antibacterial activity against *S. typhi* in the disc diffusion assay, followed by *P. aueruginosa* and least activities was registered when compared with *S. aureus* and *E. coli*. The *trans*-fagaramide showed a minimum inhibitory concentration against *P. aeruginosa* compared with the the bacterial strains.

This is the first time these compounds are reported from leaves of this plant. Lupeol is a triterpenoid that has anti inflamatory, antitumor and antimicrobial properties. *trans*-fagaramide has been recently isolated from *zanthoxylum leprieurii* stem bark used locally in Uganda in treating tuberculosis and cough related infections.

These findings coupled with bioassay studies of the crude extract justify use of *Alstonia boonei in* tradition medicine. Furthermore standardization of herbal formulation from *Alstonia boonei* leaves can be possible by using characterized compounds as markers in improved traditional medicine.

5.2 Recommendations

Further research should be made on the action of *trans*-fagaramide and its derivatives.

Biotechnological studies such as plant culturing are hereby recommended so as to generate these compounds in the laboratory and reduce on pressure imparted on natural environment.

There is need to increase on the number of microbial strain to be tested against.

Safety/cytotoxicity studies should be carried out about extracts and the isolated compounds.

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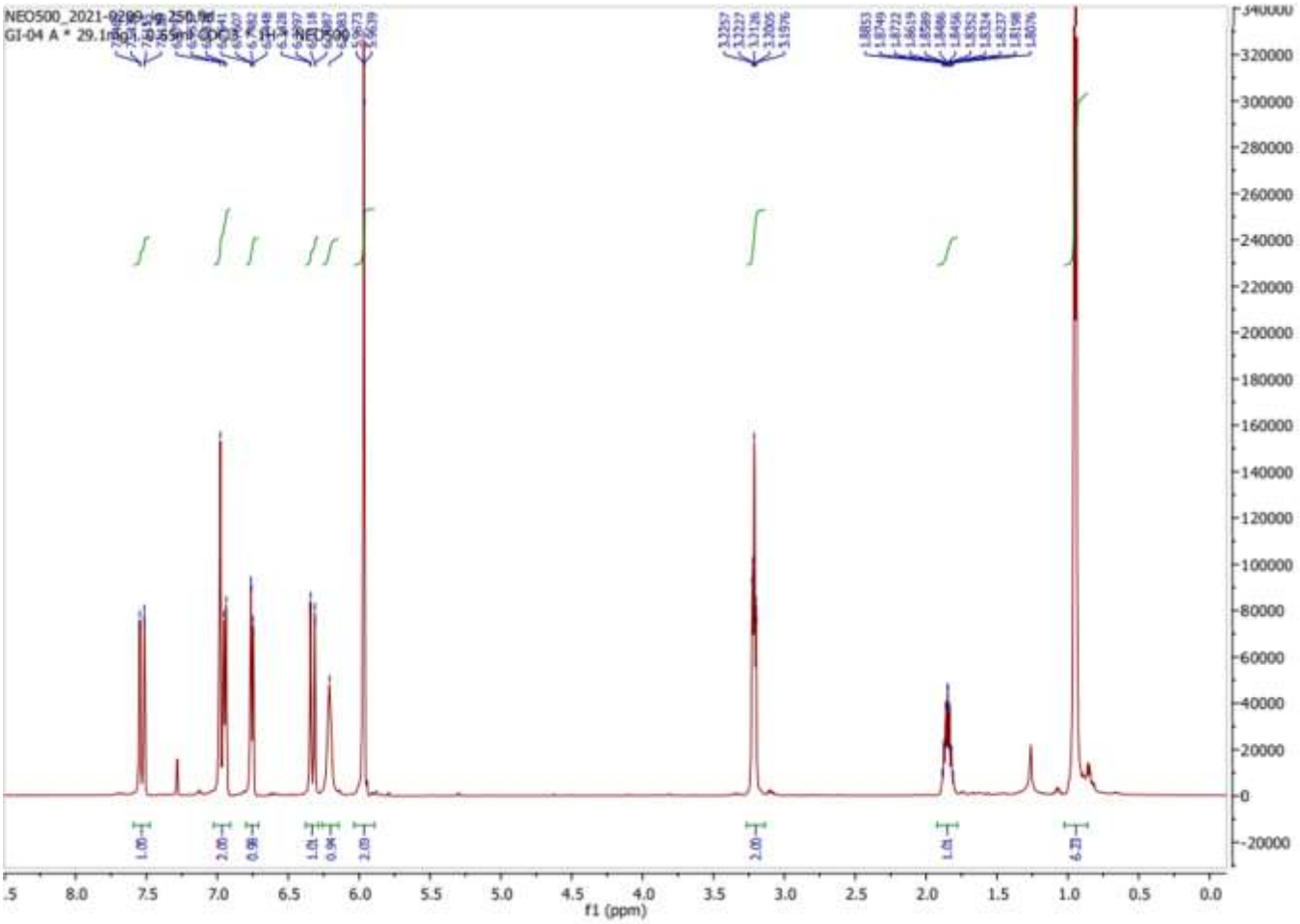
Appendices

Appendix 1: Compund 1

Compound 1 was obtained as a white powder, soluble in Dichrolomethane.

^{1}H NMR analysis in 500 MHz spectrometer with a solvent system of $CDCl_3$

Figure 4.2.1a: ^{1}H NMR spectrum of compound 1



APPENDIX 2: EXPANDED ^{1}H NMR SRECTRUM

Figure 4.2.1b: Expanded ^{1}H NMR spectrum of compound 1

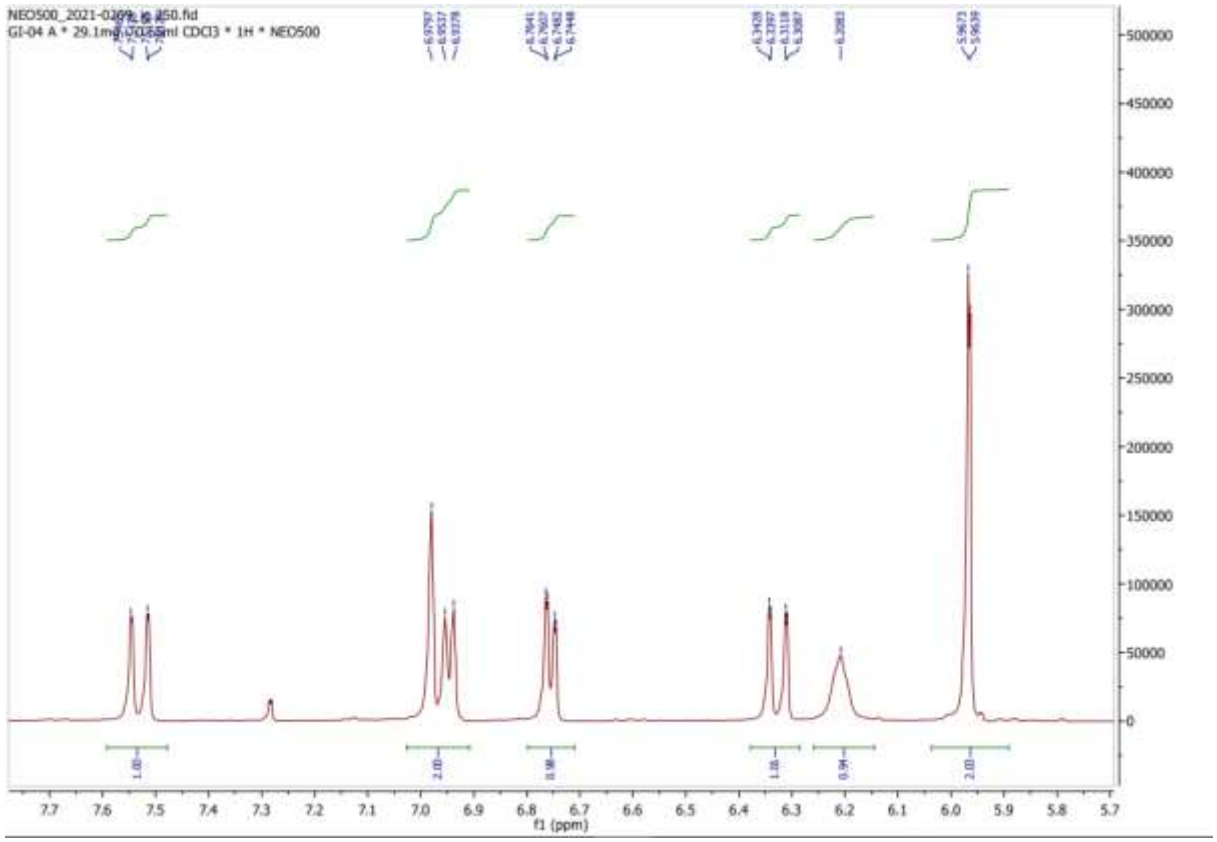


Figure 4.2.1c: ^{13}C NMR of isolated compound 1

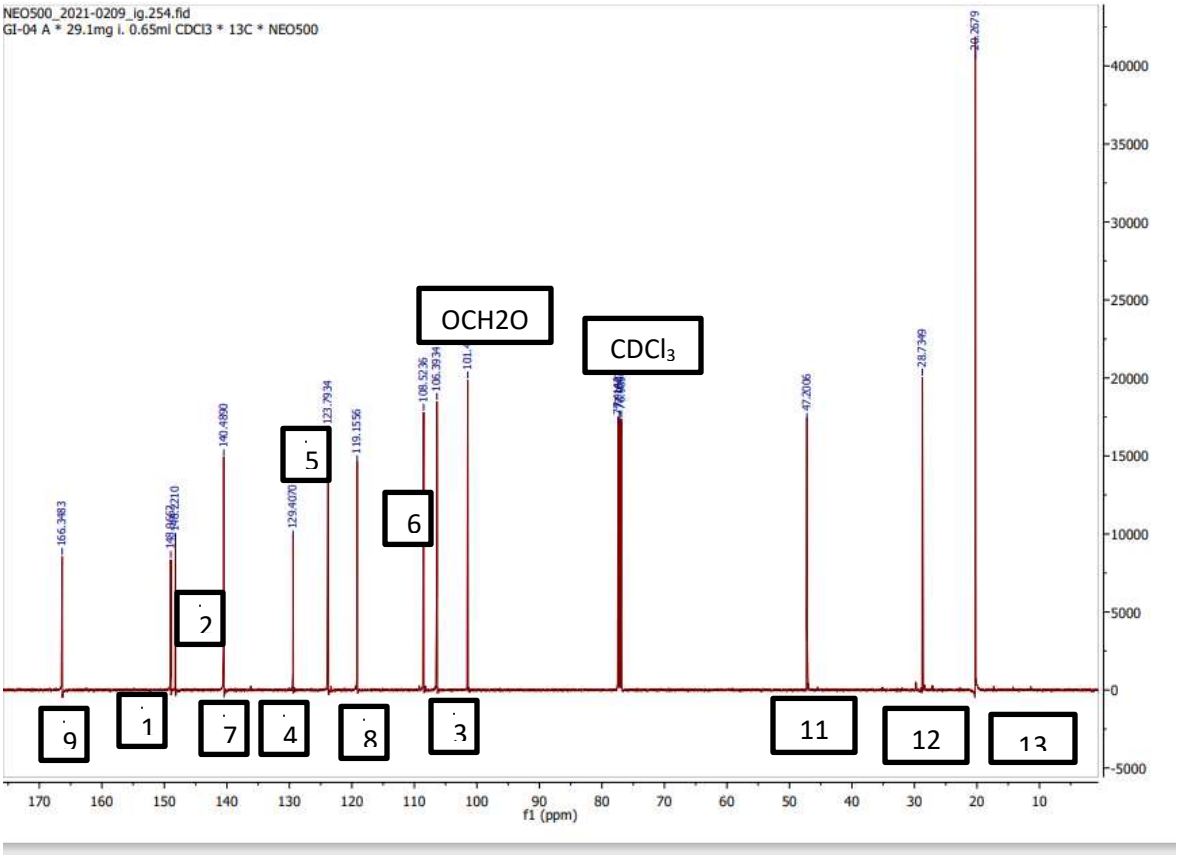

NEO500_2021-0209_ig.254.fid
GI-04 A * 29.1mg i. 0.65ml CDCl3 * 13C * NEO500
OCH2O
CDCl3
5
6
2
9
1
7
4
8
3
11
12
13
f1 (ppm)

Figure 4.2.1d: Expanded ^{13}C NMR Spectrum

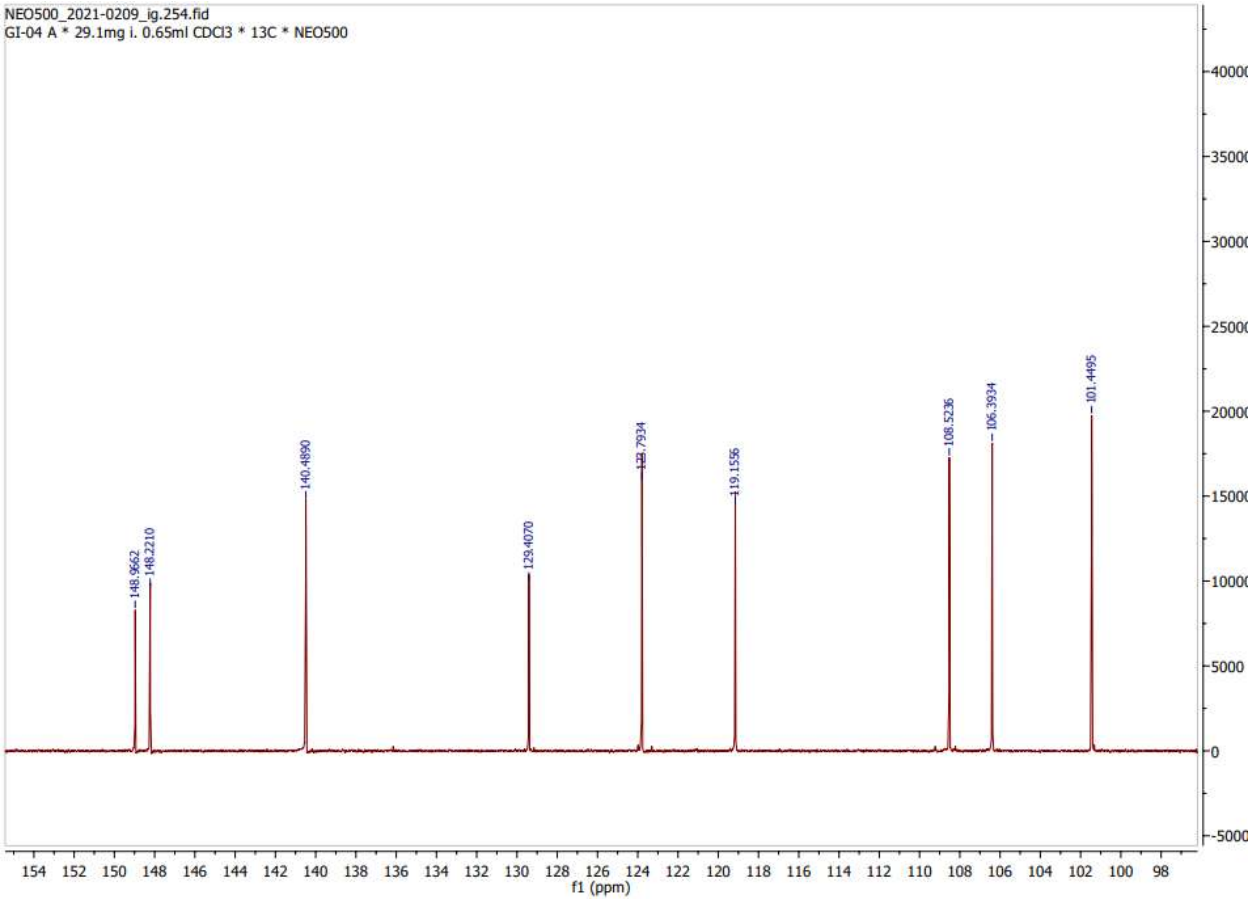


Figure 4.2.1e: ^{1}H-^{1}H COSY spectrum of compound 1

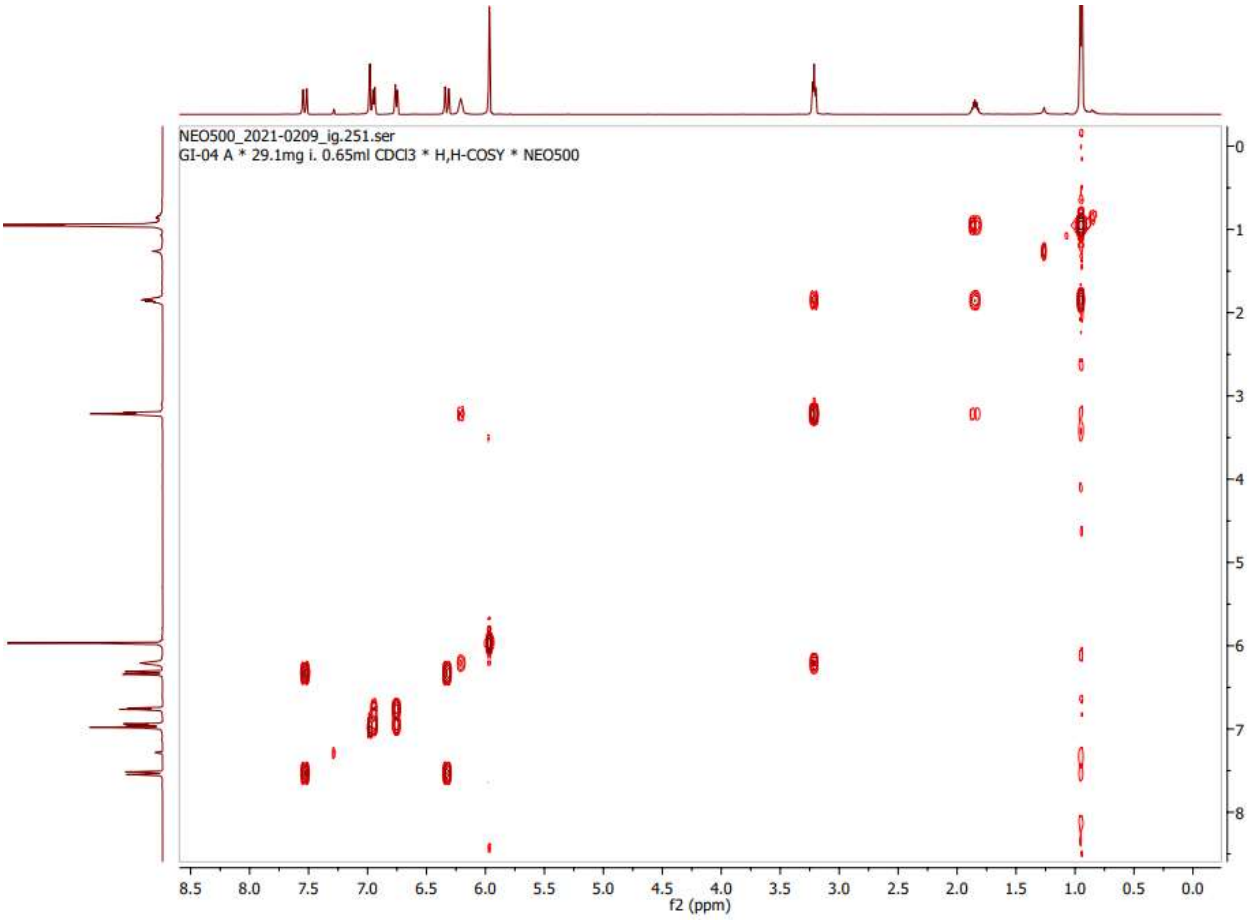


Figure 4.2.1f: ^{1}H -^{13}C HMBC spectrum of compound 1

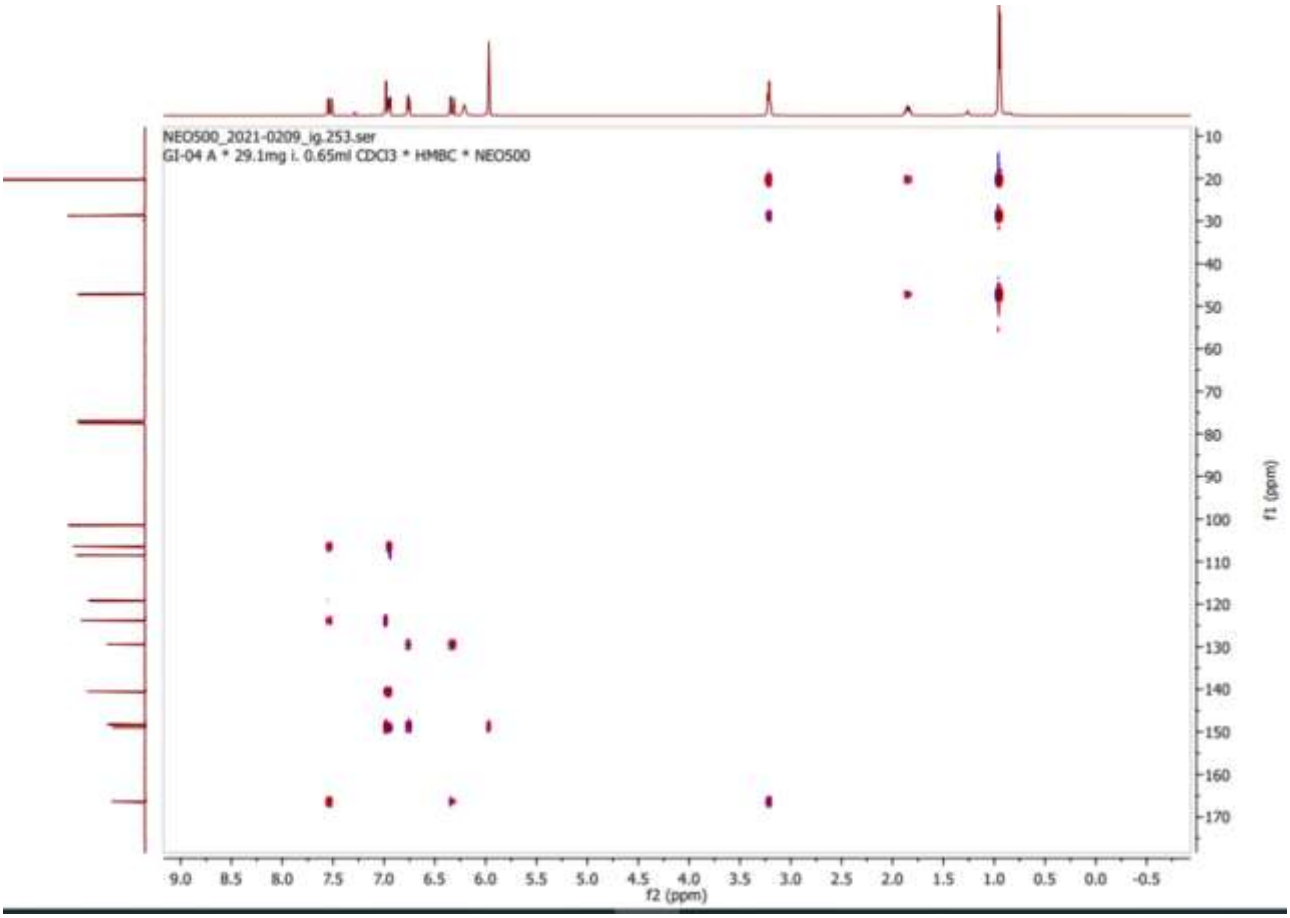
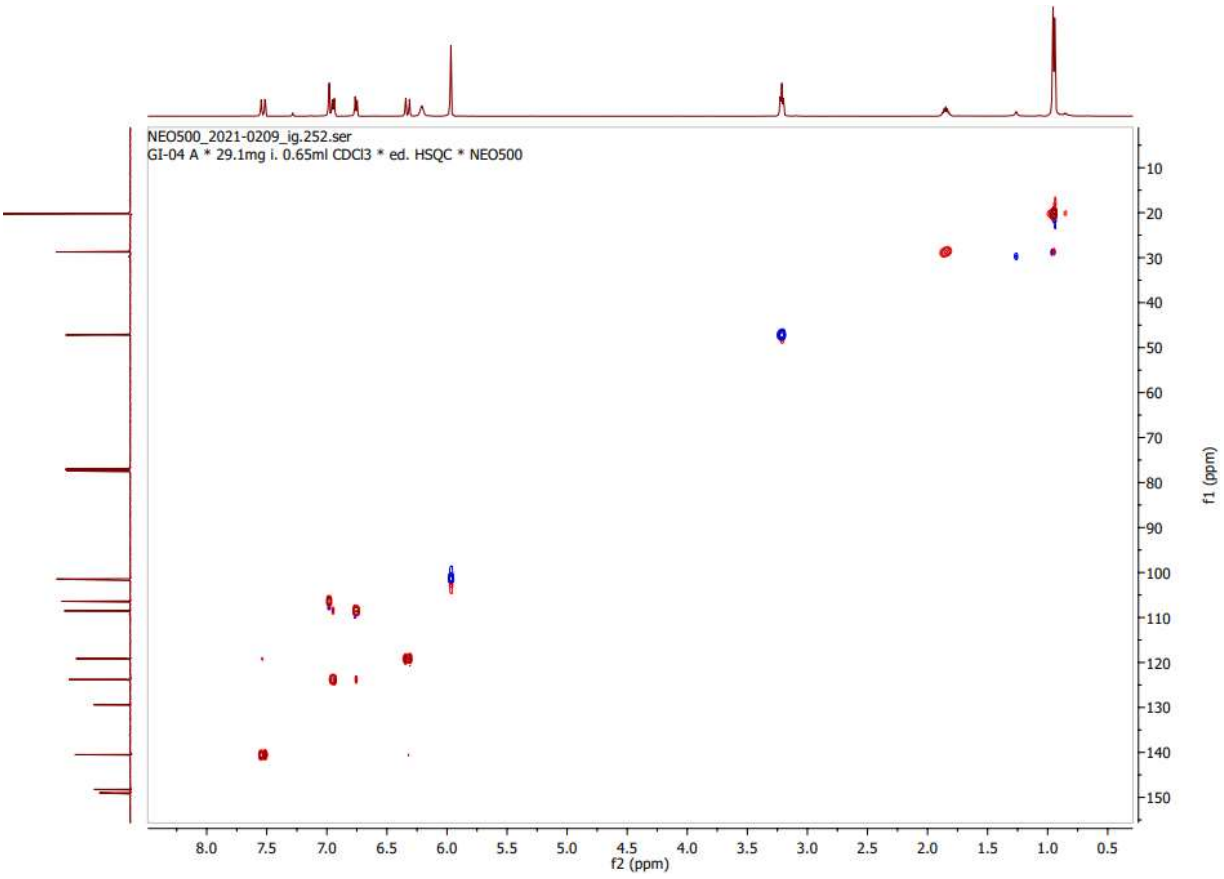


Figure 4.2.1g: ^{1}H -^{13}C HSQC spectrum of compound 1



Appendix 2: Compound 2

Figure 4.3.1a: ^{1}H - ^{13}C HMBC spectrum of compound 2

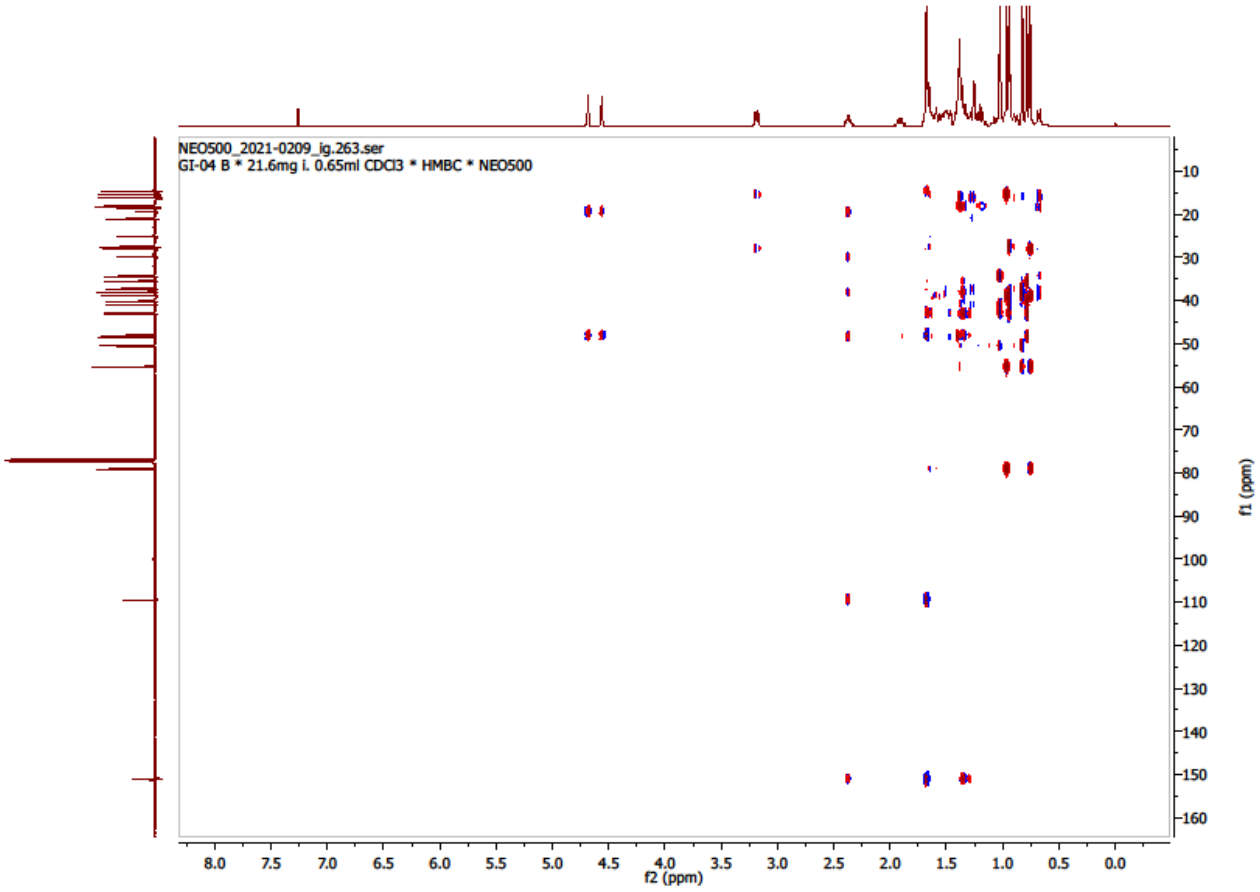


Figure 4.3.1b: ^{1}H- ^{1}H COSY spectrum of compound 2

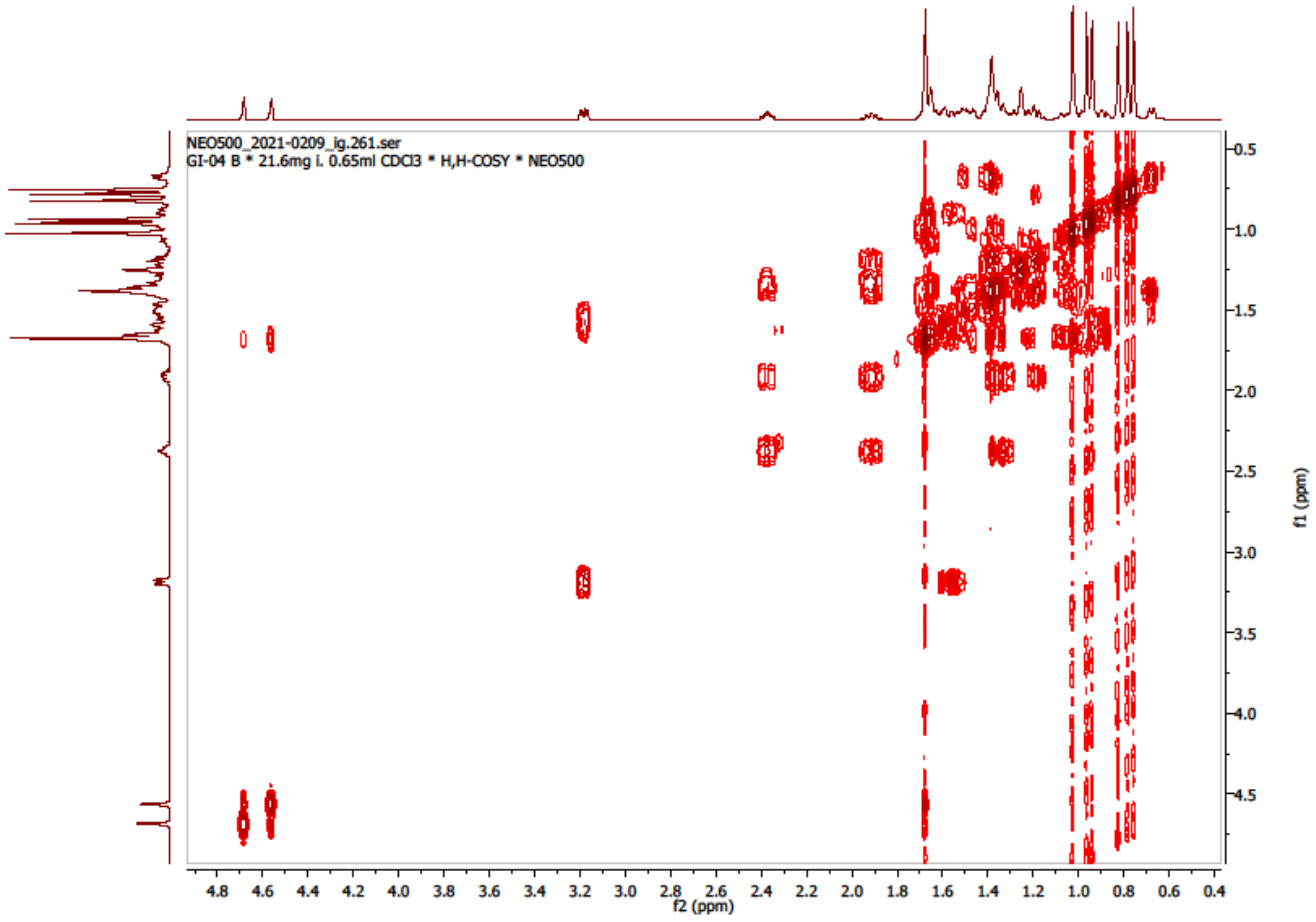


Figure 4.3.1c: ^{1}H - ^{13}C HSQC spectrum of compound 2

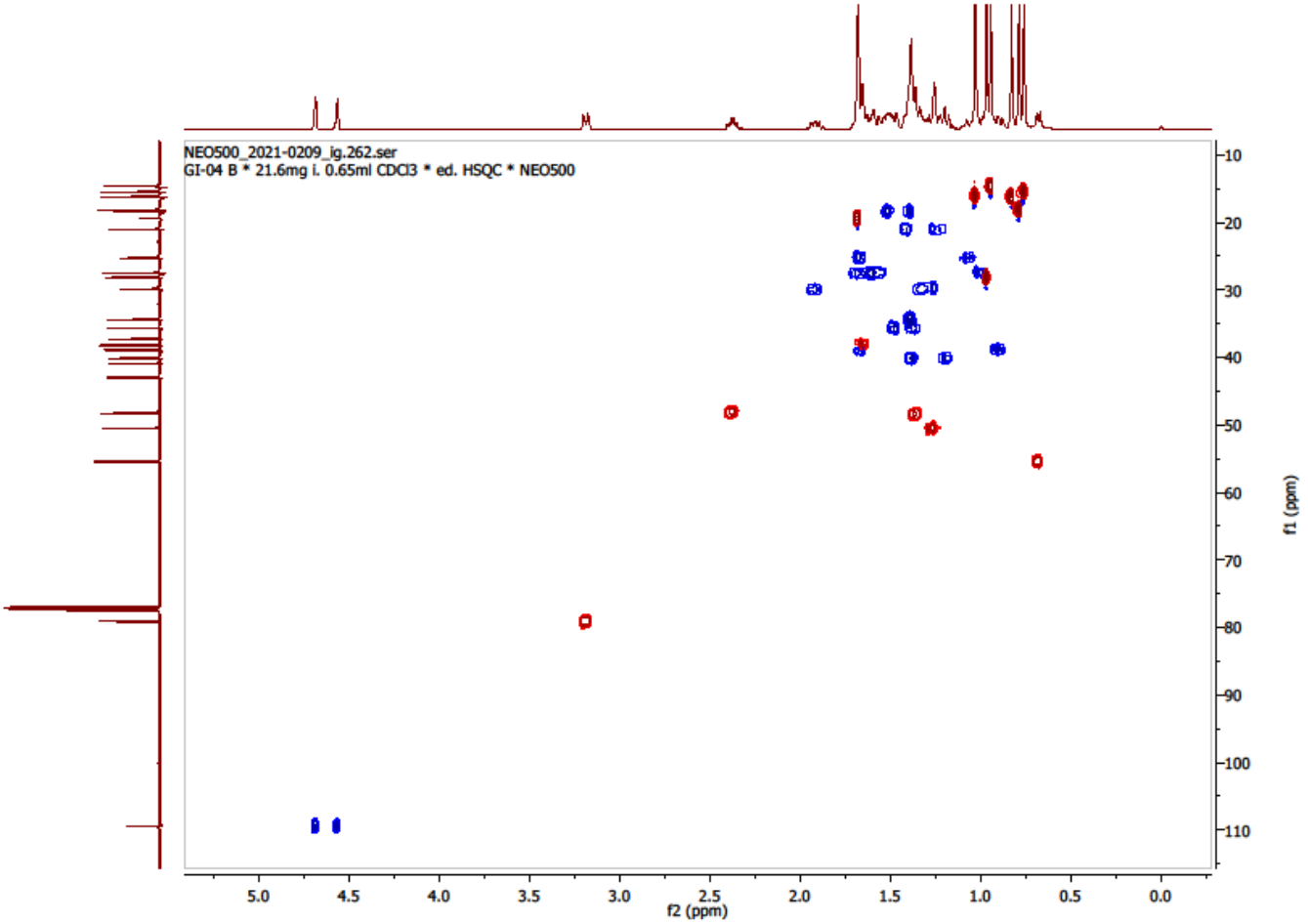


Figure 4.3.1d: ^{1}H- ^{13}C $CDC1_3$ spectrum of compound 2

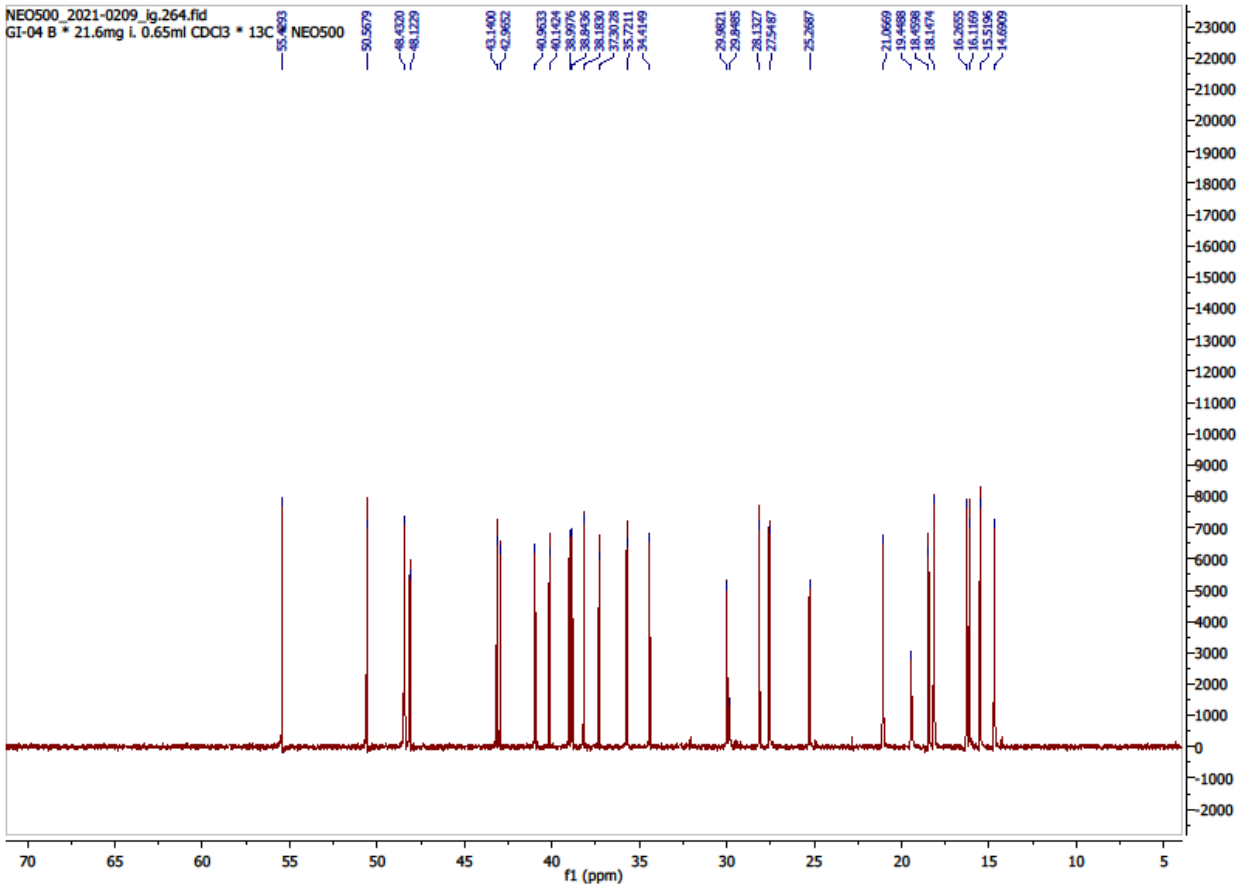


Figure 4.3.1e: ^{1}H - ^{13}C $CDC1_3$ Mass spectrum of compound 2

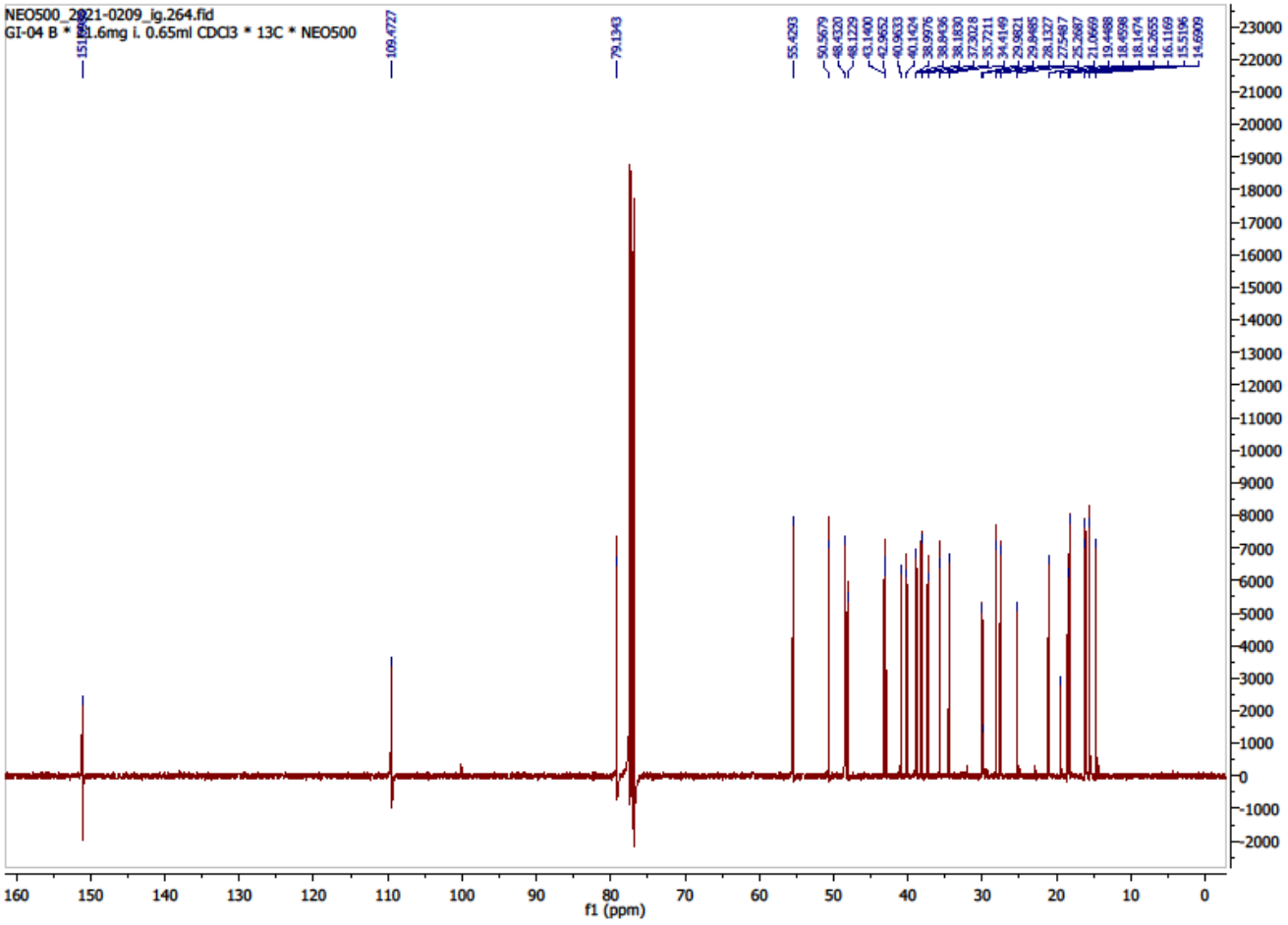
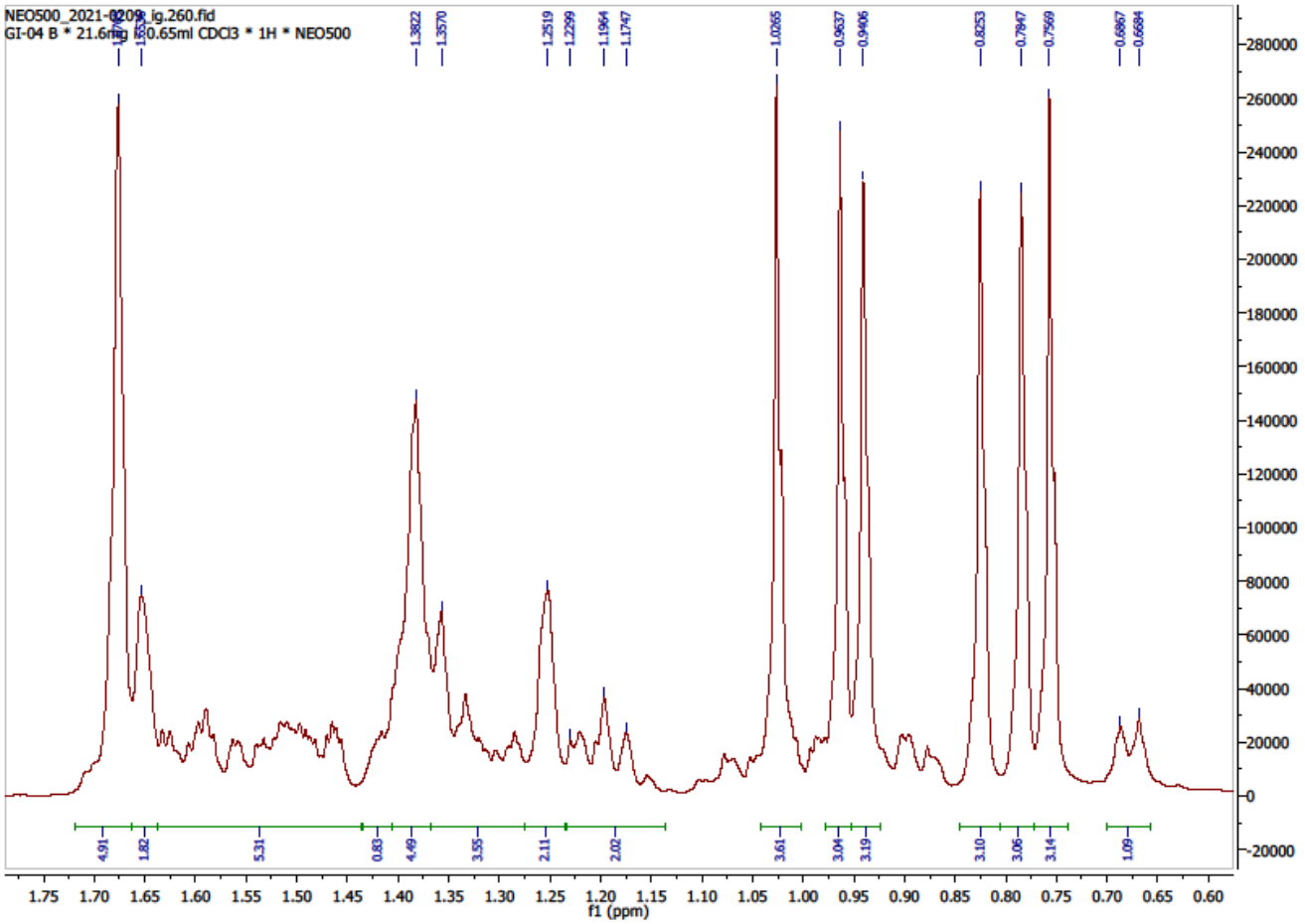


Figure 4.3.1f: ^{1}H -^{13}C $CDC1_3$ Expanded spectrum of compound 2



Appendix 3: Bioassay results of Trans fagaramide

Appendix 4: MIC results of a Trans fagaramide