



UNIVERSITY OF NAIROBI
DEPARTMENT OF CHEMISTRY

**PHYTOCHEMICAL INVESTIGATION OF *ALOE SECUNDIFLORA* FOR
ANTIPLASMODIAL AND ANTIMICROBIAL ACTIVITY**

BY:

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**A THESIS SUBMITTED FOR PARTIAL FULLFILLMET OF THE REQUIREMENT FOR THE
AWARD OF A MASTER OF SCIENCE DEGREE IN CHEMISTRY OF THE UNIVERSITY OF
NAIROBI**

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DECLARATION

This thesis is my original work and has never been presented for a degree in any university

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DEDICATION

This thesis is dedicated to my beloved wife, Larissa, baby Adriana and all my family members. Their support and encouragement enabled me to undertake this piece of work.

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LIST OF ABBREVIATIONS AND SYMBOLS

COSY	Correlated spectroscopy
CP	Chloramphenicol
<i>d</i>	Doublet
<i>dd</i>	Doublet of a doublet
EIMS	Electron Ionization Mass Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation ($^2J_{CH}$, $^3J_{CH}$, $^4J_{CH}$)
HMQC	Heteronuclear Multiple Quantum Coherence ($^1J_{CH}$)
HPLC	High Pressure Liquid Chromatography
Hz	Hertz
KEMRI	Kenya Medical Research Institute
<i>m/z</i>	Mass to charge ratio
MHz	Mega Hertz
MIC	Minimum Inhibition Concentration
Mp	Melting point
[M] ⁺	Molecular ion
<i>m</i>	Multiplet
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PTLC	Preparative Thin Layer Chromatography
RFU	Relative Fluorescence Units
<i>t</i>	Triplet
TLC	Thin Layer Chromatography
UV	Ultra Violet
WHO	World Health Organization
λ_{max}	Maximum wavelength absorption

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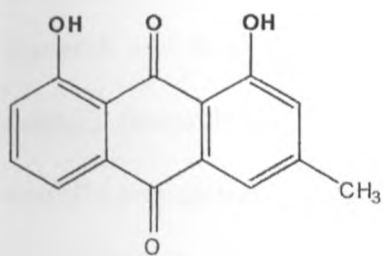
ABSTRACT

The stems of *Aloe secundiflora* was exhaustively extracted by percolation in CH_2Cl_2 -MeOH (1:1) at room temperature. The extract showed significant antiplasmodial activity against the chloroquine-resistant (W2) strain of *Plasmodium falciparum* with an IC_{50} value of 2.09 ± 0.43 $\mu\text{g/ml}$. The extract was subjected to chromatographic separations (Column chromatography and preparative thin layer chromatography) and crystallization which led to isolation of seven compounds.

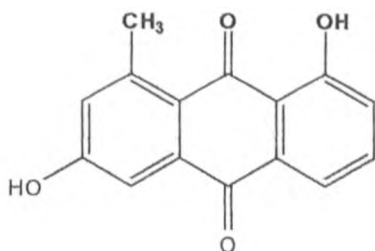
By the use of spectroscopic methods including IR, UV, MS and NMR (^1H , ^{13}C , DEPT, COSY, HMQC and HMBC) and direct TLC comparison with authentic samples in some cases, these compounds were identified as the monomeric anthraquinones chrysophanol (1), aloesaponarin II (2), aloesaponarin I (3), laccaic acid D-methyl ester (4), emodin (5), the pre-anthraquinone aloesaponol I (6); and the naphthoquinone derivative 8-hydroxy-2,7-dimethoxy-3-methylnaphthalene-1,4-dione (7). Of these, the naphthoquinone derivative (7) is a new compound. More over this is the first report on the occurrence of a naphthoquinone in the genus *Aloe*.

The isolated compounds from *Aloe secundiflora* were tested for antiplasmodial activity and aloesaponarin I (3), aloesaponarin II (2), aloesaponol I (6) and the naphthoquinone derivative (7) showed significant activities. Amongst these, aloesaponarin I (3) was the most active with an IC_{50} value of 0.92 ± 0.07 $\mu\text{g/ml}$ against the chloroquine-resistant (W2) strain. This investigation has shown the potential of the aloesaponarin I (3) as a lead structure for the development of antimalarial drugs.

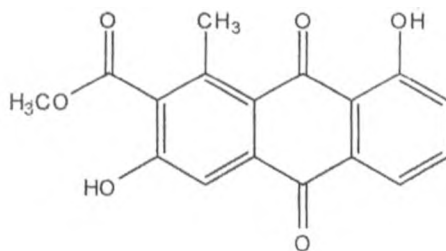
Antibacterial and antifungal tests were also carried out for some of the isolated compounds against three bacterial strains viz. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas eruginosa* and four fungal strains viz. *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton mentagrophytes* and *Microsporium gypsum*. Amongst those tested, aloesaponarin II (2) was the most active showing activity against *Staphylococcus aureus* and *Cryptococcus neoformans* with an MIC value of 18.8 µg/disc for both organisms. No significant activity was observed for any of the isolated compounds against Gram-negative bacteria, *Escherichia coli* and *Pseudomonas eruginosa*.



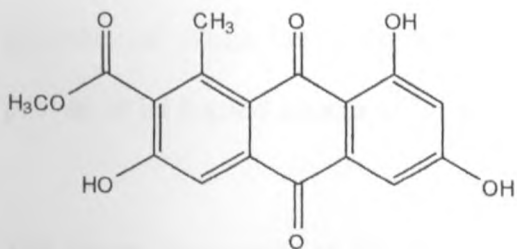
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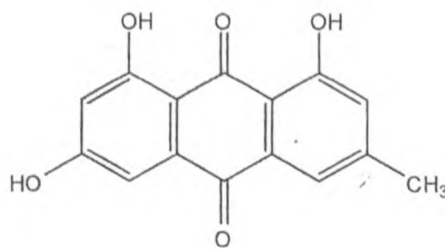
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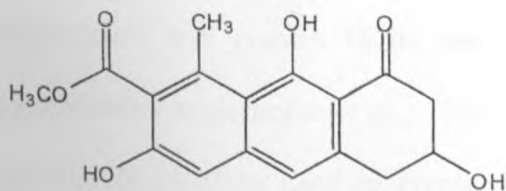
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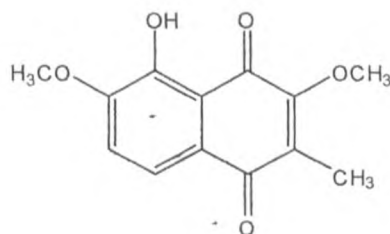
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CHAPTER ONE

1.0 INTRODUCTION

1.1 General

In the last two decades malaria has become an extremely serious threat to the health and economic prosperity of the world. It is currently restricted to the developing countries in the tropical and subtropical regions of the world, which cannot afford to pay for the high cost of research and drug development. It is hoped that, in the not to-distant future, funding for malaria research will come to reflect the major threat that this disease represents to the world's population. Strategies to control the disease involve; vector control, development of vaccines and of new drugs, to overcome the serious spread of resistance against the presently available drugs. The rapid spread of resistance against the presently used antimalarial drugs has created a desperate need for new drugs. Nature has consistently proven to be a good source of lead structures in drug development.

The recent discovery of the drug artemisinin from the plant *Artemisia annua*, has shown plants, especially those used in traditional medicine, as potential source of drugs. Artemisinin has proven to be one of the most promising antimalarial drugs and lead compounds (Angerhofer *et al.*, 1992; Casteel, 1997; Klayman, 1985). In malaria treatment, artemisinin is often used in combination with other antimalarials such as mefloquine or tetracyclines (Casteel, 1997). Currently, the majority of drugs used against malaria has been

developed from, or are, natural products. Among these is the oldest and still-used drug, quinine, which is obtained from the bark of trees belonging to the genus *Cinchonae*. The origin of the *Cinchonae* bark has been traced to South America (Eiden, 1998).

Several plants and classes of compounds are being screened for antimalarial activities amongst which quinones have showed good activities. In addition, a number of plants in the family Asphodelaceae are known to contain anthraquinones (Bringmann *et al.*, 2002). The *Aloe* species which are members of the family Asphodelaceae, are used in traditional medicine for treatment of malaria (Smith and Van Wyk, 1991). The isolation of compounds from these plants and study of the anti-malarial activity of the isolated compounds is expected to contribute to the progress towards discovery of new anti-malarial drugs.

Aloe species in addition to wide ethnomedical uses, are known to elaborate quinones especially anthraquinones (Gjerstard and Riner, 1968). The major compounds in these plants being anthraquinones and their derivatives, it is postulated that they could be responsible for medicinal use of these plants. Research on the chemical composition of these plants is thus a worthy cause. In this project, *Aloe secundiflora* is investigated for the isolation and characterization of antiplasmodial quinones.

1.2 Problem statement

Artemisinin Combination Therapy (ACT) which is recommended for treatment of malaria has turned to be unaffordable for the common man and alternative solutions are urgently

required. As expensive as this drug is, it may become ineffective as the other drugs because the parasites mutate and become resistant. There is thus an urgent need to seek for new cures for malaria and the process has to be repeated consistently because the parasites become resistant to the new cure after only a short time. The Plant Kingdom offers a good potential for identification of lead structures for subsequent drug development.

1.3 Justification of the research

Anthracene derivatives such as anthraquinones are expected to be the antiplasmodial agents in *Aloe* species, in the same way as these compounds are responsible for the wide use of *Aloe* in medicine and agriculture.

There are a number of *Aloe* species that grow wildly in Kenya. These are widely used in folk medicine. In several Kenya communities, one of the major uses of these plants is the cure of malaria and other infections. Information on the chemistry of the Kenyan species, especially their antiplasmodial activities is scanty.

Therefore there is a need to conduct phytochemical and antiplasmodial studies on Kenyan *Aloe* species. Such studies will also be useful for chemotaxonomic purposes as the relationship among different taxa is not clearly defined. Knowledge of the composition of these plants and more so on the compounds responsible for the antiplasmodial and antimicrobial activity would have far-reaching implications to the healthcare practice in the tropics.

1.4 Objectives

The general objective of this research was to establish the antiplasmodial and antimicrobial activity of the constituents of the stems of *Aloe secundiflora*.

The specific objectives of the research were;

- a) To establish the antiplasmodial and antimicrobial activity of the stem extract of *Aloe secundiflora*.
- b) To isolate the constituents of the stem of *Aloe secundiflora*.
- c) To characterize the isolated compounds.
- d) To determine the antiplasmodial and antimicrobial activity of the isolated compounds.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria

Malaria is caused by protozoa of the genus *Plasmodium*, and is transmitted through bites by female mosquitoes of the genus *Anopheles*. Four *Plasmodium* species are known to cause malaria in humans, namely; *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Amongst these, *P. falciparum* and *P. vivax* are the most common species (Kreier, 1980), with the former being the agent of most severe form of malaria that causes about 90% of malarial deaths, especially in Africa (Kreier, 1980).

2.1.1 World Malaria Situation

Severe malaria cases are being reported now than ever in the human beings lifetime. The incidence of malaria worldwide is estimated to be between 300 and 500 million clinical cases each year, principally in Africa, Asia and South America (Fig. 1). It constitutes a public health problem in more than 90 countries inhabited by about 40% of world's population (WHO, 2002). An estimated 90% of these cases are reported to occur in sub-Saharan Africa. The World Health Organisation (WHO) estimates that close to 2 million people succumb to malaria annually, where young children and expectant mothers are the groups most affected.

These childhood deaths, resulting mainly from cerebral malaria and anaemia, constitute nearly 25% of child mortality in Africa. Fatality rates of 10–30% have been reported among children referred to hospital with severe malaria, although these rates are even higher in rural and remote areas where patients have restricted access to adequate treatment. Deaths from malaria in countries outside sub-Saharan Africa, occur principally in non-immune people who become infected with *P. falciparum* in areas where diagnosis and treatment are not available (WHO, 2002).

Global malaria risk



Figure 1: Global malaria risk (WHO, 1998)

2.1.2 Current Malaria Control Measures

The malaria control strategy aimed at malaria eradication was re-oriented in 1978 to focus on the reduction of malaria to a level at which it would no longer constitute a major public health problem (WHO, 1978). This strategy was based on the combined use of vector control measures and effective treatment of malaria patients. This initial success in many tropical countries was interrupted mainly by the development of resistant mosquitoes and chloroquine-resistant *Plasmodium falciparum*, as well as lack of continuous political and financial support to the program (Bradley, 1991).

Analysing the failures during the consolidation phase, WHO recognized that the basic requirements for achieving and sustaining malaria control are; first the integration of malaria control into a reasonably well-established health care system and secondly, the establishment of an un-interrupted effort and finally research into new and improved tools. In 1978, during and following the Global Malaria Eradication Programme, which run upto 1982, 24 endemic countries were certified by the World Health Organization as malaria-free. The objectives of malaria control measures range from reducing the disease burden and maintaining it at a reasonably low level, to eliminating the disease from a defined geographical areas, and ultimately to eradicating the disease globally. These levels of control are defined as follows (WHO, 2007):

1. Malaria control: reducing the disease burden to a level at which it is no longer a public health problem.
2. Malaria elimination: interrupting local mosquito-borne malaria transmission in a defined geographical area (zero incidence of locally contracted cases, although

imported cases will continue to occur). Therefore continued intervention measures are required.

3. Malaria eradication: permanent reduction to zero levels of the worldwide incidence of malaria infection.

The practical implementation of the strategy requires two main approaches (WHO, 1998).

1. Malaria chemotherapy for early and effective treatment of malaria cases, management of severe and complicated cases, and prophylaxis for the most susceptible population (particularly expectant women and non-immune travelers).
2. Use of insecticide-treated nets for protection against mosquito bites. This strategy has, however, been increasingly confronted by serious setbacks due to the continued spread of insecticide-resistant mosquito vectors, political and socio-economic problems.

Most countries where *Plasmodium falciparum* malaria, the most dangerous type of the disease, is endemic face some degree of parasite resistance to frequently used drugs (Fig. 2), hence the need to develop new drugs on a consistent basis (Guido, *et al.*, 1999; Winstanley *et al.*, 2002).

Reported *P. falciparum* drug resistance



Figure 2: Reported *P. falciparum* drug resistance (WHO, 1998).

2.1.3 Constraints against Current Malaria Control Measures

The most effective antimalarial drugs are currently recommended as a combination of artemisinin-derived drug with another drug to delay the emergence and spread of resistance. Artemisinin-based combination therapies (ACT's) are safe and highly effective in curing infections and also are the most effective treatment yet for reducing parasite activity due to the anti-gametocyte action of the artemisinin component. The disadvantages of these drugs are that they have relatively short shelf-lives, which imparts negatively on means of supply and adherence to treatment schedules (WHO, 2008).

The usable nature and the short lifespan of the artemisinin-based combination treatment (ACT's) and the development and spread of resistance by *P. falciparum* have threatened the future use of this treatment. Deficiency is caused by both the artemisinin component and the

partner medicine. Several studies conducted at the Thai-Cambodian border, which historically has been the major point of resistance to all antimalarial medicine, confirm the increase in failure rates of the combined treatment of artesunate offered with mefloquine (WHO, 2008).

Antimalarial medicines have wider uses beyond just curing sick patients. Prevention particularly in high transmission situations and in high-risk groups such as expectant women, infants and children has been identified as an alternative use. Currently, sulfadoxine-pyrimethamine is the only medicine available for use as an intermittent preventive treatment in pregnancy. However, widespread parasite resistance seems to compromise this alternative use especially in high-burden countries in Africa (Fig. 2). Therefore, preventive treatment options are being made difficult by the absence of suitable medicines to replace sulfadoxine-pyrimethamine (WHO, 2008).

Medicines used for preventive purposes must have a long half-life and an extremely good safety profile as may be prescribed to normal and healthy subjects. Ideally they should not be the same as those prescribed for curative purposes. This will by extension reduce the risk of the parasite developing resistance that might be accelerated by the wider use in prevention (WHO, 2008).

Success in reduction of the risk associated with malaria will be achieved with better control methods and therefore the need for preventive treatment will be minimal. However, as the world moves towards malaria prevention, there are chances that there will be an increased demand for curative medicines that require steady development as well as those with the

ability to deal with the early stages of malaria transmission. The medicines will therefore be required to be safe, formulated as fixed-dose combinations and have a shelf-life of at least 3 years (WHO, 2008).

A strategic fixed-dose combination of at least three medicines, each with a different mode of action will ideally be recommended for any antimalarial medicine. This is undertaken as a precaution to delay parasite resistance. The constituents of such combinations should not be in the market in isolation, as they run the risk of parasite resistance (WHO, 2008).

One of the most commonly used drugs, chloroquine, is no longer effective. As a consequence Malawi and Kenya, in 1993 and 1996 respectively, changed their recommendations for first-line treatment of uncomplicated malaria from chloroquine to sulfadoxine/pyrimethamine. Likewise, Botswana and South Africa revised their treatment guidelines in 1997. The current use of sulfadoxine-pyrimethamine is in jeopardy with the increase in resistance to this combination treatment (Trape *et al.*, 2002; Nzila-Mounda *et al.*, 1998; Sibley *et al.*, 2001). The resistance to sulfadoxine/pyrimethamine, the main alternative to chloroquine, is widespread in south-east Asia and South America. Recent reports from Kenya and Tanzania suggest that changes in parasite susceptibility to sulfadoxine/pyrimethamine have occurred and may presage clinical resistance. Mefloquine resistance is now common in the border areas of Thailand with Cambodia and Myanmar. Parasite sensitivity to quinine is declining in several other countries of south-east Asia and in the Amazon region, where it has been used in combination with tetracycline for the treatment of uncomplicated malaria. Consequently, artemisinin and its derivatives are now increasingly being used as first-line treatment in some of these areas (WHO, 2008).

Resistance of *P. vivax* to chloroquine has now been reported from Indonesia (Irian Jaya), Myanmar, Papua New Guinea and Vanuatu. Cross-resistance to amodiaquine sometimes occurs. An increasing number of malaria epidemics have been recently documented throughout the world, particularly in Africa. According to the World Health Organisation (WHO), areas become epidemic when conditions that normally limit transmission change radically as a result of abnormally heavy rains, long periods of increased humidity and temperature, or more permanent changes of microclimate due to the development of irrigation systems, agricultural projects or tree plantations (WHO, 2008).

Another setback results from the re-emergence of malaria in areas where it had been eradicated such as Republic of Korea and Tadjikistan. Accordingly, there is reported increase of malaria in countries where it was nearly eradicated such as Azerbaijan, northern Iraq and Turkey. The current malaria epidemics in the majority of these countries are as result of a rapid deterioration of malaria prevention and control operations (WHO, 1998).

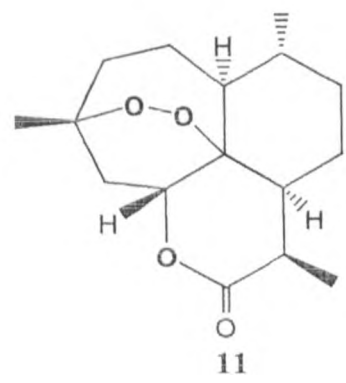
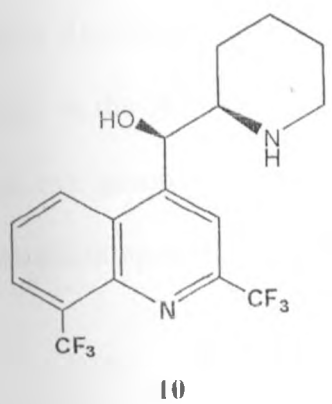
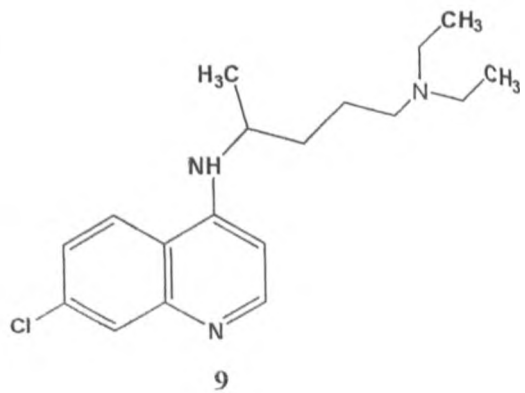
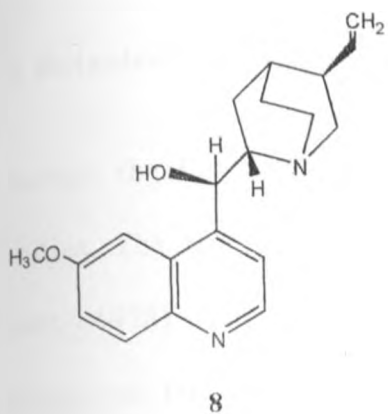
In addition, the cost of carrying out malaria diagnosis tests at present is almost as high as that of treatment. As the malaria burden increases, the demand for high-performance diagnostic tests will increase, because the disease will account for a smaller proportion of febrile illnesses. Furthermore, in situations of high transmission, the age profile may shift from children to adults, in whom confirmation of diagnosis is important for treatment. Diagnosis is even more useful in the phases of malaria elimination and eradication than in the control phase because of the need for extensive surveillance, and this will require simpler, more reliable diagnostic tools than are presently available (WHO, 2008).

2.1.4 Plants in Malaria treatment

The development of traditional medicines for treatment of malaria, would provide the following major benefits to the poorest and worst deprived populations in the world as far as health and economic development is concerned (WHO, 2008):

1. Provision of affordable and effective drugs.
2. Prevention of large number of deaths of children and expectant women.
3. Alleviation of poverty by reducing the burden of malaria and offering the population alternative commercial crops.
4. Creation and strengthening of capacities for drug production.
5. A replicable approach to the provision of effective and affordable medicines for other diseases.

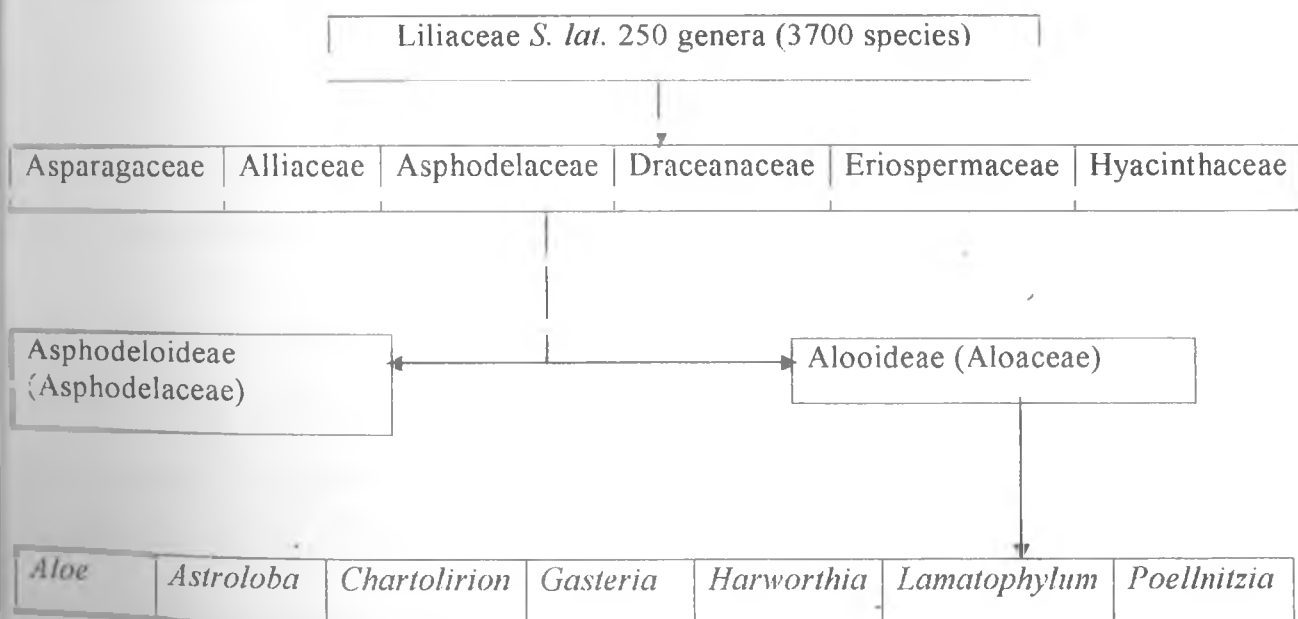
The first and one of the most important antimalarials to come from plants is quinine (8) which was isolated from the bark of *Cinchona succuriba* (Rubiaceae) and has served as a template for chloroquine (9) and mefloquine (10) (Balint, 2001; Price, 2002). The most recent lead drug is artemisinin (11) from the Chinese anti-malarial herb *Artemisia annua* (Astraceae) (Balint, 2001; Price, 2002) which has been used successively against the parasite that has become resistant to chloroquine (9). *Artemisia annua* has been used for eons by the Chinese to treat malaria (Balint, 2001).



A number of plants in the family Asphodelaceae are known to contain anthraquinones (Bringmann *et al.*, 2002). The *Aloe* species which are members of this family Asphodelaceae, are used in traditional medicine for treatment of malaria (Smith and Van Wyk, 1991). The isolation of compounds from these plants and the study of their antimalarial activity is expected to contribute towards discovery of new antimalarial drugs.

2.2 Botanical information on *Aloe*

Amongst the flowering plants, Liliaceae *S. lat.* is one of the most widely distributed families. The family is mostly composed of perennial herbs with rhizomes (Trease and Evans, 1978). This big and heterogenous family was subdivided into smaller and homogenous families (Scheme 1). The Asphodelaceae is one of these and it consists of the sub-families Asphodeloideae and Alooideae. Accordingly the genus *Aloe* along with the genera *Astroloba*, *Chartolirium*, *Gasteria*, *Harworthia*, *Lomatophylum* and *Poellnitzia* are placed in the Alooideae. The generation of chemical information on species belonging to these two groups is believed to reveal the relationships among the various taxa and to assist in establishing taxonomic classifications at various levels (Dagne and Yenesew, 1994).



Scheme 1: Taxonomic position of *Aloe* (Trease and Evans, 1978).

The *Aloe* (Asphodelaceae) comprises about 600 species, and is found in Africa (Okamura *et al.*, 1996), principally in Eastern & Southern Africa and Madagascar. However has been introduced into the West Indies and many other tropical countries (Mabberley, 1987; Demissew, 1996). The genus *Aloe* has been divided into several groups ranging from grass aloes (Reynolds, 1966), to tree aloes (group 20, tall trees, dichotomously branched and re-branched) depending on similarities in morphology (Reynolds, 1966). There are twenty six *Aloe* species in Kenya, of which six are endemic. The Kenyan species as reported by Reynolds (1996) are: *Aloe erensii*, *A. pirottae*, *A. amudatensis*, *A. graminicola*, *A. kilifiensis* (endemic), *A. lateritia*, *A. rivae*, *A. otallensis*, *A. calidophila*, *A. turkanensis*, *A. secundiflora*, *A. ruspoliana*, *A. classenii* (endemic), *A. ukambensis* (endemic), *A. tweediae*, *A. marsabitensis*, *A. volkensii*, *A. ballyi*, *A. deserti*, *A. elgonicaa* (endemic), *A. rabaiensis*, *A. dawei*, *A. kedongensis*, *A. ngobitensis* (endemic), and *A. nyeriensis* (endemic) (Gillett, 1968).

2.2.1 Morphological features of *Aloe* species

Aloe species are coarse-looking perennial plants with stem sizes varying with different species. Some species are trees, others are short-stemmed, and some have stems underground. The plants are naturally found in the wild, mostly in semi-arid areas. The common morphological features of all aloes are in the nature of their leaves. The leaves are long, erect and crowded in basal rosette. They are succulent, containing a clear sticky juice (jelly-like) between two green skins. The smooth surfaces of the skins are spotted in some

species. The margins of the leaves have spiny teeth (prickly at the margins). The leaves breaks easily and when cut it exude a yellow coloured juice (Reynolds, 1985a).

2.2.1.1 Morphological features of *Aloe secundiflora*

Habit: The rosettes are normally stemless and usually solitary.

Leaves: ca 20, 35-45 x 8-14 cm, suberect with recurving tips, dark green, slightly glossy, sometimes obscurely pale-spotted; marginal teeth 8-10 per 10 cm, 4-5 mm long, dark brown, colour sometimes continuous along margin.

Inflorescence: 1-1.5 m high, many spreading branched, lower branches always branching again, with upto 50 racemes, these 12-20 cm long, distinctly 1-sided with flowers all \pm erect, \pm lax. Bracts 2.5-5 x 1.5-2.5 (-4) mm. Pedicels 5.5-6(-10) mm long. Perianth cylindrical, 19-23(-28) mm long, c 4.5 mm wide when pressed, pale red minutely flecked with white when alive.

Ecology: open grassland and *Acacia* bushland on well drained soils occurring between altitudes of 1350-1550 m.

Distribution: known from Gamo Gofa and Sidamo provinces (Ethiopia), southern Sudan, Kenya and Tanzania (Demissew, 1996).



Figure 4: Photographs of flowers and stems of *Aloe secundiflora* at the University of Nairobi Chiromo campus, Chemistry Department gardens.

2.3 Ethnomedical Uses of *Aloe*

There are numerous reports on the medicinal importance of *Aloe*. Many manufactures of cosmetics and related products incorporate quantified amounts of *Aloe* extracts in order to have a competitive edge in the market. The dried resinous sap derived from various parts of *Aloe* species is called "ALOE" and has been described as a drug for all seasons (Haller, 1990). The leaf exudate of this species has found ethnoveterinary use for treatment of diseases and ectoparasites, and in the treatment of some viral diseases. In poultry for example, the exudates has been extensively used as a prophylaxis for Newcastle disease virus, and as a therapeutic for fowl typhoid, coccidiosis and other enteric conditions (Tringali, 2001).

Aloe has been used in folk medicine for treatment of constipation, burns and dermatitis (Man *et al.*, 1996). It's for these reasons that aloe is recognized throughout the world as a traditional and folk medicine aid, for healing of skin injuries, such as cutaneous wounds and sunburns (Bouthet *et al.*, 1995). In Kenya, traditional medicine men always incorporate aloe

extracts (water, exudate or succulent fluid) in their herbal concoctions. Around the world *Aloe vera* gel obtained from the inner thin walled mucilaginous parenchyma cells has been used for a host of treatment purposes (Gjerstad and Riner, 1968; Tritter, 1981). Other uses of aloe include in cosmetics (skin lotions and hair products) and food industries (making jam) (Van der Bank *et al.*, 1995). *A. marlothii* has been used traditionally for treatment of roundworm infections; the powdered leaves snuffed for stomach troubles, and hastening weaning of children (Van der Bank *et al.*, 1995). In South Africa, the leaf juice of *Aloe ferox* is collected and dried by a traditional method to produce a dark brown solid substance known as bitter aloes or Cape aloes (Van Wyk *et al.*, 1995).

In Somalia, the leaf juice of *Aloe microdonta* (vernacular name *Dacaar Qaraar*) is traditionally used as a remedy for jaundice and skin diseases (Mohamed *et al.*, 1992). A hot glycerine extract of *Aloe barbadensis* was found to inactivate herpes simplex virus type 1 (Sydiskis *et al.*, 1991). When damaged, aloe is known to close any wounds to its leaf almost immediately so that the plant does not lose any of its precious life-sustaining fluids. It's probably for this reason that aloe has gained its reputation as a moisturizer in the cosmetic industry (Van Wyk *et al.*, 1995).

2.4 Biological activities of *Aloe*

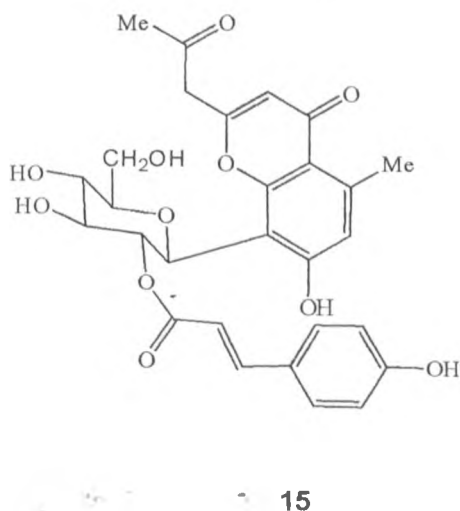
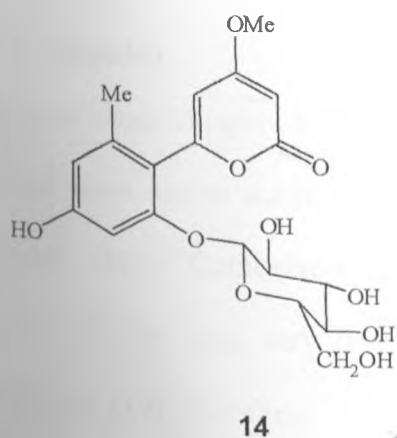
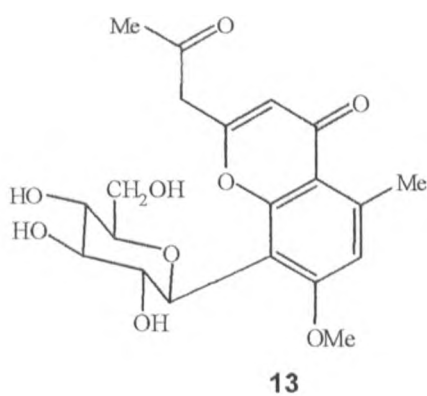
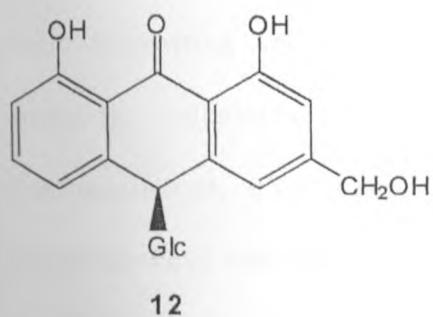
Aloe plant extracts have a long history of use as a therapeutic agent with many reported medicinal properties. Among its therapeutic properties, anti-inflammatory (Azfal *et al.*, 1991; Malterud *et al.*, 1993), immunostimulatory (Ramamoorthy and Tizard, 1998), and cell growth stimulatory activities (Tizard *et al.*, 1994; Rodriguez-Bigas, 1988). Furthermore,

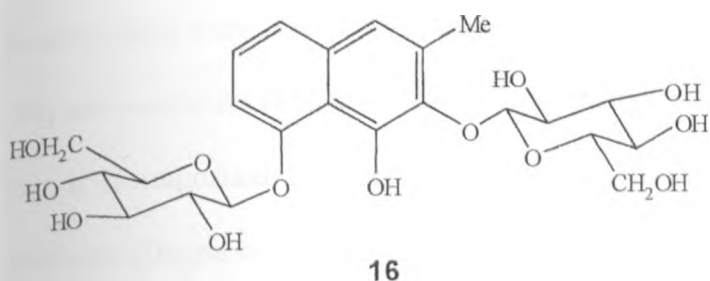
activity against bacterial (Ferro *et al.*, 2003), antiviral (Kahlon *et al.*, 1991) and antifungal (Kawai *et al.*, 1998) activities have been reported. There are also some reports on the antimicrobial effects on pure components and extracts obtained from *Aloe* species. It has been shown that aloe leaf gel can inhibit the growth of two Gram-positive bacteria, *Shigella flexneri* and *Streptococcus progenies* (Ferro *et al.*, 2003). Specific plant metabolites such as anthraquinones (Garcia-Sosa *et al.*, 2006; Dabai *et al.*, 2007) and saponins (Reynolds and Dewick, 1999) have been proposed to be responsible for the antimicrobial activity of the aloe leaf gel. Acemannan, a polysaccharide component from whole plant material, has been proposed to show indirect antimicrobial activity through its ability to stimulate phagocytic leukocytes (Pugh *et al.*, 2001). Wang *et al.* (1998) have reported on the effect of the anthraquinone aloe-emodin on arylamine N-acetyl transferase activity in *Helicobacter pylori*, and hence its antimicrobial activity.

2.5 Compounds of *Aloe*

Aloe species have been known for their medicinal properties since the fourth century, and the leaf components have been credited as the active components possessing anti-bacterial, antifungal and antiviral activities amongst others (Grindlay and Reynolds, 1986; Groom and Reynolds, 1987; Reynolds and Dewick, 1999). The major components of the leaf consist of the gel from inner thin-walled parenchymatous cells and the yellow exudates obtained from the pericyclic cells. The gel consists mainly of polysaccharides (Fermentia *et al.*, 1999), of which the acetylated mannose sugar is the major bioactive component (Manna and McAnnelley, 1993). The exudates of *Aloe* species consist of phenolic compounds, mainly

chromones and anthrones (Zonta *et al.*, 1995; Okamura *et al.*, 1996; Viljoen and Van Wyk, 1996; Kuzuya *et al.*, 2001) of which an anthrone C-glucoside, barbaloin (**12**), is the major component in these plants (Zonta *et al.*, 1995). Other phenolic compounds identified in exudates from *Aloe* species include aloesin (**13**), aloenin (**14**), aloeresin (**15**) and plicataloside (**16**) (Van Der Bank *et al.*, 1995; Okamura *et al.*, 1996; Reynolds, 1996; Speranza *et al.*, 1986; Park *et al.*, 1998a; Viljoen *et al.*, 1998a; Van Heerden *et al.*, 2000). Some of the compounds, such as plicataloside (**16**), have been used for taxonomic surveys (Viljoen *et al.*, 1998b). The generic variation between different *Aloe* species has been studied on the basis of the phenolic constituents of their leaves (Van Der Bank *et al.*, 1995).



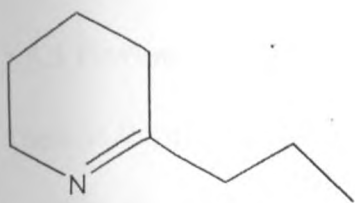


The chemistry of *Aloe* is widely studied, mostly due to its medicinal value and also for chemotaxonomic purposes. In the 1950s and 1960s, research was largely directed towards the physiologically active components, but it has now taken a wider scope (Reynolds, 1985). Compounds have been characterized from the dried exudates prepared for drug purposes but only from a few species (Reynolds, 1985). The leaves and roots of *Aloe* species elaborate many interesting secondary metabolites belonging to different classes of compounds including alkaloids, steroids, anthraquinones, pre-anthroquinones, anthrones, bianthaquinones, chromones, coumarins, pyrones and pyrans (Dagne, 1996). A few flavonoids have been reported from *Aloe* (Dagne *et al.*, 2000). A brief account of each class and a few representative compounds are given in the following sub-sections.

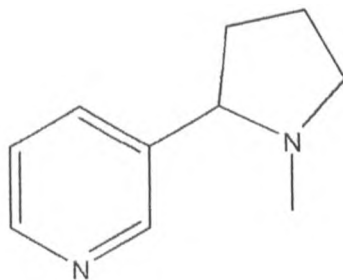
2.5.1 Alkaloids

The term alkaloid refers to naturally occurring basic compounds (apart from simple amines) derived from amino acids and N-heterocycles of the pyrrole, pyrimidine, purine type etc. (Torssel, 1983). Coniceine (**17**) (a piperidine alkaloid) has been reported to occur in six species of *Aloe* with very restricted distribution (Dring, *et al.*, 1984). Nicotine (**18**) and strychnine (**19**) also form part of these prominent alkaloids. Screening of 224 species of

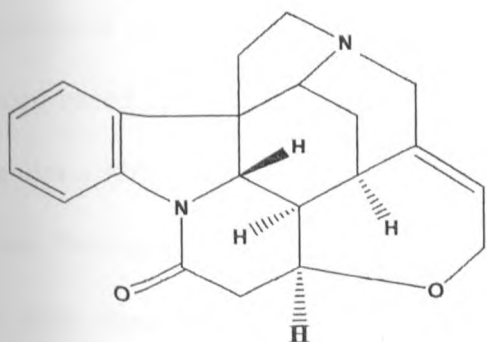
Aloe for alkaloids showed that 21% of the species were positive (Dagne *et al.*, 2000). The positive tests were due to the alkaloid; N-methyltyramine, O, N-dimethyltyramine, coniine (20) and coniceine (17). In view of the potential toxicity of alkaloids, it has been pointed out that it is important to screen for alkaloids prior to recommending any *Aloe* species in medicine (Dagne *et al.*, 2000).



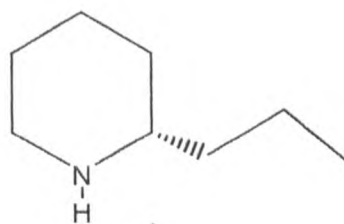
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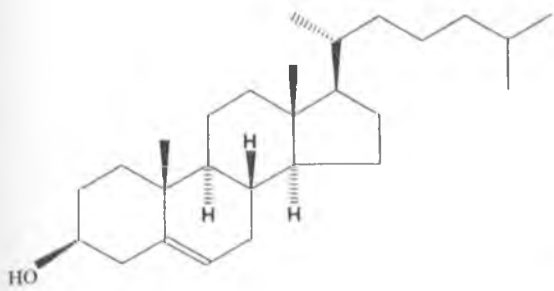
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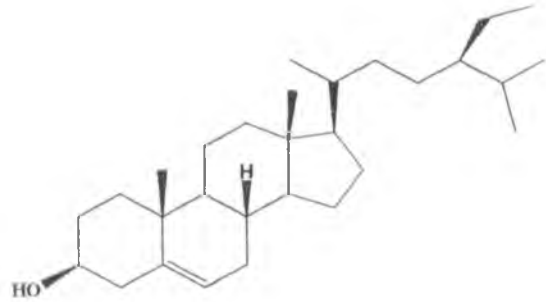
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2.5.2 Steroids

The steroids reported in *Aloe* are those that are commonly present in the plant kingdom. These include: cholesterol (21) and β -sitosterol (22). They have been claimed to be responsible for the anti-inflammatory activity of the gel (Davis, 1997).



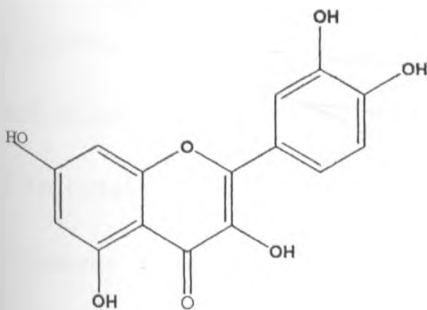
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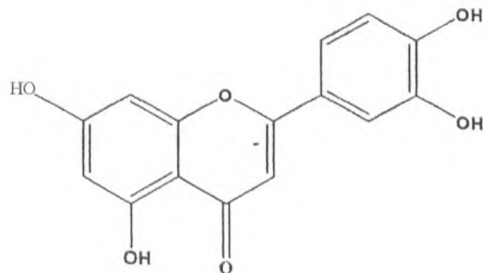
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2.5.3 Flavonoids

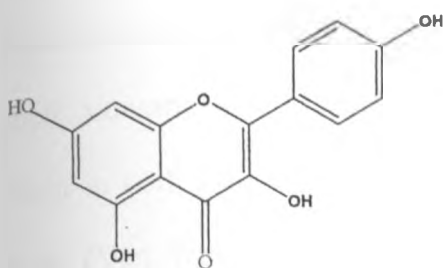
There is limited information on the occurrence of flavonoids in the genus *Aloe*. The reason could be due to the fact that most of the flavonoid-containing species are either difficult to obtain (grass-like *Aloes* which are Malagasy endemics) or they do not perform well under cultivation once transplanted from nature (Viljoen *et al.*, 1998a). Williams (1975) did broad-based screening for the flavonoids, quercetin (23), luteolin (24), kaempferol (25), and isovitexin (26). Eight out of eighteen species of *Aloe* screened contained trace amounts of these flavonoids. In a chemotaxonomic survey of flavonoids in *Aloe*, significant quantities of these compounds were detected in at least 31 species of the 380 species investigated (Viljoen *et al.*, 1998a).



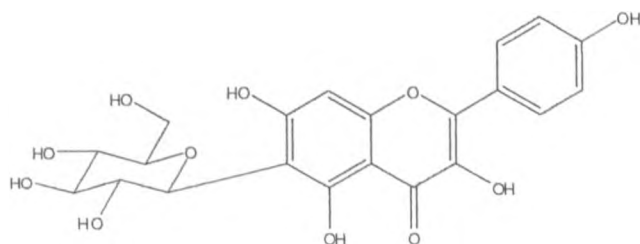
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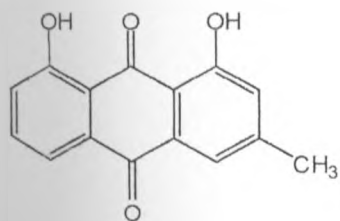
2.5.4 Anthraquinones

Anthraquinones are the largest group of quinones (Torsell, 1983). They have been used as mordant dyes, e.g. alizarin from *Rubia tinctorium*; as purgatives e.g. emodin (5) from *Rheum*, or *Rhamnus spp* (Torsell, 1983). Anthraquinones are widely spread in lower and higher plants and occur also in animal kingdom (Torsell, 1983). They are represented as glycosides in young plants (Torsell, 1983). Studies on fungal metabolites (Takeda *et al.*, 1973) showed that the colouring matter produced by *Penicillium islandicum* were generally anthraquinones.

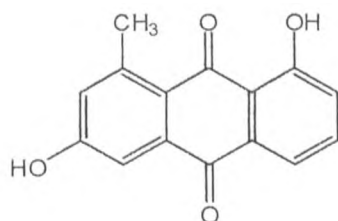
The taxon *Aloe* is widely associated with the presence of anthraquinone derivatives (Yenesew, 1991). The anthraquinones including anthrones found in *Aloe* species appear to have been derived through the polyketide pathway (Yagi, *et al.*, 1978), through cyclization of an octaketide chain (Manito, 1981). Aloe-emodin is the most widespread constituent of the leaves of *Aloe*, otherwise the leaves are known to contain anthraquinones in form of C-glycosides.

The anthraquinones found in *Aloe* are generally of two types, 1,8-dihydroxy-3-methylanthraquinones such as chrysophanol (**1**) and 1,6-dihydroxy-8-methylanthraquinone such aloesaponarin I (**3**) and II (**2**). Anthraquinone of the chrysophanol-type are known to occur in both shrine leaves and roots of *Aloe* but the aloesaponarin II-type are confined to roots (Van Wyk *et al.*, 1995). Other common anthraquinones include iridoskyrin (**27**), dianhydrorugulosin (**28**) and islandicin (**29**).

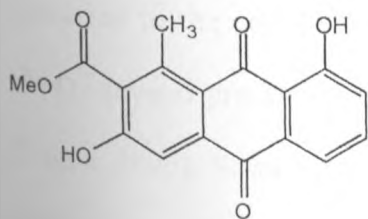
The wide occurrence of the otherwise rare 1-methyl-1,8-hydroxyanthraquinones, such as aloesaponarin I (**3**), aloesaponarin II (**2**) and the corresponding pre-anthraquinones aloesaponol I (**6**) and aloesaponol II (**32**) in the roots of *Aloe*, indicates that these compounds are characteristic constituents of the genus. Studies have also shown that these types of compounds are conspicuously absent from the roots of the genera *Kniphofia* and *Bulbin*, which have been closely associated with *Aloe* by some taxonomist (Dagne *et al.*, 1994).



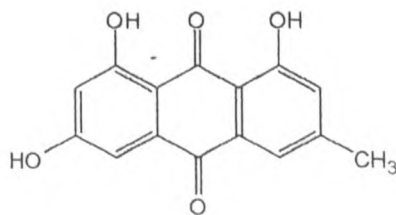
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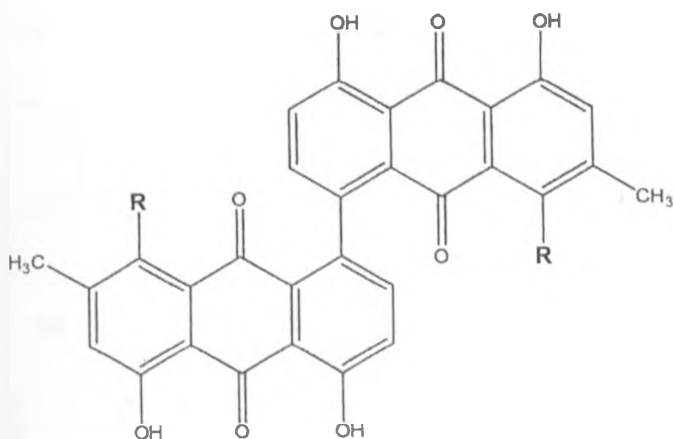
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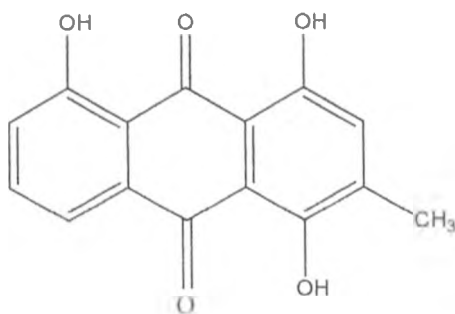


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Iridoskyrin, R - OH (27)

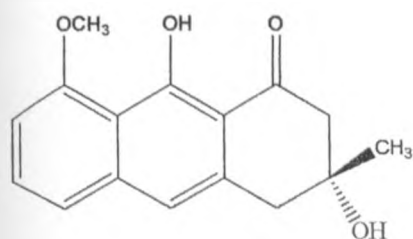
Dianhydrugulosin, R = H (28)



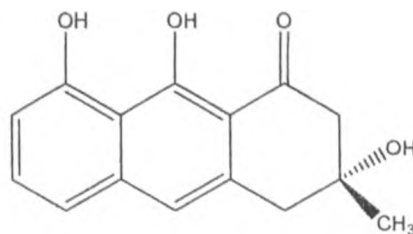
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2.5.5 Pre-anthraquinones

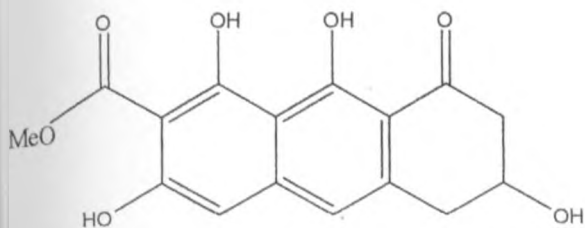
Pre-anthraquinones could be considered as precursors to anthraquinones and they are readily converted to the corresponding anthraquinones by treatment with a base (Yenesew *et al.*, 1994). Several pre-anthraquinones have been isolated from the subterranean parts of *Aloe* (Dagne, 1996). Some of these pre-anthraquinones are aloechryson (30), pre-chrysophanol (31) and aloesaponols I (6) and II (32).



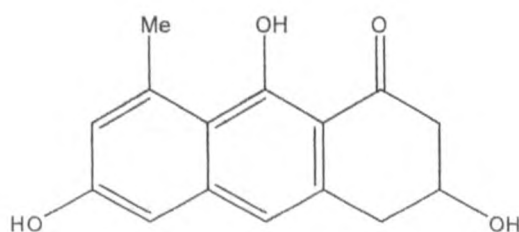
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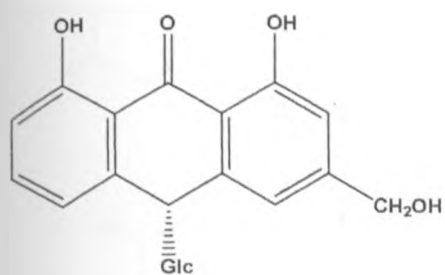


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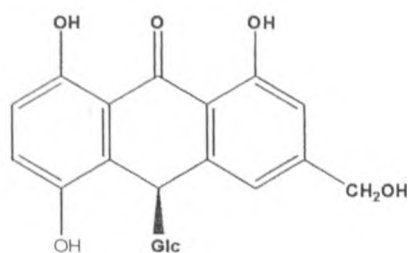
2.5.6 Anthrones

Anthrones are biosynthetic intermediates leading to the anthraquinone structure *via* simple oxidation. They are responsible for the bitter and purgative properties of aloe drugs (Dagne, 1996). The anthrones have been reported to be the most important of all classes of compound present in aloe (Dagne *et al.*, 2000). In *Aloe pubescence* and *Aloe jucunda*, it was found that the highest percentage of the anthrone barbaloin (**12**) occurs in the leaves near the plant apex and the lowest percentage in the older and lowest leaves (Groom and Reynolds, 1987). The study of *Aloe aborescens*, by Chauser-Volfson and Gutteman (1996), showed that barbaloin (**12**) is concentrated in the top of each leaf and the leaves near the apex contain much more barbaloin (**12**) than those at the lower parts of the branch. Previous studies have showed that barbaloin (**12**) could be a repellent to leaf-eating animals (it is logical to say that the highest content will be in young leaves and at the top of the leaves,

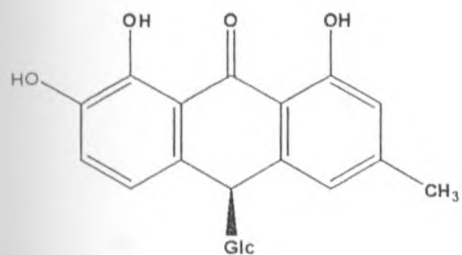
possibly as a defense strategy). Some of the other common anthrones include; aloin (33), 5-hydroxyaloin (34) and nataloin (35).



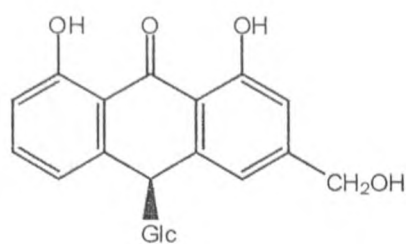
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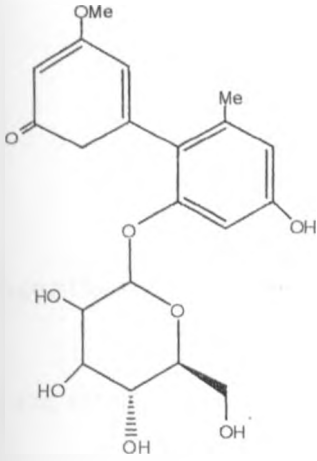


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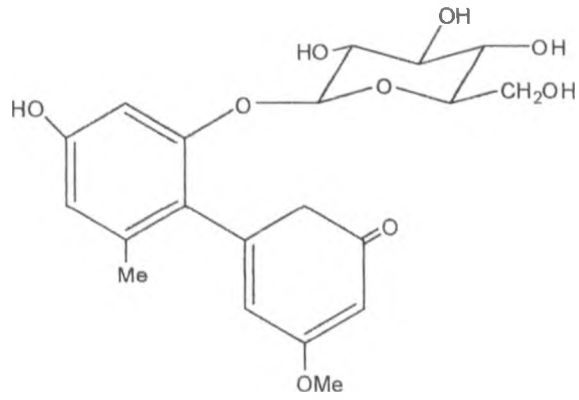
2.5.7 Pyrones

The pyrones isolated from aloe include the phenylpyrone derivatives aloenin A (36) and B (37) which are not frequently encountered in leaf exudate (Suga *et al.*, 1974). Aloenin aglycone and aloenin coumaroyl ester have been isolated from *Aloe nyeriensis* together with aloenin (Conner *et al.*, 1987). Aloenin is one of the major constituents of commercial Kenyan aloe (Speranza *et al.*, 1986). Other pyrones reported from *Aloe* include aloenin aglycone (38), aloenin acetal (39) dihydroisocoumarin glycoside (a coumarin from *Aloe*

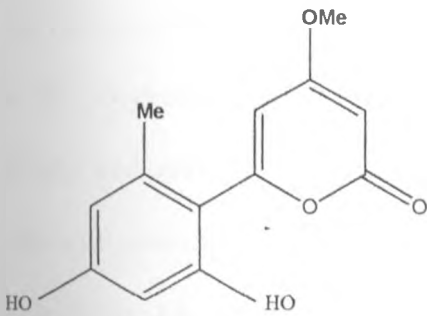
hildebrandtii) (40) (Veitch *et al.*, 1994), feralolide (41) (a coumarin from Cape aloë) (Speranza *et al.*, 1993).



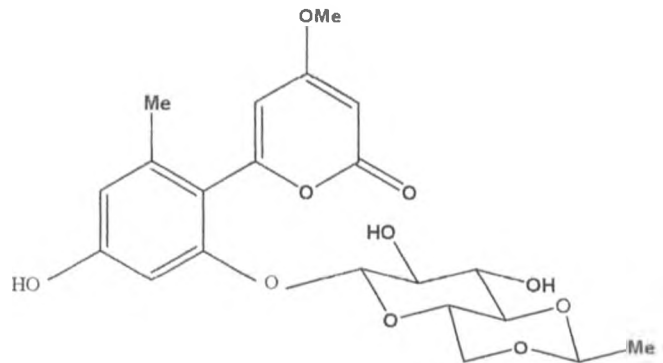
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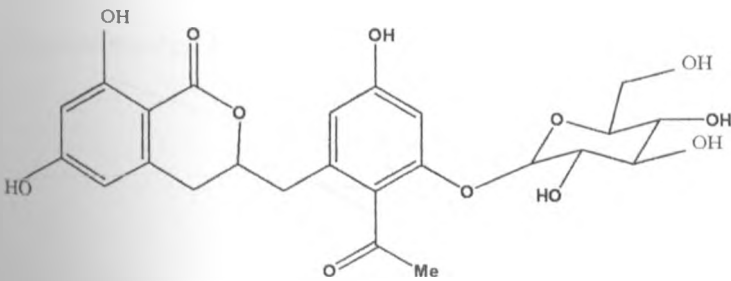
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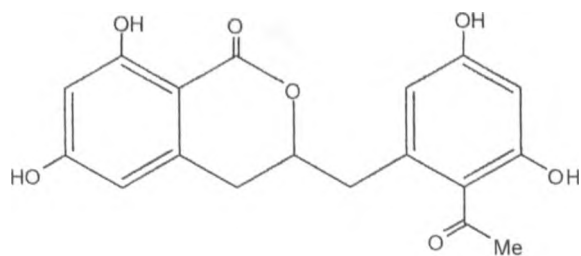
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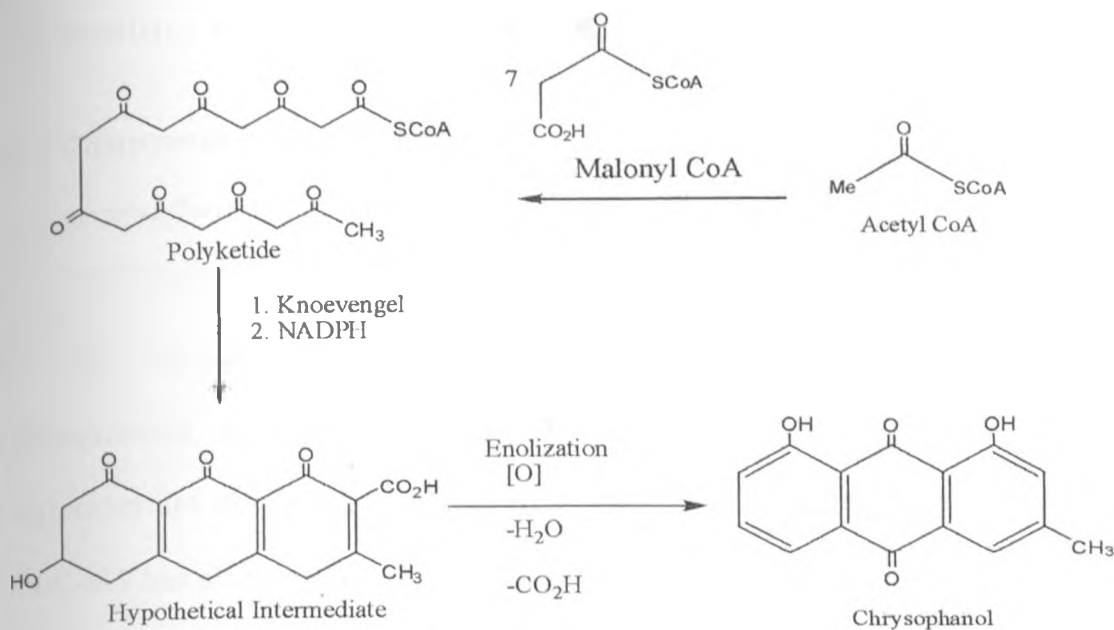


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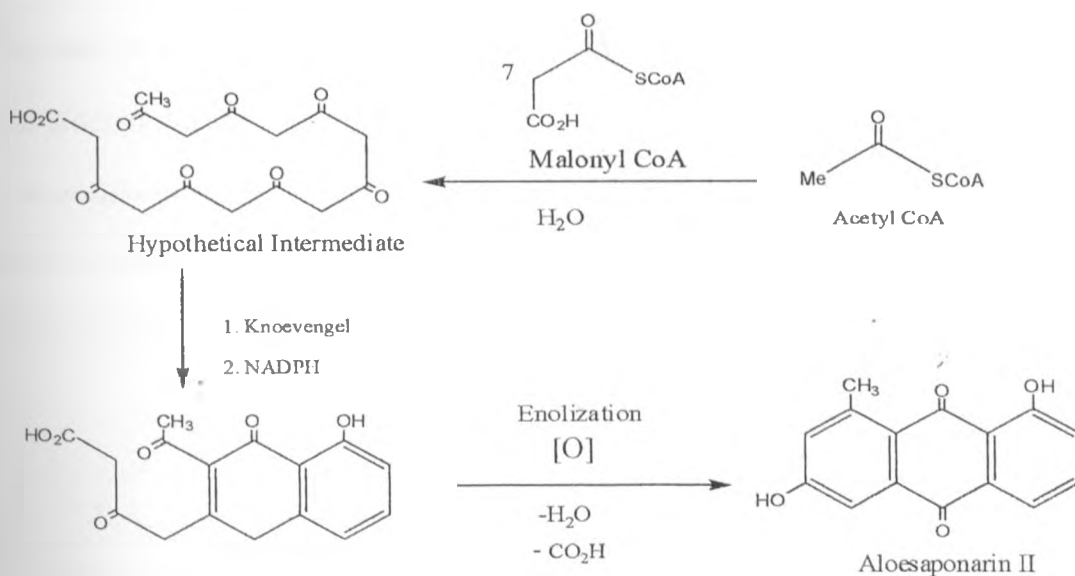
2.6 Biosynthesis of anthraquinones

One of the remarkable features of anthraquinones biosynthesis in higher plants is that they are derived from a variety of precursors and pathways (Leistner, 1985). There are two main pathways leading to anthraquinones in higher plants, the polyketide pathway (Van de Berg and Labadie, 1989) and the chorismate/ O-succinylbenzoic acid pathway (Leistner, 1985).

In the polyketide pathway, anthraquinones are biosynthesized from acetyl-CoA ($\text{CH}_3\text{CO-SCoA}$) and malonyl-CoA ($\text{HOOCCH}_2\text{CO-SCoA}$) via an octaketide chain. These types of anthraquinones often exhibit a characteristic substitution pattern, i.e. they are substituted in both rings (A and C of the anthraquinone). In some families, such as Rubiaceae, anthraquinone from species like *Morinda*, *Rubia*, *Galium* are considered to be formed via the chorismate/O-succinylbenzoic acid pathway (Ying-Shan *et al.*, 2002).



A: Biosynthesis of the 1,8-dihydroxy-3-methylanthraquinone, chrysophanol



B: Biosynthesis of the 1,6-dihydroxy-8-methylanthraquinone, Aloesaponarin II

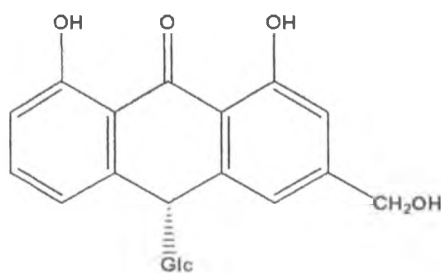
Scheme 2: Biosynthetic pathways leading to some anthraquinones in the genus *Aloe*.

2.7 Chemistry of *Aloe secundiflora*

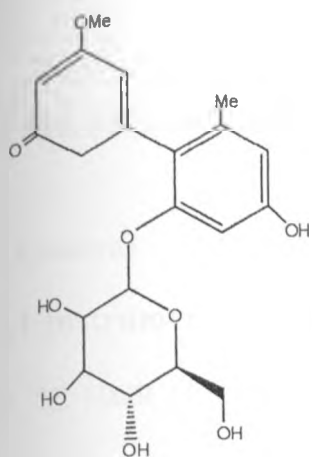
2.7.1 Compounds of *Aloe secundiflora*

Aloe secundiflora occurs in semi-arid areas and open grasslands of both Kenya and Tanzania. Phytochemical analysis of *Aloe secundiflora* var *secundiflora* (Aloaceae) using HPLC-MS revealed it comprises of a mixture of phenolic compounds mainly anthraquinones, anthrones, pyrones and chromones together with a low content of polysaccharides or aliphatic compounds (Carter, 1994). Analysis of *A. secundiflora* using HPLC-MS has also revealed the presence of phenolic compounds similar to those of other *Aloe* species (Okamura *et al.*, 1996; Reynolds, 1997; Park *et al.*, 1998b; Viljoen *et al.*, 1998a; Viljoen and Van Wyk, 2000; Kuzuya *et al.*, 2001).

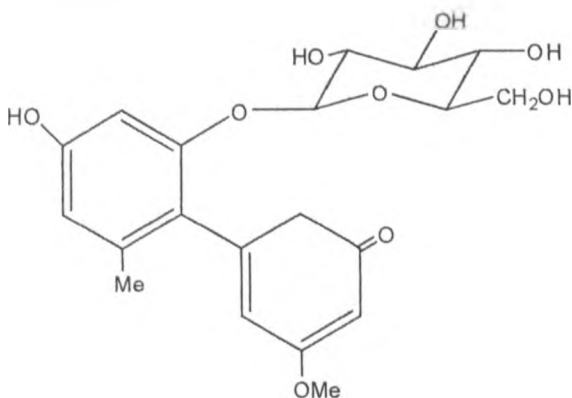
Leaf exudate of *A. secundiflora* has been reported to contain significant amounts of aloin (33) and aloenin A (36) and B (37). The high amounts of aloenin and aloin in the crude exudate of *A. secundiflora* could explain the medicinal principle behind the ethnomedical practice. Nevertheless other phenolic constituents could be working synergistically with aloin giving greater antimicrobial effect (Waihenya *et al.*, 2001).



(33)



(37)



(38)

2.8 Ethnomedical Uses of *Aloe secundiflora*

The leaf exudate of *Aloe secundiflora* has found ethnoveterinary use for the treatment of bacterial diseases and ectoparasites, and in the management of some viral diseases. In poultry, for example, the exudate has been extensively used as a prophylaxis for Newcastle disease virus, and as a therapeutic for fowl typhoid, coccidiosis and other enteric conditions. (*Ethnoveterinary Medicine in Kenya*, 1996). Studies have shown Aloenin B (37) to exhibit antibacterial activity against *Salmonella gallinarum* and other bacterial isolates (Waihenya *et al.*, 2001).

CHAPTER THREE

3.0 METHODOLOGY

3.1 General

3.1.1 Instrumentation

The ^{13}C NMR (125 or 50 MHz) and ^1H NMR (500 or 200 MHz) were run on Bruker or Varian-Mercury spectrometers using TMS as the internal standard. Homonuclear correlation spectroscopy (COSY), Nuclear Overhauser Enhancement Spectroscopy (NOESY), Heteronuclear Multiple Bond Correlation HMBC; ($^2J_{\text{CH}}$, $^3J_{\text{CH}}$) and Heteronuclear Multiple Quantum Coherence HMQC ($^1J_{\text{CH}}$) were acquired using standard Bruker software. UV/VIS spectra were recorded using a pre-Unicam SPS-150 spectrophotometer. EI-MS spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV) with perfluorkerosine as reference substance for EI-HRMS. The stems were ground using Willey mill.

3.1.2 Collection of Plant Material

Whole plant of *Aloe secundiflora* were collected from Ndaragwa district in central Kenya in September, 2008 and the parts were then separated into leaves, subterranean stems and roots. Voucher specimens were deposited at the herbarium, School of Biological Sciences, University of Nairobi. The plant was identified by Mr. Simon Mathenge of the University of Nairobi, School of Biological Science herbarium.

3.1.3 Chromatographic Conditions

Compounds were isolated by using various chromatographic techniques including column chromatography (oxalic acid impregnated Silica gel) and Sephadex LH-20. Preparative thin layer chromatography (PTLC) using silica gel (Merck, 70-230 mesh ASTM) as the adsorbent and crystallisation was used in the final purification. Analytical TLC was done on Merck pre-coated silica gel 60 F₂₅₄ plates, with UV (254, 366 nm) and iodine vapour to visualise spots.

The spectroscopic methods used to determine the molecular structures of pure compounds isolated were Ultra Violet spectroscopy (UV), NMR spectroscopy and mass spectroscopy (MS) techniques.

3.2 Extraction and Isolation of Compounds

The subterranean stems of *Aloe secundiflora* were air dried, ground into powder (2.2 kg) and then extracted by soaking in dichloromethane/methanol (1:1) in a 5L conical flask. The extraction was repeated three times, each extraction taking 24 hours. Removal of the solvent under reduced pressure yielded 135g of the crude extract. About 100g of the extract was subjected to column chromatography on oxalic acid impregnated silica gel (400 g) eluting with hexane containing increasing amounts of ethylacetate to afford 19 major fractions (A-S).

Fraction D of the column eluting with 5% hexane in ethylacetate was passed over Sephadex LH-20 column [$\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1)] and PTLC (30% ethyl acetate in hexane) which gave, 1,8-dihydroxy-3-methylantraquinone, trivial name chrysophanol (1, 35mg).

Fractions F-G of the column eluting with hexane containing 5% ethylacetate was passed over Sephadex LH-20 column [$\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1)] which gave aloesaponarin II (2, 35mg). Aloesaponol I (6, 30mg) was separated after subjecting combined fractions P-R (20% ethyl acetate in dichloromethane to Sephadex LH-20 [$\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1)]).

Further Sephadex LH-20 [$\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1)] and PTLC (30% ethyl acetate in hexane) separation of fraction N gave laccaic acid D- methyl ester (4, 20 mg) and emodin (5, 15 mg).

The combined fractions H-J eluting with 20% ethyl acetate in hexane were subjected to Sephadex LH-20 [$\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1)] and further purified using PTLC (30% ethyl acetate in hexane) which gave 8-hydroxy-2,7-dimethoxy-3-methylnaphthalene-1,4-dione (7, 25mg).

3.3 Physical and Spectroscopic Data for the isolated Compounds

Chrysophanol (1)

Yellowish-red crystals, melting point 195-197⁰C; UV (λ_{max} , MeOH): 270, 288 and 430 nm; ¹H NMR (CDCl_3 , 200 MHz): δ_{H} 6.85 (1H, *br s*, H-2), 7.55 (1H, *d*, $J = 1.2$ Hz, H-4), 7.73 (1H, *dd*,

$J = 7.5, 1.2$ Hz, H-5), 7.70 (1H, *t*, $J = 8.1$ Hz, H-6), 7.28 (1H, *dd*, $J = 7.2, 1.0$ Hz, H-7), 2.35 (3H, *s*, Me-3), 11.98 (OH-8, *s*), 12.08 (OH-1, *s*).

Aloesaponarin II (2)

Orange needles, melting point 264-265⁰C; UV (λ_{\max} , MeOH) 280, 310, 388, 410, 478 nm;

¹H NMR (acetone-d₆, 200 MHz): δ_{H} 7.57 (1H, *dd*, $J = 7.8, 1.0$ Hz, H-4), 7.42 (1H, *t*, $J = 7.8$ Hz, H-3), 7.46 (1H, *d*, $J = 3.0$ Hz, H-5), 7.10 (1H, *dd*, $J = 7.8, 1.0$ Hz, H-2), 6.87 (1H, *d*, $J = 2.6$ Hz, H-7) 2.62 (3H, *s*, Me-8), 9.27 (OH-6, *s*), 12.93 (OH-1, *s*); ¹³C NMR (acetone-d₆, 50 MHz): δ_{C} 145.96 (C-8), 137.53 (C-13/14), 135.45 (C-11), 133.19 (C-3), 124.80 (C-2), 124.34 (C-7), 118.62 (C-4), 112.69 (C-5), 104.163 (C-12), 162.22 (C-6), 163.0 (C-1), 182.69 (C-10), 190.13 (C-9), 24.03 (Me-8).

Aloesaponarin I (3)

Orange crystals, melting point 190-192⁰C; UV (λ_{\max} , MeOH) 216.5, 279.0, 411.0 nm; ¹H NMR (CDCl₃, 500 MHz): δ_{H} 7.62 (1H, *dd*, $J = 8.0, 7.2$ Hz, H-3), 7.80 (1H, *s*, H-5), 7.77 (1H, *t*, $J = 8.0$ Hz, H-4), 7.31 (1H, *dd*, $J = 8.4, 1.4$ Hz, H-2), 4.06 (3H, *s*, OMe-7), 3.95 (3H, *s*, CH₃-8), 10.39 (OH-6, *s*), 12.90 (OH-1, *s*); ¹³C NMR (CDCl₃, 50 MHz): δ_{C} 144.13 (C-14), 138.96 (C-13), 136.03 (C-12), 132.83 (C-11), 125.26 (C-3), 124.69 (C-4), 117.18 (C-5), 115.27 (C-2), 170.72 (C-7'), 163.47 (C-1), 162.68 (C-6), 182.39 (C-10), 189.80 (C-9), 53.42 (C-OMe), 22.07 (Me-7).

Laccaic acid D-methyl ester (4)

Red crystalline solid; UV (λ_{\max} , MeOH) 431, 287, 221 nm; ^1H NMR (CDCl_3 + acetone- d_6 , 200 MHz): δ_{H} 7.72 (1H, *s*, H-5), 7.20 (1H, *d*, $J = 2.4$ Hz, H-4), 6.67 (1H, *d*, $J = 2.4$ Hz, H-2), 3.91 (3H, *s*, OMe-7), 2.70 (3H, *s*, Me-8), 13.16 (OH-1, *s*), ^{13}C NMR (CDCl_3 +acetone- d_6 50MHz): * (C-9), * (C-10), δ_{C} 167.97 (C-9'), 165.23 (C-1), 163.95 (C-6), 158.57 (C-3), * (C-13), 142.40 (C-14), 137.00 (C-12), 134.00 (C-8), 126.00 (C-7), 112.71 (C-11), 108.88 (C-4), 108.88 (C-5), 107.56 (C-2), 52.43 (OMe-9'), 20.13 (Me-8).

Emodin (5)

Yellowish red crystals; UV spectrum (λ_{\max} 431, 522 nm); ^1H NMR (CDCl_3 , 200 MHz): δ_{H} 13.01 (OH-8, *s*) 12.71 (OH-1, *s*), 7.82 (1H, *d*, $J = 7.5, 1.2$ Hz, H-5), 7.63 (1H, *d*, $J = 1.2$ Hz, H-4), 7.30 (1H, *d*, $J = 7.2, 1.0$ Hz, H-7), 7.11(1H, *s*, H-2).

Aloesaponol I (6)

Colourless needles, blue fluorescence under UV light (366 nm), melting point 235 $^{\circ}$ C; UV (λ_{\max} , MeOH) 280, 281, 377 nm; ^1H NMR (acetone- d_6 , 500 MHz): δ_{H} 6.98 (1H, *s*, H-10) 6.91 (1H, *s*, H-5), 2.78 (1H, *dd*, $J = 6.9, 17.6$ Hz), 2.88 (1H, *d*, $J = 17.7$ Hz,) 2.99 (1H, *dd*, $J = 3.5, 15.1$ Hz) 3.21 (1H, *dd*, $J = 3.3, 15.7$ Hz) , 3.91 (3H, *s*, OMe-7), 2.80 (3H, *s*, H-8) 9.57 (OH, *s*, H-6), 15.38 (OH, *s*, H-9); ^{13}C NMR (acetone- d_6 , 125 MHz): δ_{C} 169.84 (C-9), 143.00 (C-6), 139.68 (C-7), 118.27 (C-5), 112.27 (C-8), 109.50 (C-10), 66.83 (C-3), 48.38 (CH $_2$ -2), 39.7 (CH $_2$ -4), 53.07 (OMe-1'), 21.84 (Me-8), 205.07 (C-1'), 206.81 (C-1).

8-Hydroxy-2,7-dimethoxy-3-methylnaphthalene-1,4-dione (7)

Orange crystals, melting point of 125-128⁰C; HRMS [M+1]⁺ at *m/z* 248.2000, EIMS (70 Ev): M⁺ (100, C₁₃H₁₂O₅), 233 (45, [M-Me]⁺), 230 (18), 205 (38), 177 (17), 151 (18); UV (λ_{max} , MeOH) 280, 336, 390, 430, 511 nm; ¹H NMR (CDCl₃, 300 MHz): δ_H 12.15 (OH-8, s), 7.57 (1H, *d*, *J* = 8.4 Hz, H-5), 7.31 (1H, *d*, *J* = 8.4 Hz, H-6), 4.08 (3H, *s*, H-2) 3.97 (3H, *s*, H-7), 2.03 (3H, *s*, H-3); ¹³C NMR (CDCl₃, 75 MHz): δ_C 117.3 (C-6), 121.2 (C-5), 125.5 (C-10), 135.6 (C-3), 153.7 (C-8), 155.4 (C-7), 116.1 (C-9), 159.00 (C-2), 185.1 (C-4), 188.4 (C-1), 62.1 (OMe-2), 57.4 (OMe-7), 10.2 (Me-3).

3.4 *In vitro* Antiplasmodial Activity Assay.

In vitro antiplasmodial activities were done at the Kenya Medical Research Institute (KEMRI). The crude extract and pure compounds were assayed using a non-radioactive assay technique with modifications to determine 50% growth inhibition of cultured parasites. Two different strains, chloroquine-sensitive Sierra Leone I (D6) and chloroquine-resistant Indochina I (W2), of *P. falciparum* were grown.

The culture-adapted *P. falciparum* were added on to the plate containing dose range of drugs and incubated in a gas mixture (5 % CO₂, 5 % O₂, and 90 % N₂) at 37 °C for 72 hours and frozen at -80 °C. After thawing, lysis buffer containing SYBR green I (1 x final concentration) was added directly to the plates and gently mixed by using the Beckman Coulter Biomek 2000 automated laboratory workstation. The plates were then incubated for 5-15 minutes at room temperature in the dark. Parasite growth inhibition was quantified by measuring the per-well relative fluorescence units (RFU) of SYBR green I dye using the Tecan Genios Plus with

excitation and emission wavelengths of 485 nm and 535 nm, respectively and with the gain set at 60. Differential counts of relative fluorescence units (RFUs) were used in calculating IC₅₀ values for each drug using Prism 4.0 software for Windows. A minimum of three separate determinations was carried out for each sample. Replicates had narrow data ranges hence presented as mean ± standard deviation (Yenesev, 2009).

3.4.3 Antimicrobial testing

The microbial assays were carried out at the Kenya Medical Research Institute (KEMRI) according to Biyiti *et al* (1988), with variety of micro-organisms. Bacterial and fungal strains were used to carry out the microbial assays.

Pathogenic bacteria

Gram-positive bacteria: *Staphylococcus aureus*.

Gram-negative bacteria: *Escherichia coli* and *Pseudomonas eruginosa*.

Pathogenic fungi

Yeast: *Candida albicans*.

Dermatophytes: *Cryptococcus neoformans*, *Trichophyton mentagrophytes* and *Microsporum gypsum*.

Weighed aliquots of each dry sample was dissolved in acetone and placed on a 6mm Whatman paper disc to give different concentrations. Discs of Chloramphenicol (standard) were also used for comparison. The discs were air dried and placed on agar plates seeded with micro-organisms and, after incubation the zones of inhibition were measured.

The incubation conditions depend on the micro-organism tested. Bacteria were incubated for 24 hrs at 37°C on Mueller Hinton agar. Fungi were grown on Sabourand dextrose agar for 3 days at 37°C (for yeast) and 24 hrs at 28°C (for dermatophytes).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

4.1 Preliminary Test

The extract of the subterranean stems of *Aloe secundiflora* was analysed by thin layer chromatography (TLC) and showed the presence of several coloured components. Most of the spots on TLC changed colour from yellow to orange. From biogenetic point of view, these compounds were presumed to be anthraquinone derivatives. The major components were isolated and characterised. The crude extracts and some of the isolated pure compounds were tested for biological activity.

4.2 Characterization of the Isolated Compounds

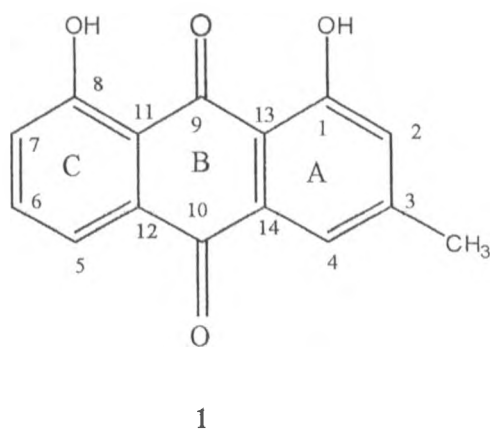
The ground and dried stems of *A. secundiflora* were extracted using dichloromethane and methanol (1.1) mixture. The extract was chromatographed over silica gel yielding seven compounds. The characterization of these compounds is discussed below.

4.2.1 Chrysophanol (1)

Compound 1 was isolated as yellowish red crystals with melting point of 195-197 °C and an R_f value of 0.50 (20% EtOAc in hexane). The ^1H NMR (200 MHz) spectrum of this compound showed two downfield shifted hydroxyl protons at δ_{H} 12.72 and δ_{H} 12.31, which is typical of a 1,8-dihydroxyanthraquinone skeleton where the de-shielding results from chelation of hydroxyl protons with carbonyl. Three mutually coupled signals at δ_{H} 7.73 (*dd*, $J = 7.5, 1.2$ Hz), δ_{H} 7.70 (*t*, $J = 8.1$ Hz), and δ_{H} 7.28 (*dd*, $J = 7.2, 1.0$ Hz) were assigned to protons at C-5, C-6 and C-7 of ring C respectively of a 1,8-dihydroxyanthraquinone. In ring

A, the proton at C-4 appeared at δ_H 7.55 (*d*, $J = 1.2$ Hz), while H-2 resonate as a broad singlet at δ_H 6.85. The biogenetically expected methyl group at C-3 resonated at δ_H 2.35.

Based on these data, compound **1** was identified as 1,8-dihydroxy-3-methylantraquinone, trivial name chrysophanol (**1**). The identity of this compound was confirmed by TLC comparisons with an authentic sample of chrysophanol (Achieng', 2009).



This compound is widely distributed in plants and is common in the genus *Aloe* (Van wyk *et al.*, 1995). It is commonly used as an antimicrobial and as a purgative agent (Thomson, 1987).

4.2.2 Aloesaponarin II (2)

Compound **2** was isolated as orange needles with a melting point of 264-265°C and with an R_f value of 0.60 (30% EtOAc in hexane). The UV spectrum (λ_{max} 280, 310, 388, 410, 478 nm) showed a characteristic absorption bands for an anthraquinone (Dagne *et al.*, 1994). The 1H NMR spectrum (200 MHz) of compound **2** showed mono-substituted (OH at C-1) ring A with the three aromatic protons appearing at δ_H 7,10 *dd* (1H, $J = 7.8, 1.0$ Hz for H-

2), δ_{H} 7.42 *t* ($J = 7.53$ Hz for H-3) δ_{H} 7.57 *dd* (1H, $J = 7.8, 1.0$ Hz for H-4). In ring C the presence of two *meta*-coupled protons at δ_{H} 7.46 *d* (1H, 2.6 Hz for H-5) and δ_{H} 6.87 *d* (1H, $J = 3.0$ Hz for H-7) suggests the presence of substitution at C-6 and C-8 on a 9,10-anthraquinone skeleton. These substituents are a methyl (δ_{H} 2.62 at C-8) and hydroxyl (δ_{H} 9.27 at C-6). In the ^{13}C NMR spectrum, the presence of two carbonyl carbons at C-9 (δ_{C} 190.13) and C-10 (δ_{C} 182.69) and two oxygenated aromatic carbon atoms at C-1 (δ_{C} 162.22) and C-6 (δ_{C} 161.75) is consistent with a 1,6-dihydroxy-9,10-anthraquinone skeleton.

In agreement with this structure, the ^1H NMR spectrum of compound 2 (Table 4.1) showed only one chelated hydroxyl group at δ_{H} 12.93 ppm indicating that the second hydroxyl group is not chelated. The methyl group at C-8 is deshielded at δ_{H} 2.62 which is due to the adjacent carbonyl at C-9. Therefore, the structure of compound 2 was characterized as 1,6-dihydroxy-8-methylanthracene-9, 10-dione (trivial name aloesaponarin II).

This compound has been reported from the roots of several *Aloe* species (Van Wyk *et al.*, 1995). The ^{13}C NMR data of this compound is reported here (Table 4.1) for the first time.

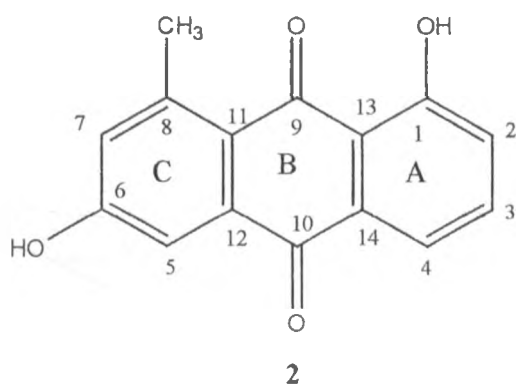


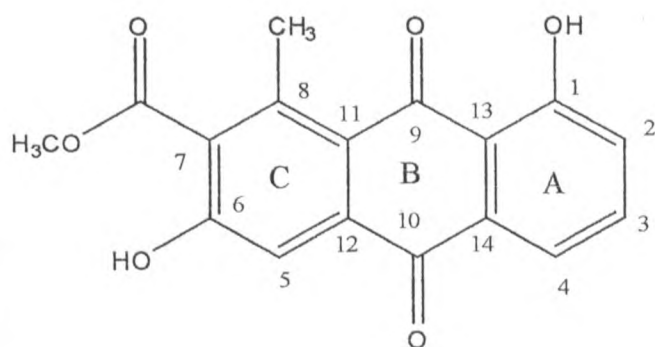
Table 4.1: ^1H (200 MHz) and ^{13}C (50 MHz) NMR data for compound **2** (acetone- d_6).

POSITION	δ_{H} <i>m</i> (J in Hz)	δ_{C}
1		162.22
2	7.10 <i>dd</i> (7.8, 1.0)	124.80
3	7.42 <i>t</i> (7.8)	133.19
4	7.57 <i>dd</i> (7.8, 1.0)	118.62
5	7.46 <i>d</i> (3.0)	112.69,
6		161.75
7	6.87 <i>d</i> (2.6)	124.34
8		145.96
9		190.13
10		182.69
11		135.45
12		104.16
13		137.53
14		137.53
CH ₃	2.62 <i>s</i>	24.03
6-OH	9.27 <i>s</i>	
1-OH	12.93 <i>s</i>	

4.2.3 Aloesaponarin I (**3**)

Compound **3** was isolated as orange crystals with a melting point of 190-192 $^{\circ}\text{C}$ and with an R_{f} value of 0.50 (10% acetone in dichloromethane). The UV spectrum showed characteristic absorption bands of anthraquinone chromophore (λ_{max} 217, 279, 411 nm) (Dagne *et al.*, 1994). Accordingly the ^1H NMR spectrum (200 MHz) of compound **3** (Table 4.2) showed

one chelated hydroxyl group (δ_{H} 12.91), one free hydroxyl group (δ_{H} 10.41) as well as the corresponding oxygenated carbon atoms (δ_{C} 163.47 and δ_{C} 162.68) respectively. The presence of an aromatic methyl group (δ_{H} 2.95, δ_{C} 22.07) suggests a 1,6-dihydroxy-8-methylanthraquinone skeleton as in compound 2. The ^1H NMR spectrum further showed the presence of four aromatic protons at δ_{H} 7.31 *dd* (1H, $J = 8.4, 1.4$ Hz), δ_{H} 7.62 *t* (1H, $J = 8.0$ Hz), δ_{H} 7.77 *dd* (1H, $J = 8.0, 1.2$ Hz), corresponding to protons at C-2, C-3 and C-4 respectively of ring A. The fourth aromatic proton at δ_{H} 7.80 was assigned to H-5 of a tri-substituted ring C. The substituent at C-7 was identified as a methyl ester from the methoxy signal which appeared at (δ_{H} 4.06; δ_{C} 53.42) and δ_{C} 170.72 for the ester carbonyl. Therefore, the structure of compound 3 was identified as 3,8-dihydroxy-1-methyl-2-anthraquinone methyl ester (trivial name aloesaponarin I). This compound is also a typical *Aloe* root metabolite (Van Wyk *et al.*, 1995).



3

Table 4.2: ^1H (500 MHz) and ^{13}C (50 MHz) NMR data for compound **3** (CDCl_3)

POSITION	δ_{H} <i>m</i> (<i>J</i> in Hz)	δ_{C}
1		163.47
2	7.31 <i>dd</i> (8.4, 1.4)	115.27
3	7.62 <i>t</i> (8.0)	125.26
4	7.77 <i>dd</i> (8.0, 1.2)	119.18
5	7.80 <i>s</i>	124.69
6		162.68
7		170.72
11		132.83
12		136.03
13		138.96
14		148.13
9		189.80
10		182.39
9'-OCH ₃	4.06 <i>s</i>	53.42
8-CH ₃	2.95 <i>s</i>	22.07
6-OH	10.41 <i>s</i>	
1-OH	12.91 <i>s</i>	

4.2.4 Laccaic acid D-methyl ester (**4**)

Compound **4** was isolated as a red crystalline solid. The UV spectrum (λ_{max} 431, 287, 221 nm) suggested an anthraquinone skeleton (Dagne *et al.*, 1994). The ^1H NMR spectrum (200 MHz) of **4** showed a de-shielded singlet proton at δ_{H} 13.16 ppm, due to the hydrogen-bonded proton of the hydroxyl group at C-1 of a 1,6-dihydroxy-8-methyl-9,10-anthraquinone. Evidence of a substituent at C-8 was deduced from the presence of a methyl signal (δ_{H} 2.70; δ_{C} 20.13). The presence of a methyl ester at C-7 was also evident from a

methoxy signal at (δ_{H} 3.91; δ_{C} 52.43) and δ_{C} 167.97 for ester carbonyl. In fact ring C is identical to that of compound 3 where the proton singlet at δ_{H} 7.72 was due to H-5. In ring A the presence of *meta*-coupled protons at δ_{H} 6.67 *d* ($J = 2.4$ Hz for H-2) and δ_{H} 7.20 *d* ($J = 2.4$ Hz for H-4) is consistent with the presence of a hydroxyl group at C-3. The presence of three hydroxyl group substituents in this compound is supported by the presence of ^{13}C peaks at (δ_{C} 158.57, δ_{C} 163.95 and δ_{C} 165.24 for C-3, C-6 and C-1, respectively).

Thus compound 4 was identified as 1,3,6-trihydroxy-8-methyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid methyl ester (trivial name laccaic acid D-methyl ester).

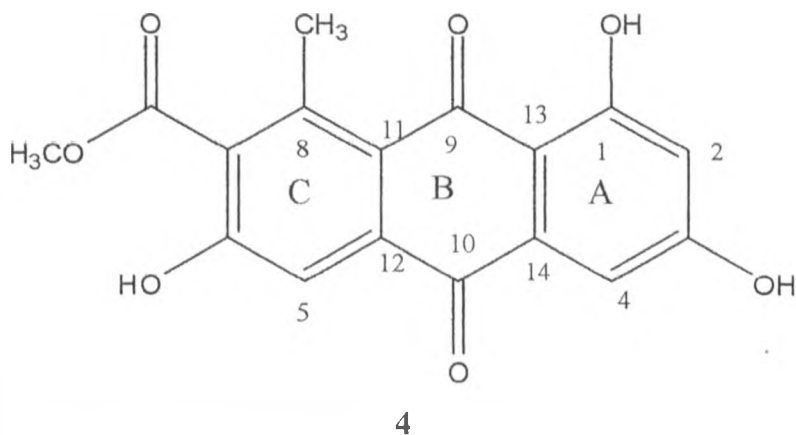


Table 4.3: ^1H (200 MHz), ^{13}C (50 MHz) NMR data for compound 4 ($\text{CDCl}_3 + \text{acetone } d_6$)

POSITION	δ_{H} <i>m</i> (<i>J</i> in Hz)	δ_{C}
1		165.24
2	6.67 <i>d</i> (2.4)	107.56
3		158.57
4	7.20 <i>d</i> (2.4)	108.90
5	7.72 <i>s</i>	108.90
6		163.95
7		126.00
8		134.00
9		167.97
9		*
10		*
11		112.71
12		137.00
13		*
14		142.40
7-OCH ₃	3.91 <i>s</i>	52.43
8-CH ₃	2.70 <i>s</i>	20.13
1-OH	13.16 <i>s</i>	

* Not observed

4.2.5 Emodin (5)

Compound **5** was isolated as yellowish red crystals. The UV spectrum (λ_{max} 431, 522 nm) again suggests an anthraquinone skeleton (Dagne *et al.*, 1994). This compound showed two downfield shifted hydroxyl protons at δ_{H} 13.01 ppm and δ_{H} 12.71, which is consistent with a 1,8-dihydroxyanthraquinone skeleton where the de-shielding results from chelation. In fact the ^1H NMR data for this compound is consistent with a 1,6,8-trihydroxy-3-methyl-9,10-anthraquinone (trivial name emodin). Thus, the aromatic protons at C-5 and C-7 resonated at δ_{H} 7.63 *d* ($J = 1.2$ Hz) and δ_{H} 7.30 *d* ($J = 7.2, 1.0$ Hz), respectively. The proton at C-4 appeared at δ_{H} 7.82 *d* ($J = 7.5, 1.2$ Hz), while H-2 resonates as a broad singlet at δ_{H} 7.11. The methyl group at C-3 resonated at δ_{H} 2.70. A summary of the NMR data is given in table 4.4 below.

Emodin (**5**) is widely distributed in plants as well as fungi. However, this appears to be the first report on its occurrence in the genus *Aloe*.

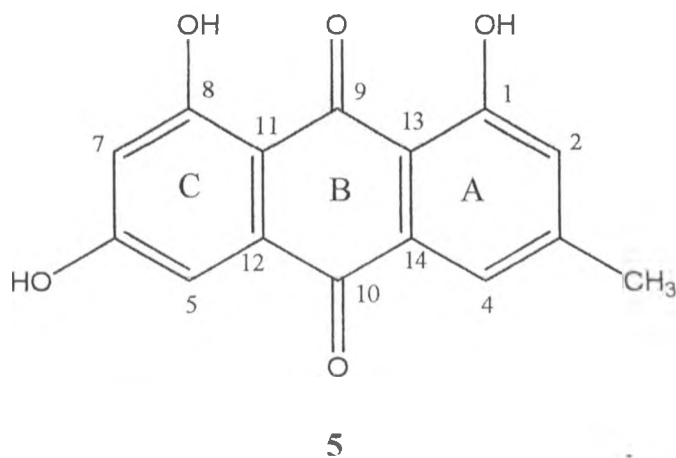


Table 4.4: ^1H NMR data for compound **5** (CDCl_3 , 200 MHz).

POSITION	δ_{H} <i>m</i> (<i>J</i> in Hz)
2	7.11 <i>s</i>
4	7.82 <i>d</i> (7.5, 1.2)
5	7.63 <i>d</i> (1.2)
7	7.30 <i>d</i> (7.2, 1.0)
3- CH_3	2.70 <i>s</i>
1-OH	12.71 <i>s</i>
8-OH	13.01 <i>s</i>

4.2.6 Aloesaponol I (**6**)

Compound **6** was isolated as colourless needles with a melting point of 235-237°C and with an R_f value of 0.40 (30% EtOAc in hexane). The colourless spot on TLC showed a strong blue fluorescence under UV light (366 nm). The UV spectrum of **6** (λ_{max} 280, 281, 377 nm) is typical of pre-anthraquinones (Yagi *et al.*, 1974). The ^1H NMR (500 MHz) spectrum showed a strongly chelated hydroxyl group (δ_{H} 15.36) at C-9 (δ_{C} 169.84), a hydroxyl group at C-3 (δ_{C} 66.83) and a carbonyl at C-1 (δ_{C} 206.81) of ring A. Accordingly, the oxymethine at C-3 appeared as a multiplet centered at δ_{H} 4.40. The methylene protons at C-2 and C-4 resonated at δ_{H} 2.76 *dd* ($J = 6.9, 17.6$ Hz), δ_{H} 2.93 *d* ($J = 17.7$ Hz), δ_{H} 2.99 *dd* ($J = 3.5, 15.1$ Hz) and δ_{H} 3.21 *dd* ($J = 3.3, 15.7$ Hz).

The NMR spectra further showed the presence of a methyl ester (δ_{H} 3.91, δ_{C} 53.07) and a methyl group (δ_{H} 2.80, δ_{C} 21.84) which were located at C-7 and C-8 respectively. Finally

two aromatic singlets at δ_H 6.90 and δ_H 6.98 can be assigned to H-5 and H-10 respectively. The structure of compound **6** was therefore characterized as 3,6,9-trihydroxy-8-methyl-1-oxo-5,6,7,8-tetrahydro-anthracene-2-carboxylic acid, trivial name aloesaponol I. However the absolute configuration of C-3 has not been determined here.

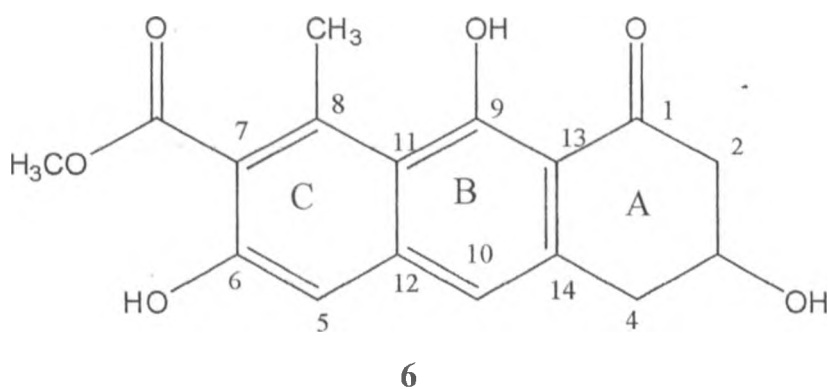


Table 4.5: ^1H (500 MHz) ^{13}C (125 MHz) NMR data for compound **6** (acetone d_6).

POSITION	δ_{H} <i>m</i> (J in Hz)	δ_{C}
CH ₂ - 2	2.78 <i>dd</i> (6.9, 17.6) 2.88 <i>dd</i> (17.7)	48.38
C-3	4.40 <i>m</i>	66.83
CH ₂ - 4	2.99 <i>dd</i> (3.5, 15.1) 3.21 <i>dd</i> (3.3, 15.7)	39.70
H-5	6.91 <i>s</i>	118.27
C-6		143.00
C-7		139.68
C-8		112.27
C-9		169.84
H-10	6.98 <i>s</i>	109.50
1'		205.07
1		206.81
1' - OCH ₃	3.91 <i>s</i>	53.07
8-CH ₃	2.80 <i>s</i>	21.84
6-OH	9.57 <i>s</i>	
9-OH	15.38 <i>s</i>	

4.2.7 Compound (7)

Compound **7** was isolated as orange crystals with a melting point of 125-128^oC and with an R_f of 0.5 (30% ethyl acetate in Hexane). The HRMS showed a molecular ion peak at m/z 248.2000 of compound **7** established the molecular formula to be $\text{C}_{13}\text{H}_{12}\text{O}_5$. In agreement with this, the ^{13}C NMR spectrum showed the presence of thirteen carbon atoms. The UV

spectrum (λ_{\max} 280, 336, 390, 430, 511 nm) suggests a naphthoquinone chromophore (Bringmann *et al.*, 2008). The presence of a chelated hydroxyl (δ_{H} 12.15; δ_{C} 153.7), two methoxyls (δ_{H} 4.08 and δ_{H} 3.97; δ_{C} 62.1 and δ_{C} 57.4), an aromatic methyl (δ_{H} 2.03; δ_{C} 10.2) were evident from the ^1H and ^{13}C NMR spectra (Table 4.6).

The ^1H NMR displayed *ortho*-coupled protons at δ_{H} 7.57 ($J = 8.4$ Hz) and δ_{H} 7.31 ($J = 8.4$ Hz) with the chelated hydroxyl being at C-8, these *ortho*-coupled protons correspond to H-5 (δ_{H} 7.57, $J = 8.4$ Hz) and H-6 (δ_{H} 7.31, $J = 8.4$ Hz). This also required the presence of a substituent at C-7, which in this case is one of the two methoxyls (δ_{H} 3.97; δ_{C} 57.4). The chemical shift values of C-7 (δ_{C} 155.4) and C-8 (δ_{C} 153.7) is in agreement with *ortho*-dioxygenated aromatic ring. The substitution pattern in ring B was confirmed from the HMBC spectrum (Table 4.6).

The remaining two substituents (OCH_3 and CH_3) must be in ring A at C-2 and C-3. HMBC correlation of the methyl protons (δ_{H} 2.03) with C-3 (δ_{C} 135.6), C-4 (δ_{C} 185.1) and C-2 (δ_{C} 159.0) is consistent with the placement of the methyl at C-3 and then the second methoxy at (δ_{H} 4.08; δ_{C} 62.1) should be at C-2. Therefore this compound is 8-hydroxy-2,7-dimethoxy-3-methylnaphthalene-1,4-dione (7).

This naphthaquinone derivative is a new compound. More over this is the first report on the occurrence of a naphthaquinone from the genus *Aloe*. This naphthaquinone appear to have been derived through the polyketide pathway (Scheme 3) as the anthraquinones of the genus *Aloe*.

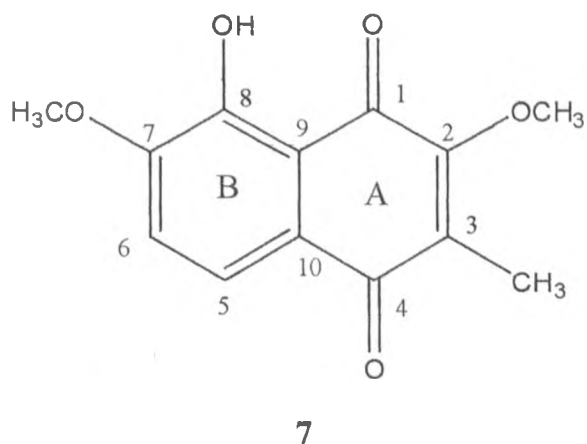
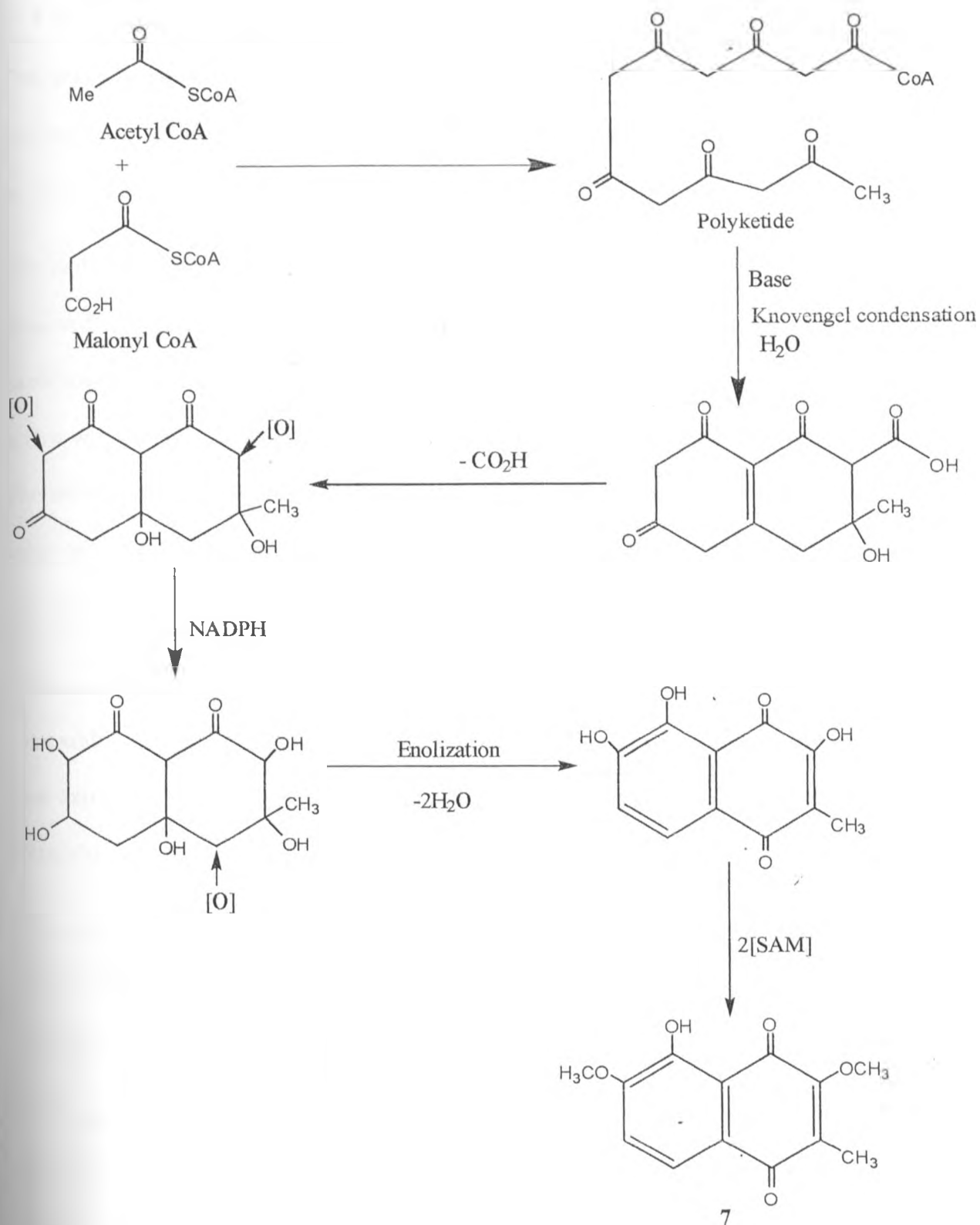


Table 4.6: ^1H (300 MHz), ^{13}C (75 MHz) NMR data for compound 7 (CDCl_3)

POSITION	δ_{H} <i>m</i> (<i>J</i> in Hz)	δ_{C}	HMBC
1		188.4	
2		159.0	
3		135.6	
4		185.1	
5	7.57, <i>d</i> (8.4 Hz)	121.2	C-6, C-7, C-10, C-9
6	7.31, <i>d</i> (8.4 Hz)	117.3	C-5, C-7, C-8, C-10
7		155.4	
8		153.7	
9		116.1	
10		125.5	
2-OCH ₃	4.08 <i>s</i>	62.1	C-2
7-OCH ₃	3.97 <i>s</i>	57.4	C-7
3-CH ₃	2.03 <i>s</i>	10.2	C-2, C-3, C-4
8-OH	12.15 <i>s</i>		C-8



Scheme 3: Proposed biogenesis of compound 7 through the polyketide pathway

4.3 ANTIPLASMODIAL ACTIVITIES

The crude extract of the stems of *Aloe secundiflora* showed significant antiplasmodial activity against the chloroquine-resistant (W2) and strains of *Plasmodium falciparum* with an IC_{50} value of $2.09 \pm 0.43 \mu\text{g/ml}$.

The isolated compounds from this extract were tested and antiplasmodial activities were observed for aloesaponarin I (**3**), aloesaponarin II (**2**), aloesaponol I (**6**) and the naphthoquinone derivative (**7**) (Table 4.7). Amongst these, aloesaponarin I (**3**) was the most active with an IC_{50} value of $0.92 \pm 0.07 \mu\text{g/ml}$ against the chloroquine-resistant (W2) strain. The monomeric anthraquinone, aloesaponarin II (**2**) only showed weak activity with an IC_{50} value of $14.39 \pm 0.83 \mu\text{g/ml}$ against the chloroquine-resistant (W2) strain of *P. falciparum*.

The pre-anthraquinone aloesaponol I (**6**) was moderately active against the chloroquine-resistant (W2) strain of *P. falciparum* with an IC_{50} value of $8.53 \pm 0.58 \mu\text{g/ml}$. This is comparable with an IC_{50} value of $8.5 \mu\text{g/ml}$ reported for the same compound from the crude stem extract of *Aloe kilifiensis* against the chloroquine-resistant (W2) strain of *P. falciparum* (Wanjohi, 2006).

The naphthaquinone derivative, 8-Hydroxy-2,7-dimethoxy-3-methylnaphthalene-1,4-dione (**7**) was marginally active with an IC_{50} value of $14.50 \pm 3.80 \mu\text{g/ml}$ against the chloroquine-resistant (W2) strain of *P. falciparum*.

The major compounds in *Aloe* species being anthraquinones and their related derivatives show these compounds could be responsible for the antiplasmodial activity of the crude extract. The antiplasmodial activities of some quinones have been reported earlier (Bringmann *et al.*, 2002).

This investigation therefore illustrates the potential of the monomeric anthraquinone aloesaponarin I (**3**) as a lead structure for the development of antimalarial drugs.

Table 4.7: *In vitro* IC₅₀ values against the chloroquine-resistant (W2) strain of *P.*

falciparum

Sample	Mean IC ₅₀ µg/ml ± Std deviation
Stem extract of <i>Aloe secundiflora</i>	2.09 ± 0.43
Aloesaponarin II (2)	14.39 ± 0.83
Aloesaponarin I (3)	0.92 ± 0.07
Aloesaponol I (6)	8.53 ± 0.58
8-Hydroxy-2,7-dimethoxy-3-methylnaphthalene-1,4-dione (7)	14.50 ± 3.80
Chloroquine	0.0108 ± 0.0013

4.4 ANTIBACTERIAL AND ANTIFUNGAL TESTS

Antibacterial and antifungal tests were also carried out for some of the isolated compounds against Gram positive-bacteria (*Staphylococcus aureus*), two Gram-negative bacteria (*Escherichia coli*, *Pseudomonas eruginosa*) and four species of fungus (*Candida albicans*, *Cryptococcus neoformans*, *Trichophyton mentagrophytes* and *Microsporum gypsum*).

Amongst the compounds tested, aloesaponarin II (2) exhibited the most pronounced antibacterial and antifungal activities; showing activity against *Staphylococcus aureus* (inhibition zone of 12mm) and *Cryptococcus neoformans* (inhibition zone of 7mm) with an MIC value of 18.8 µg/disc against both organisms. This was comparable to an MIC value of 25 µg/disc reported from the same compound isolated from *Aloe kilifiensis* against *Staphylococcus aureus* (Wanjohi, 2006).

Significant activity was also found for another monomeric anthraquinone; laccaic acid D-methyl ester (4) which showed moderate activity against *Staphylococcus aureus* (inhibition zone of 7mm), *Cryptococcus neoformans* (inhibition zone of 12mm) and *microsporum gypsum* (inhibition zone of 7mm) with an MIC value of 94 µg/disc.

The monomeric anthraquinone, aloesaponarin I (3) showed weak activities against; *Staphylococcus aureus* (inhibition zone of 9mm), *Trichophyton mentagrophytes* (inhibition zone of 7mm), *Microsporum gypsum* (inhibition zone of 11mm) with MIC value of 98 µg/disc.

No activity was reported for the pre-anthraquinone aloesaponol I (6) against any of the bacterial and fungal strains. Likewise, no significant activity was observed for any of the

isolated compounds against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas eruginosa*.

The roots of *Aloe* species are used traditionally to treat bacterial infections and therefore anthraquinones, especially aloesaponarin II, present in these plants could be responsible for the reported uses. Earlier studies have shown the potential of the crude extract of the leaves of *Aloe secundiflora* to inhibit the growth of *Candida albicans* among other fungal strains (Waihenya *et al.*, 2002).

Table 4.8: Compounds of *Aloe secundiflora* and their zone of inhibition against different bacterial and fungal strains.

Sample	MIC (in µg/disc)	Microorganisms						
		Sau	Eco	Pseu	Can	Cry	Tm	Mg
Aloesaponarin II (2)	18.8	20	0	0	0	15	10	0
Aloesaponarin I (3)	98	15	0	0	0	0	13	11
Laccaic acid D-methy ester (4)	94	7	0	0	0	12	0	7
Aloesaponol I (6)	0	0	0	0	0	0	0	0

Key: Micro-organisms: Bacterial strains; Sau = *Staphylococcus aureus* (ATCC 25922), Eco = *Escherichia coli*, Pseu = *Pseudomonas eruginosa* (ATCC 25923) and fungal strains; Can = *Candida albicans* (ATCC 90028), Cry = *Cryptococcus neoformans* (clinical isolate), Tm = *Trichophyton mentagrophytes* (clinical isolate) and Mg = *Microsporum gypsum* (clinical isolate).

5.0 CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

- ❖ The CH₂Cl₂-MeOH (1:1) extract of the stem of *Aloe secundiflora* showed significant antiplasmodial activity against the chloroquine-resistant (W2) strain of *Plasmodium falciparum* and thus its use as an antimalarial agent traditionally is justified.
- ❖ From the stem of *Aloe secundiflora*, a total of seven compounds were isolated and characterized as the monomeric anthraquinones chrysophanol (1), aloesaponarin II (2), aloesaponarin I (3), laccaic acid D-methyl ester (4), emodin (5); the pre-anthraquinone aloesaponol I (6) and the naphthoquinone derivative 8-hydroxy-2,7-dimethoxy-3-methylnaphthalene-1,4-dione (7). Among these, compound 7 is new and is the first naphthoquinone reported from the genus *Aloe*.
- ❖ Among the isolated compounds from *Aloe secundiflora*, aloesaponarin I (3), aloesaponarin II (2), aloesaponol I (6) and the naphthoquinone derivative (7) showed significant antiplasmodial activities with aloesaponarin I (3) being the most active with an IC₅₀ value of 0.92±0.07µg/ml against the chloroquine-resistant (W2) strain. This investigation was able to show the potential of the monomeric anthraquinone aloesaponarin I (3) as a lead structure for the development of antimalarial drugs.
- ❖ Antibacterial and antifungal tests were also carried out for some of the isolated compounds against three bacterial strains viz. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas eruginosa* and four fungal strains viz. *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton mentagrophytes* and *Microsporum gypsum*. Amongst those tested, aloesaponarin II (2) another *Aloe* root metabolite was the most

active showing activity against *Staphylococcus aureus* and *Cryptococcus neoformans* with an MIC value of 18.8 µg/disc for both organisms. No significant activity was reported for any of the isolated compounds against Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*.

- ❖ This investigation has shown the potential of the monomeric anthraquinone aloesaponarin II (**2**) as a lead structure for the development of antimicrobial drugs.

5.2 RECOMMENDATIONS

- ❖ Further studies are required to establish the *in vivo* antiplasmodial activity of the crude extract and the active ingredients of *Aloe secundiflora*.
- ❖ It is recommended the compounds isolated in this study to be tested against other strains of *Plasmodium falciparum*.
- ❖ Further phytochemical work on *Aloe secundiflora* and other *Aloe* species could lead to the isolation and characterization of more novel compounds with biological activities.

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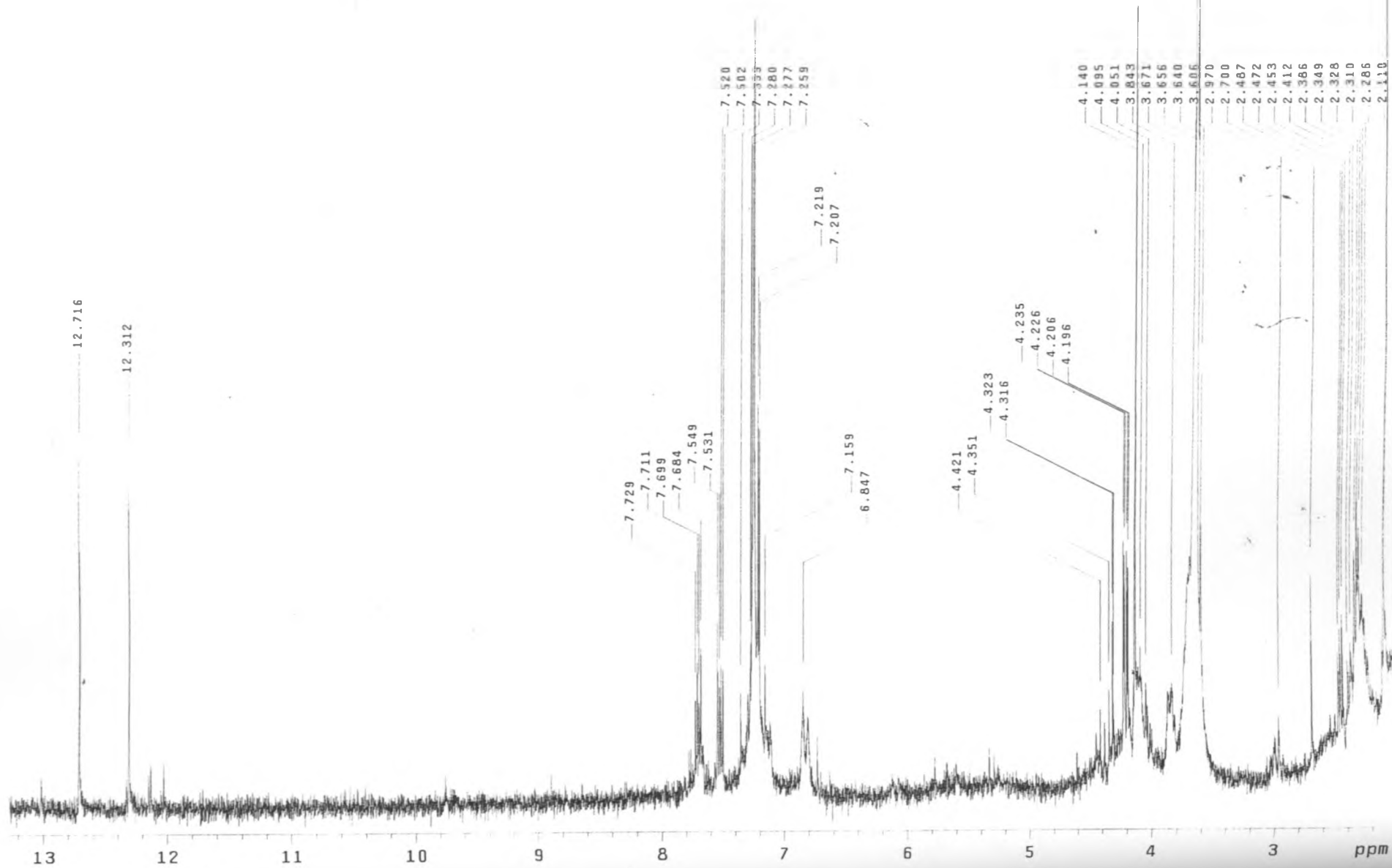
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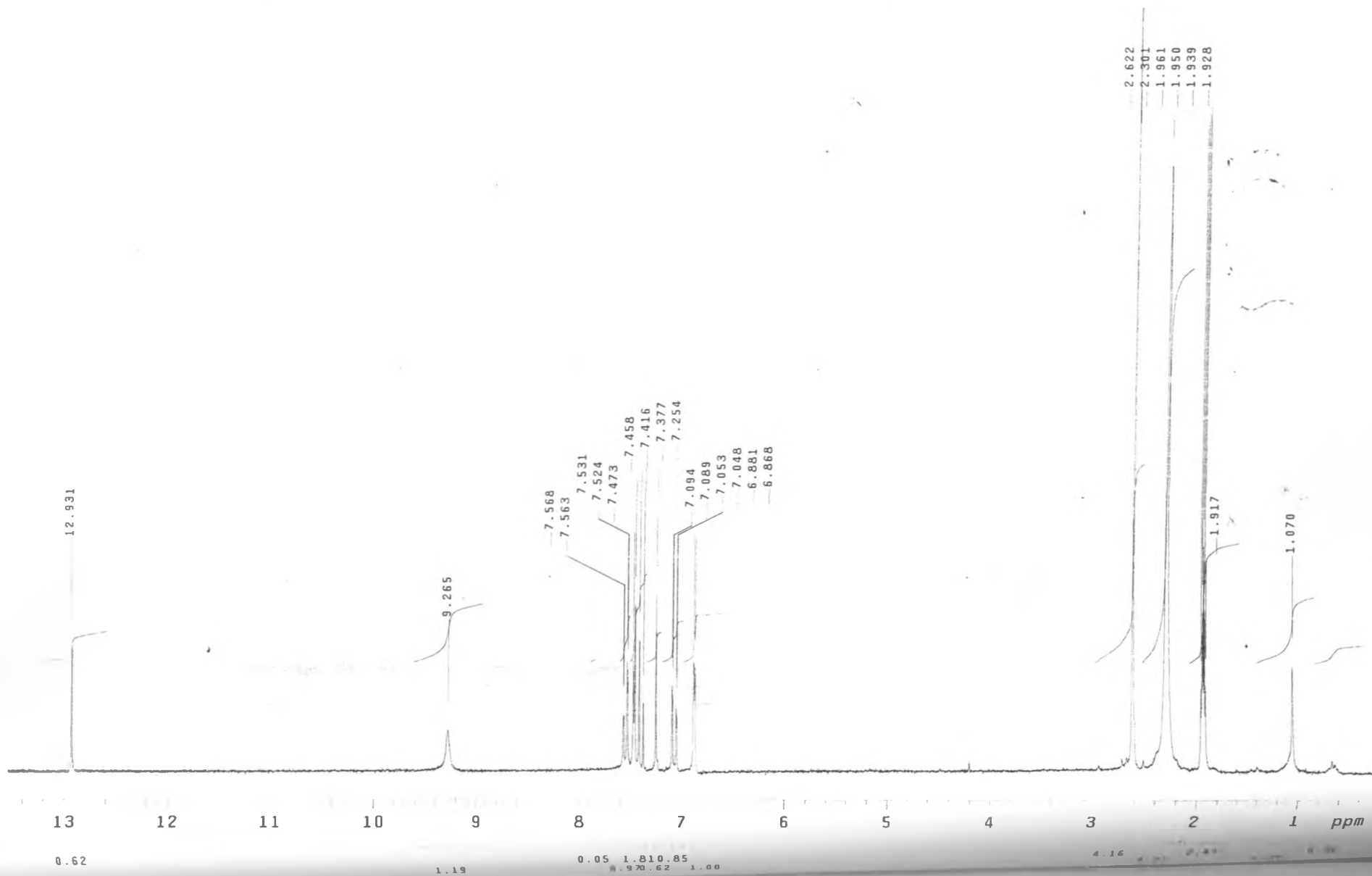
SPECTRA FOR COMPOUND 1

¹H NMR SPECTRUM FOR COMPOUND 1 (SOLVENT; CDCl₃, 200 MHz)

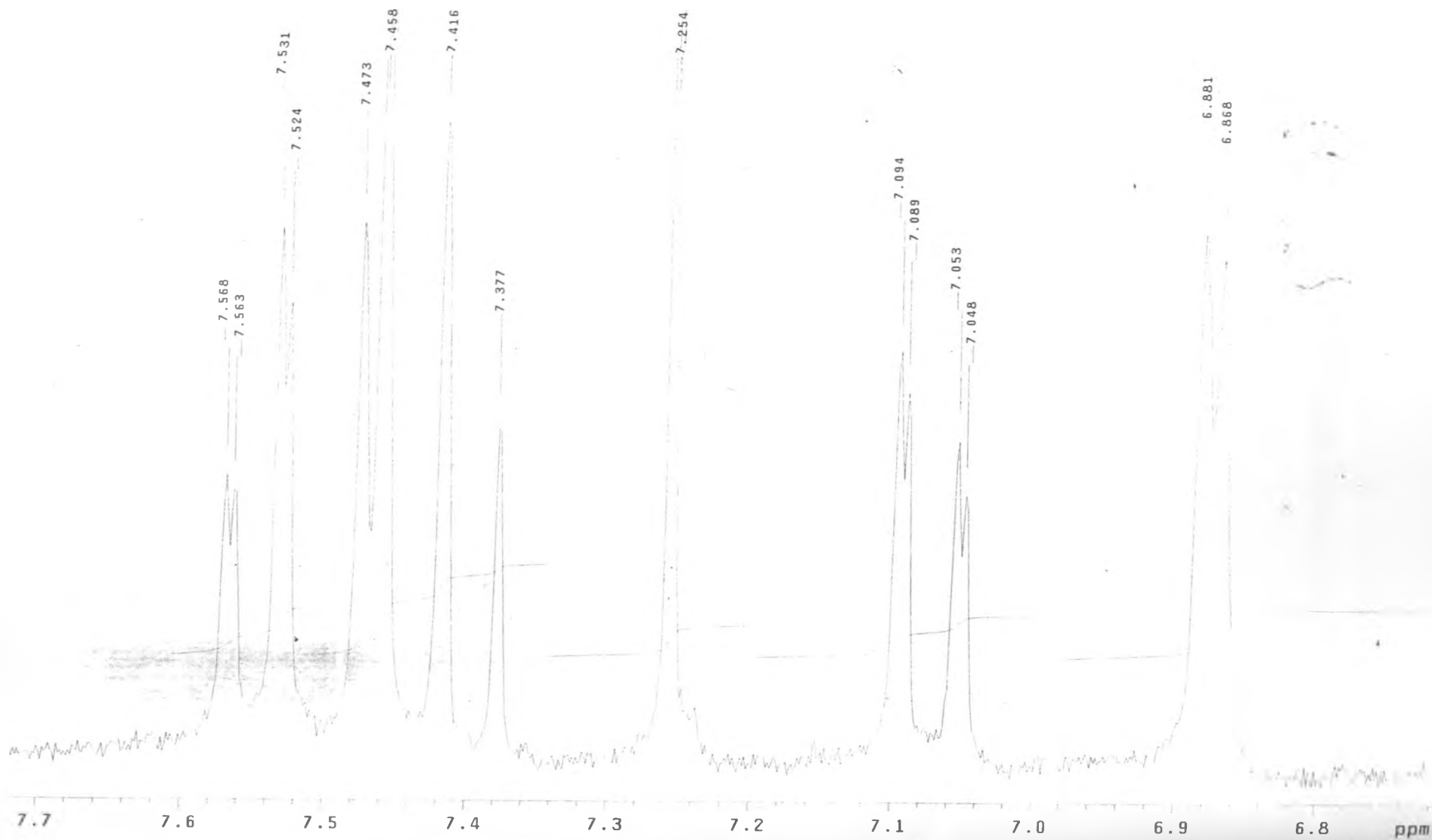


SPECTRA FOR COMPOUND 2

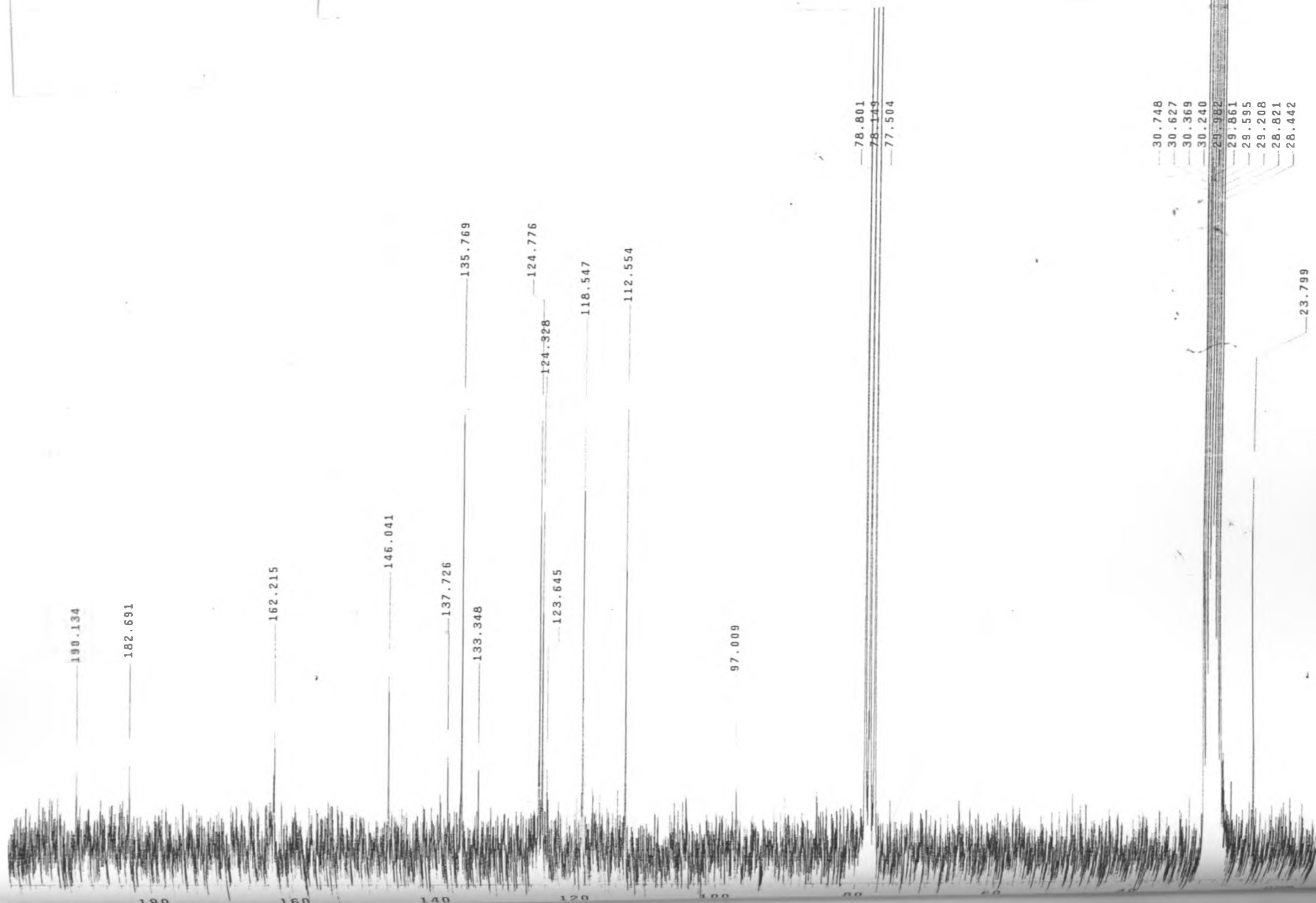
¹H NMR SPECTRUM FOR COMPOUND 2 (SOLVENT; CDCl₃ - ACETONE-d₆, 200 MHz)



¹H NMR SPECTRUM FOR COMPOUND 2 (SOLVENT; CDCl₃ - ACETONE-d₆, 200 MHz)

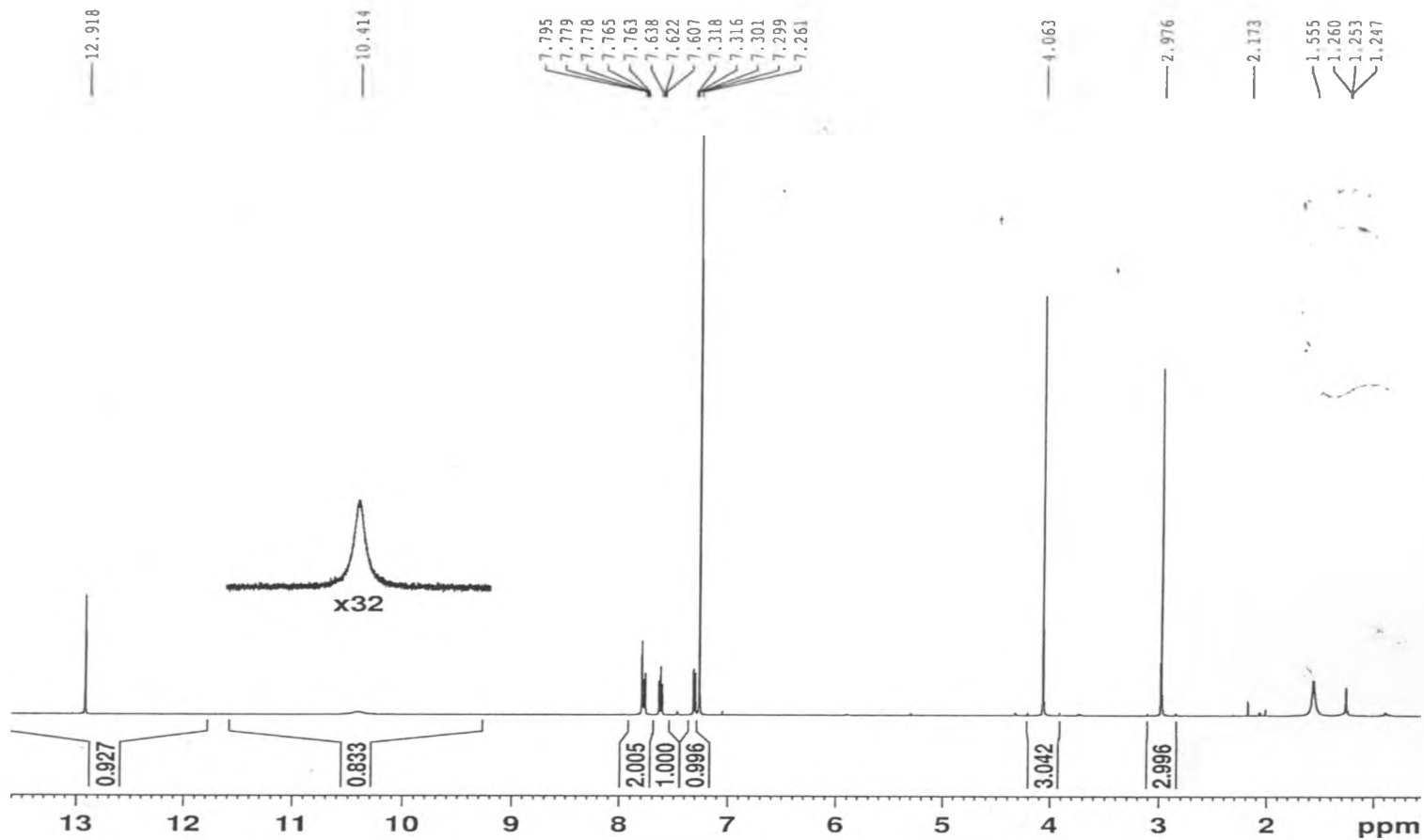


¹³C NMR SPECTRUM FOR COMPOUND 2 (SOLVENT: CDCl₃-ACETONE-d₆, 50 MHz)

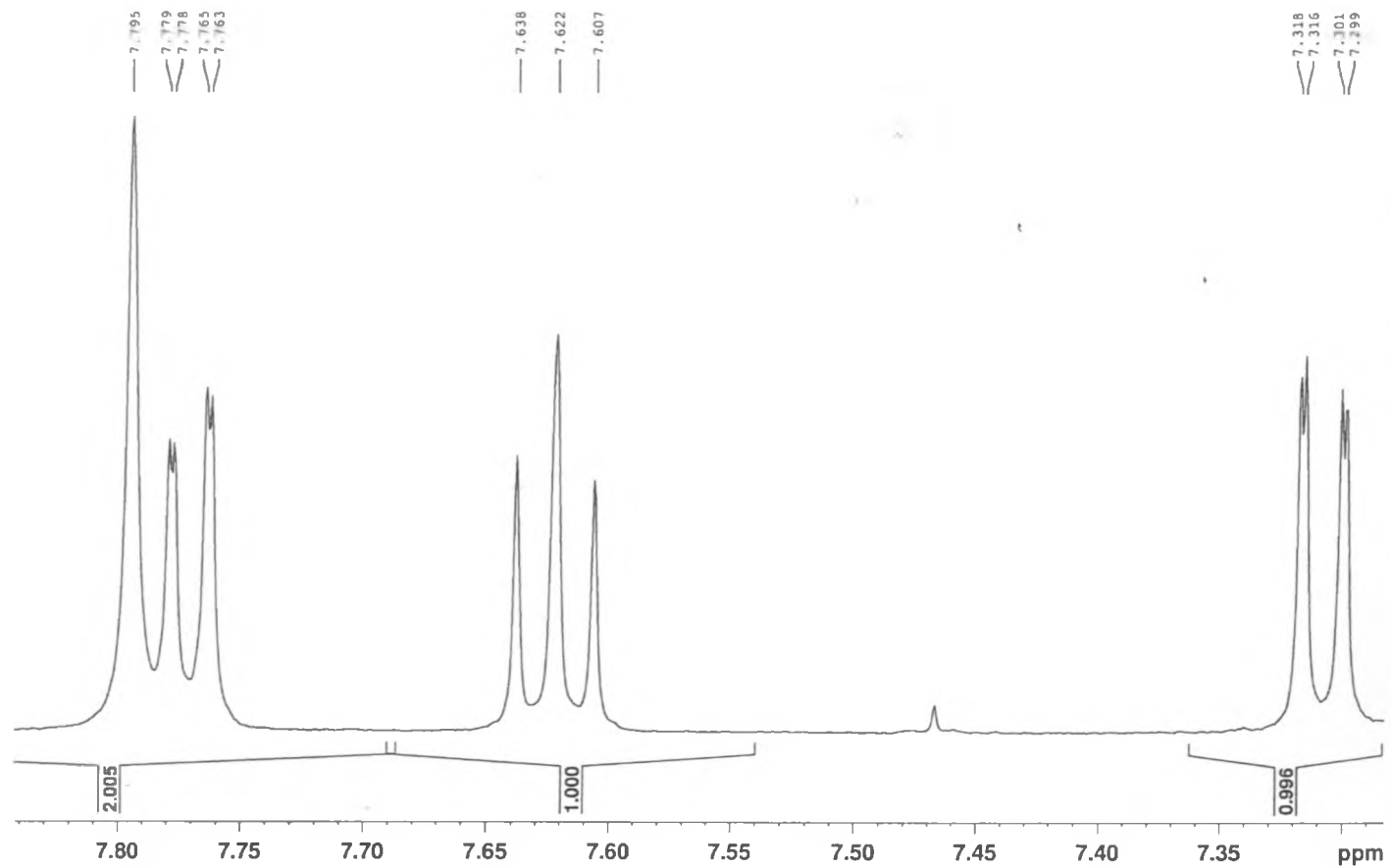


SPECTRA FOR COMPOUND 3

¹H NMR SPECTRUM FOR COMPOUND 3 (SOLVENT: CDCl₃, 500 MHz)



¹H NMR SPECTRUM FOR COMPOUND 3 (SOLVENT: CDCl₃, 500 MHz)



¹³C NMR SPECTRUM FOR COMPOUND 3 (SOLVENT: CDCl₃, 50 MHz)

CDCl₃ L₁ L₂ L₃ L₄ L₅ L₆ L₇ L₈ L₉ L₁₀ L₁₁ L₁₂ L₁₃ L₁₄ L₁₅ L₁₆ L₁₇ L₁₈ L₁₉ L₂₀ L₂₁ L₂₂ L₂₃ L₂₄ L₂₅ L₂₆ L₂₇ L₂₈ L₂₉ L₃₀ L₃₁ L₃₂ L₃₃ L₃₄ L₃₅ L₃₆ L₃₇ L₃₈ L₃₉ L₄₀ L₄₁ L₄₂ L₄₃ L₄₄ L₄₅ L₄₆ L₄₇ L₄₈ L₄₉ L₅₀ L₅₁ L₅₂ L₅₃ L₅₄ L₅₅ L₅₆ L₅₇ L₅₈ L₅₉ L₆₀ L₆₁ L₆₂ L₆₃ L₆₄ L₆₅ L₆₆ L₆₇ L₆₈ L₆₉ L₇₀ L₇₁ L₇₂ L₇₃ L₇₄ L₇₅ L₇₆ L₇₇ L₇₈ L₇₉ L₈₀ L₈₁ L₈₂ L₈₃ L₈₄ L₈₅ L₈₆ L₈₇ L₈₈ L₈₉ L₉₀ L₉₁ L₉₂ L₉₃ L₉₄ L₉₅ L₉₆ L₉₇ L₉₈ L₉₉ L₁₀₀

189.800

182.388

170.720

163.649

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148.127

138.963

136.034

132.833

125.261

124.685

119.177

115.270

77.860

77.000

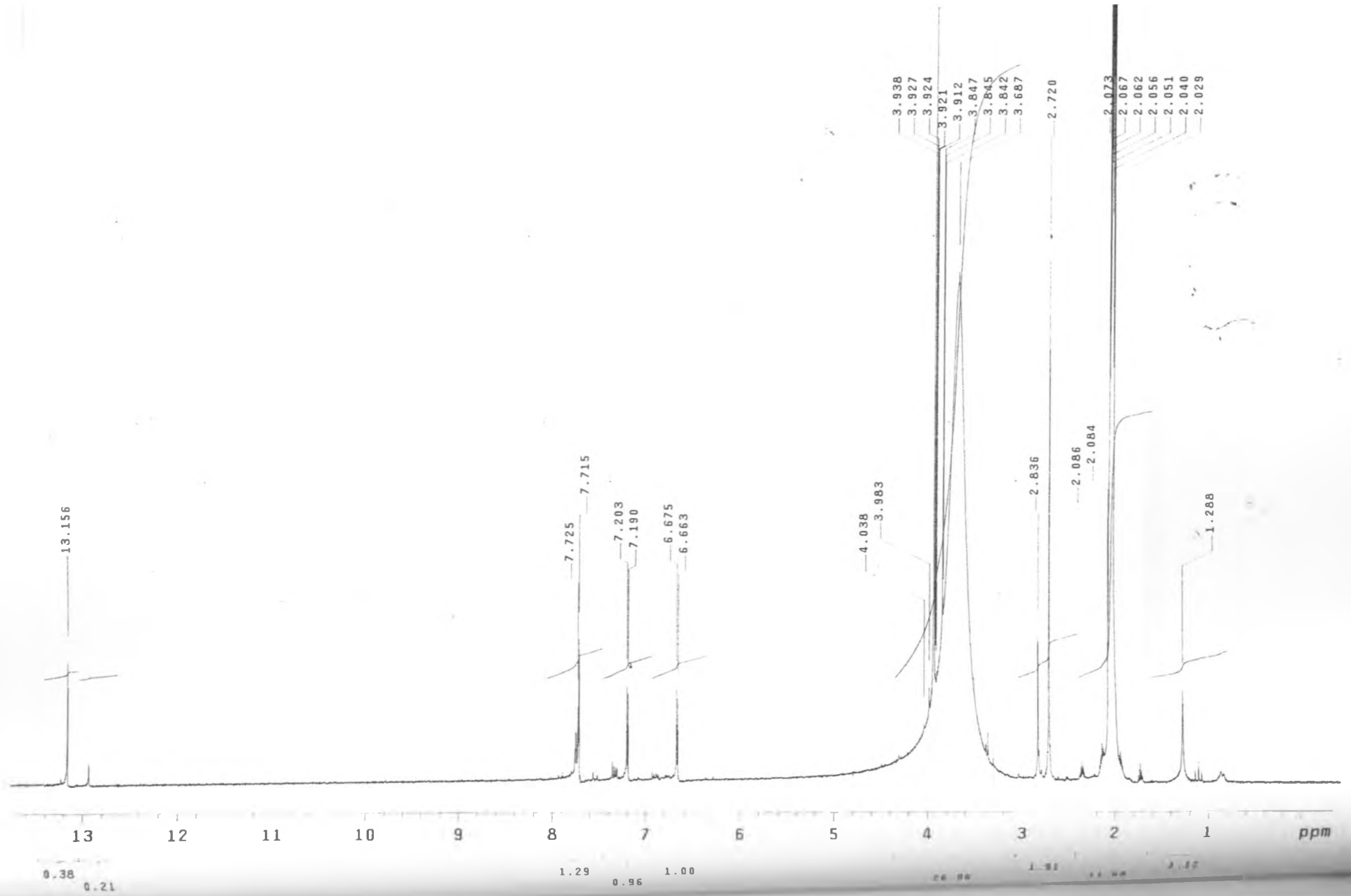
76.593

53.416

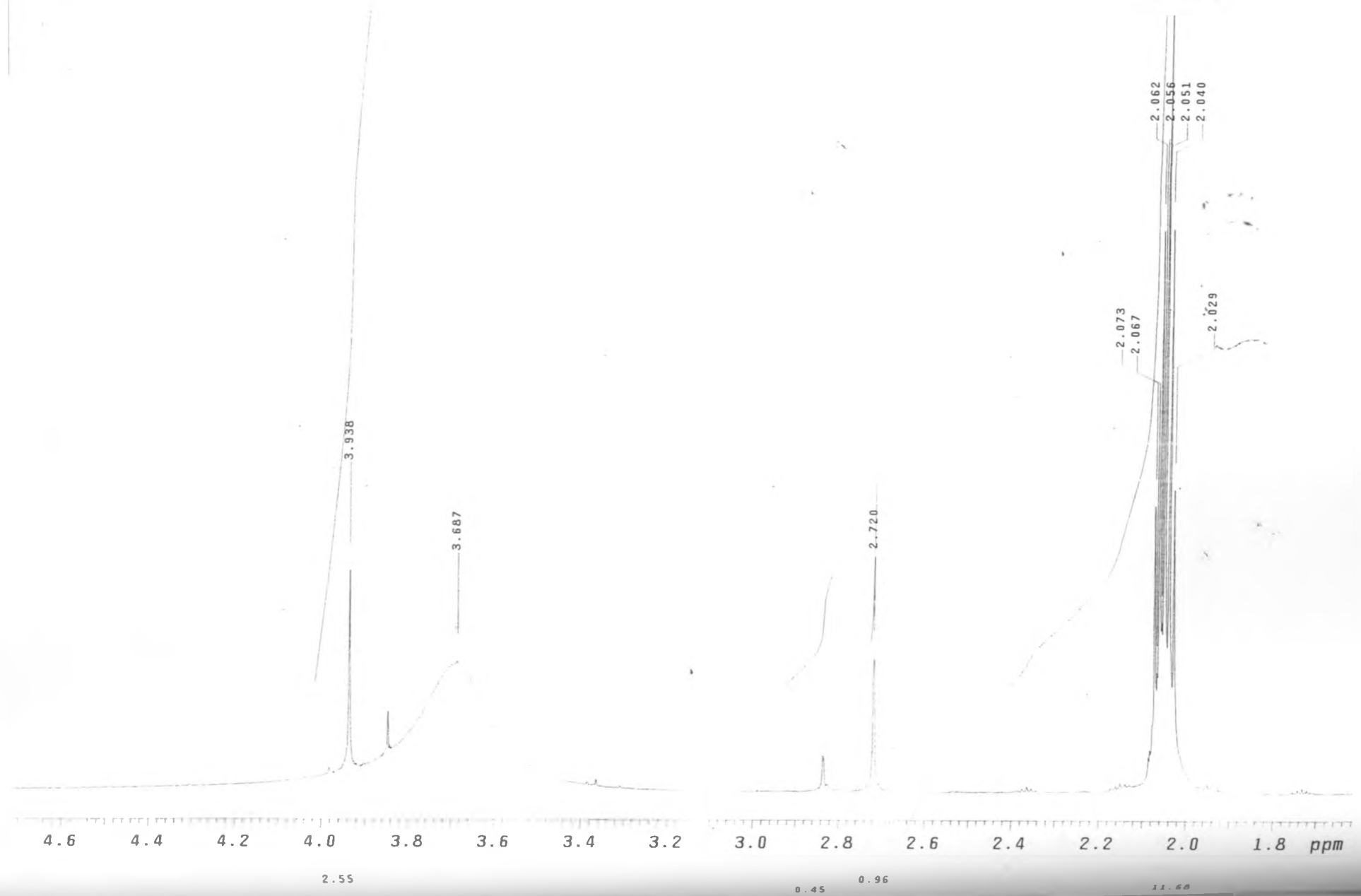
22.069

SPECTRA FOR COMPOUND 4

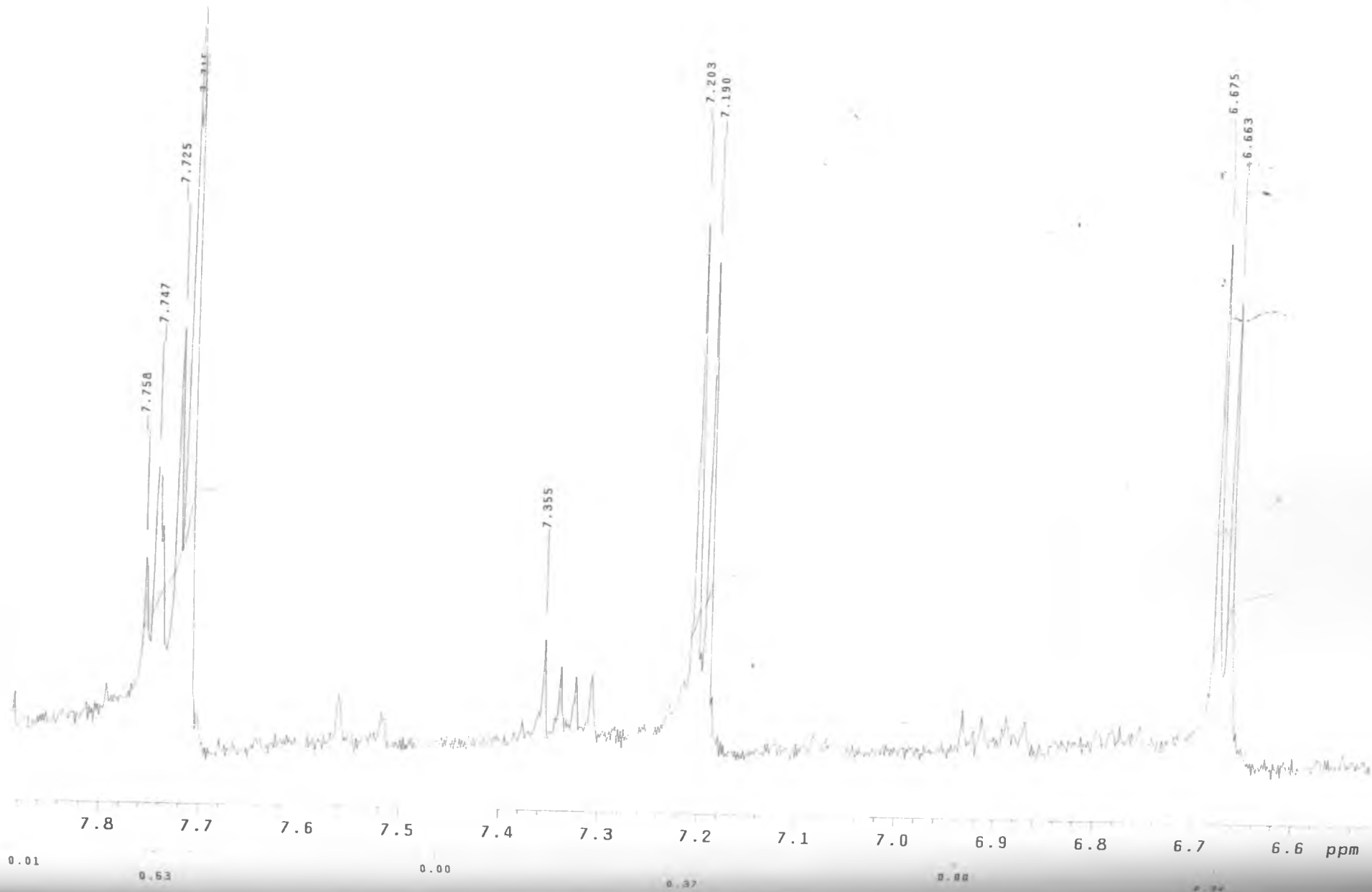
¹H NMR SPECTRUM FOR COMPOUND 4 (SOLVENT; ACETONE-d₆, 200 MHz)



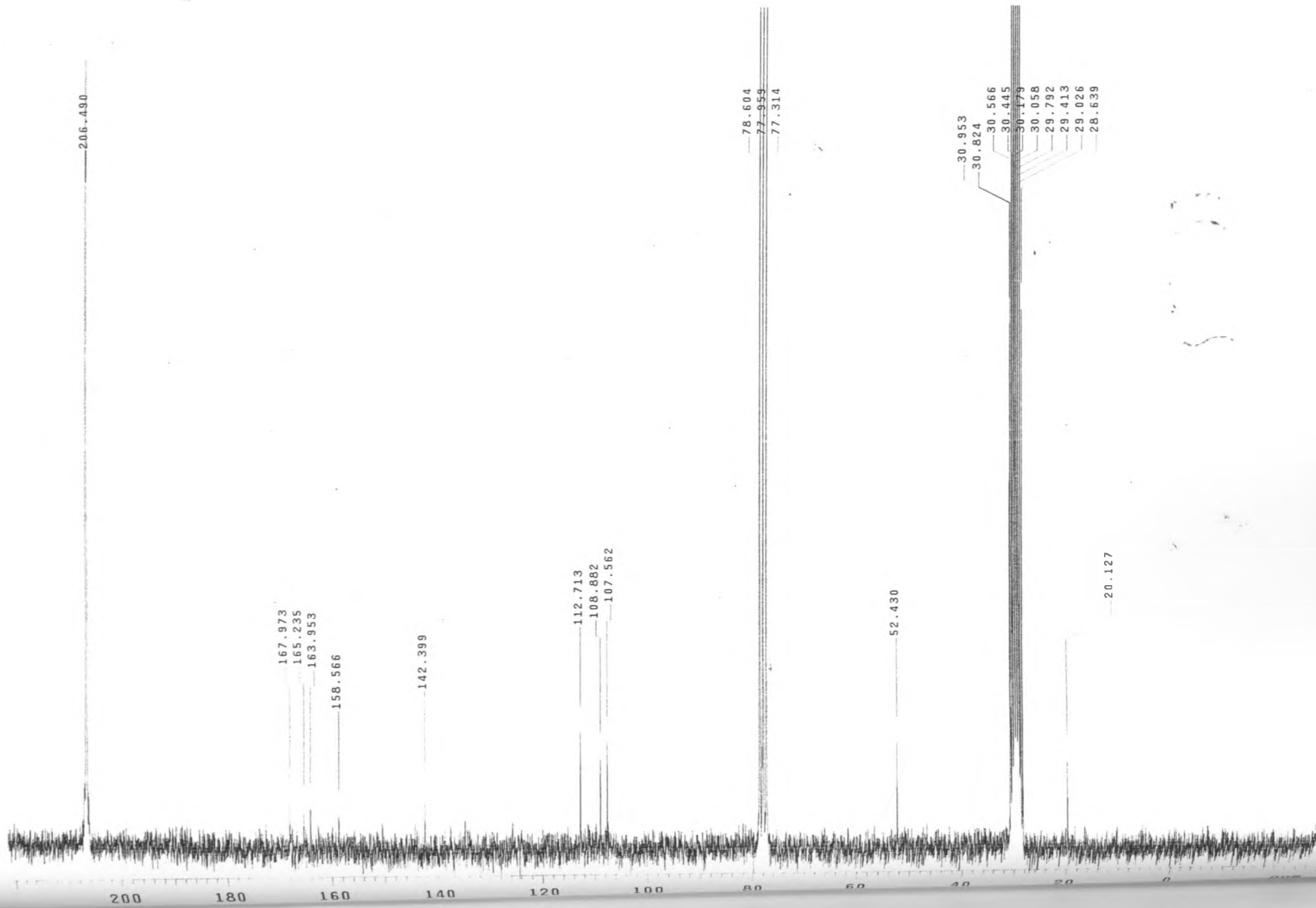
¹H NMR SPECTRUM FOR COMPOUND 4 (SOLVENT; ACETONE-d₆, 200 MHz)



¹H NMR SPECTRUM FOR COMPOUND 4 (SOLVENT; ACETONE-d₆, 200MHz)

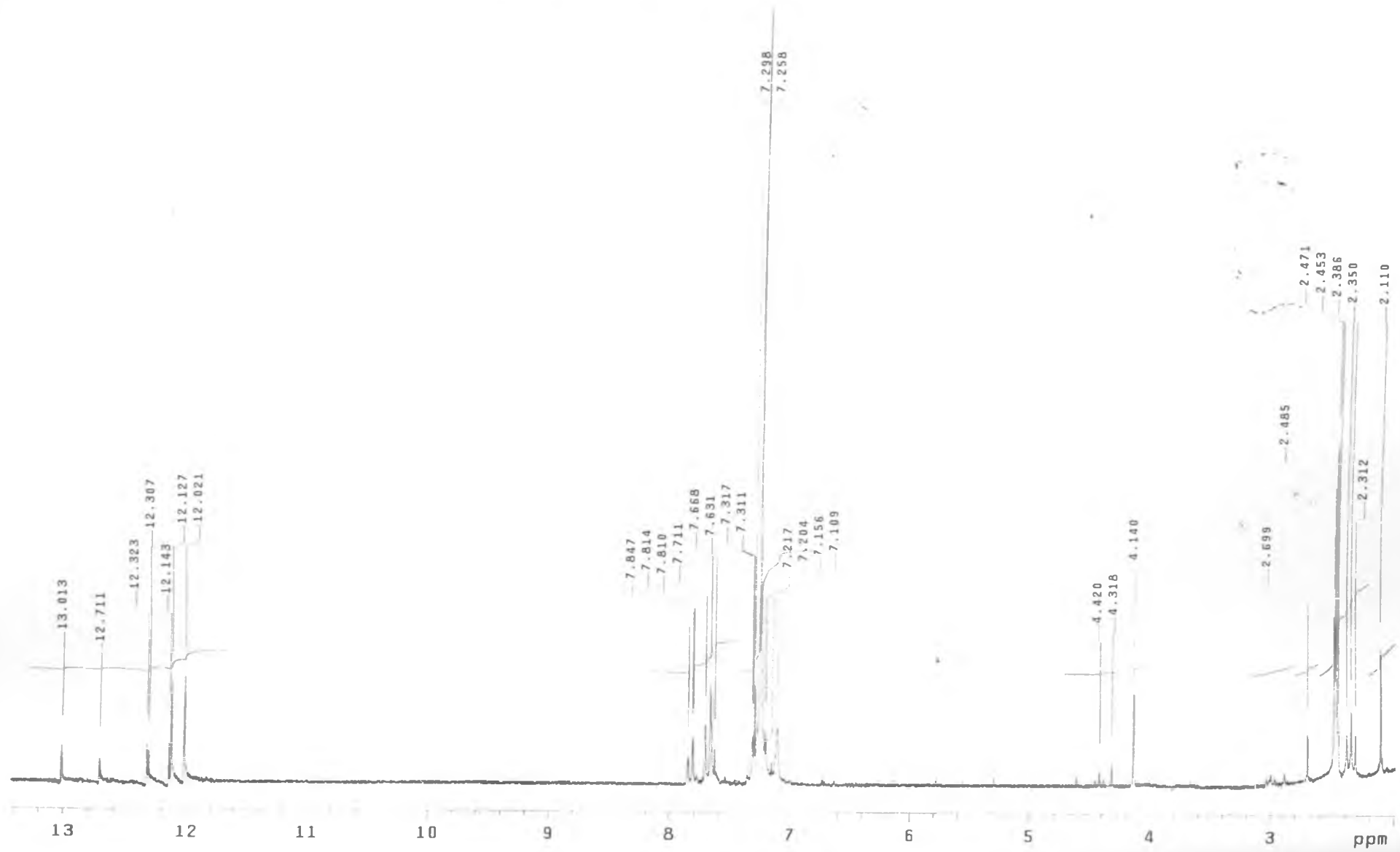


¹³C NMR SPECTRUM FOR COMPOUND 4 (SOLVENT; ACETONE-d₆, 50MHz)



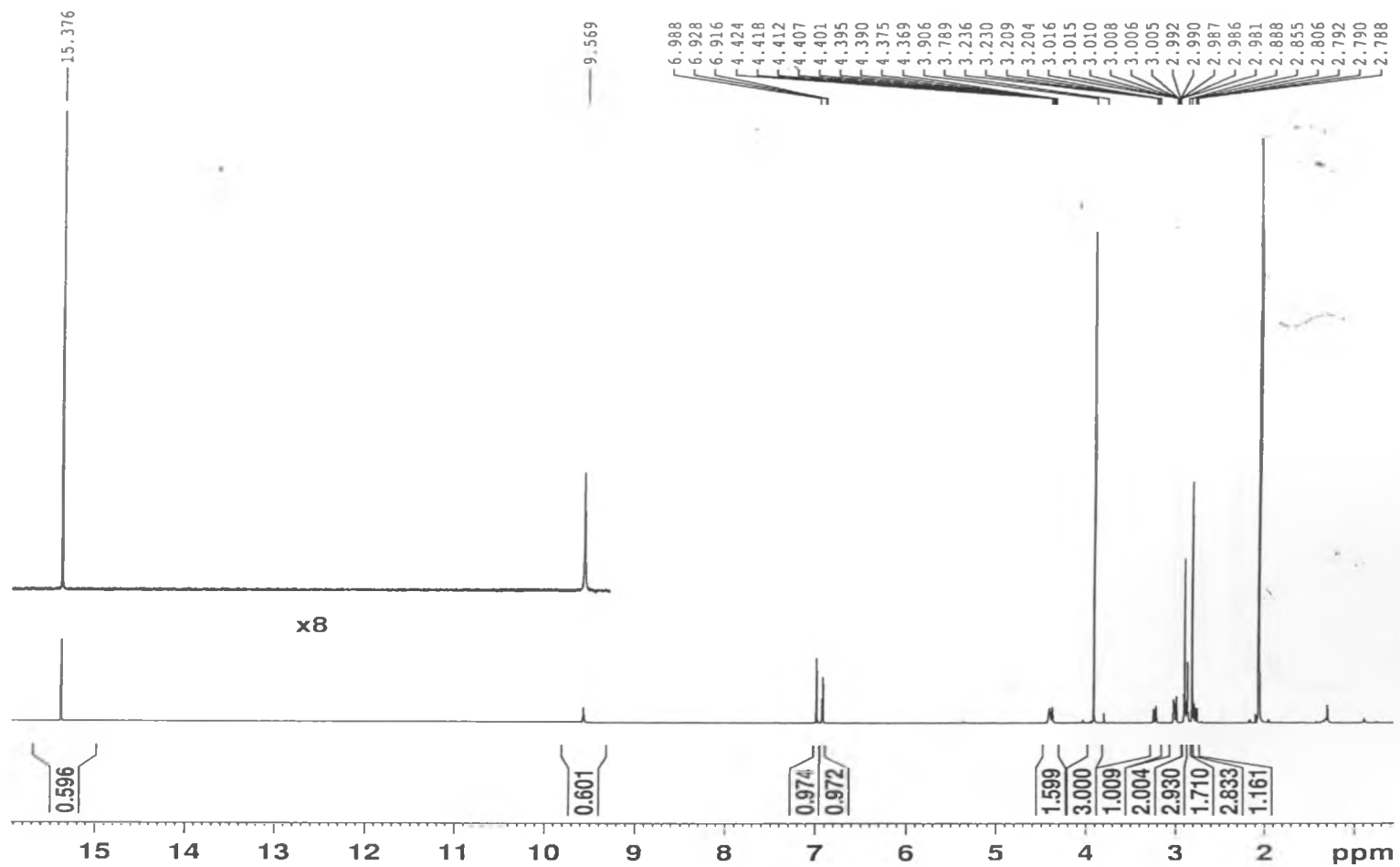
SPECTRA FOR COMPOUND 5

¹H NMR SPECTRUM FOR COMPOUND 5 (SOLVENT; CDCL₃, 200MHz)

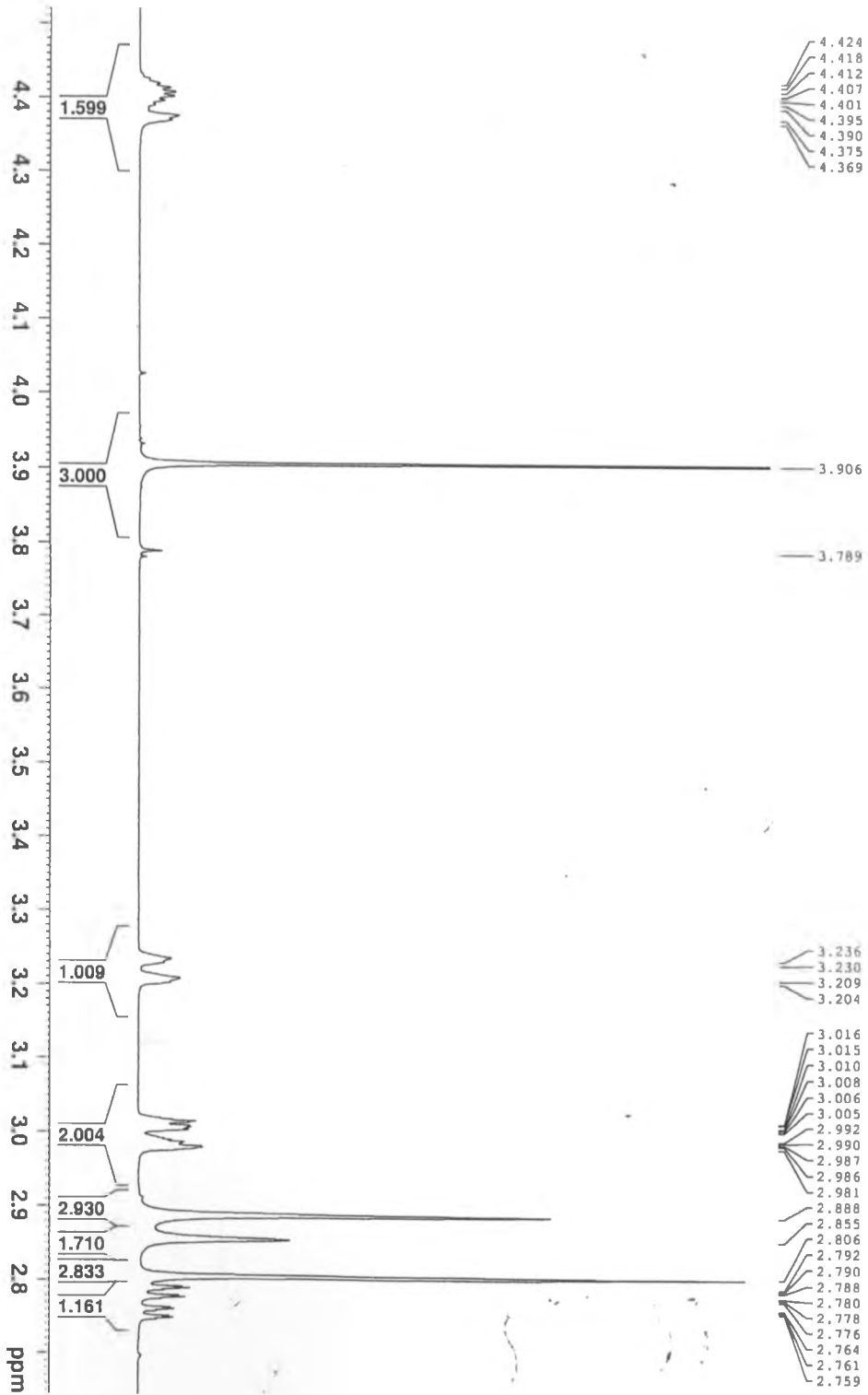


SPECTRA FOR COMPOUND 6

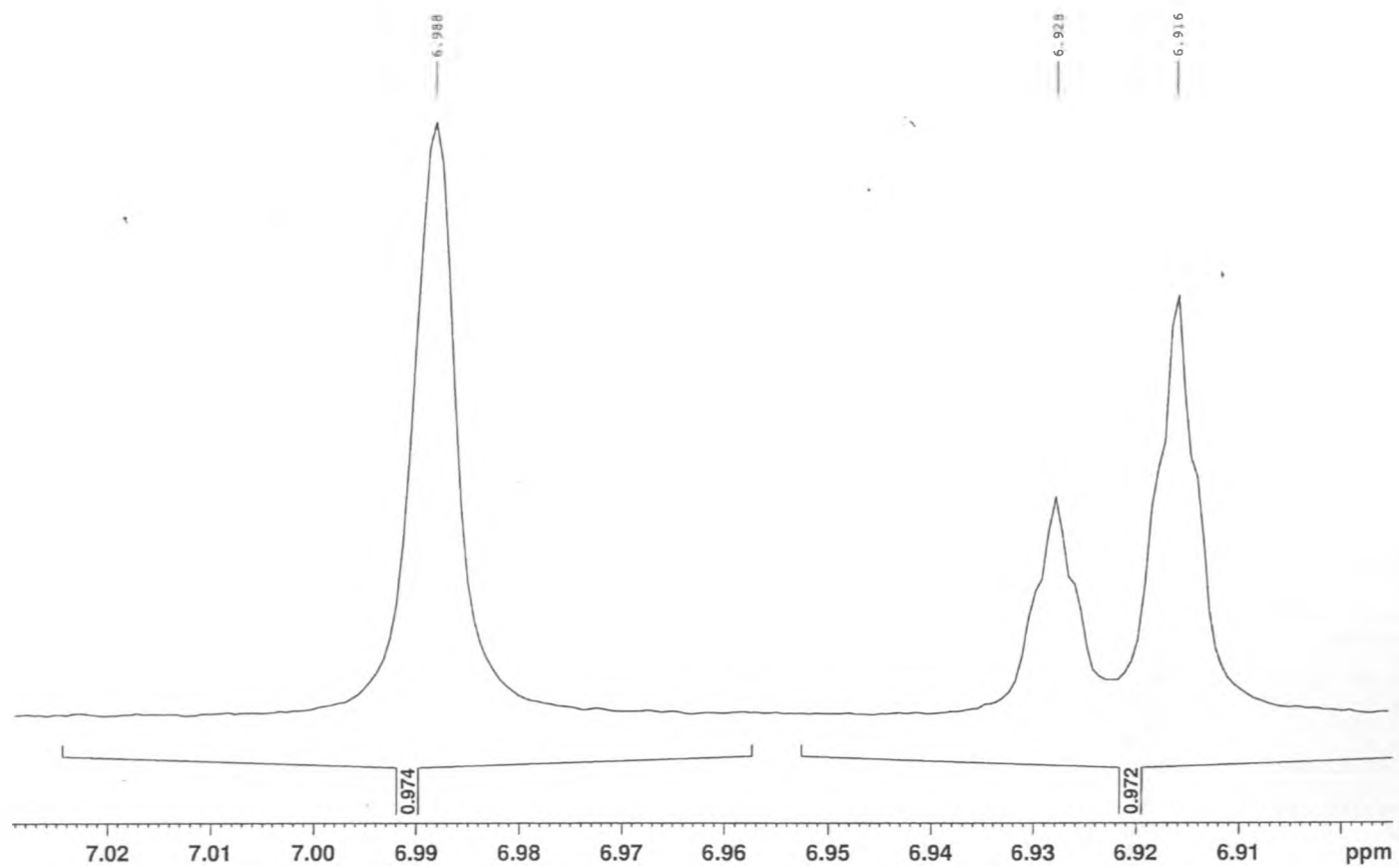
¹H NMR SPECTRUM FOR COMPOUND 6 (SOLVENT: CDCl₃, 500 MHz)



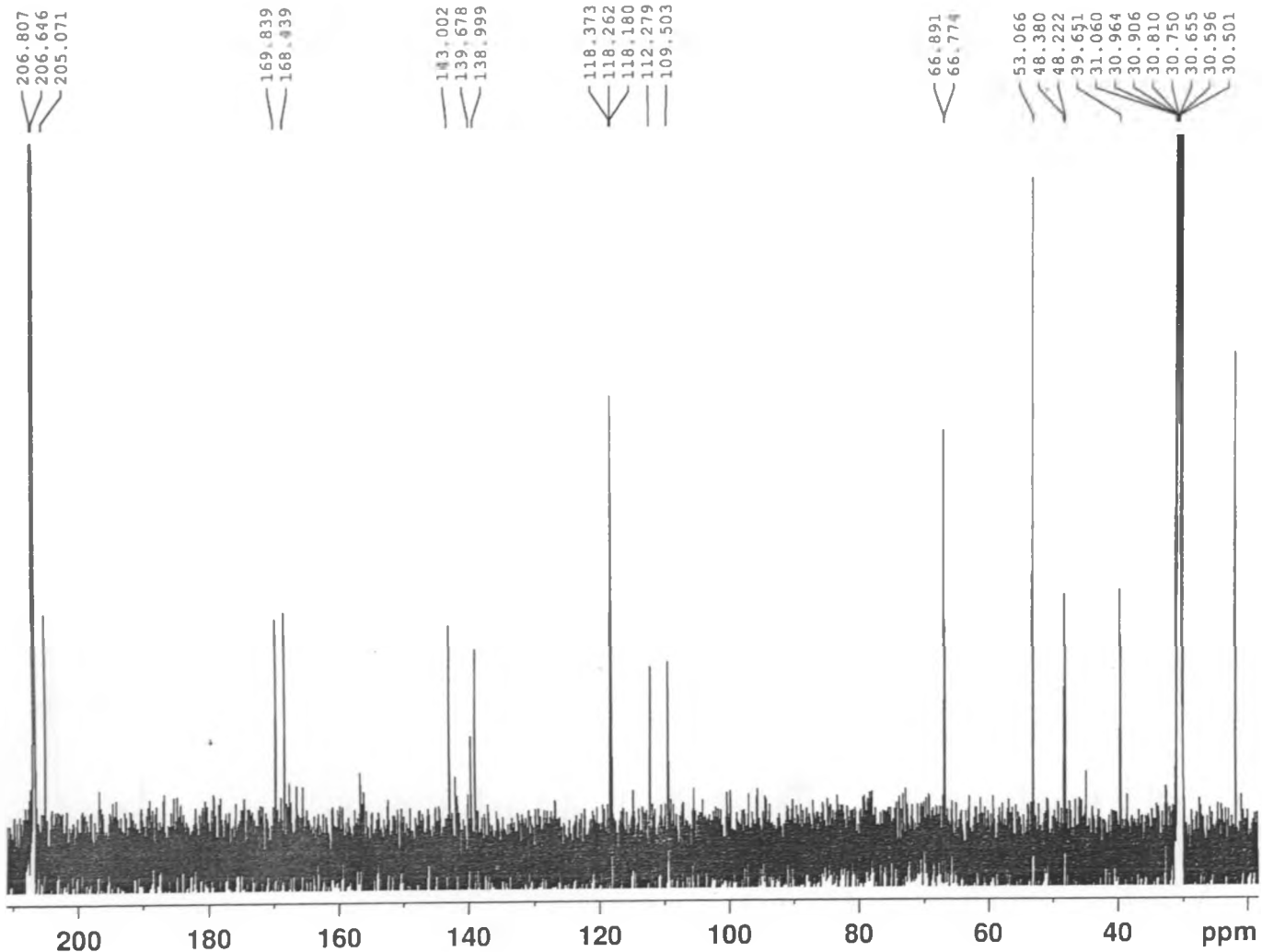
¹H NMR SPECTRUM FOR COMPOUND 6 (SOLVENT: CDCl₃, 500 MHz)



¹H NMR SPECTRUM FOR COMPOUND 6 (SOLVENT: CDCl₃, 500 MHz)



¹³C NMR SPECTRUM FOR COMPOUND 6 (SOLVENT: CDCl₃, 125 MHz)



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PROCNO    1
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TD         65536
SOLVENT   Acetone
NS         14336
DS         4
SWH        30030.029 Hz
FIDRES     0.458222 Hz
AQ         1.0912410 sec
RG         574.7
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DE         6.00 usec
TE         298.9 K
D1         2.00000000 sec
D11        0.03000000 sec
TD0        1
    
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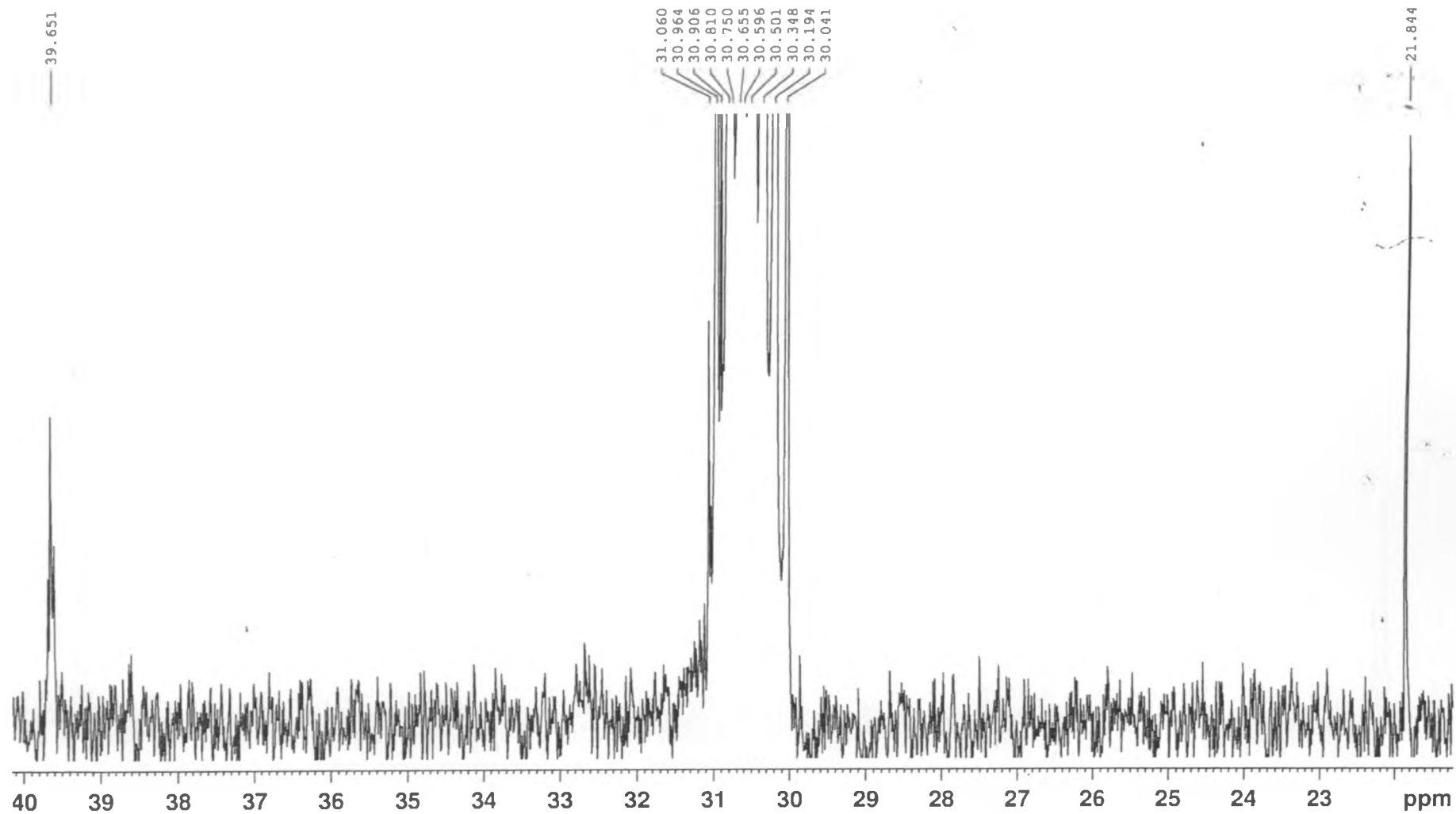
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PL1       3.00 dB
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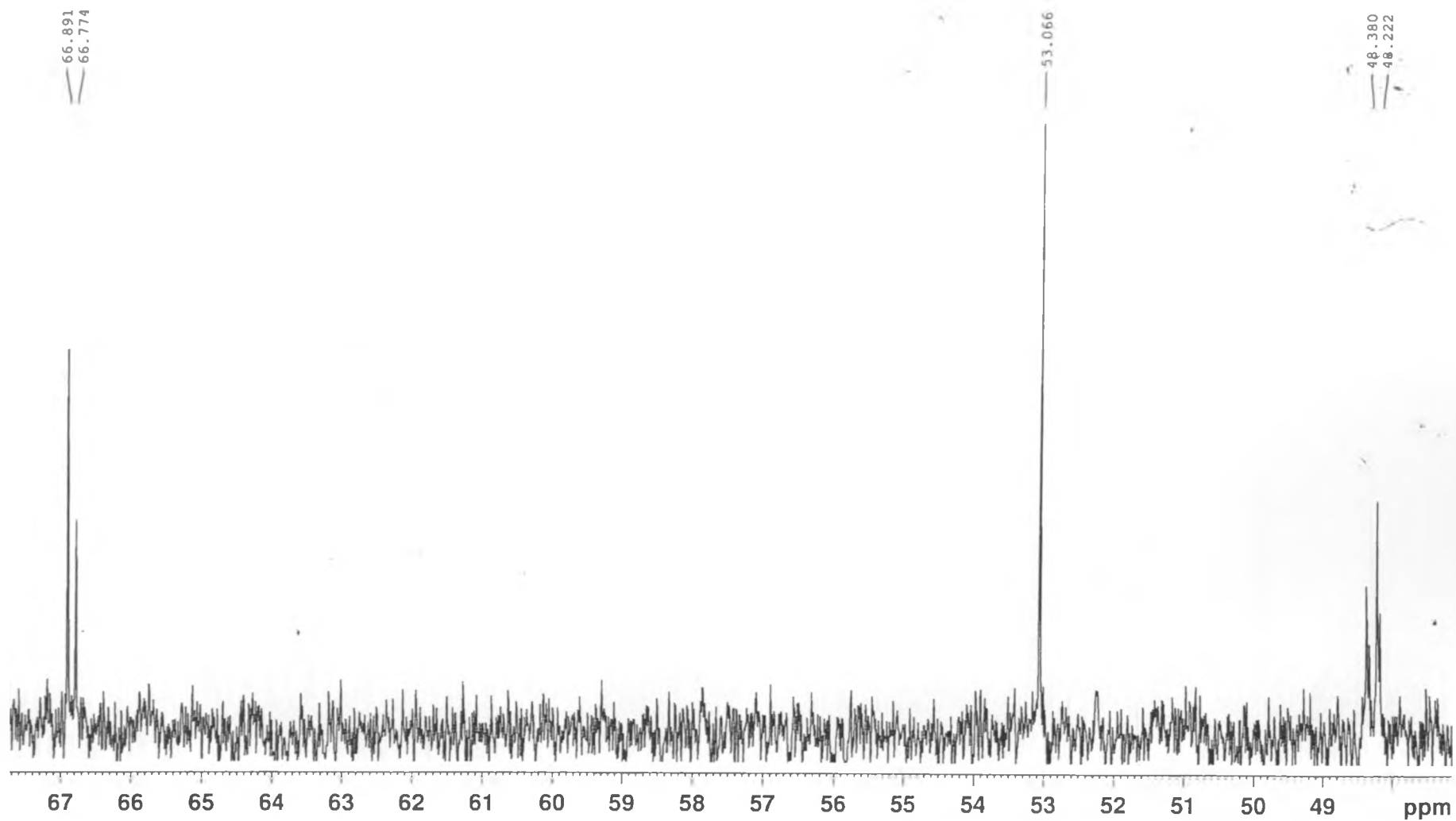
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PCPD2     70.00 usec
PL2       -3.00 dB
PL12      15.00 dB
PL13      18.00 dB
SFO2      500.1720007 MHz
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PC        1.40
    
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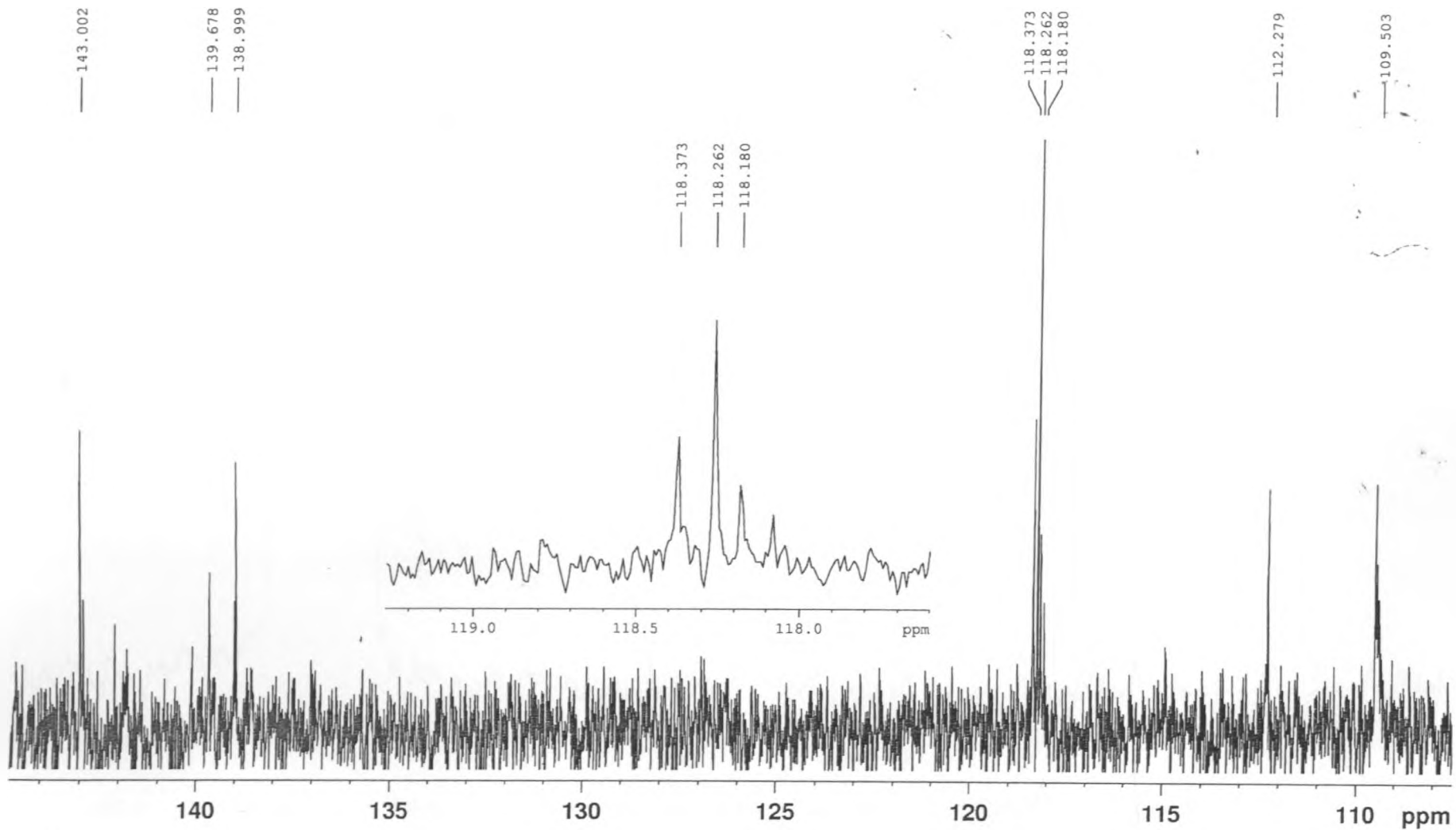
¹³C NMR SPECTRUM FOR COMPOUND 6 (SOLVENT: CDCl₃, 125 MHz)



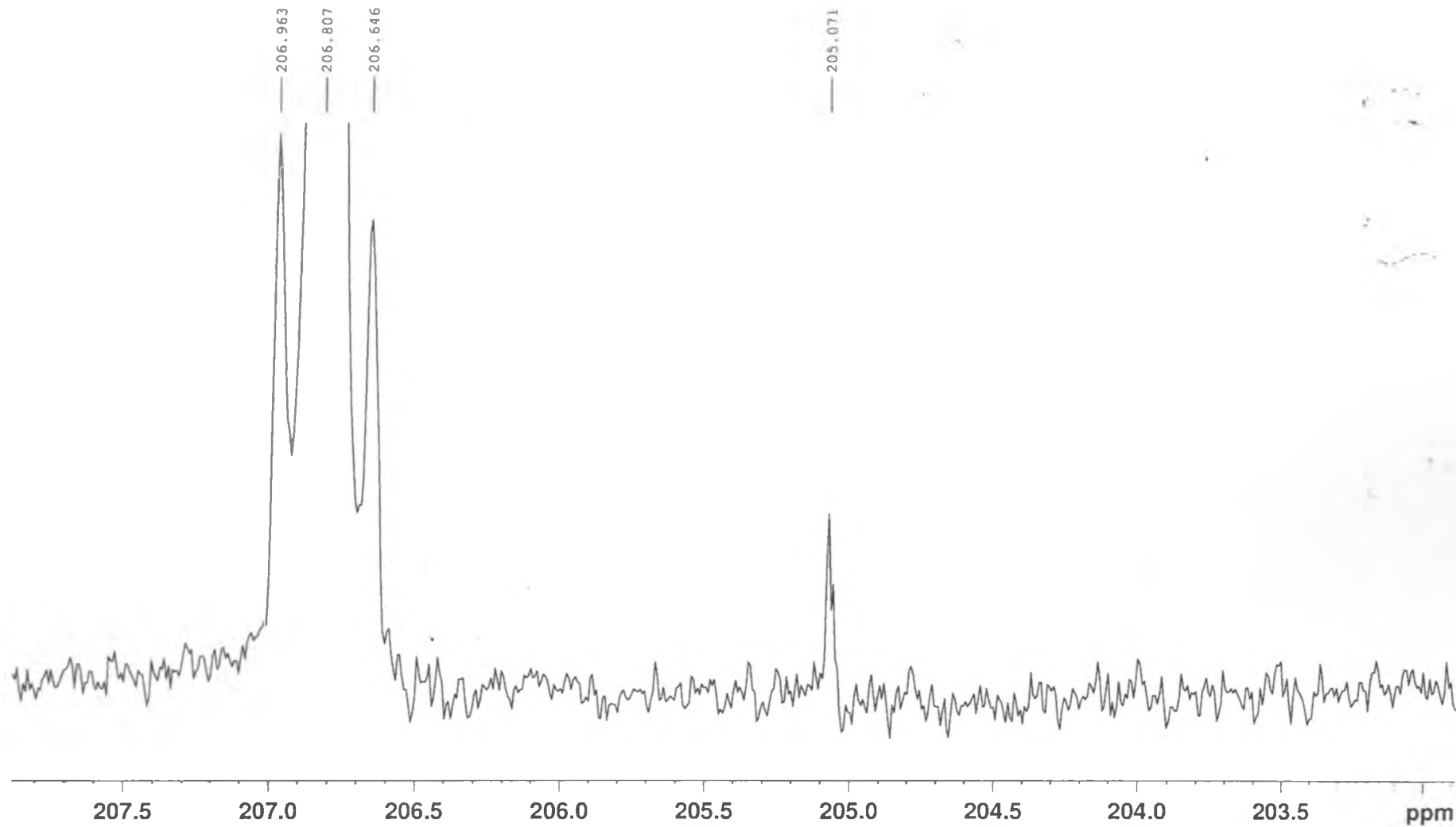
¹³C NMR SPECTRUM FOR COMPOUND SOLVENT: CDCl₃, 125 MHz)



¹³C NMR SPECTRUM FOR COMPOUND 6 (SOLVENT: CDCl₃, 125 MHz)

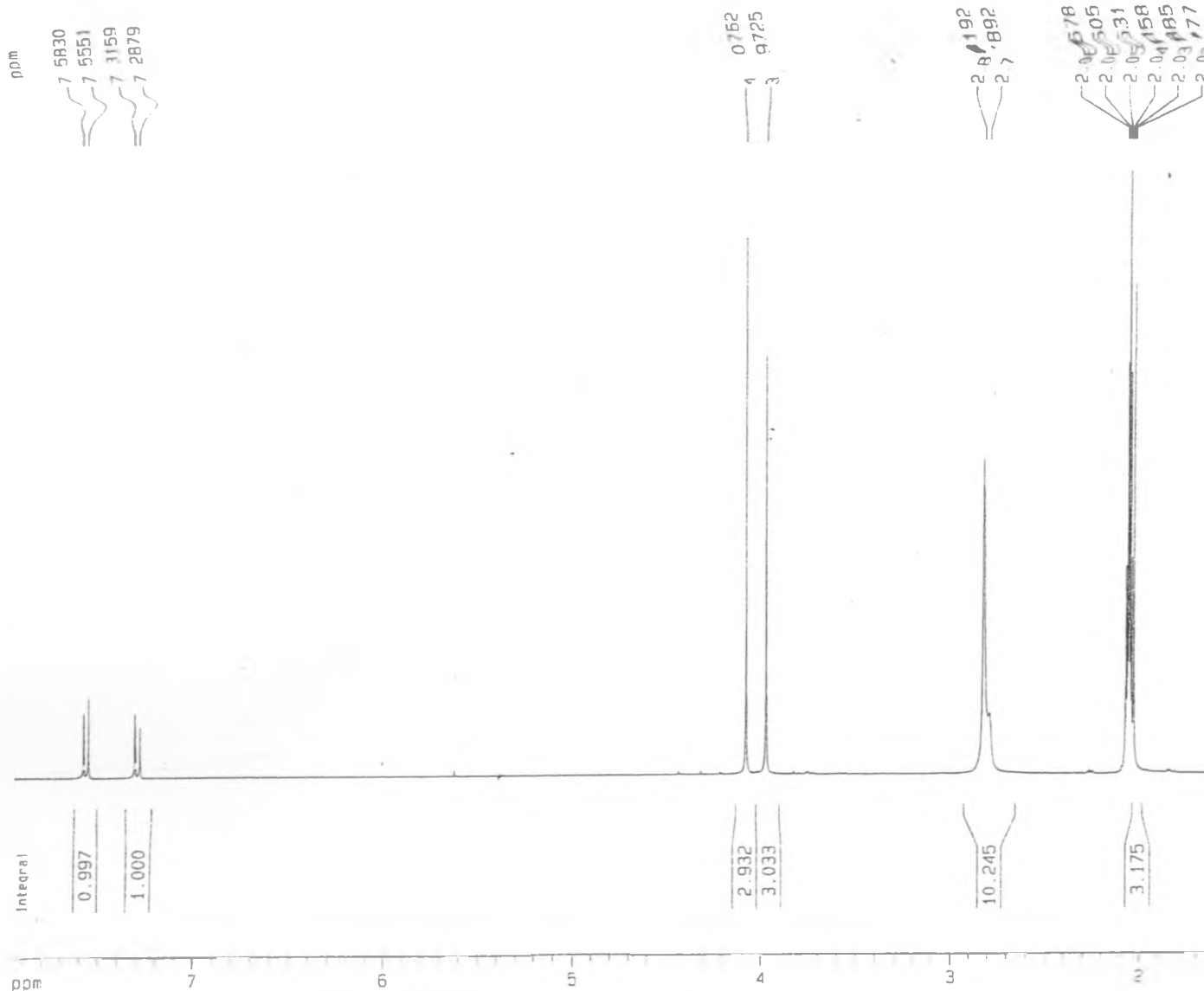


¹³C NMR SPECTRUM FOR COMPOUND 6 (SOLVENT: CDCl₃, 125 MHz)



SPECTRA FOR COMPOUND 7

¹H NMR SPECTRUM FOR COMPOUND 7 (SOLVENT: CDCl₃, 300 MHz)



Current Data Parameters:
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 EXPNO 40
 PROCNO :

F2 - Acquisition Parameters

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 Time 14 06
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 TO 65536
 SOLVENT CD2Cl2
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 JS 2
 SWH 6172.839 Hz
 FIDRES 0.094190 Hz
 AQ 5.3084660 sec
 RG 812.7
 DW 81.000 usec
 DE 10.00 usec
 TE 300.0 K
 D1 1.00000000 sec

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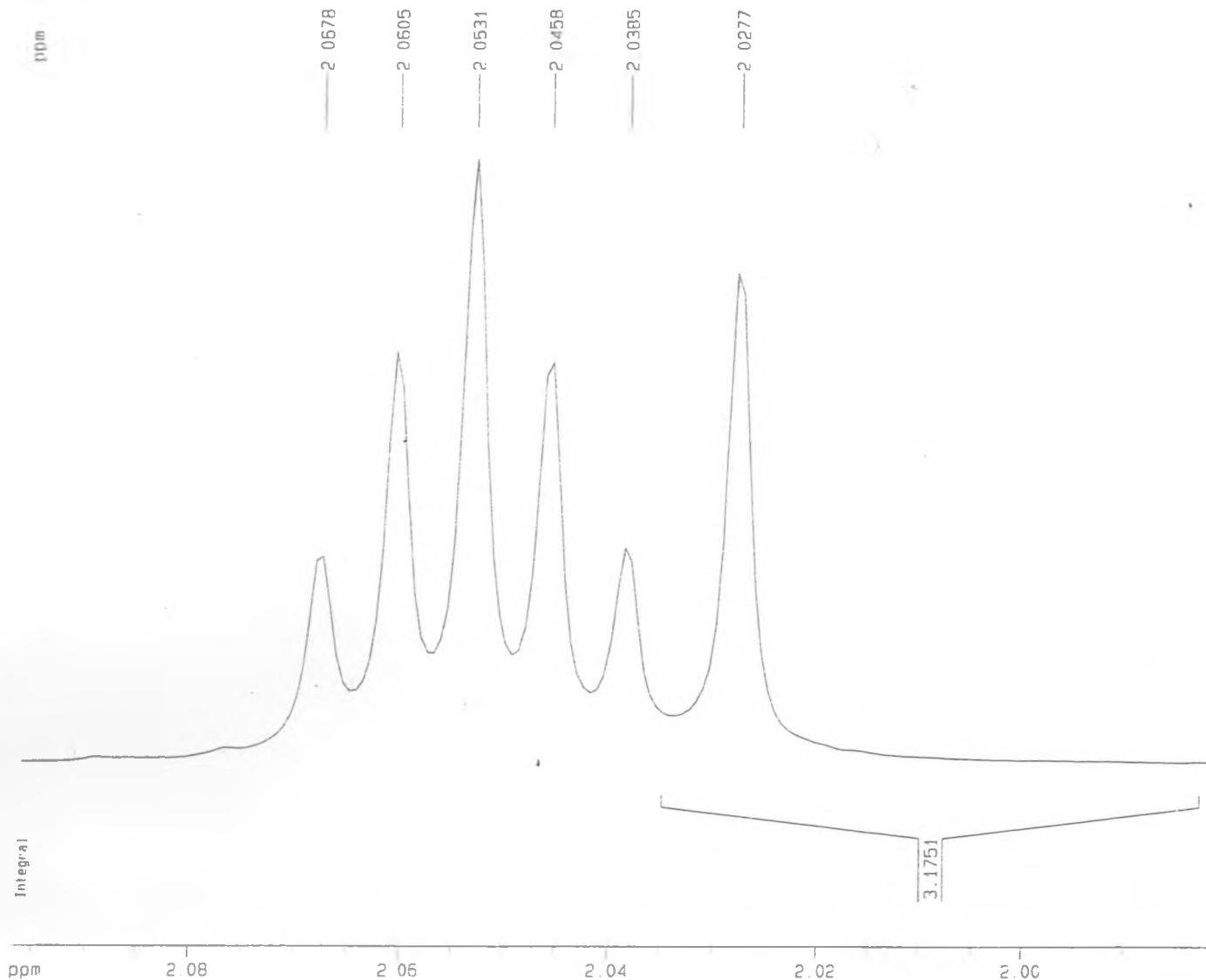
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 B 0.30 Hz
 GB 0
 PC 1.00

1D NMR plot parameters

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 CY 10.00 cm
 F1P 7.952 ppm
 F1 2386.75 Hz
 F2P 1.624 ppm
 F2 487.41 Hz
 FPCMC 0.31642 ppm/cm
 FZCM 94.98677 Hz/cm

¹H NMR SPECTRUM FOR COMPOUND 7 (SOLVENT: CDCl₃, 300 MHz)



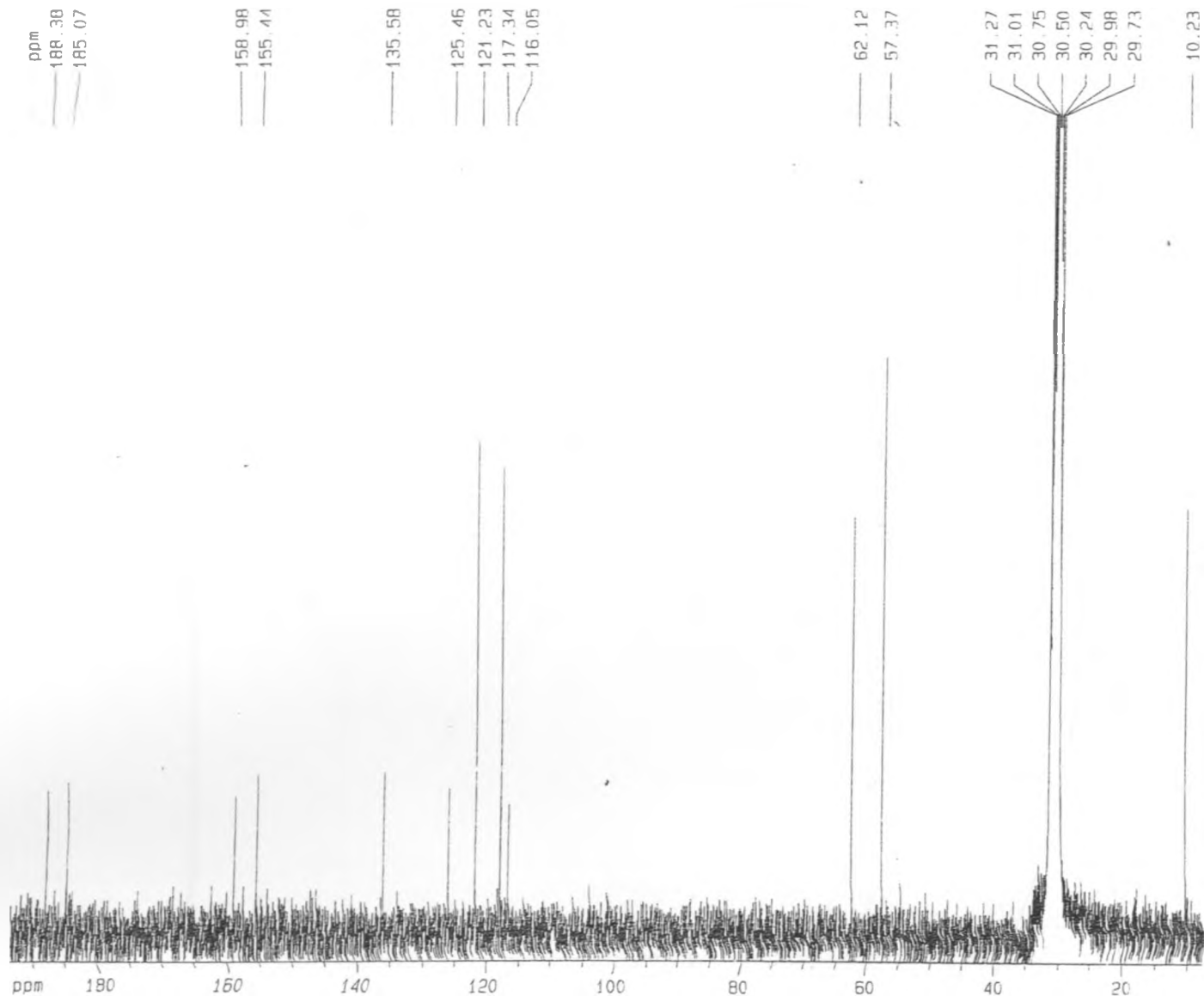
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SOLVENT CDCl3
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DS 2
SWH 6172.839 Hz
FIDRES 0.094190 Hz
AQ 5.3084660 sec
RG 812.7
RM 81.000 usec
RE 10.00 usec
FE 300.0 K
D1 1.00000000 sec

***** CHANNEL f1 *****
NUC1 1H
P1 9.00 usec
PL1 0.00 dB
SFO1 300.1318534 MHz

F2 - Processing parameters
SI 32768
SF 300.1309876 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

1D NMR plot parameters
CX 20.00 cm
CY 10.00 cm
F1P 2.097 ppm
F1 629.36 Hz
F2P 1.981 ppm
F2 594.50 Hz
PPMCM 0.00581 ppm/cm
HZCM 1.74301 Hz/cm

¹³C NMR SPECTRUM FOR COMPOUND 7 (SOLVENT: CDCl₃, 75 MHz)



2 - Acquisition Parameters
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 PULPROG zgpg30
 TD 65536
 SOLVENT Acetone
 NS 10240
 DS 4
 SWH 17985.611 Hz
 FIDRES 0.274439 Hz
 AQ 1.821950E sec
 RG 4096
 JW 27.800 usec
 JE 10.00 usec
 TE 300.0 K
 D1 2.0000000 sec
 d11 0.0300000 sec
 d12 0.0002000 sec

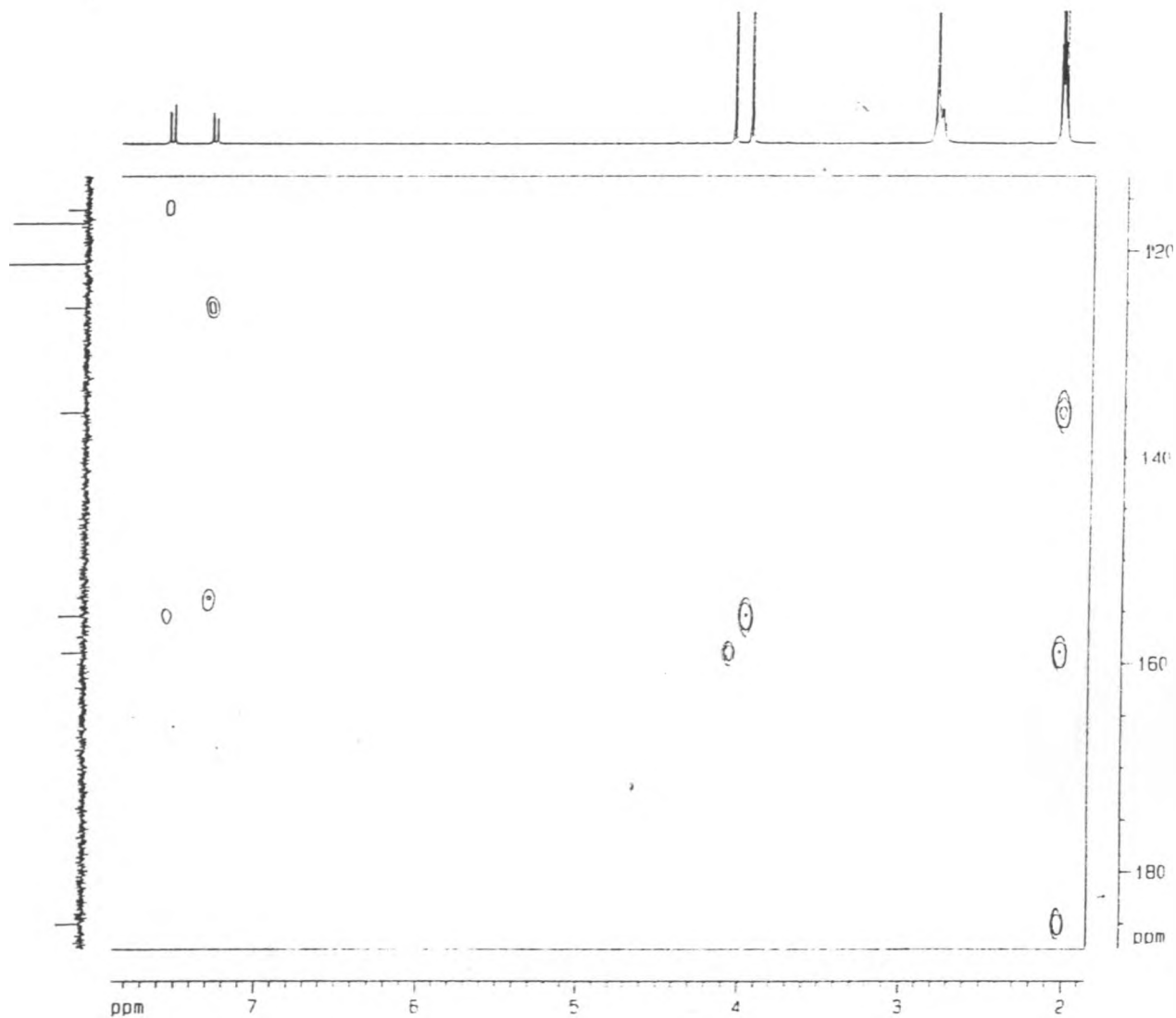
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 PL1 0.00 dB
 SFO1 75.4752653 MHz

----- CHANNEL f2 -----
 CPOPRG2 waltz16
 NUC2 1H
 PCPD2 110.00 usec
 PL2 0.00 dB
 PL12 22.00 dB
 PL13 28.00 dB
 SFO2 300.1312005 MHz

2 - Processing parameters
 SI 32768
 SF 75.4676290 MHz
 WDW EM
 SSB 0
 GB 1.00 Hz
 GE 0
 GC 1.40

1D NMR plo: parameters
 CX 20.00 cm
 CY 745.17 cm
 CP 193.708 ppm
 F1 14618.69 Hz
 F2 7.519 ppm
 F3 567.44 Hz

HMBC SPECTRUM FOR COMPOUND 7 (SOLVENT: CDCl₃, ¹H-300 and ¹³C-75 MHz)



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NUC2: 1H
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FIDRES: 0.751000 MHz
AQ: 0.111612 sec
RG: 18384
DS: 128.000 user
DE: 10.00 user
TE: 300.2 K
CMT1: 14.000000 sec
AQ: 0.000000 sec
D1: 2.033191 sec
d2: 0.000000 sec
D3: 1.070000 sec
d13: 0.000000 sec
d14: 0.001000 sec
d15: 0.000000 sec
d16: 0.000000 sec
d17: 0.000000 sec
d18: 0.000000 sec
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d78: 0.000000 sec
d79: 0.000000 sec
d80: 0.000000 sec
d81: 0.000000 sec
d82: 0.000000 sec
d83: 0.000000 sec
d84: 0.000000 sec
d85: 0.000000 sec
d86: 0.000000 sec
d87: 0.000000 sec
d88: 0.000000 sec
d89: 0.000000 sec
d90: 0.000000 sec
d91: 0.000000 sec
d92: 0.000000 sec
d93: 0.000000 sec
d94: 0.000000 sec
d95: 0.000000 sec
d96: 0.000000 sec
d97: 0.000000 sec
d98: 0.000000 sec
d99: 0.000000 sec
d100: 0.000000 sec

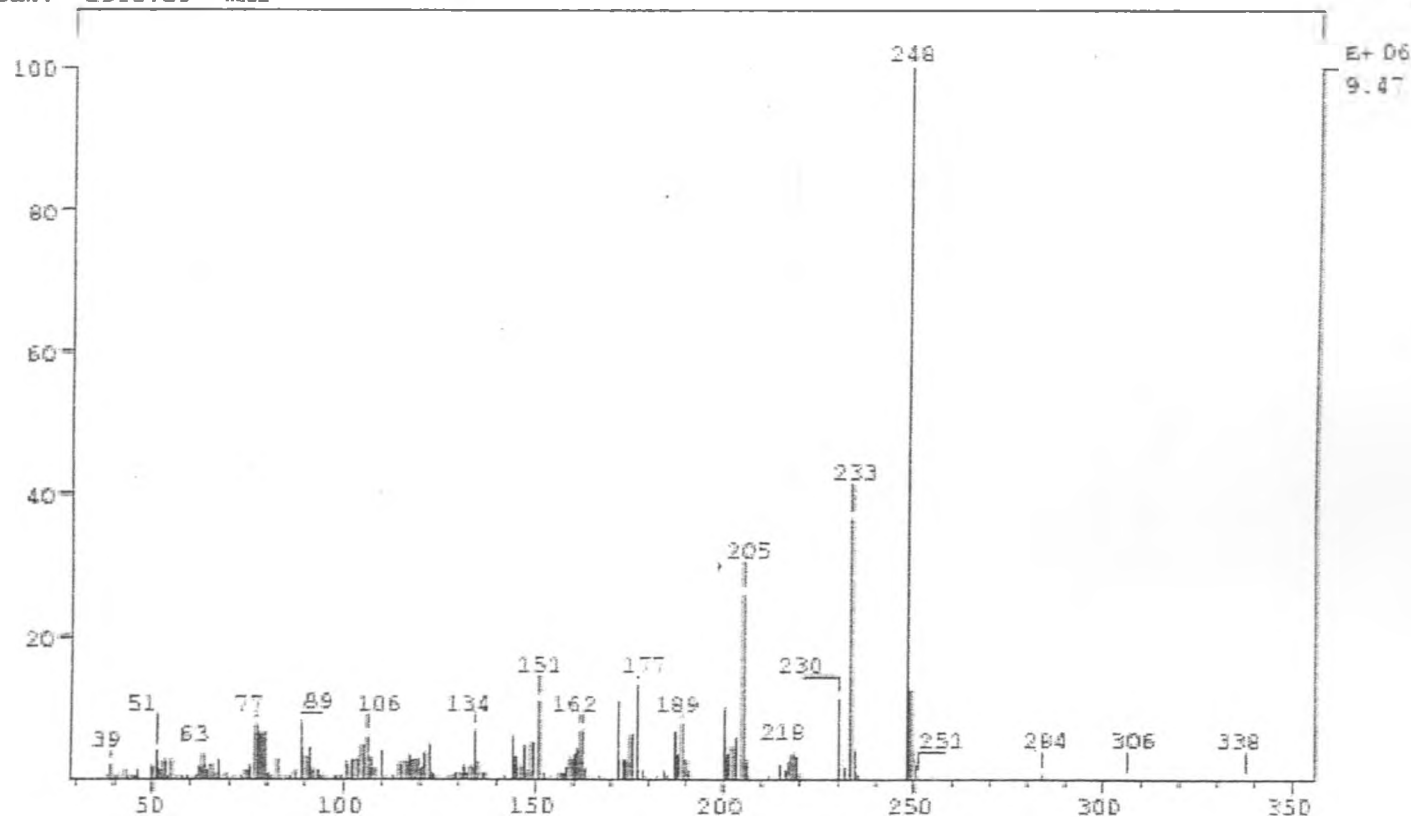
```

MS FOR COMPOUND 7

SPEC: heydl31
Samp: ENM 16A
Comm: M 248
Node: EI +QMS LMR UP LR
Oper: st+sf
Base: 248.2
Norm: 248.2
Peak: 1000.00 muu

Client: Haydenreich
Inten: 9468826
RIC: 49378984

Elapsed: 00:00:40.9 41
Start: 02:57:13 173
Study: DEP/EI
Inlet: DEP
Masses: 35 > 350
Spikes: 275



HRMS FOR COMPOUND 7

HEYD131EXM 5 (0.202) AM (Cen,5, 80.00, Ar,5000 0.556.28,0.00), Cm (3:9)

TOF MS ES+
899

