Phytochemical and anti-microbial studies of isolates from *Senecio lyratipatitus* (Asteraceae)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

This work is dedicated to my loving parents Mr. Jacob Marete and Mrs. Harriet Marete.

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
C_{18}	Octadecylsilane
CC	Column Chromatography
CH_2Cl_2	Dichloromethane
Cm	Centimetre
DCM	Dichloromethane
DF	Degrees of freedom
EtOH	Ethanol
FT-IR	Fourier Transform Infrared Spectrophotometer
g	Grams
GLC	Gas Liquid Chromatography
GLM	General Linear Model
H_2SO_4	Sulphuric acid
HIV	Human Immuno-deficiency Virus
HPLC	High Performance Liquid Chromatography
KBr	Potassium bromide
Kg	kilograms
L	Litres
LD_{50}	Lethal dose at 50% concentration

MIC	Minimum Inhibitory Concentration
ml	Millilitres
mm	millimetre
nm	nanometer
^{o}C	Degrees Celsius
PA	Pyrrolizidine alkaloids
ppm	Parts per million
Prep-TLC	Preparative Thin Layer Chromatography
RPCC	Reverse Phase Column Chromatography
SAS	Statistical analysis system
TLC	Thin Layer Chromatography
tRNA	transfer Ribonucleic acid
UV	Ultra-Violet

ABSTRACT

Infections caused by bacteria and fungi account for approximately one-half of all deaths in the developing countries. Prevention of the microbial infections includes vaccination and development of anti-microbial agents. Although anti-microbial drugs have saved many lives, the high production cost of these drugs and development of resistance in the responsible pathogens are challenges facing the healthcare system. Developing countries have the potential of plant resources that exhibit a wide range of biological activities which may help in the development of cheaper and more potent anti-microbial agents. *Senecio lyratipatitus* has been used traditionally for the treatment of wounds and sexually transmitted diseases. The anti-microbial activities of the crude extracts and compounds of *S. lyratipatitus* were investigated. Dried and powdered roots of *S. lyratipatitus* were sequentially extracted using *n*-hexane, dichloromethane and methanol. The extracts were separated using Column Chromatography (normal and reverse phase) and Preparative Thin Layer Chromatography (Prep-TLC). Toxicity tests were carried out against brine shrimp. Anti-microbial activities against gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were carried out. Anti-fungal activity against *Candida albicans* was also done. The brine shrimp lethality test results indicated that the crude extracts and dichloromethane fractions possessed high toxicity against the brine shrimp larvae. The dichloromethane extract showed a significant level of toxicity with LD_{50} = 450.33 ppm. Further toxicity tests on the dichloromethane fractions, showed the highest toxicity was exhibited by the dichloromethane/ethyl acetate (1:1) fraction with LD_{50} = 175.89 ppm and ethyl acetate/ methanol (1:1) fraction was least toxic with LD_{50} = 1158

ppm. The crude extracts and dichloromethane fractions exhibited significant activity against bacteria compared to the standard anti-biotics. Two (2) compounds were isolated from the *n*-hexane extract, three (3) from the dichloromethane extract and one (1) from the methanol extract. Compounds SLRD1 and SLRH1 isolated from dichloromethane and *n*-hexane respectively, exhibited anti-microbial activity at a concentration of 20 μg/disc. Compound SLRD1 exhibited inhibition diameter ranging from 7.7 to 11.0 mm while that of SLRH1 ranged from 8.6 to 10.6 mm. On the basis of spectroscopic and physical data obtained, compound SLRD1 was identified as β-sitosterol. The anti-bacterial and anti-fungal activity exhibited by extracts and compounds of *S. lyratipatitus* is an indication of its potential in the development of anti-microbial agents.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Bacterial and fungal infections

Micro-organisms of every type continually surround the environment. The skin, gut and mucosal surfaces are colonised by many bacterial and fungal species and may be exposed to potential infectious agents through the air, food, water, contact with other people or in some circumstances through insect vectors (Max, 2002a). Some of the micro-organisms live in peaceful co-existence with human beings or lead to local or disseminated infection causing death. These infections account for approximately one-half of all deaths in tropical countries.

In the 1970s the concept that infectious diseases were eradicable was entertained by many physicians in the developed nations; the success of sanitation, vaccination, public health and a battery of anti-biotics reassured many that humanity's greatest scourges were on the verge of extinction (Max, 2002a).

In industrialised nations, despite the progress made in the understanding of microbiology and their control, incidents of epidemics due to drug resistant micro-organisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns. The increases in infections are attributed to increases in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in anti-biotic resistance in community acquired infections. These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention.

1.2 Economic impact of bacterial and fungal infections

Increased microbial resistance to existing anti-biotics and related models has underestimated the force of major killers such as HIV/AIDS, tuberculosis and malaria. The economic costs of infectious diseases, especially HIV/AIDS and malaria are significant and have an increased heavy toll on productivity, profitability and foreign investment. Infectious diseases are likely to slow socio-economic development in the developing countries and regions. This will challenge democratic development transitions that may contribute to humanitarian emergencies and civil conflicts (National intelligence council, 2000).

The infectious disease burden weakens the military capabilities of some countries as well as international peacekeeping efforts. The severe social and economic impact of infectious diseases is likely to intensify the struggle for political power to control scarce state resources (National intelligence council, 2000). Approaches like prevention (such as vaccination) and development of new treatment, which encompasses development of new anti-microbials, would help solve these negative effects.

1.3 Anti-microbial agents

Anti-microbial agents are chemical substances that can either kill or inhibit the growth of micro-organisms. Agents that kill micro-organisms are termed cidal (bactericidal), whereas those that do not kill but merely inhibit growth are referred to as static agents (bacteriostatic). The essence of anti-microbial chemotherapy is selective toxicity. The invading organism is regarded as being killed or its growth inhibited without the active agent causing harm to the patient. In order to be effective, anti-microbial agents must

posses a selective action on micro-organisms without causing toxicity to the cells of the host (Max, 2002b).

The use of anti-microbial agents has greatly contributed to improvements in health. Anti-microbial agents have been used for decades to treat communicable diseases and prevent infections. These agents can either be produced synthetically or naturally bacteria, fungi or plants. However, their use has also resulted in certain selected microbes developing resistance. This has in turn to led increased danger, prolonged suffering of the individual and enhanced costs for health care and society.

1.4 Effects of synthetic anti-microbials

Medicines have an important role in the treatment and prevention of diseases in both humans and animals. It is because of the very nature of medicines that they may also have unintended effects on animals and micro-organisms in the environment. Although the side effects on human and animal health are usually investigated in thorough safety and toxicological studies, the potential environmental impacts of the manufacture and use of medicine are less well understood.

This is further complicated by the fact that pharmaceuticals can cast effects on bacteria and animals well below the concentrations that are usually used in safety and efficacy tests. In addition, breakdown products and the combination of different biologically active compounds may have unanticipated effects on the environment (Alistain, 2004).

Pharmaceutical substances may be degraded by biological organisms in treatment systems, water bodies and soils as well as abiotic reactions. Although these processes reduce the toxicity of medicines, some breakdown products may also have similar effects to their parent compounds (Halling *et al.,* 2002).

Degradation varies significantly depending on the chemistry, biological and climatic conditions. For example, the half-life of the anti-parasitic ivermectin under winter conditions is six times greater than in the summer and the compound degrades faster in sandy soils than in sandy loam soils (Halley *et al.,* 1993). These pressures have accelerated the search for more toxicologically safe and more selective and efficacious anti-microbials. Thus natural products have increasingly become the focus in the discovery of anti-microbials.

1.5 Use of natural products from plants

A key challenge to the anti-biotic industry is that constant innovation is necessary not only because of the resistance but also side effects associated with the use of the anti-biotics, including bacterial resistance to the said drugs. Most resistant bacteria can also produce substances that destroy the anti-biotic (Frere, 1995).

Two factors that have contributed to the ongoing problems of bacterial resistance to anti-biotics are unnecessary use and the misuse of anti-biotics to treat diseases for which an anti-biotic is not effective. Thus, efforts have been aimed at modifying the molecular characteristics of existing anti-biotics to make them better in terms of reduced side effects and avoidance of resistance (Nature biotechnology, 2000).

Plants have several uses to mankind, which include food, building construction, furniture woodworking, fuel and medicine. Plants have almost limitless ability to synthesize aromatic substances most of which are phenols or their oxygen substituted derivatives (Mariorie, 1999).

The shikimic acid pathway is responsible for the biosynthesis of many aromatic compounds in plants. This pathway is considered to lack in animals as this is demonstrated by their dietary requirement for shikimate-drived aromatic amino acids (Starcevic, *et al*., 2008). Most are secondary metabolites of which at least 12,000 have been isolated, and estimated to be less than 10% of the total. In many cases these substances serve as plant defence mechanisms against predation by micro-organisms, insects and herbivores. Some, such as terpenoids give plants their odours, while quinones and tannins are responsible for plant pigments (Mariorie, 1999).

The secondary metabolites of plants are a vast repository of compounds with a wide range of biological activities. Isolation and chemical characterization of the active compounds from plants with strong anti-microbial activities can be a major effort compared to synthesizing a new synthetic anti-biotic. The use of plants as medicines goes back to the early man (David, 2001). Great civilizations of the ancient Chinese, Indians and North Africans provided written evidence of man's ingenuity in utilizing plants for the treatment of a wide variety of diseases (David, 2001).

Egyptian pharmaceutical record documents over 700 drugs (mostly plants though animal organs were included with some minerals) and includes formulae such as gargles, snuffs, infusions, pills and ointments with beer, milk, honey, and wine commonly used as

vehicles (Newman *et al*., 2000). In the 19th century man began to isolate the active principles of medicinal plants with the discovery of quinine from cinchona bark, morphine from poppy plant and aspirin from willow bark (David, 2001). The plant-based traditional medicine systems continue to play an essential role in healthcare and it has been estimated by World Health Organisation that approximately 80% of the World's inhabitants rely mainly on traditional medicine for their primary healthcare (Newman *et al.*, 2000).

Plants have provided a good source of anti-infective agents, for example emetine and berberine that remain highly effective instruments in the fight against microbial infections. Phytomedicines derived from plants have shown great promise in the treatment of intractable infectious diseases including opportunistic AIDS infections. A series of natural products isolated from higher plants have been used as clinical agents. Thus, plants have continued to be used world-wide for the treatment of disease and novel drug entities continue to be developed through phytochemical research. In the developed countries, high-throughput screening tests are used for bioassay guided fractionations leading to isolations of active principles that may be developed into clinical agents either as natural products or a synthetic modification or a synthesised analogue with reduced side effects (David, 2001).

Thousands of secondary metabolites from plants represent a large reservoir of chemical substances with valuable biological activities. The mode of extraction of these substances depends on the texture and water content of the plant material being extracted and on the type of substance being isolated. The classical chemical procedure for obtaining organic constituent from dried plant tissue involves continuous extraction of

powdered material in a Soxhlet apparatus with a range of solvents for example ether and chloroform for lipids and terpenoids (Harborne, 1973). Modern methods of extraction involve cold extraction with solvents of increasing polarity. This can also be done by extracting with a polar solvent then partitioning with less polar solvents. The extract is filtered and concentrated *in vacuo.*

Separation of the constituents includes; thin layer chromatography (TLC), column chromatography (CC), reverse phase column chromatography (RPCC), high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC). The technique of separation depends on the solubility properties and volatilities of the compounds to be separated (Harborne, 1973).

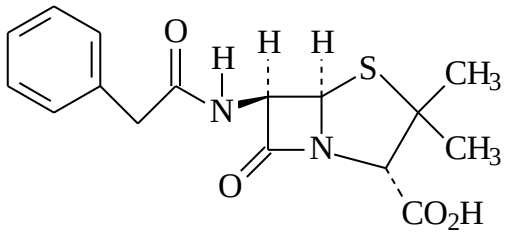
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Anti-bacterial compounds

Concerted and systematic programs to discover and develop new anti-biotics and anti-fungals have been driven to a considerable extent by the development of resistance by these organisms to the drugs commonly used against them. Ideas about new anti-biotics come from a variety of sources. The historical background of anti-biotics began with the discovery of penicillin (**1**) in 1928 by Fleming. The base structure of penicillin molecule acted by a mechanism that targeted a structure unique to the bacterial cell wall (Newman *et al.*, 2000). With immediate realization that microbes produced protective enzymes, penicillinases or β-lactamases that degraded the penicillin by opening the β-lactam ring, there was need to produce other non lactam anti-biotics. The basic penicillin nucleus could be chemically modified to reduce or eliminate the enzymatic activity (David *et al.*, 2000).

In 1948, cephalosporin (**2**) a ring expanded molecule, was reported from an isolate of a *pseudo* marine fungus, *Cephalosporium acremonium* (Nature biotechnology, 2000). In 1985, anti-biotic peptides released by normal human neutrophils were characterized. These peptides also called defensils kill such bacteria as; *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli in vitro* (Nature biotechnology, 2000).



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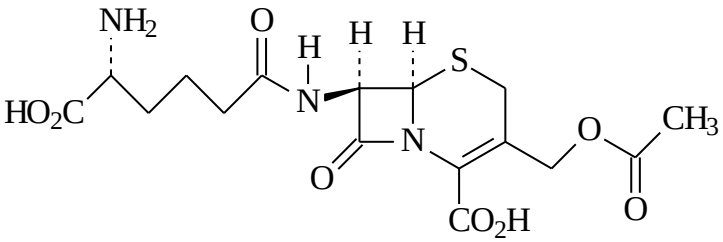

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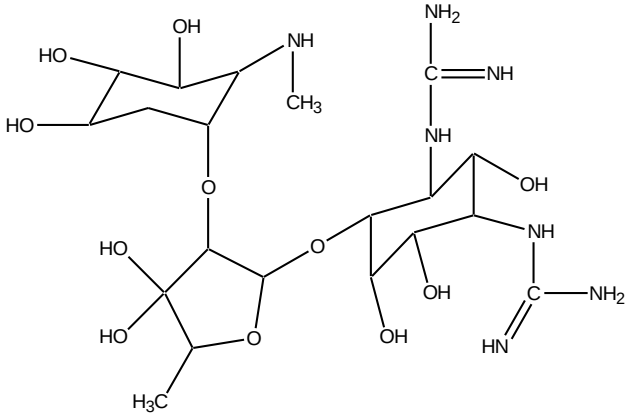
A search for microbial metabolites against tuberculosis led to the isolation of the amino glycoside anti-biotic streptomycin (**3**) from *Streptomyces griseus*, which was active against *Mycobacterium tuberculosis* and other bacterial infections (Newmann *et al*., 2000).

Another class of anti-bacterials that have been used extensively are the tetracyclines, for example aureomycin (**4**). These were produced by various *Streptomyces* species and have the skeleton of four linear fused rings. Tetracyclines are broad spectrum agents that inhibit binding of the aminoacyl tRNA to the 30S ribosomal sub-unit in bacteria (Newmann *et al*., 2000).



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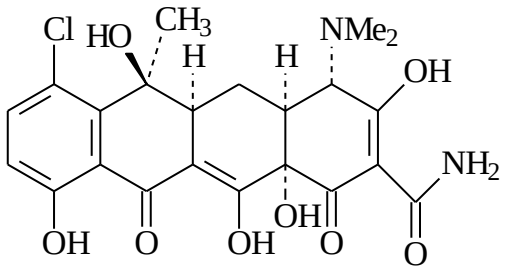

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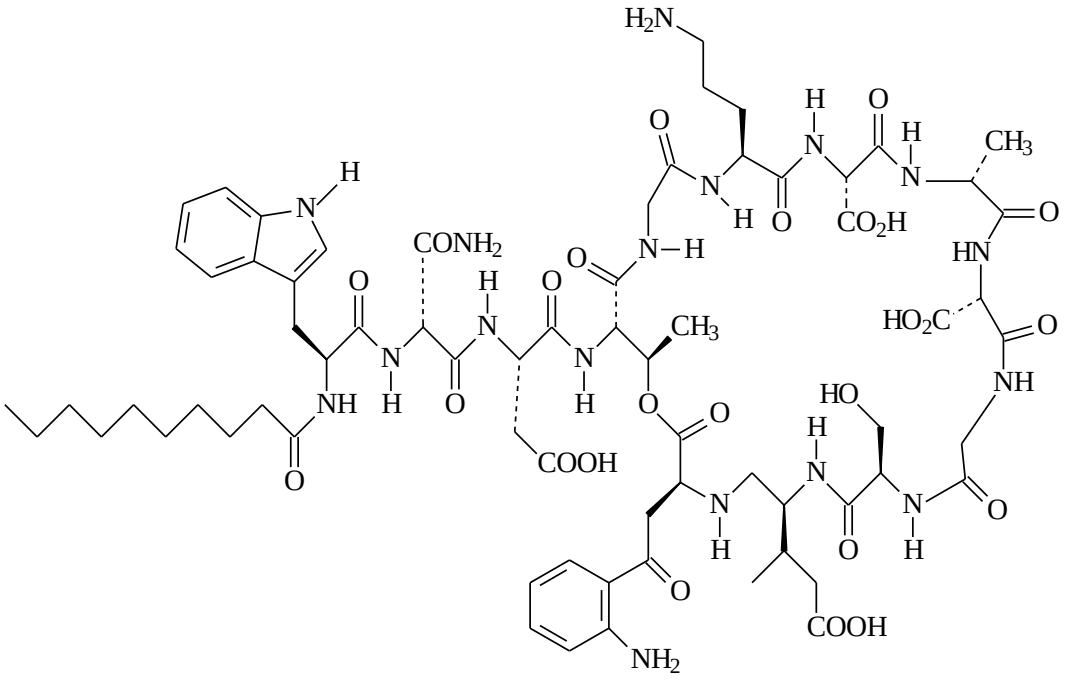
In search of natural products with a broad spectrum of anti-fungal activity as lead compounds for novel treatments of mycoses, lipopeptides were isolated. Daptomycin (**5**), an acidic lipopeptide anti-biotic produced by *Streptomyces roseosporus* possesses potent bactericidal activity against many clinically important gram positive pathogens (Lee-Jon *et al.*, 2004). An echinocandin type lipopeptidolactone (**6**) isolated from an unknown fungus inhibits the synthesis of 1,3-betaglucan, a key component of the fungal cell wall (Barrett, 2002).

Some clinically important anti-biotics isolated from some organisms have exhibited activity against gram-positive bacteria, fungi and gram-negative bacteria (Table 2.1). In gram-positive bacterium the cell wall mainly consists of peptidoglycan and lacks the outer membrane while gram negative bacterium consists mainly of lipids and has a secondary outer membrane. When gram staining, the gram-positive bacterium appears purple as the violet stain is absorbed in the cell wall, while the gram-negative bacterium appears pink since the outer membrane prevents the dye from penetrating the cell wall (David *et al.*, 2000; Alcamo, 2001).

Table 2.1: Some clinically important anti-biotics

Anti-biotic	**Producer organism**	**Potency**
Penicillin	*Penicillium chrysogenum*	Gram positive bacteria
Cephalosporin	*Cepharosporium acremonium*	Broad spectrum
Griseofulvin	*Penicillium grisefulvum*	Dermatophytic fungi
Bacitracin	*Bacillus subtilis*	Gram positive bacteria
Polymyxin B	*Bacillus polymyxa*	Gram negative bacteria
Amphotericin B	*Streptomyces nodosus*	Fungi
Erythromycin	*Streptomyces erythreus*	Gram positive bacteria
Neomycin	*Streptomyces fradiae*	Broad spectrum
Streptomycin	*Streptomyces griseus*	Gram negative bacteria
Tetracycline	*Streptomyces nimosus*	Broad spectrum
Vancomycin	*Streptomyces orientalis*	Gram positive bacteria
Gentamicin	*Micromonospora purpurea*	Broad spectrum
Rifamycin	*Streptomyces mediterranei*	Tuberculosis



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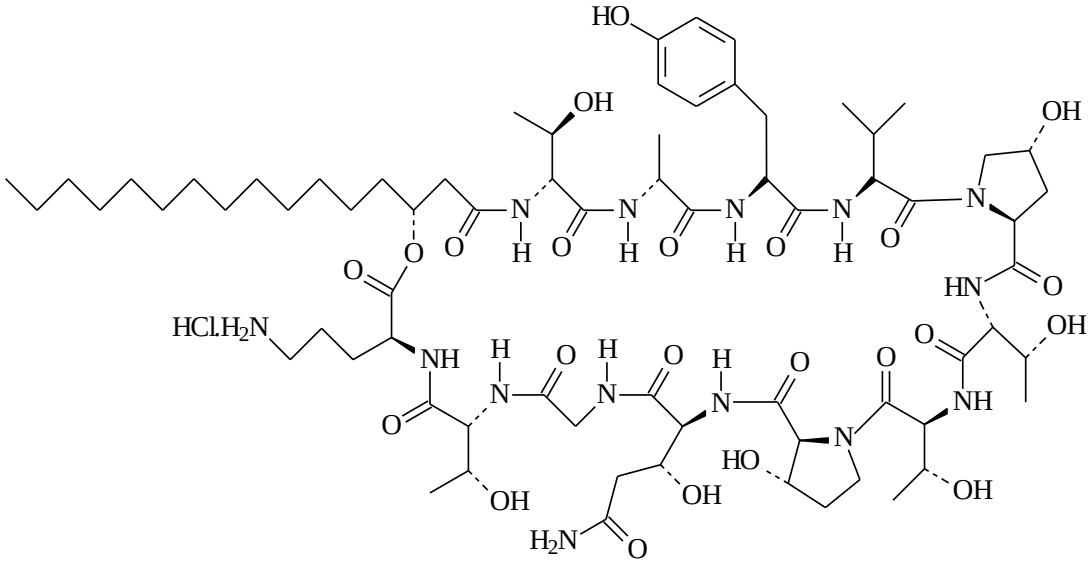

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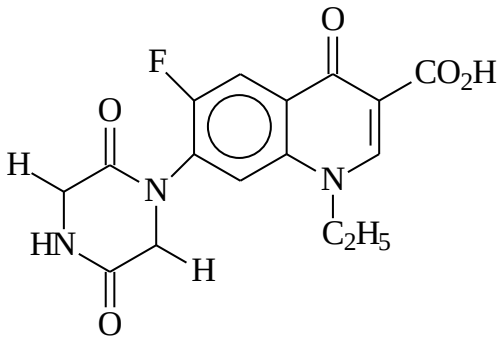
Floroquinolines with 7-piperazinyl,1-ethyl and 1-benzyl have been reported to possess anti-bacterial, anti-fungal and anti-viral activities (Natesh *et al.*, 2003). Synthetic 1-ethyl-6-fluoro-7-(2,5-dioxo-piperazin-1-yl)1,4-dihydro-4-oxo-quinoline-3-carboxylic acid (**7**) exhibited significant anti-bacterial and weak anti-fungal activities (Natesh *et al.*, 2003). Hydrazide-hydrazones have displayed anti-bacterial, anti-convulsant and anti-tubercular activities. Nifuroxazide (**8**), which possesses a hydrazide-hydrazone structure, is used as an intestinal antiseptic. Several diflusinal hydrazide-hydrazone derivatives synthesised from diflusinal as the starting compound have exhibited anti-microbial activity (Guniz *et al.*, 2003).



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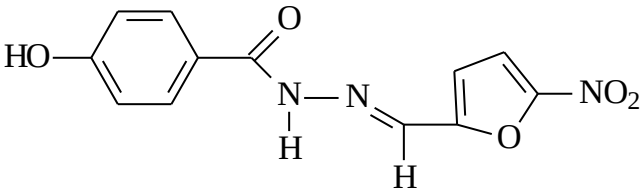

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Soil micro-organisms and fungi produce most of the clinically useful anti-biotics for example erythromycin, neomycin and vancomycin. Higher plants have also been used as a source of anti-biotics. The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives offering profound therapeutic benefits and more affordable treatment (Iwu *et al.*, 1999).

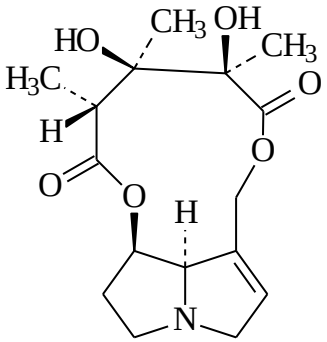
Plant based anti-microbials have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side

effects often associated with synthetic anti-microbials. It is estimated that there are about 500,000 species of plants on earth and relatively high percentages are used for medicinal purposes. The family Asteraceae, being the second largest family has a high therapeutic potential.

2.2 The Asteraceae family

The family Asteraceae is one of the largest plant families in the world, with about 900 genera and almost 20,000 species distributed over most of the earth and in almost all habitats. It consists of mainly herbs, shrubs and a few real trees (Blundell, 1982; Beentje, 1994). Members of this family exhibit anti-microbial activity (Table 2.2). Phytochemical research has revealed that sesquiterpene lactones are the principal secondary metabolites responsible for the anti-microbial activity of these plants (Rai and Acharya, 1999). About 22 genera in Asteraceae contain pyrrolizidine alkaloids that have generated much interest for many years because of their diverse range of biological activities, including hepatotoxicity (Harborne and Dey, 1993). However, some alkaloids and their derivatives do have beneficial physiological activities (Harborne and Dey, 1993). The 1,2-unsaturation in the base portion of the pyrrolizidine nucleus is responsible for hepatotoxicity, for example in monocrotaline (**9**) (Harborne and Dey, 1993). The pyrrolizidine alkaloids containing 1,2-unsaturation are metabolised by oxidase enzymes in the liver to the corresponding pyrrole derivatives.

These pyrrole derivatives are reactive alkylating agents and cause most damage at their site of manufacture in the liver. Transportation of the reactive metabolites to the lungs is also possible, leading to pulmonary damage. The useful alkaloids usually contain a saturated pyrrolizidine nucleus. For example platyphylline (**10**), isolated from Senecio genus, have been used for the treatment of hypertension and internal ulcers (Harborne and Dey, 1993). Other useful alkaloids for example, indicine oxide (**11**) an N-oxide has anti-tumour activity (Harborne and Dey, 1993).



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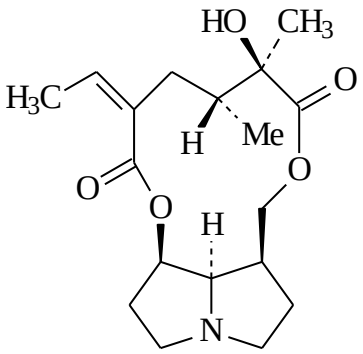

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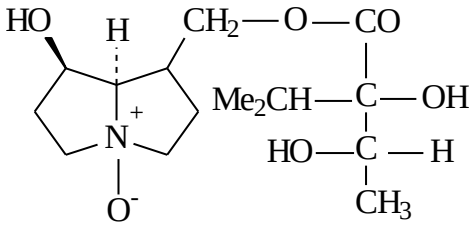

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Table 2.2: Anti-microbial activity of some plants of Asteraceae family

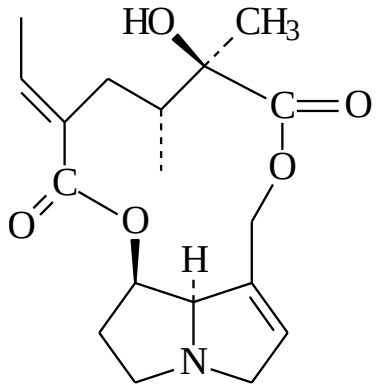
Plant	Extract	Activity MIC (mg/ml)	Organism	Reference
Helichrysum bellium	Acetone	1.0	*Psedomonas Aeruginosa*	Abbey, 2001
H. argyosphaerum	Acetone	0.01	*Bacillus subtilis*	Abbey, 2001
H. bellium	Acetone	1.0	*B. subtilis*	Abbey, 2001
H. argyosphaerum	Acetone	1.0	*P. aeruginosa*	Abbey, 2001
Arctium lappa	EtOH : H_2O (9:1)	0.5	*Staphylococcus aureus*	Fabiola *et al*., 2002
A. lappa	EtOH : H_2O (9:1)	0.5	*B. subtilis*	Fabiola *et al*., 2002
A. lappa	EtOH : H_2O (9:1)	0.5	*Escherichia coli*	Fabiola *et al*., 2002
A. lappa	EtOH : H_2O (9:1)	> 1.0	*Candida albicans*	Fabiola *et al*., 2002
A. lappa	EtOH : H_2O (9:1)	0.5	*P.aeruginosa*	Fabiola *et al*., 2002

2.3 The genus, Senecio

The genus Senecio is the largest in the family Asteraceae comprising of 1500 species widespread all over the world (Assem *et al*., 2002; Vaselin *et al.*, 2002). It is a well-known source of pyrrolizidine alkaloids (PA), sesquiterpenes, in particular eremophilanolide derivatives and benzofurans (Azucena *et al.*, 1999, Assem *et al*.,

2002). More than 180 Senecio species contain pyrrolizidine alkaloids as the most characteristic secondary metabolites (Harborne and Dey, 1993, Liddell, 2000).

Pyrrolizidine alkaloids have been found to possess important medicinal properties, including anti-tumour activity (Hartmann and Witte 1995; El-Shazly, 2002). Pyrrolizidine alkaloids are also responsible for the hepatotoxic and carcinogenic effects of this genus (Cheng *et al*., 1992). Some N-oxides of pyrrolizidine alkaloids, particularly indicine N-oxide exhibit anti-tumour activity (Harborne and Dey, 1993). *In-vitro* assays have demonstrated that pyrrolizidine alkaloids can interfere with neuroreceptors and protein synthesis (Schmeller *et al*., 1997; Wink *et al*., 1998). Pyrrolizidine alkaloids isolated from *Senecio macedonicus* have shown activity on murine lymphocytes with suppressive effect on the proliferation (Vaselin *et al.*, 2002). Integerrimine (**12**) and its N-oxide isolated from *S. miser* showed strong insect anti-feedant activity against *Leptinotarsa decemlineta* (Colorado Potato Beetle) (Azucena *et al.*, 1999; Liddell, 2002).



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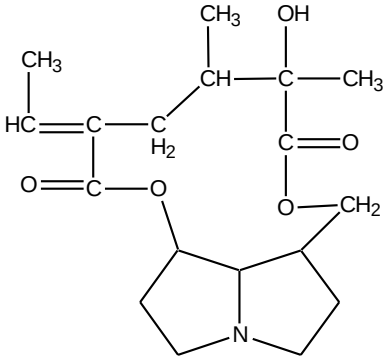
There are hepatotoxic alkaloids known to occur in tansy ragwort (*Senecio jacobaea* L.). The total alkaloid content amounts between 0.2 and 0.3% of the dry weight in kilograms. These alkaloids include senecionine (**13**), seneciphylline, eruciflorine (**14**), jacoline (**15**), jaconine, jacobine, senecivernine, retrosine, spartoidine and jacozine (Witte *et al*., 1992; Roeder, 1995; Hol and Veen, 2002). Pyrolizidine alkaloids isolated from *S. jacobaea* have been reported to show a variable effect on fungal growth (Hol and Veen, 2002).

Flower and leaf volatile oils of *S. aegyptius* have been reported to show significant level of anti-fungal activity against *Candida albicans* and moderate effect against gram-positive bacteria (Assem *et al*., 2002). The isolated sesquiterpene (1,10-epoxyfuranoeremophilane) displayed marked inhibition activity against gram-negative bacteria, *Bacillus subtilis* and fungi and no effect against *Staphylococcus aureus* (Assem *et al*., 2002). *S. vulgaris* contains a large number of alkaloids up to a content of 0.16% dry weight. These alkaloids include senecionine, integerimine, retrosine (**16**), usamarine, seneciphylline, spartoidine, riddeline and the corresponding N-oxides (Roeder, 1995). *S. bicolor* contains a total concentration of alkaloids of 0.9% dry weight. These alkaloids include senecionine, retrosine, seneciphylline, otosenine, jaconine and jacobine (Roeder, 1995).



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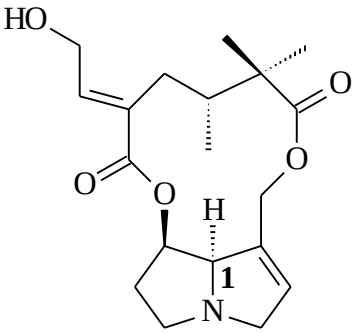

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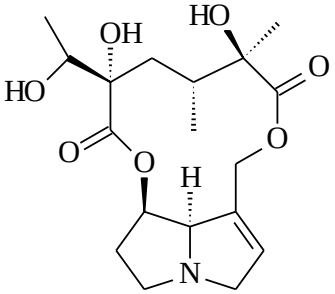

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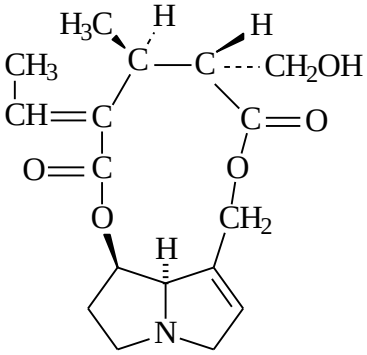

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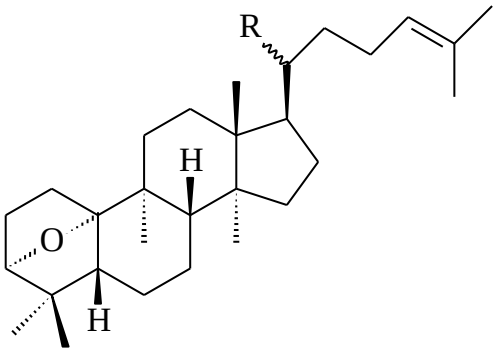
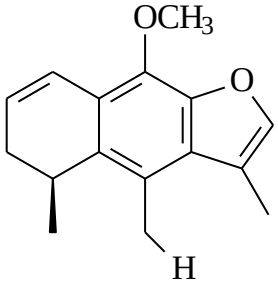
Pyrrolizidine alkaloids retrosine, senecionine and 18-hydroxyjaconine isolated from *S. selloi* are considered to be responsible for its toxicity against cattle, sheep and horses (Rucker *et al.*, 1996). Five sesquiterpene peroxides isolated from the aerial parts of *S. selloi* displayed anti-malarial activity against *Plasmodium falciparum* (Rucker, *et al.*, 1996). Two triterpenes have been isolated from *S. selloi* and identified as (20R)-3α,10α-Epoxy-9-epi-cucurbita-24-ene (**17**) and (20S)-3α,10α-Epoxy-9-epi-cucurbita-24-ene

(**18**) (Rucker *et al.,* 1999). Some species of genus Senecio have been reported to exhibit anti-microbial activity (Table 2.3). The aerial parts of *S. canescens* are used against infections and rheumatism. Abdo *et al.,* (1992) reported furanoeremophilanes and other constituents from *S. canescens.* These include cacalohastine (**19**), maturinone (**20**), cacalonol (**21**), dammariadienone (**22**) and geranicone (**23**).



17 R = β-CH_3

18 R = α-CH_3



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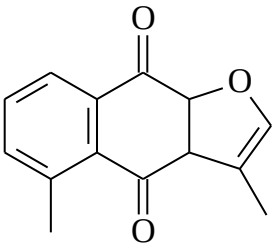

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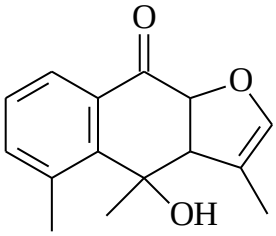

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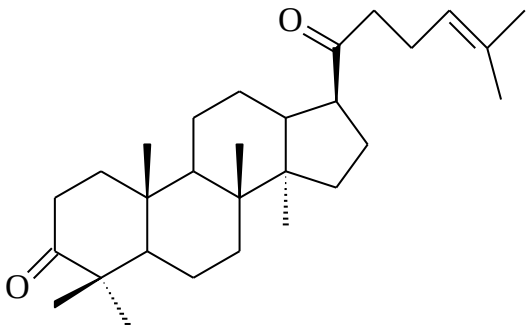

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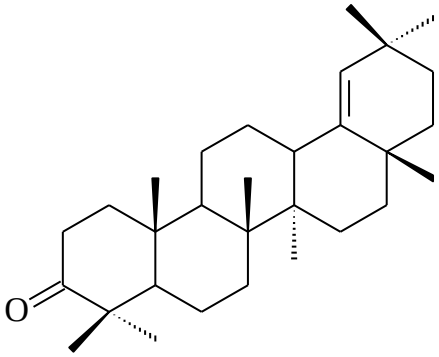

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Table 2.3: Anti-microbial activity of some plants from genus Senecio

Plant	Extract	Activity MIC (mg/ml)	Test organism	Reference
Senecio vulgaris	*n*-hexane	0.031	*Trichophyton tonsurans*	Loizzo *et al.*, 2004
S. graveolens	Essential oil	8.73	*Micrococcus luteus*	Perez *et al.*, 1999
S. graveolens	Essential oil	10.91	*Staphylococcus aureus*	Perez *et al.*, 1999
S. graveolens	Essential oil	0.0213	*Candida albicans*	Perez *et al.*, 1999

2.4 The *Senecio lyratipatitus* species

Senecio lyratipatitus is a wiry trailing climber (Plate 2.1), long hairy on all young parts with triangular or ovate toothed leaves often lobed at base (Waliaula, 1988). In Kenya the plant is distributed in dry upland forest and woodland of altitudes between 1500 m

and 2760 m above sea level, around Mt. Kenya, Cherangani hills, Mau forest, Kericho district and Kisii highlands among others (Agnew and Shirley, 1994). In the traditional medicine, the roots of this plant were washed then ground to fine powder, and applied to wounds. The leaves alone are used as an emetic by the Maasai tribe of Kenya (Kokwaro, 1993).

The extracts from leaves and roots have also been used for the treatment of sexually transmitted infections (Syphilis and Gonorrhoea) by the Kipsigis and the Maasai tribes (Kiprono *et al.*, 2000). Table 2.4 shows the pyrrolizidine alkaloids isolated from other species in the genus Senecio while Table 2.5 lists the traditional use of some other species in the genus Senecio.

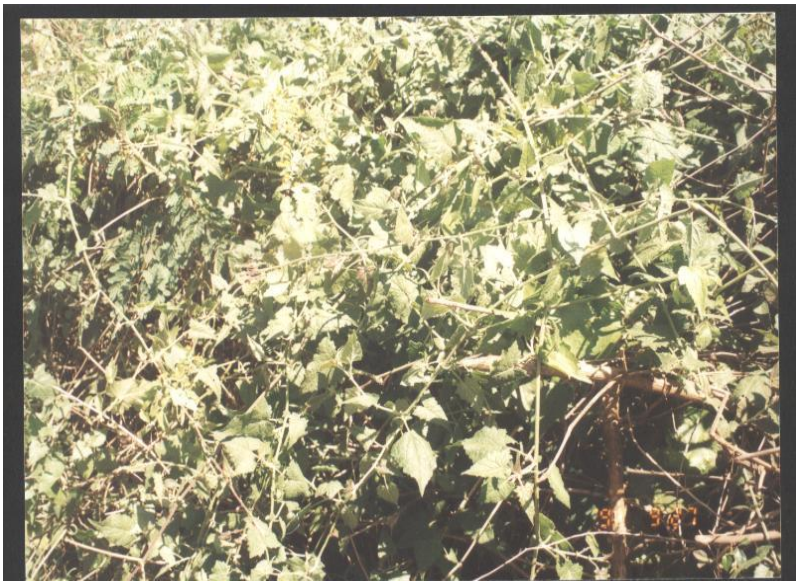


Plate 2.1: The aerial parts of *Senecio lyratipatitus* (Asteraceae)

Table 2.4: Pyrrolizidine alkaloids from the genus Senecio

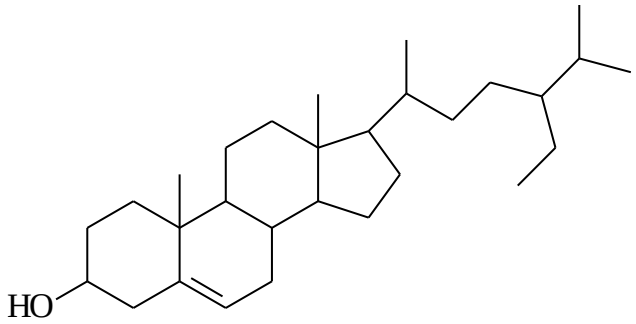
Species	**Pyrrolizidine alkaloids**	**Reference**
Senecio mulgediifolius	Mulgediifoline, retroisosenine, bulgarsenine, isoretroisosenine	Liddell, 1997
S. jacalensis	Senecionine, platyphylline	Liddell, 1997
S. iodanthus	Iodanthine, retroisosenine, bulgarsenine, mulgediifoline	Liddell, 2001 Ana-L *et al.*, 1999
S. argumensis	Seneciphylline, senecionine	Robins, 1994
S. madrensis	Platyphylline, platyphylline N-oxide, senecionine	Liddell, 2000
S. gallicus	Ligularizine, senkirkine, senecionine N-oxide	Julio *et al*., 1988
S. adonidifolius	Retrocenine, florosenine	Julio *et al*., 1988
S. ruwenzoriesis	Ruwenine, ruzorine	Were and Benn, 1992
S. runcinatus	Rosmarinine, rosmarinine N-oxide	Liddell, 2000

Table 2.5: Traditional use of some species in the genus Senecio

Species	**Medicinal purpose**	**Reference**
Senecio argumensis	Dysentry	Roeder, 1995
S. discifolius	Sore eyes	Kokwaro, 1993
S. nemorensis	Hepatitis, boils	Roeder, 1995
S. syringifolius	Coughs and colds	Kokwaro, 1993
S. aureus	Diurectic, diaphroretic	Roeder, 1995
S. dronicum	Asthma	Roeder, 1995
S. jacobea	Functional amenorrhoea	Roeder, 1995
S. vulgaris	Toothache	Lestrange, 1977

2.5 Compounds isolated from *S. lyratipatitus*

An anti-fungal and anti-bacterial compound, β-sitosterol (**24)** was isolated from a methanol extract of dried ground aerial parts of *S. lyratipatitus.* β-Sitosterol has been reported to exhibit anti-bacterial activity against *Salmonella typhii, Corynebacterium diphtheria, Bacillus subtilis, Shigella dysenteriae* and *Vibrio cholerae* and anti-fungal activity against *Fusarium spp* has also been reported (Kiprono *et al.*, 2000).



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2.6 Rationale and Justification

Sesquiterpene lactones and pyrrolizidine alkaloids isolated from the family Asteraceae are responsible for its biological activities (Harborne and Dey 1993). *S. lyratipatitus* has been used traditionally to treat various diseases. To investigate the anti-microbial activity of the extracts and compounds of this plant, will provide a scientific proof of its medicinal use. In addition, these studies will evaluate the extracts/compounds responsible for the biological activity. Synthetic anti-biotics and especially anti-fungal principles are only useful for local treatment because of their toxic nature. Moreover, they are costly and exhibit side effects including increased allergic reactions. Some fungi and bacteria have also become resistant to synthetic anti-biotics and anti-fungal compounds. Therefore, there is also a need to search for cheap, more potent anti-biotics and anti-fungals with reduced side effects.

2.7 Statement of the problem

The roots of *S. lyratipatitus* have been used traditionally to treat wounds and sexually transmitted diseases. There is little or no information in literature on the anti-microbial activity of the root extracts and compounds of *S. lyratipatitus.*

2.8 Hypothesis

The medicinal plant, *S. lyratipatitus* that has been used traditionally to treat various infections may contain bio-active compounds that may inhibit the growth of some strains of gram positive bacteria, gram negative bacteria and fungus.

2.9 Objectives

2.9.1 Main Objective

To isolate and characterize biologically active principles from the root bark of *Senecio lyratipatitus.*

2.9.2 Specific objectives

i) To extract the root bark of *Senecio lyratipatitus* using *n*-hexane, dichloromethane and methanol.

ii) To carry out bioassay of crude extracts and fractions thereof against brine shrimp (*Artemia salina),* gram positive and gram negative bacteria and the fungus, *Candida albicans*.

iii) To carry out bioassay guided separation of the active constituents through Column Chromatography and Preparative Thin Layer Chromatography (Prep-TLC).

iv) To elucidate structures of the pure compounds using spectroscopic techniques (FT-IR and NMR) and physical data e.g. melting point.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant material

The roots of *Senecio lyratipatitus* were collected from Sotik in Bomet district, Rift Valley Province of Kenya. The samples were identified by a taxonomist from Botany department and voucher specimen JKUATHK/15/2/03/1 was deposited in the herbarium of Botany department, Jomo Kenyatta University of Agriculture and Technology (JKUAT).

3.2 Extraction

The roots of *S. lyratipatitus* were dried at room temperature and ground into fine powder. A sample (1.3 kg) of powdered roots was sequentially extracted with *n*-hexane (1.5 L), dichloromethane (1.5 L) and methanol (1.5 L) for three days respectively (Scheme. 3.1). The respective extracts were filtered and the filtrate dried *in vacuo* using a rotary evaporator (Heidolph Laborota 4000) available in the Chemistry Laboratory, JKUAT.

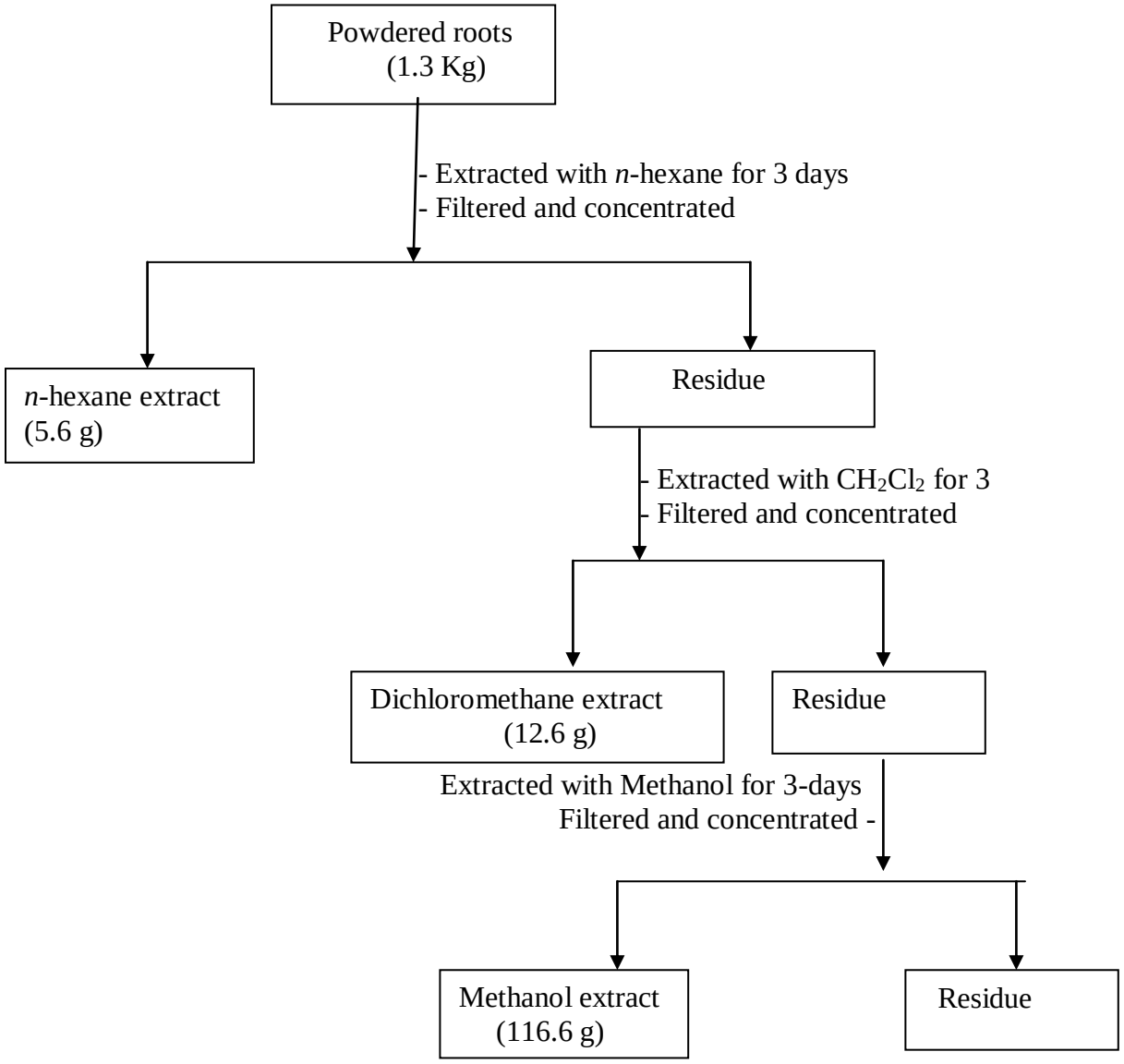
3.3 Separation Techniques

3.3.1 Normal and Reverse phase Column Chromatography

Normal column chromatography was done with silica gel 60 (0.063 - 0.2 mm, 70 - 230 mesh, ASTM, Kobian Kenya Ltd) and eluting the sample with a non-polar solvent (*n*-hexane/dichloromethane/diethyl ether). Reverse phase column chromatography was done by packing a column with silica gel 100 C_{18} 0.015 - 0.035 mm, $\geq$ 400 mesh (Fluka Chemie GmbH- Switzerland) and eluting the sample with a polar solvent (methanol). Thin Layer Chromatography (TLC) was used to monitor separation of fractions obtained from various columns. It involved the application of samples by spotting on pre-coated plates (silica gel 0.20 mm, Macherey-Nagel, Germany). The plates were developed in tanks containing a solvent system that gave a good separation during the fractionation process. UV active compounds were detected using a UV (254 and 366 nm) lamp. Detection of other compounds was carried out by spraying with 2% H_2SO_4 in methanol.

3.3.2 Preparative Thin Layer Chromatography (Prep-TLC)

This was done using silica gel (0.25 mm, 200 - 400 mesh, Merck, Germany). The sample was applied continuously using a capillary tube. After developing, bands of separated constituents observed were recovered by scrapping off the adsorbent at the appropriate places. The adsorbent was eluted with dichloromethane, filtered and evaporated to obtain compound SLRM1.



Scheme 3.1: Sequential crude extraction of the root bark of S. *lyratipatitus* using *n*-hexane, dichloromethane and methanol

3.4 Bioassay Methods

3.4.1 Brine Shrimp (*Artemia salina*) Lethality test

Toxicity susceptibility of brine shrimp (*Artemia salina)* larvae to bioactive substances was used with some modifications (Keriko, 1996). Artificial sea water was prepared by dissolving 33 g of sea salt in 1.0 L of water. Brine shrimp eggs were added to about 80 ml of the artificial sea water in a plastic hatchery. This was incubated for 48 hrs at room temperature. Crude extracts were tested in triplicates at various concentrations (0 - 1000 ppm) in calibrated vials containing 2.0 ml of artificial sea water and 10 brine shrimps. The control vials contained 10 brine shrimps and sea water alone. The assay was done in triplicate. The surviving brine shrimps were counted after every 24 hrs and hence number of the dead brine shrimps was obtained. The LD_{50} was determined using SAS 8.0 at 95% confidence interval (Alkofahi *et al.,* 1989; Jerry *et al.,* 1998).

3.4.2 Anti-Bacterial assay Test

Pure cultures of the bacteria (*Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*) were obtained from Kenya Medical Research Institute (KEMRI). *Bacillus subtilis* was locally isolated from a soil sample. The bacteria (20 μl) were introduced in a Petri-dish containing nutrient agar (CM3, Oxoid Ltd, England). 'Wells' were made in the medium of the growing organisms. Different concentrations (0 - 1000 ppm) of the various fractions and the crude extracts were introduced into the 'wells' using a micro-syringe. The Petri-dishes were incubated at 37^{o}C for 24 hrs after which the inhibition diameters were measured (Guniz *et al.,* 2003). Since the bacteria used were human

pathogens, the tests were carried out at 37^{o}C so as to mimic the human body temperature. Standard anti-biotics in the form of anti-biotic discs (ampicillin, tetracycline, nitrofurantoin, nalidixic acid, streptomycin, sulphamethaxazole, cotrimoxazole and gentamicin) were used for comparison with the compounds isolated. The standard anti-biotics were obtained from Himedia Laboratories Ltd, India. Dimethylsulphoxide (about 2 drops) dissolved in water were used as negative controls.

3.4.3 Anti-Fungal assay Test

The fungus (*Candida albicans*) was obtained from KEMRI. The fungus (20 μl) was introduced in a Petri-dish containing potato dextrose agar (CM139, Oxoid Ltd, England). 'Wells' were made in the medium of growing organisms. Different concentrations (0 - 1000 ppm) of the various fractions and the crude extracts were introduced into the 'Wells' using a micro-syringe. The Petri-dishes were incubated at 25^{o}C for 4 days after which the inhibition diameters were measured (Collee *et al.,* 1996). The tests were carried out at lower temperature compared to that of the anti-bacterial tests since fungi takes a longer time to grow and incubation at higher temperature may destroy the spores. Dimethylsulphoxide (about 2 drops) dissolved in water was used as negative controls.

3.5 Spectroscopic methods

3.5.1 Fourier Transform Infrared Spectroscopy (FT-IR)

Infra-red data of the compounds was obtained on Fourier Transform Infrared Spectrophotometer (FT-IR 8400 - Shimadzu) in the Chemistry Laboratory, JKUAT. The spectra were determined by producing a disc which was made by grinding 1 mg of the sample with 100 mg of potassium bromide (KBr) using a mortar and pestle. The powder was then put in a circular die under a mechanical pressure to produce the transparent discs which were then placed at the prescribed position of the IR machine.

3.5.2 Nuclear Magnetic Resonance (NMR) Data

Proton, ^{13}C and COSY NMR spectra were recorded on 300 MHz Bruker Advance Equipment available in the Department of Chemistry at the University of Botswana in August 2004. Trimethyl Silane (TMS) was used as the internal standard.

3.6 Physical Characteristics of the Compounds

Melting points (Mpt) of the compounds were obtained using a melting point apparatus (Gallenkamp, UK) available in the laboratory of Chemistry department at JKUAT and were not corrected.

3.7 Data Analysis

The toxicity effects of different extracts and fractions with varying concentrations were tested on brine shrimp larvae. Repeated measures analysis of variances (ANOVA) was applied so as to test the effects of different dosages (DOSE), different extracts (CHEM) and their interaction (DOSE*CHEM) on the response variable (per cent mortality) on brine shrimp. Abbot's formula was used to adjust mortality in treatment with mortality in control, that is:

$$\% \text{ mortality (adjusted)} = \frac{\% \text{ Alive in control} - \% \text{ Alive in treatment}}{\% \text{ Alive in control}}$$

The adjusted mortality was then transformed to scale to fit a general linear model (GLM). Turkey test was used for comparison of means. Probit analysis was used to compute LD_{50} values. Statistical analysis system (SAS) version 8.0 was used.

CHAPTER FOUR

4.0 RESULTS

4.1 General Highlights

In this chapter, the brine shrimp lethality test, anti-fungal and anti-bacterial tests of the crude extracts, chromatographic fractions and some of the isolated compounds are discussed. The crude extracts, fractions and compounds exhibited varying anti-microbial activities which were then compared to those exhibited by the standard anti-biotics.

4.2 Crude Extraction

The powdered root bark of *S. lyratipatitus* was extracted with *n*-hexane, dichloromethane and methanol successively. The colours and percentage yields of the crude extracts are shown in Table 4.1. Methanol yielded the highest amount (9.23%) of the crude extract followed by the dichloromethane (0.97%) and *n*-hexane yielded the least amount (0.43%).

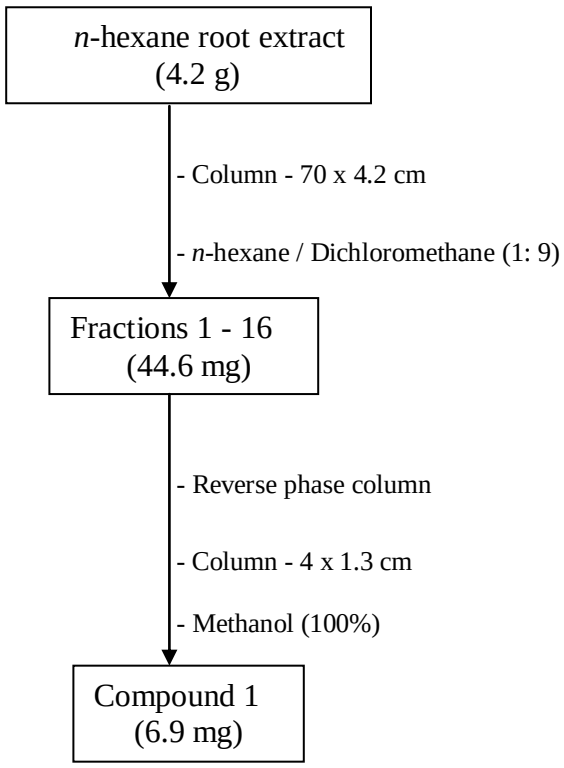
Table 4.1: Colour and percentage yield of the crude extracts

Extract	Colour of the extract	Amount (g)	Percentage yield (%)
n-hexane	Yellow	5.6	0.43
Dichloromethane	Yellowish-brown	12.6	0.97
Methanol	Brown	116.6	9.23

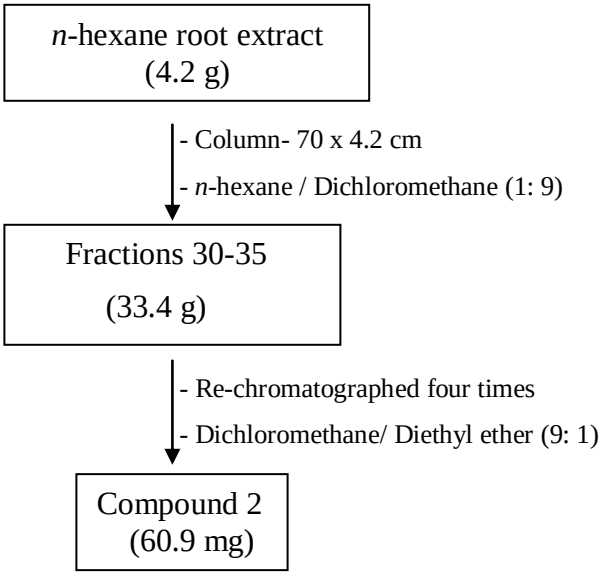
4.3 Fractionation and isolation of compounds

4.3.1 Isolation and purification of compounds 1 and 2 from the *n*-hexane root extract

Isolation of compounds **1** and **2** from the *n*-hexane extract is shown in Schemes 4.1 and 4.2. A sample (4.2 g) of *n*-hexane root extract was chromatographed on Silica gel (0.063 - 0.2 mm, 70 - 230 mesh) using *n*-hexane/dichloromethane (1:9). As shown in Scheme. 4.2, Compound **1** was obtained through reverse phase chromatography of fractions 1-16 by using methanol as the eluting solvent. Compound **2** was obtained through repeated normal phase chromatography of fractions 30 - 35 by using dichloromethane /ethyl ether (9:1) as the eluting solvent.



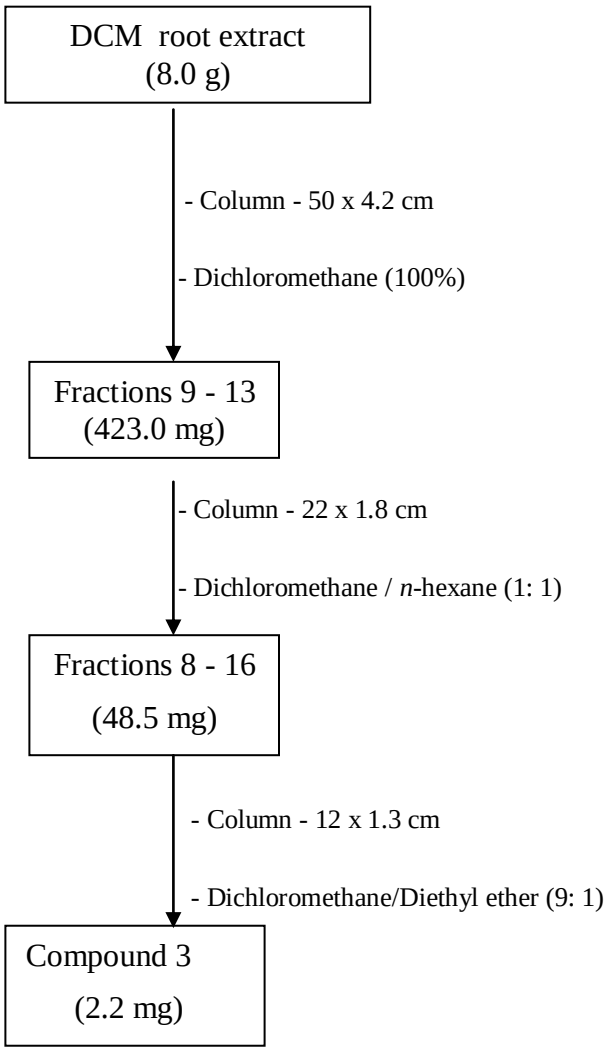
Scheme 4.1 Isolation of compound 1 (SLRH1) from the *n*-hexane extract



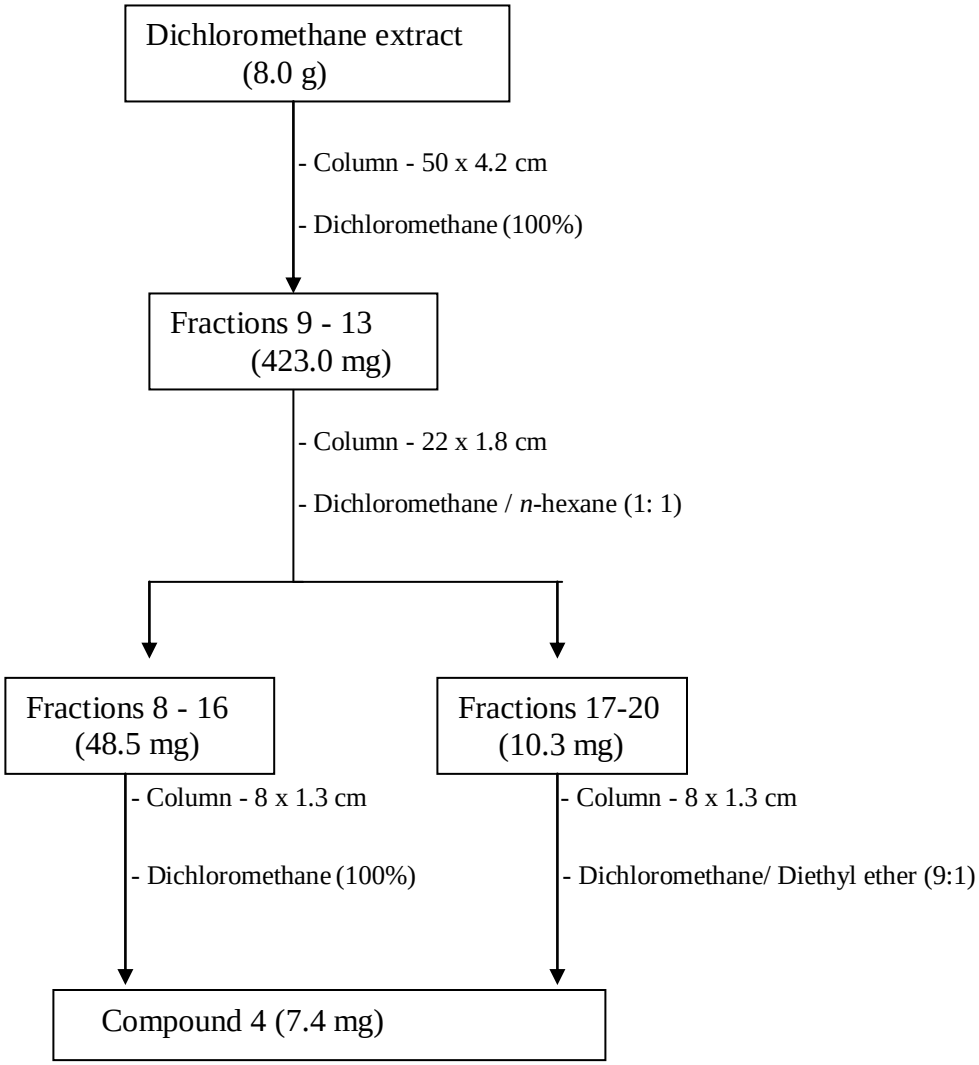
Scheme 4.2: Isolation of compound **2** (SLRH2) from the *n*-hexane root extract

4.3.2 Isolation and purification of compound 3, 4 and 5 from the dichloromethane crude extract

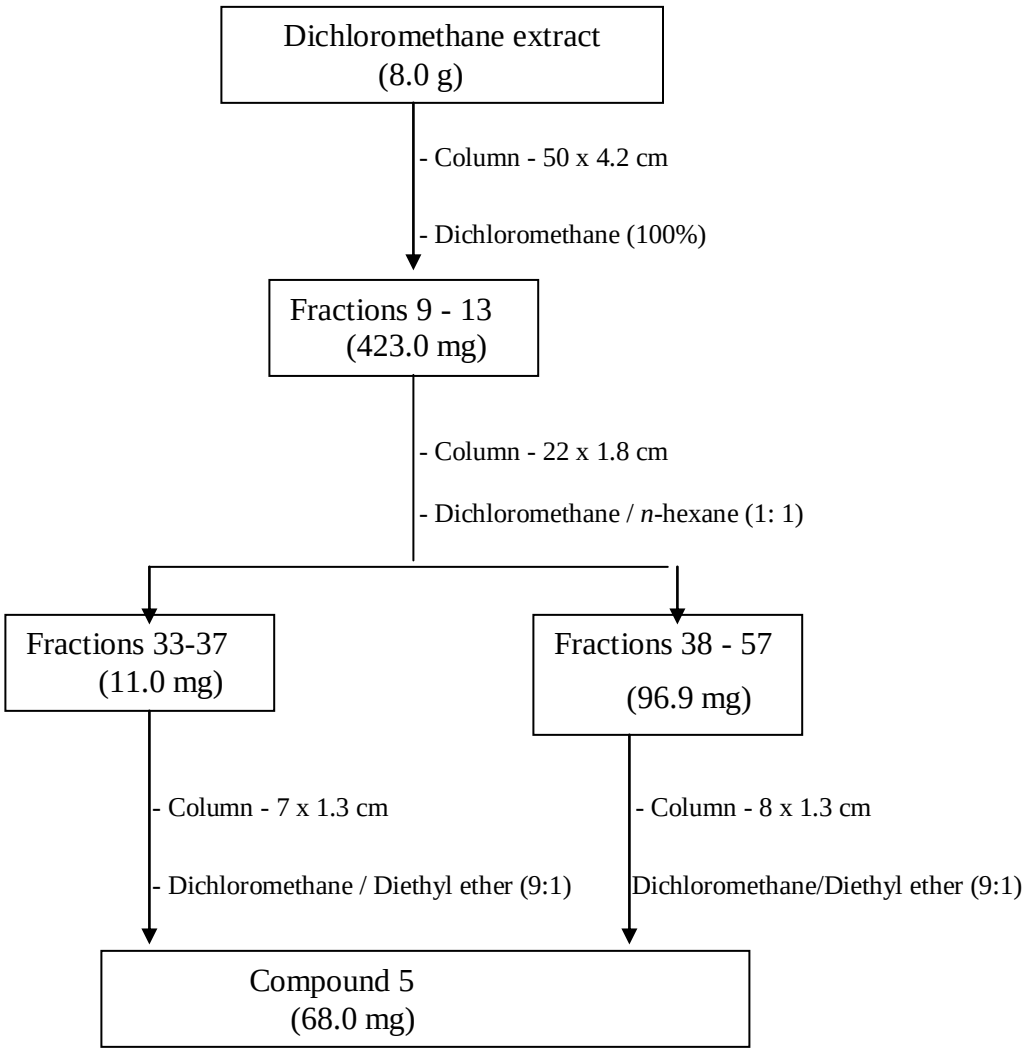
A sample (8 g) of the dichloromethane crude extract was chromatographed on Silica gel (0.063 - 0.2 mm, 70 - 230 mesh). Flush chromatography was carried out starting with n-hexane followed by dichloromethane and then methanol as the eluting solvent. Further chromatographic separation of the fractions obtained by using dichloromethane as the eluting solvent was carried out. Compounds **3**, **4** and **5** were obtained through normal phase chromatography by using dichloromethane, *n*-hexane and diethyl ether at various ratios (Schemes 4.3, 4.4, 4.5). Compound **5** yielded the highest amount (68.0 mg) followed the compound **4** with a yield of 7.4 mg. Compound **3** yielded the least amount of 2.2 mg.



Scheme 4.3: Isolation of compound **3** (SLRD3) from the dichloromethane crude extract



Scheme 4.4: Isolation of compound **4** (SLRD2) from the dichloromethane root extract

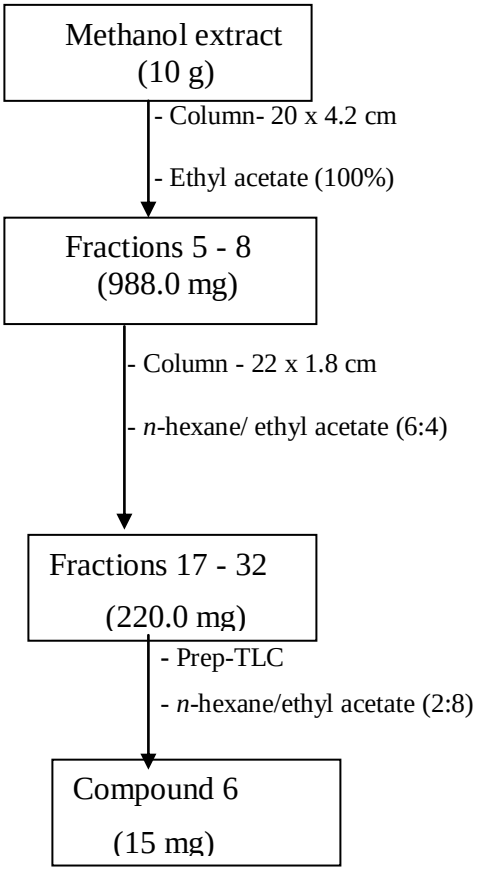


Scheme 4.5: Isolation of compound **5** (SLRD1) from the dichloromethane extract

4.3.3 Isolation and purification of compound 6 from the methanol crude extract

Isolation and purification of compound **6** from the methanol extract is shown in Scheme 4.6. A quantity of 10.0 g of the methanol crude extract was chromatographed on Silica gel (0.063 - 0.2 mm, 70 - 230 mesh) using ethyl acetate. This was further chromatographed through normal phase chromatography by using *n*-hexane/ethyl

acetate (6:4). Preparative Thin layer Chromatography (0.25 mm, Merck) was carried out on fractions 17 - 32 using *n*-hexane/ethyl acetate (2:8) to yield compound **6** (15 mg).



Scheme 4.6: Isolation of compound **6** (SLRM1) from the methanol crude extract

4.4 Bioassay tests

4.4.1 Brine shrimp (*Artemia salina*) lethality test for the crude extracts and fractions

A two way analysis of variance was carried out to evaluate the combined effects of the extracts and fractions thereof, and the dose on the *nauplii* mortality. In addition to the

single factors, that is (extract, F = 117.98; DF = 2; P < 0.0001 and (dose, F = 84.74; DF = 6; P < 0.0001) effect of interaction between the extracts and the doses (F = 5.43; DF = 12; P < 0.0001) and (fraction, F = 438.41; DF = 5; P < 0.0001 and (dose, F = 283.5; DF = 4; P < 0.0001) effect of interaction between the fractions and the doses (F = 18.02; DF = 20; P < 0.0001) was significant. Consequently, the mortality induced by each dose was evaluated for each extract and fraction.

The brine shrimp test was used to evaluate the toxicity of the crude extracts and chromatographic fractions as it is a simple, fast and low cost test. The effect of crude extracts against brine shrimp is showed in Table 4.2. The dichloromethane extract showed a significant level of toxicity with LD_{50} = 450.33 ppm (P = 0.05). The mortality rate was dose dependent. However, there was no significant difference at 800 ppm and 900 ppm (P < 0.0001) for dichloromethane extract and at 700 ppm and 800 ppm for the methanol extract. Also there was no significant difference (P < 0.0001) observed at 600 ppm, 700 ppm and 800 ppm for the *n*-hexane extract.

Since the toxicity is associated with pharmacological properties, it was deduced that the dichloromethane extract had the highest bioactivity. The effect of dichloromethane fractions on the brine shrimp larvae is shown in Table 4.3. Brine shrimp tests on the dichloromethane fractions, showed that the highest toxicity was exhibited by the dichloromethane/ethyl acetate (1:1) fraction (No. 3) with LD_{50} = 175.89 ppm and the ethyl acetate/methanol (1:1) fraction was least toxic with LD_{50} = 1158 ppm. A dose response was observed in the dichloromethane fractions, however no significant difference (P < 0.0001) was observed at 200 ppm and 300 ppm for fraction 3.

Table 4.2: Mean percent mortality ± standard error for the crude extracts at different concentrations

Dose (ppm)	*n*-hexane	Dichloromethane	Methanol
900	70.0 ± 5.8^{Ba}	96.7 ± 3.3^{Aa}	36.7 ± 6.7^{Ca}
800	46.7 ± 3.3^{Bb}	86.7 ± 6.7^{Aa}	30.0 ± 5.8^{Bab}
700	30 ± 5.8^{Bbc}	70.0 ± 5.8^{Aab}	23.3 ± 3.3^{Bab}
600	26.7 ± 3.3^{Bc}	53.3 ± 3.3^{Abc}	10.0 ± 5.8^{Bbc}
400	13.3 ± 3.3^{ABdc}	33.3 ± 8.8^{Acd}	0 ± 0^{Bc}
200	3.3 ± 3.3^{Abd}	20.0 ± 5.8^{Ade}	0 ± 0^{Bc}
100	0 ± 0^{Ad}	3.3 ± 3.3^{Ae}	0 ± 0^{Ac}
0	0 ± 0^{Ad}	0 ± 0^{Ae}	0 ± 0^{Ac}
LD_{50}	804.84	450.33	972.48

A-D, a-d Mean values with the same capital letters within the same row and same small letters within the same column are not significantly different at 95% confidence level.

Table 4.3: Mean percent mortality ± standard error for the dichloromethane fractions at different concentrations

	Dose (ppm)						
Fractions	**0**	**100**	**200**	**300**	**400**	**800**	**LD_{50}**
1	0 ± 0^{Ac}	0 ± 0^{Bc}	0 ± 0^{Cc}	3.3 ± 3.3^{Cc}	16.7 ± 3.3^{Cb}	33.3 ± 3.3^{Ba}	1001.0
2	0 ± 0^{Ad}	6.7 ± 3.3^{Bd}	16.7 ± 3.3^{Bd}	33.3 ± 3.3^{Bc}	63.3 ± 3.3^{Bb}	96.7 ± 3.3^{Aa}	337.47
3	0 ± 0^{Ad}	33.3 ± 3.3^{Ac}	53.3 ± 3.3^{Ab}	60 ± 0^{Ab}	83.3 ± 3.3^{Aa}	96.7 ± 3.3^{Aa}	175.89
4	0 ± 0^{Ac}	0 ± 0^{Bc}	0 ± 0^{Cc}	3.3 ± 3.3^{Cbc}	13.3 ± 3.3^{Cb}	33.3 ± 3.3^{Ba}	1004.0
5	0 ± 0^{Ab}	0 ± 0^{Bb}	0 ± 0^{Cb}	0 ± 0^{Cb}	3.3 ± 3.3^{Cb}	20.0 ± 5.8^{Ba}	1158.0
6	0 ± 0^{Ac}	0 ± 0^{Bc}	0 ± 0^{Cc}	0 ± 0^{Cc}	10.0 ± 0^{Cb}	30.0 ± 0^{Ba}	1027.0

$^{A\text{-}D,\ a\text{-}d}$ Mean values with the same capital letters within the same column and same small letters within the same row are not significantly different at 95% confidence level.

4.4.2 Anti-bacterial tests on the crude extracts

The crude extracts exhibited significant ($P < 0.0001$) anti-bacterial activities. The effect of *n*-hexane and dichloromethane extracts against bacterial growth is shown in Figs 4.1 and 4.2 respectively. The extracts exhibited relatively high inhibition against *S. aureus*. A dose response was observed, although in a few exceptional cases the lower concentration showed a higher inhibition than that observed for the higher concentration. For example, activity of dichloromethane extract at 125 ppm against *B. subtilis* was higher than at 250 ppm (Fig. 4.2).

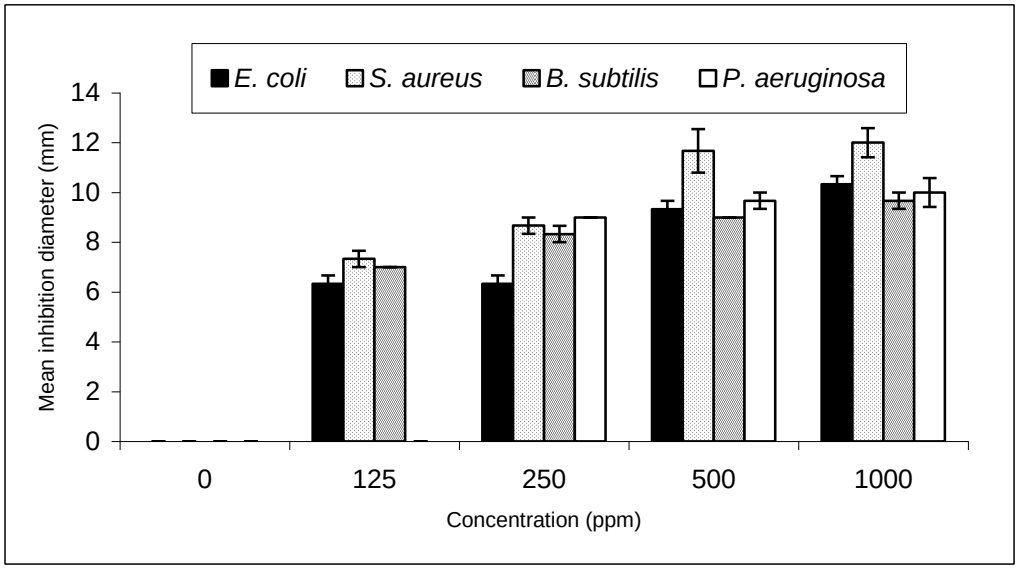


Figure 4.1: Effects of *n*-hexane crude extract on bacterial growth

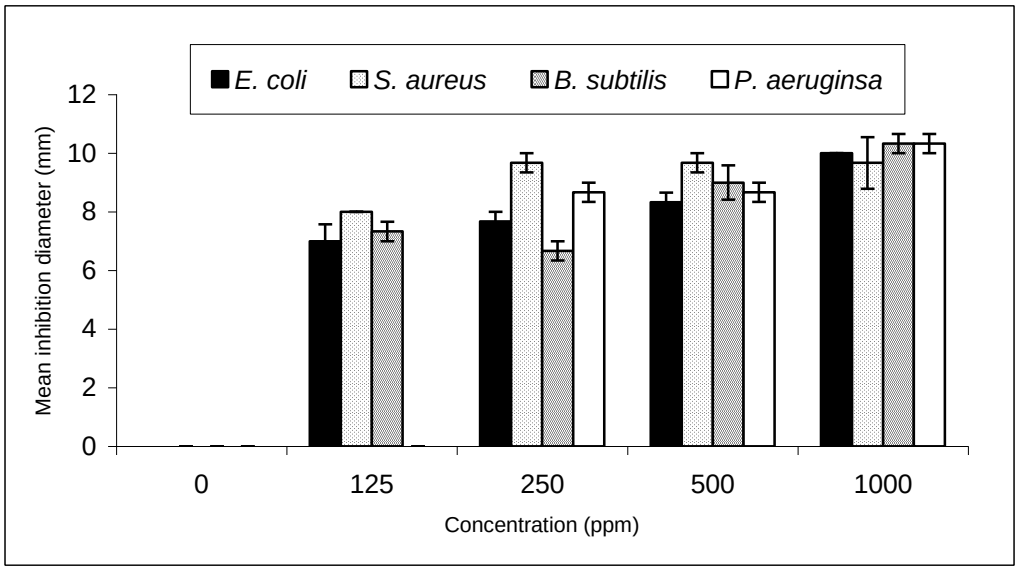


Figure 4.2: Effects of dichloromethane extract on bacterial growth

The effect of methanol extract on bacterial growth is shown in Fig. 4.3. Generally, inhibition zone increased with increase in concentration. Methanol extract exhibited relatively higher activity against *E. coli* and *P. aeruginosa* than against *S. aureas* and *B. subtilis*.

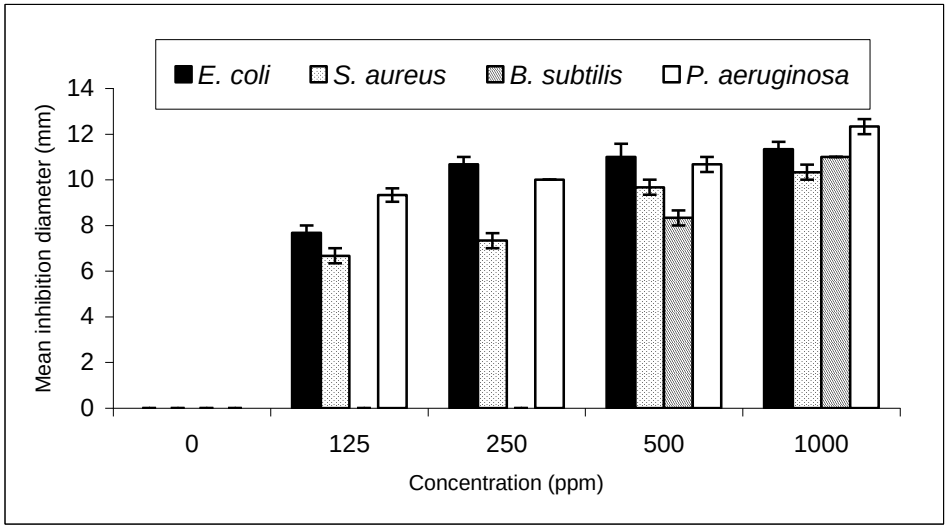


Figure 4.3: Effects of methanol extract against bacterial growth

4.4.3 Effects of dichloromethane fractions on bacterial growth

The effect of the six dichloromethane fractions on bacterial growth is shown in Figs. 4.4 - 4.9. Generally, increasing concentration of the extract resulted to an increase in the inhibition diameter. However, an increase in concentration of fractions 1, 2, 5, and 6 resulted to a decrease in the inhibition diameter against. *E. coli*. In fraction 1, 3, 5 and 6 there was enhanced activity at 1000 ppm against *P. aeruginosa*. A similar observation was made for fraction 2, with the highest inhibition (9.7 ± 0.3) exhibited at 1000 ppm against *B. subtilis* and *P aeruginosa*.

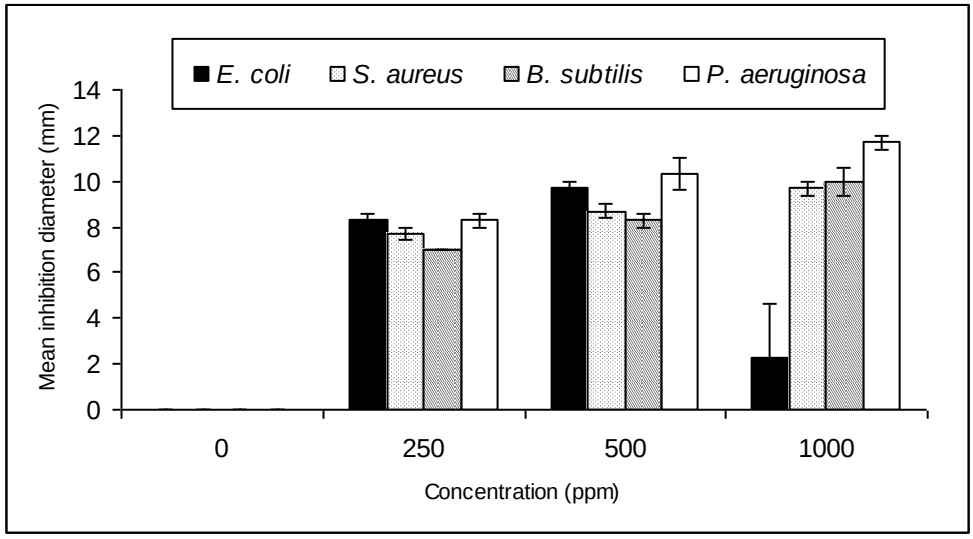


Figure 4.4: Effects of various concentrations of dichloromethane fraction 1 on bacterial growth

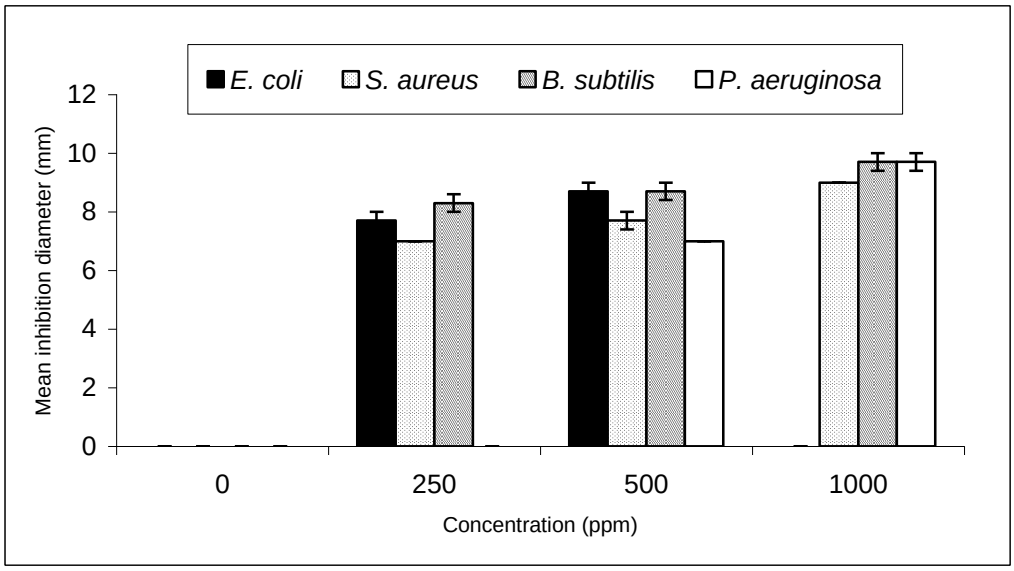


Figure 4.5: Effects of various concentrations of dichloromethane fraction 2 on bacterial growth

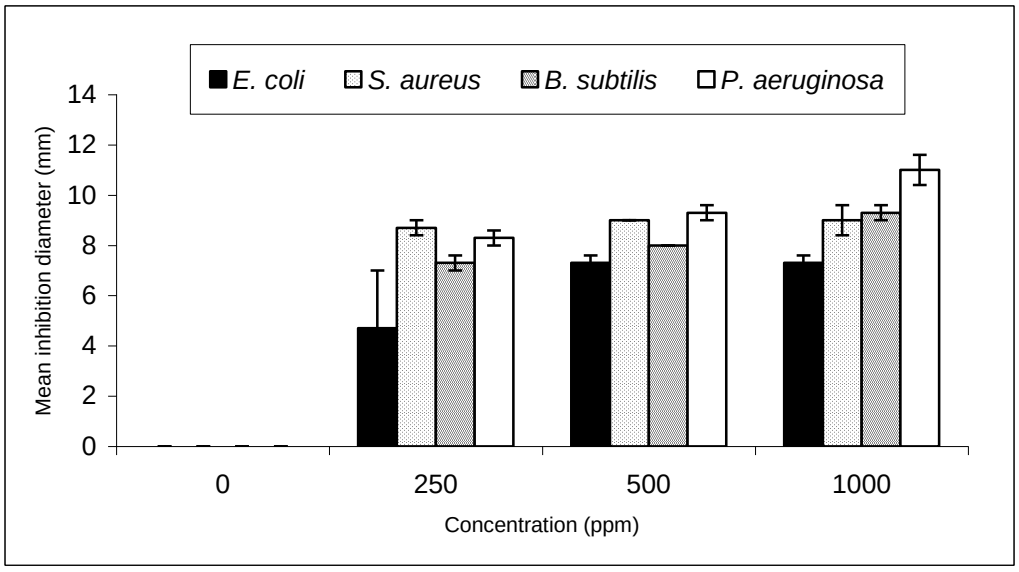


Figure 4.6: Effects of various concentrations of dichloromethane fraction 3 on bacterial growth

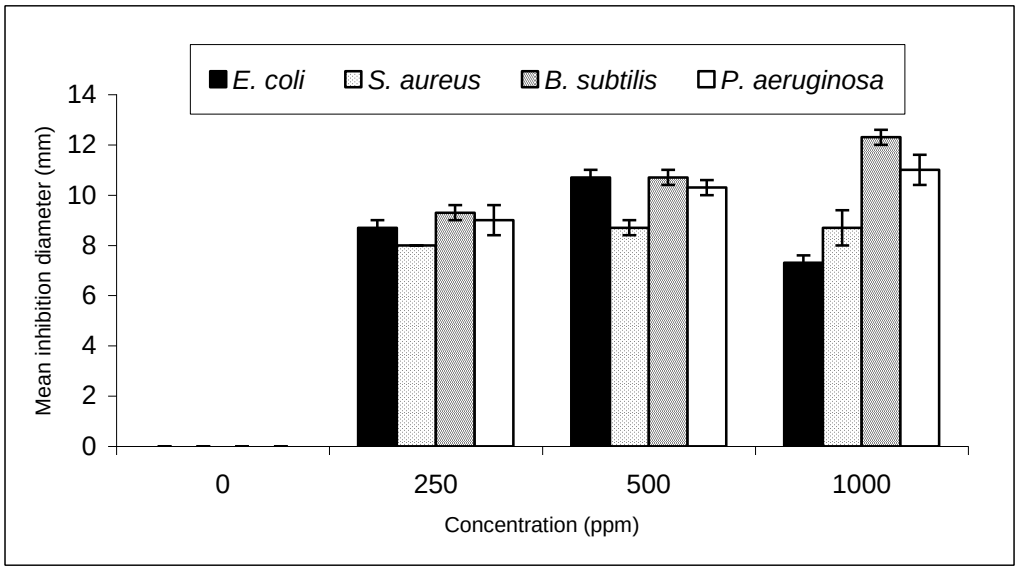


Figure 4.7: Effects of various fractions of dichloromethane fraction 4 on bacterial growth

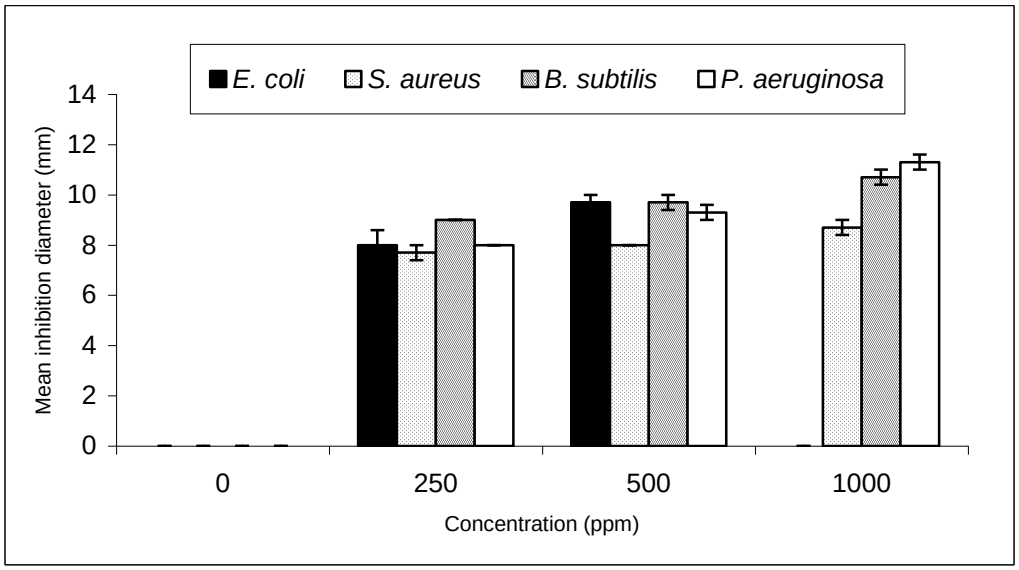


Figure 4.8: Effects of various concentrations of dichloromethane fraction 5 on bacterial growth

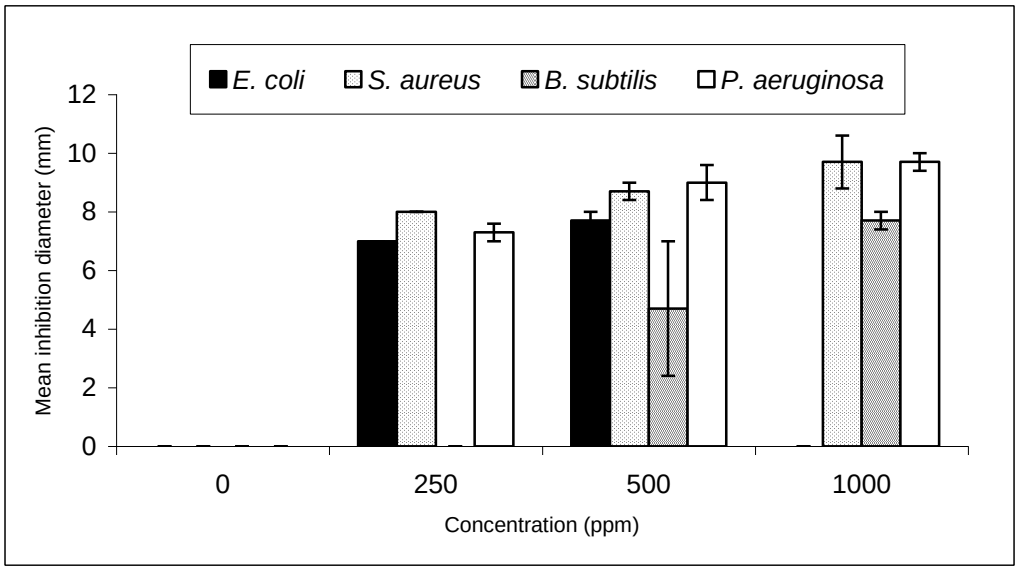


Figure 4.9: Effects of various concentrations of dichloromethane fraction 6 on bacterial growth

4.4.4 Anti-fungal tests for the crude extracts

The crude extracts exhibited significant ($P < 0.0001$) anti-fungal activities. The methanol extract exhibited relatively high inhibition against *Candida albicans* compared to *n*-hexane and dichloromethane extracts (Fig. 4.10). Anti-fungal activity against *C. albicans* of ethyl acetate fractions of *Senecio inaequidens* and *S. vulgaris* has been reported although the methanol and *n*-hexane fractions exhibited no activity (Loizzo, *et al*., 2004). At 125 ppm there was no significant difference ($P < 0.0001$) in activity for the three extracts. No significant difference ($P < 0.0001$) was observed for dichloromethane extract at 125, 250 and 500 ppm.

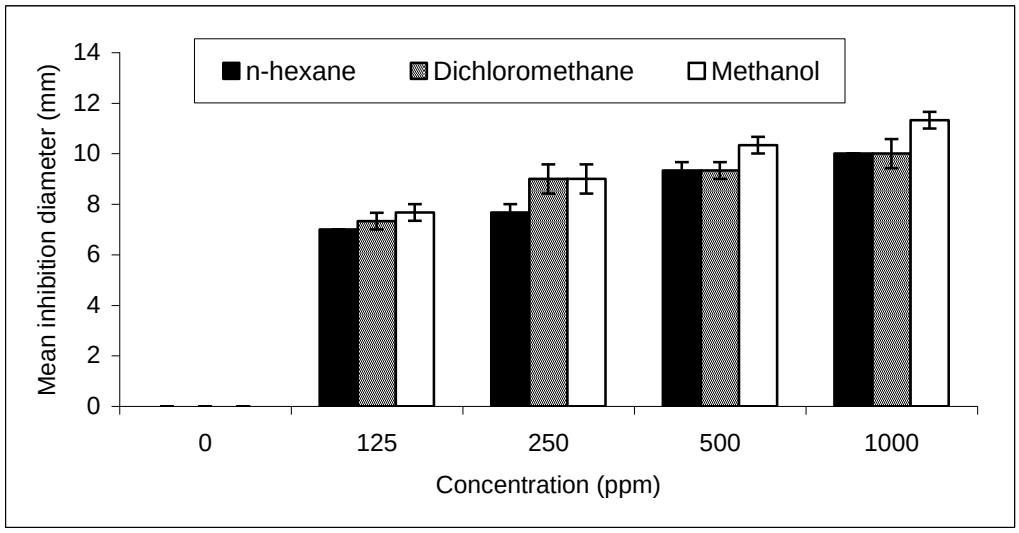


Figure 4.10: Effects of *n*-hexane, dichloromethane and methanol crude extracts on the growth of the *Candida albicans.*

4.4.5 Anti-fungal test on the dichloromethane fractions

The dichloromethane fractions also exhibited activity against *C. albicans.* At 1000 ppm there was no significant difference ($P < 0.0001$) in mean inhibition for the six fractions. There was no significant difference ($P < 0.0001$) across the dose range for Fraction 1, 3 and 5 (Fig. 4.11).

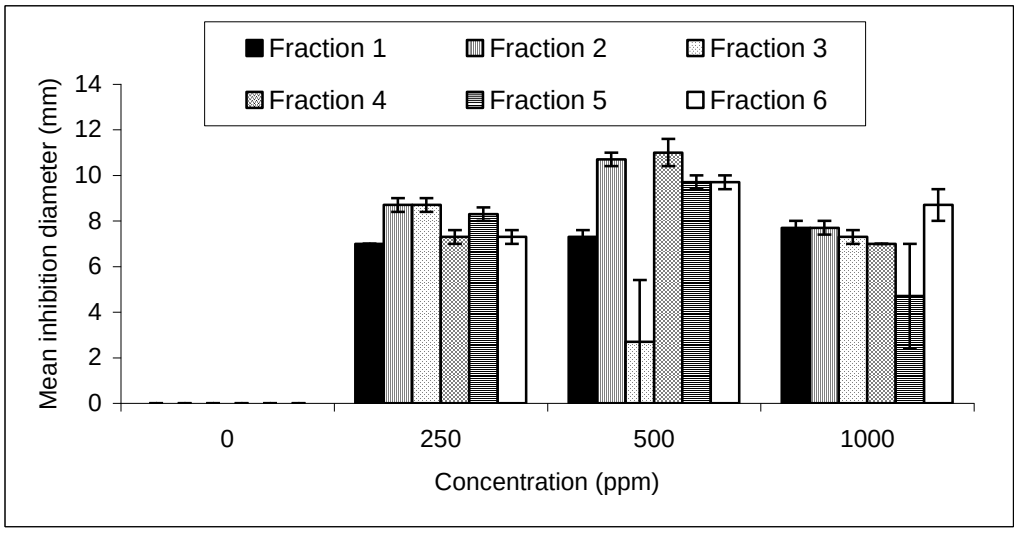


Figure 4.11: Effects of various fractions of dichloromethane on the growth of the *C. albicans*

4.4.6 Anti-bacterial and anti-fungal tests on standard anti-biotics and isolated compounds SLRD1 and SLRH1

The inhibition diameter of standard anti-biotics, crude extracts and compounds against *E. coli, P. aeruginosa, B. subtilis, S. aureus and C. albicans* is shown in Table 4.4. The crude extracts compounds exhibited a higher inhibition zone against the bacteria tested in comparison to anti-biotics; ampicillin, tetracycline, nitrofurantoin and sulphamethaxazole. In addition the anti-biotics; ampicillin, nitrofurantoin, sulphamethaxazole and cotrimoxazole did not exhibit and activity against *P. aeruginosa*.

Table 4.4: Comparison of anti-microbial activity of anti-biotics, extracts and isolated compounds

Mean inhibition diameter (mm)

Anti-biotic/extract/ compounds	***B. subtilis***	***S. aureus***	***E. coli***	***P. aeruginosa***	***C. albicans***
Ampicillin (25 μg/disc)	0.0	12.0	0.0	0.0	-
Tetracycline (100 μg/disc)	25.0	24.0	30.0	14.0	-
Nitrofurantoin (200 μg/disc)	14.0	22.0	20.0	0.0	-
Nalidixic acid (30 μg/disc)	24.0	30.0	26.0	24.0	-
Streptomycin (25 μg/disc)	24.0	30.0	26.0	24.0	-
Sulphamethaxazole (200 μg/disc)	20.0	20.0	16.*0*	0.0	-
Cotrimoxazole (25 μg/disc)	20.0	18.0	26.0	0.0	-
Gentamicin (10 μg/disc)	26.0	240-.0	28.0	20.0	-
n-hexane extract (20 μg/disc)	10.3 ± 0.3	12 ± 0.6	9.7 ± 0.3	10 ± 0.6	10 ± 0
DCM extract (20 μg/disc)	10.0 ± 0	9.7 ± 0.9	10.3 ± 0.3	10.3 ± 0.3	10 ± 0.6
Methanol extract (20 μg/disc)	11.3 ± 0.3	10.3 ± 0.3	11 ± 0	12.3 ± 0.3	11.3 ± 0.3
SLRD1 (20 μg/disc)	8.7 ± 0.3	9.7 ± 0.3	10.7 ± 0.3	8.3 ± 0.3	8.0 ± 0.3
SLRH1 (20 μg/disc)	8.7 ± 0.3	9.3 ± 0.7	10.3 ± 0.3	7.7 ± 0.7	8.6 ± 0

4.5 Summary of compounds isolated from *S lyratipatitus*

The physical properties of the isolated and purified compounds are shown in Table 4.5.

Table 4.5: The physical properties of the isolated and purified compounds

Compound	**Percentage yield (%)**	**Physical appearance**	**Melting point (^{o}C)**
1	1.45	White needle like crystals	136.5 – 137.7
2	0.16	White powder	176.0 – 177.7
3	0.028	White powder	nd
4	0.1	White crystals	nd
5	0.85	White needle-like crystals	136.4-138.0
6	0.15	Yellowish oil	nd

nd - not determined

4.6 Functional groups assignment from the IR data

The IR spectra of the compounds, SLRD1 (compound **5**) and SLRH2 (compound **2**) are shown in Fig. 8.1 and 8.2 respectively and their proposed functional group assignment is given in Table 4.6 and 4.8. The functional group assignment for data of compound SLRD1 is shown in Table 4.7.

Table 4.6: Functional groups assignment for compound SLRD1 from the FT-IR spectra (Fig. 8.1)

Group frequency (cm^{-1})	**Functional group assignment**
3445.6	O-H stretch
2932.6	C-H stretch
1634.6	C=C stretch
1461.9	C-H stretch
1056.0, 1381.9	C-O bend

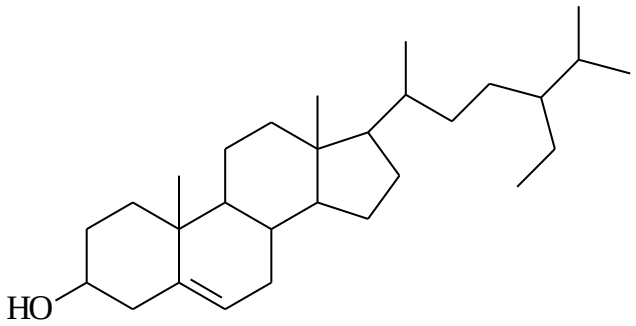
Table 4.7: Functional groups assignment of NMR data for compound SLRD1 (Compound **5**) (Fig. 8.3 – 8.10)

Chemical shift (δ)	**Assignments**
δ 0.722 - 2.351 ppm (*m*) (34 H)	- methyl and methylene protons
δ 3.543 ppm (*septet*) (1 H)	- possibly an isopropyl methine proton (*J* = 0.018Hz) strong ^{1}H-^{1}H COSY correlation with methyl protons at δ1.5 ppm and at δ 2.249 - 2.338 ppm
δ 5.111 ppm (*m*) (1 H)	- No clear ^{1}H-^{1}H COSY correlation
δ 5.378 ppm (*d, J* = 0.07 Hz) (1 H)	Unsaturated proton, ^{1}H-^{1}H COSY correlation with methyl protons at δ 1.992 -2.054 ppm

Compound 5 was isolated from the dichloromethane extract as white needle like crystals with a melting point of 136.4 - 138.0°C. A band at ν_{max} = 1634.6 cm^{-1} in the IR spectrum of this compound is an indication of unsaturation. In the ^{1}H-NMR spectrum one proton at δ 5.378 is an indication of the presence of an olefinic proton and a correlation with the ^{1}H-^{1}H COSY correlation with methyl protons at δ 1.992 -2.054 was observed.

The IR spectrum also showed a band at ν_{max} = 2932.6 cm^{-1} is an indication of methyl groups. In the ^{1}H-NMR spectrum showed the presence of methyl protons at δ 0.722-0.869 for the methyl groups. The IR spectrum showed a band at ν_{max} = 3445.6 cm^{-1} exhibiting a hydroxyl group absorption and the ^{1}H-NMR showed a one proton signal of oxygenation at δ 3.543. ^{13}C-NMR exhibited 30 signals (Fig 8.5 and 8.6) with 11 methyl carbons, two olefinic carbon signals (δ 140.76 and 121.72) and one oxygen attached carbon signal (δ 71.82).

From the above spectroscopic data and that from literature (Subhadhirasakul and Pechpongs, 2005; Habib-Ur-Rehman, 2008), the structure of compound 5 is proposed to be that of β-sitosterol (**24**).



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The IR analysis suggest the presence of O-H, C-H, C=C band stretching and C-O bending in both compounds. Also the presence of nitrogen was observed in the region of 2363.6 cm^{-1} in the case of compound SLRH2.

Table 4.8: Functional groups assignment from the FT-IR data of compound SLRH2 (Fig. 8.2)

Group frequency (cm^{-1})	**Functional group assignment**
3450.4	O-H stretch
2925.8	C-H stretch
2363.6	N-H stretch
1648.1	C=C stretch
1562.2	C-H stretch
1039.6-1398.3	C-O bend

CHAPTER FIVE

5.0 DISCUSSION

5.1 Extraction

Extraction of *S. lyratipatitus* showed that the methanol extract contain a higher proportion by mass of the components while the hexane extract contained the least proportion. *S. lyratipatitus* has been shown to exhibit anti-microbial activities. The medicinal properties of this plant could be attributed to a number of compounds. The high extraction yield obtained by using methanol as the extraction solvent is an indication that more polar compounds are present in the plant *S. lyratipatitus*.

5.2 Bioassay tests

Senecio lyratipatitus exhibited activity against brine shrimp, bacteria and fungi. The toxicity exhibited by dichloromethane fraction 3 (LD_{50} = 175.89 ppm) against brine shrimp may be comparable to the values for ethanolic extract (LD_{50} = 102.7 ppm) of *Sesuvium verrucosum* (Taha and Alsayed, 2000) and those for *Myrsine Africana,* LD_{50} = 114 ppm (Oladimeji *et al*., 2007), a plant from which a potent 2-hydroxychrysophanol was isolated (McLaughlin, 1991). Furthermore, Oladimeji *et al*., (2006) showed that the LD_{50} values of less than 200 ppm against brine shrimp lethality assay are generally considered significant. The significant lethality of the dichloromethane extract and fractions thereof against brine shrimp indicated the presence of a potent cytotoxic component in the plant, *S. lyratipatitus.*

The *n*-hexane, dichloromethane and methanol extracts exhibited relatively high activity against gram positive bacteria (*B. subtilis* and *S. aureus*) and gram negative bacteria (*E. coli* and *P. aeruginosa*). Previous studies have shown that plant extracts are more effective against gram positive bacteria than against gram negative bacteria due to the nature of the gram negative cell membrane which is made up of high amounts of phospholipids resulting in a cell envelope (Negi *et al.*, 1999; Negi and Jayaprakasha, 2001; Negi *et al*., 2002). However, the results obtained in this study revealed that, extracts from *S. lyratipatitus* may contain certain constituents with significant anti-bacterial activities which enable them to overcome the barrier in gram-negative cell wall.

Although the activity of the extracts was dose dependent, it was observed that a lower concentration of the dichloromethane extract at 125 ppm was more effective in inhibiting the growth of *B. subtilis* than a concentration of 250 ppm. This observation suggest that a concentration 125 ppm perhaps would be more effective for the treatment of systemic treatments and a concentration of 250 ppm might be more effective against localized or topical treatments.

The dichloromethane fractions exhibited significant growth activity against *E. coli, S. aureus, B. subtilis* and *P. aeruginosa*. A dose response was observed except for fractions 1, 2, 5 and 6 where activity decreased with increase in concentration against *E. coli* (Figs. 4.5, 4.5, 4.8 and 4.9). This observation on the decrease in activity against *E. coli* activity with increasing concentration of the extract was also made by White *et al*., (2007). This may be explained by the fact that *E. coli* produces defense mechanisms as a

way of responding to the environmental signals such as high chemical concentrations, pH and temperature.

The dichloromethane fractions exhibited a higher activity than the crude extracts. This enhanced spectrum of activity by the fractions is an indication of enrichment of some active metabolites during the fractionation process. This could be due to the fact that the amount of the active constituents in the crude extracts may be small or diluted and when fractionated, they become concentrated and therefore, exhibiting a higher activity. This demonstrates the importance of bioassay guided fractionation when assaying the anti-microbial activity of plant extracts.

It was also observed that the crude extracts exhibited a higher inhibition diameter against the organisms tested than the pure compounds. This could be due to synergistic effects. The compounds exhibited a higher activity than the standard anti-biotics. This is an indication that the compounds of *S. lyratipatitus* exhibited significant anti-microbial activity. It was also clear that the anti-biotic, Ampicillin (25 μg/disc) did not exhibit any activity against *B. subtilis, E. coli* and *P. aeruginosa*. Resistance of ampicillin to bacteria has been reported (Fei *et al*., 2008). This has been attributed to β-lactamases produced by bacteria and portions of bacteria producing β-lactamases increases year after year (Fei *et al*., 2008). Bacteria develop resistance to β-lactam anti-biotics for example ampicilin through β-lactamase enzymes. These enzymes render the bacteria resistance by hydrolyzing the β-lactam ring (Frere, 1995). *P. aeruginosa* did not exhibit any activity against ampicillin, nitrofurantoin, sulphamethaxazole and cotrimoxazole. Antibiotic resistance against *P. aeruginosa* has been reported in many parts of the world,

in particular to β-lactams, aminoglycosides and fluoroquinolines. This is because *P. aeruginosa* has been found to demonstrate all known enzymic and mutational mechanisms to bacterial resistance (Strateva and Yordanov, 2009).

This study supports the possible effectiveness of *S. lyratipatitus* in traditional medicine for the treatment of sexually transmitted diseases and wound infections (caused by *S. aureus*). The sexually transmitted diseases may be categorized as candidiasis and vaginitis which are caused by *C. albicans*. The inhibitory activity of the extracts and compounds against bacteria confirmed the potential use of the plant in the treatment of microbial induced ailments.

5.3 Structural elucidation of the isolated compound

Structural elucidation of Compound 5 isolated from the dichloromethane extract was based on the spectroscopic and physical data. The data was also compared to that reported in literature. The functional groups obtained from the IR, ^{1}H NMR, ^{13}C NMR and ^{1}H-^{1}H COSY correlated well. The compound was confirmed to be β-sitosterol (**24**).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. Use of methanol as an extracting solvent yielded the highest amount of the components whereas *n*-hexane yielded the least amount.

ii. The dichloromethane extract exhibited significant activity against brine shrimp. The brine shrimp test has been used as a biological test to guide fractionation process of plant extracts. Thus the dichloromethane fraction was further fractionation to yield six fractions.

iii. The dichloromethane fraction obtained by eluting with dichloromethane/Ethyl acetate (1:1) exhibited the highest activity against brine shrimp.

iv. The crude extracts and compounds of *S. lyratipatitus* exhibited both antifungal and anti-bacterial activity. A higher anti-bacterial activity was also exhibited by the extracts and compounds compared to some of the standard antibiotics. Anti-bacterial activity of extracts and compounds against *P. aeruginosa* is an interesting phenomena with regard to the development of new anti-biotics capable of treating resistant strains of bacteria.

v. The demonstration of anti-microbial activity of *S. lyratipatitus* has provided a scientific basis for its local usage as a medicinal plant for the treatment of sexually transmitted infections.

6.2 Limitations

The breakdown of the Nuclear Magnetic Resonance Spectrophotometer available in the country led to incomplete analysis of the isolated compounds.

6.3 Recommendations

(a) Potential effects of the extracts and fractions against other strains of bacteria and fungi need to be studied.

(b) More fractionation of the potent fractions need to be carried out to identify the compounds responsible for the activity in the extracts of *S. lyratipatitus* and complete the phytochemical characterisation.

(c) There is also need to evaluate the *in-vivo* biological activities of isolated pure compounds.

(d) The medicinal plant, *S. lyratipatitus* can be rehabilitated through proper agronomical methods to ensure its sustainability.

(e) The toxicity of the extracts and pure compounds can be investigated using systems other than the brine shrimp test e.g. cell culture by using human cell lines and other toxicity tests such as hepatoxicity are recommended.

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8.0 APPENDICES

Appendix 1: Spectroscopic data

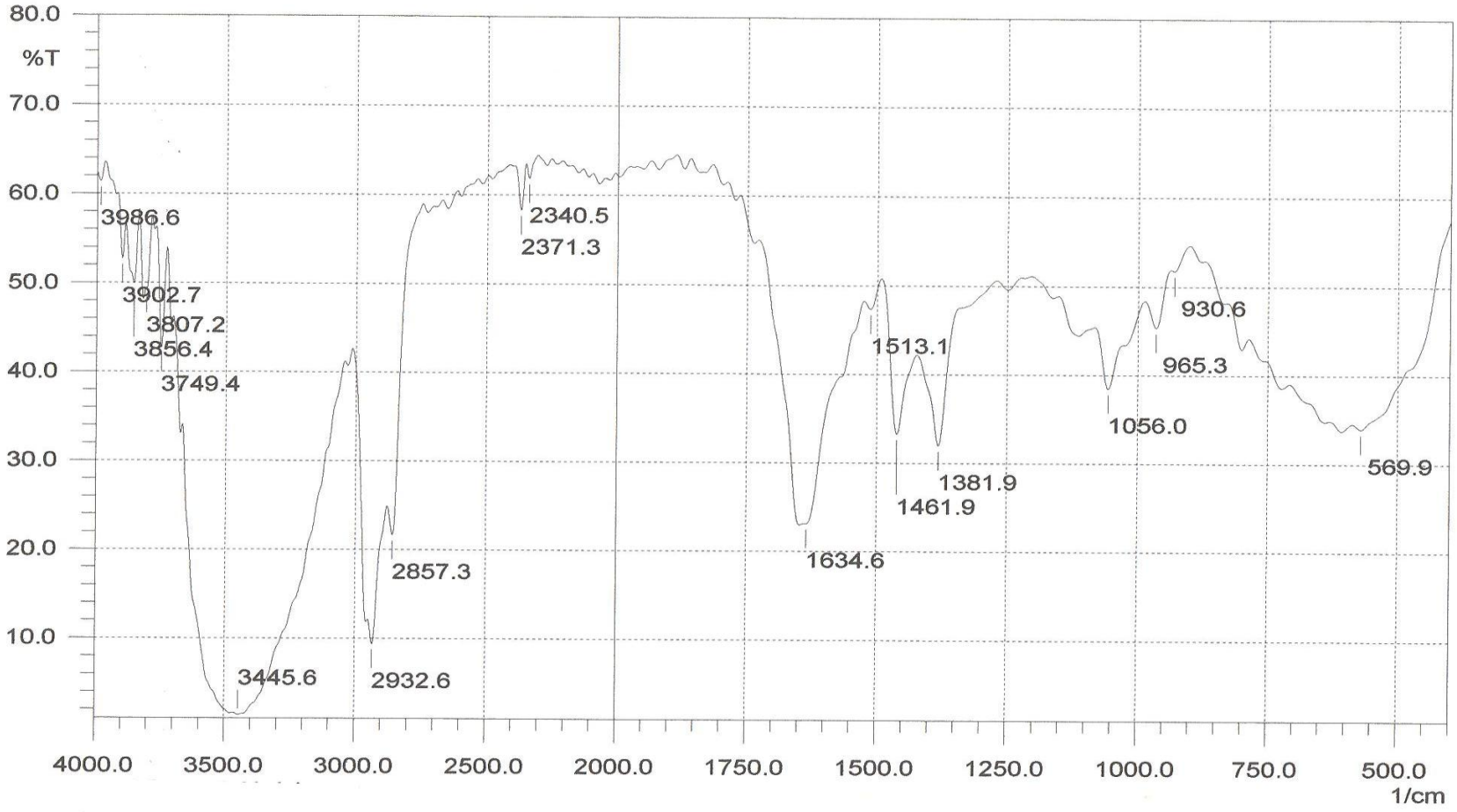

Figure 8.1: FT-IR spectrum of compound **5** (SLRD1)

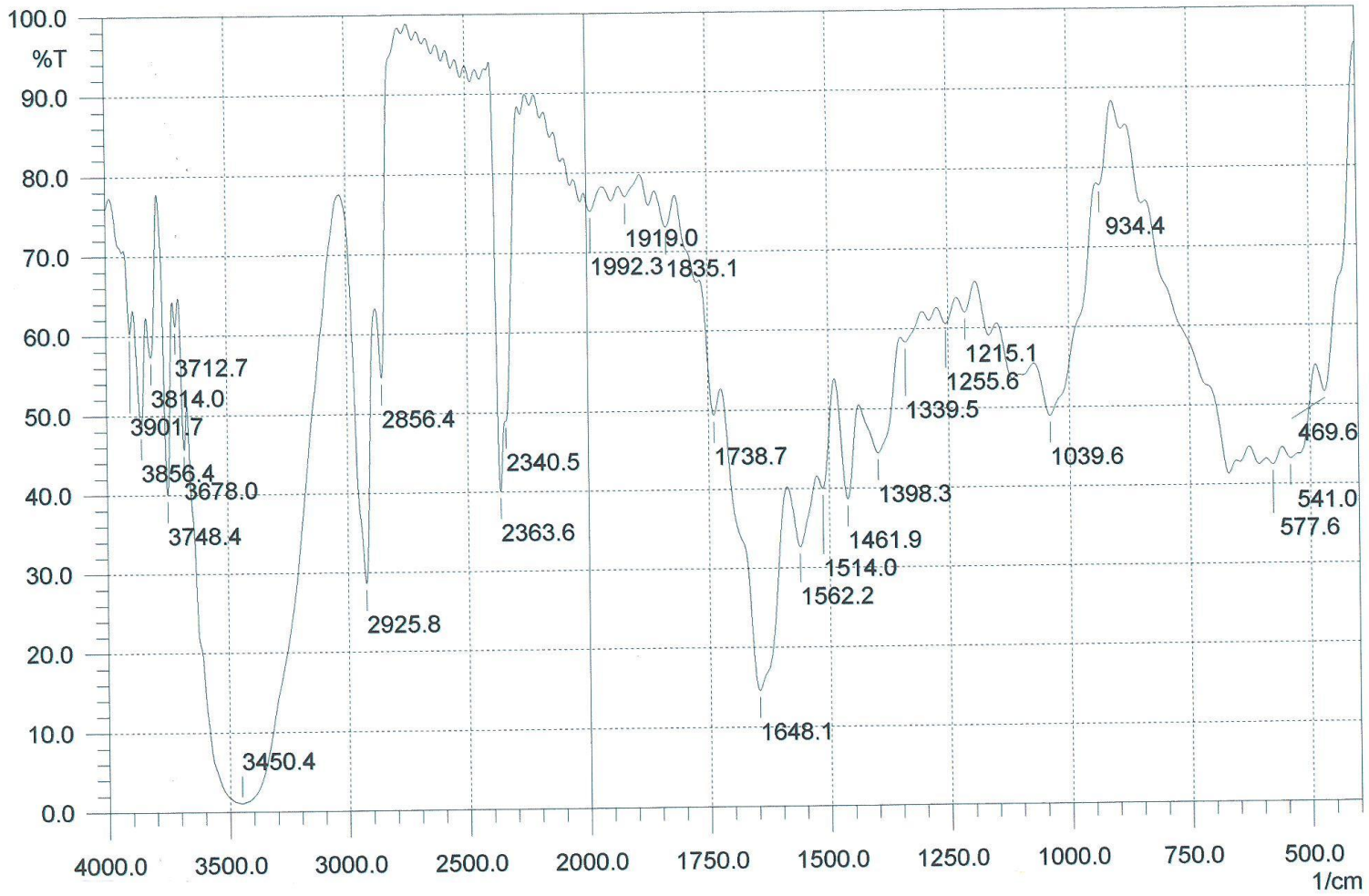

Figure 8.2: FT-IR spectrum of compound **2** (SLRH2)

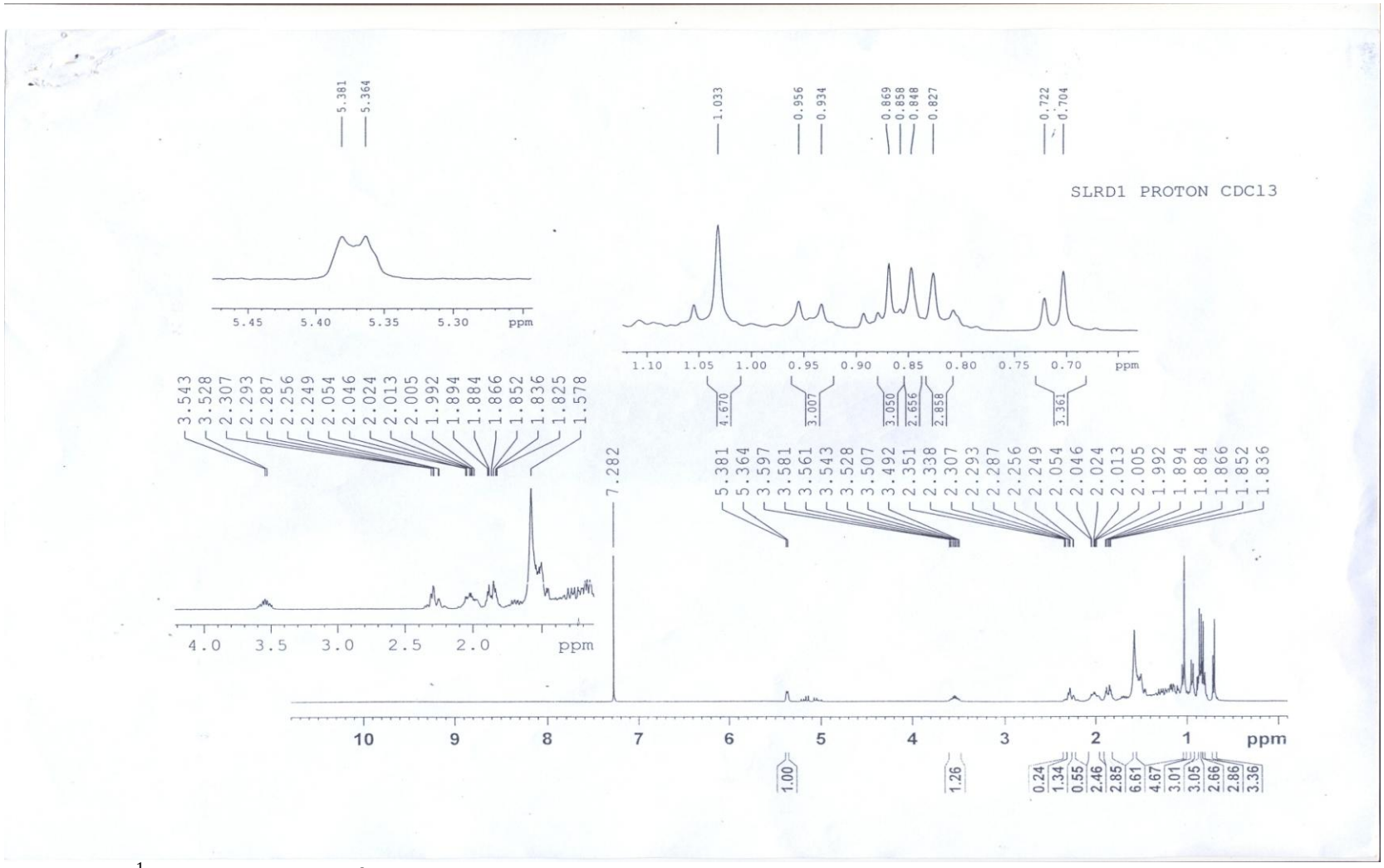

Figure 8.3: ^{1}H NMR spectrum of compound SLRD1

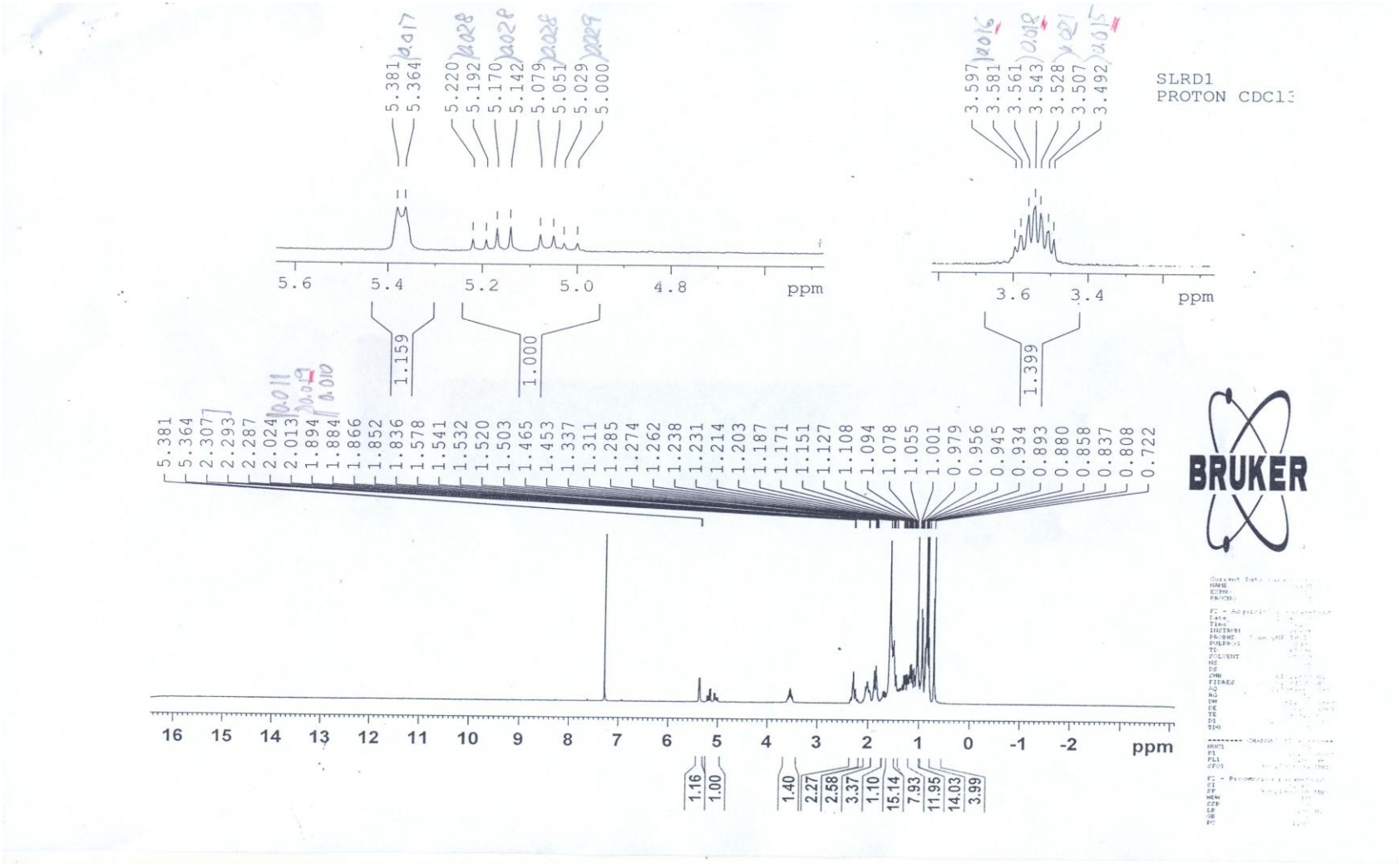

Figure 8.4: ^{1}H NMR spectrum of compound SLRD1

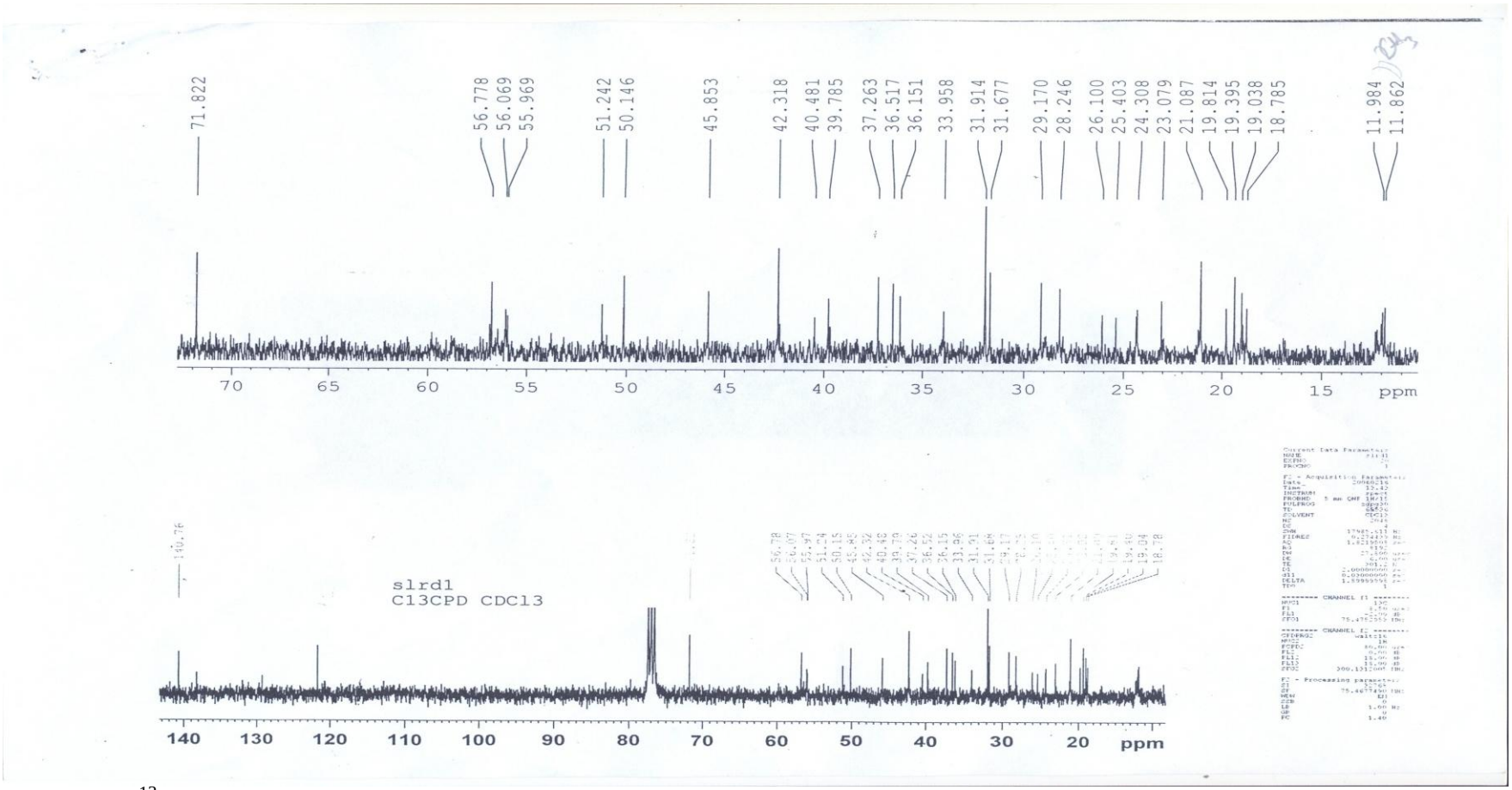

Figure 8.5: ^{13}C NMR spectrum of compound SLRD1

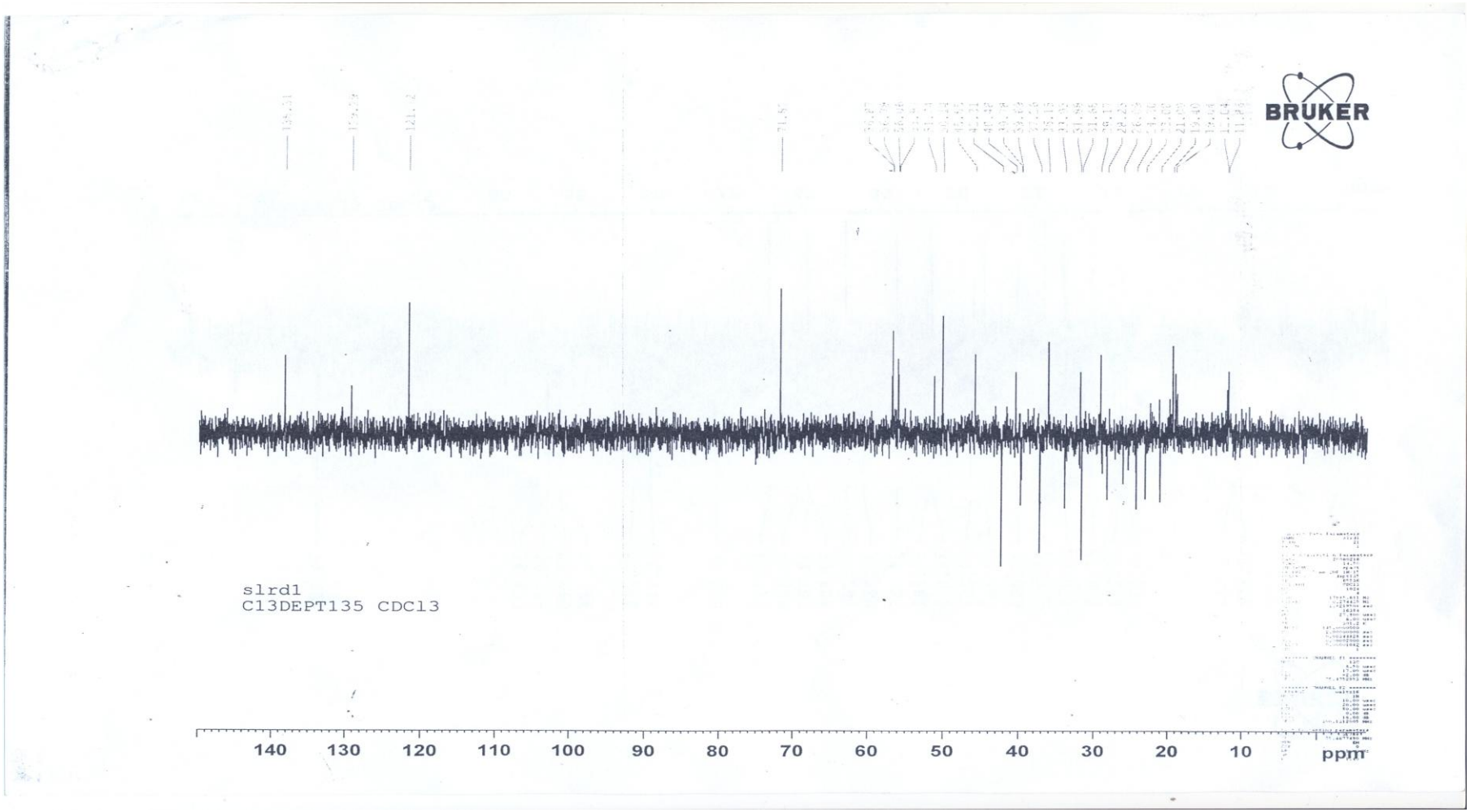

Figure 8.6: ^{13}C NMR DEPT spectrum of compound SLRD1

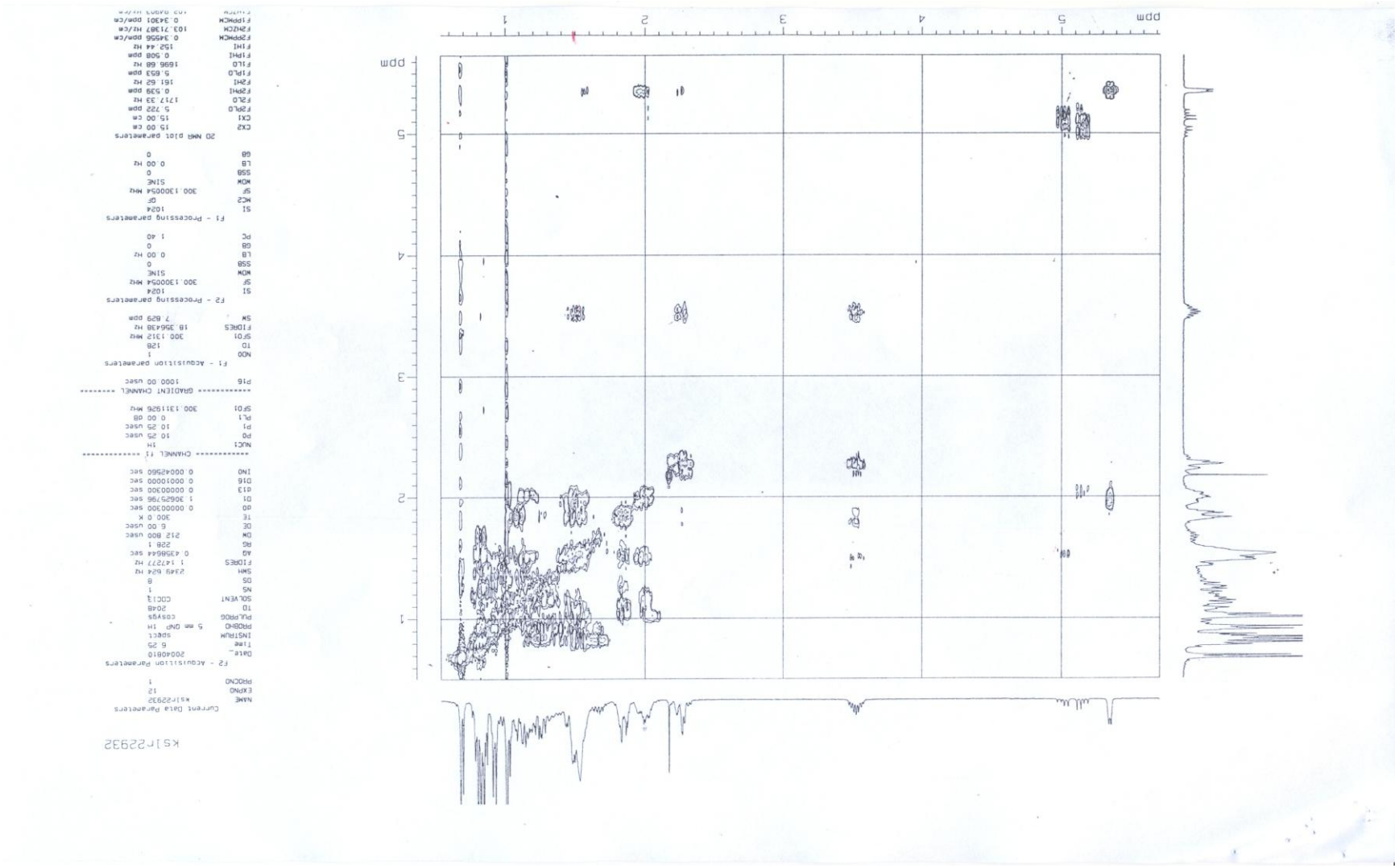

Figure 8.7: ^{1}H-^{1}H COSY spectrum of compound SLRD1

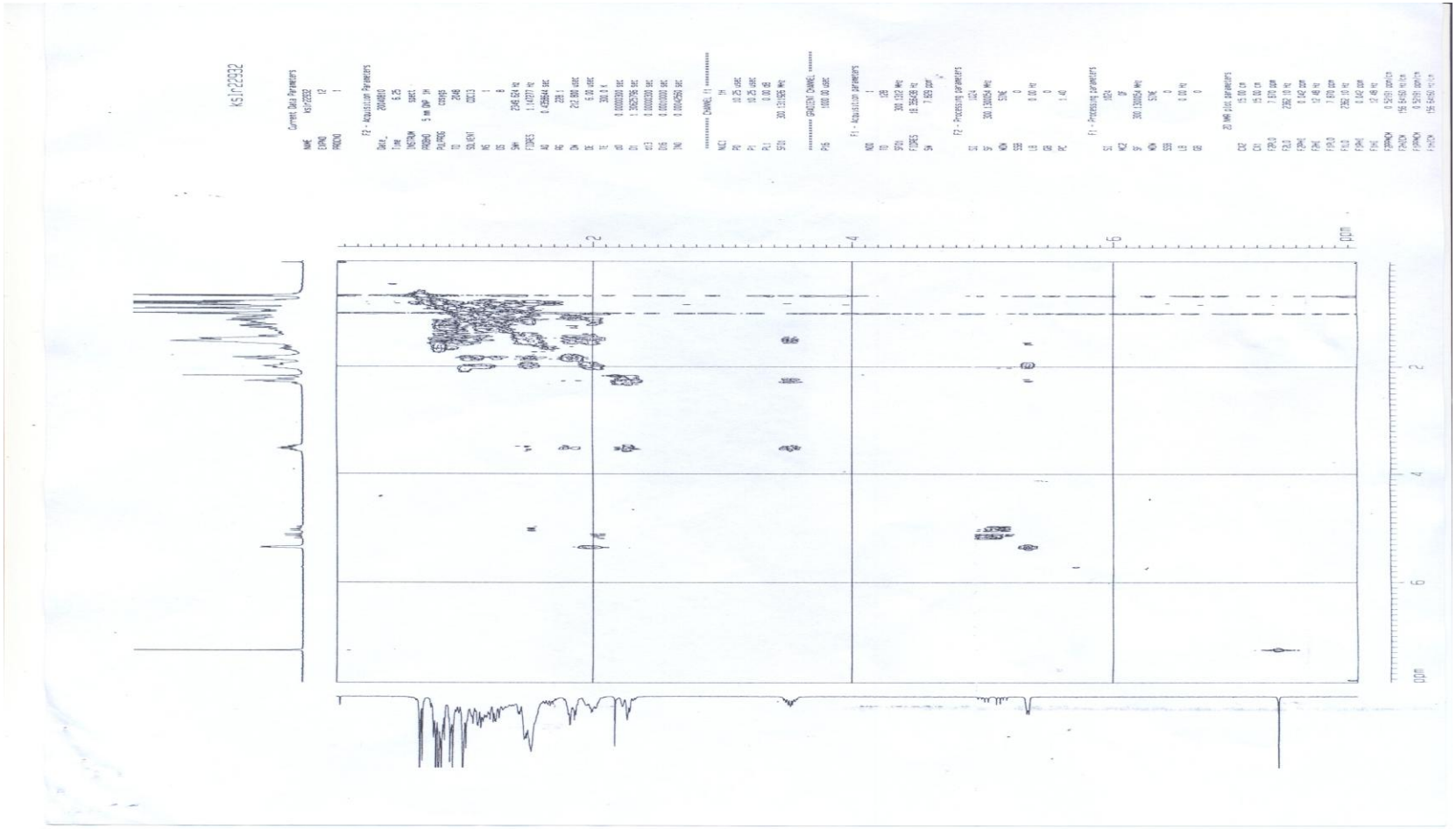

Figure 8.8: ^{1}H-^{1}H COSY spectrum of compound SLRD1

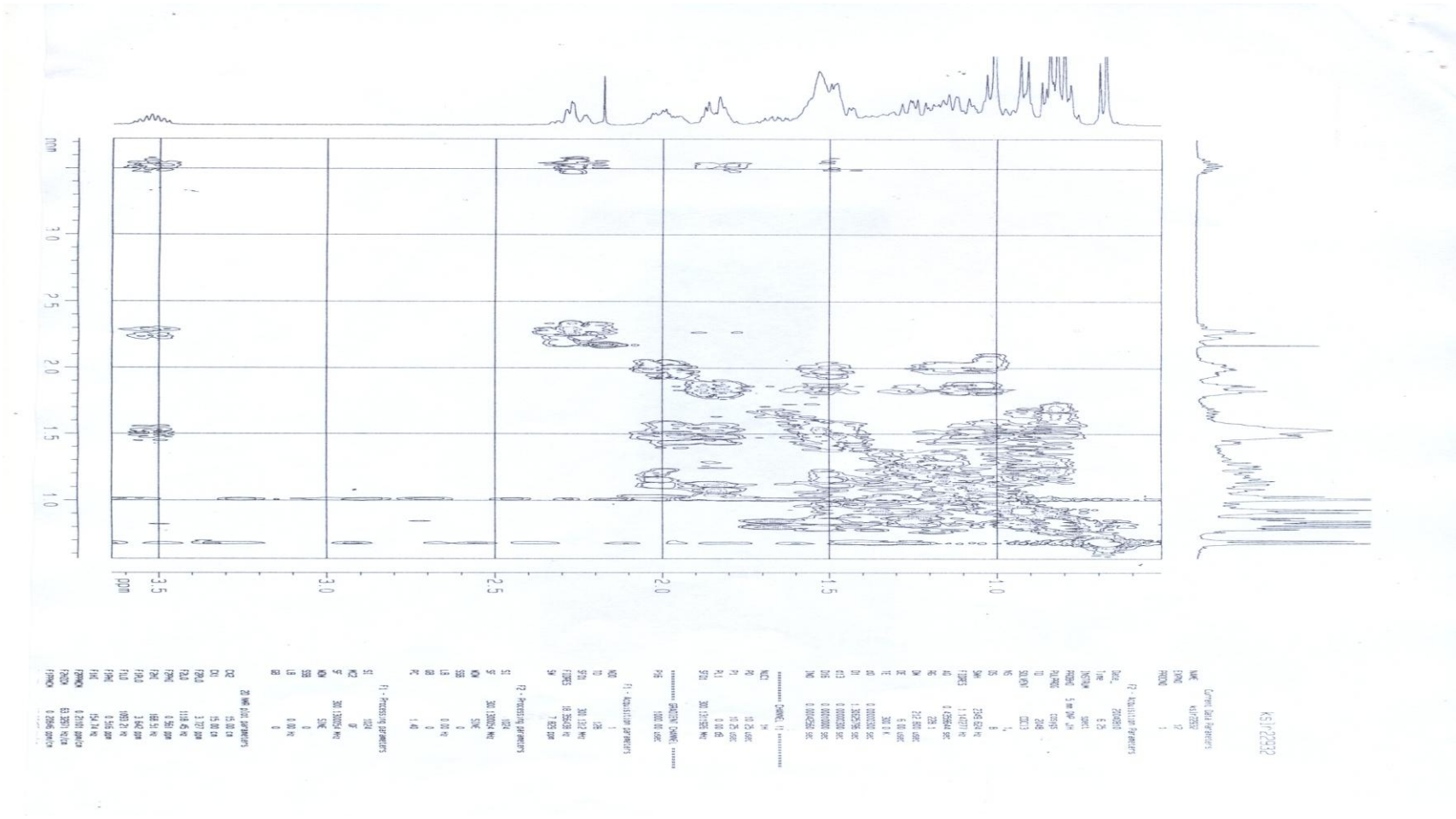

Figure 8.9: ^{1}H-^{1}H COSY expanded spectrum of compound SLRD1

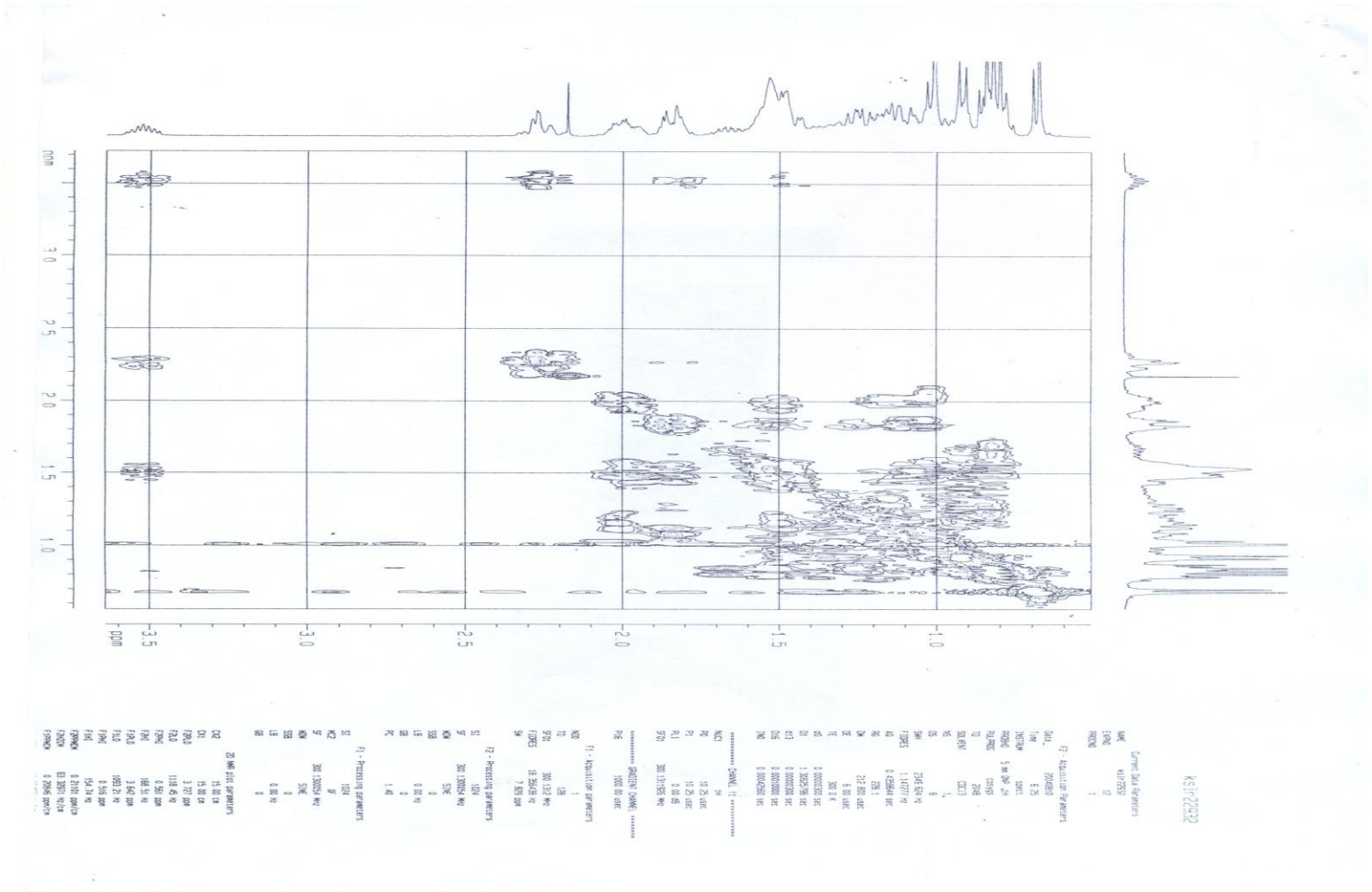

Figure 8.10: ^{1}H-^{1}H COSY spectrum of compound SLRD1