PHYTOCHEMICAL AND TOXICOLOGICAL STUDIES OF SOME BOTSWANAN PLANTS USED IN TRADITIONAL MEDICINE

Thesis submitted by

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in fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

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March 2003

To my Mum and Dad

ACKNOWLEDGEMENTS

One does not complete work of this magnitude without the contribution of many other people. First, I would like to express my sincere gratitude to my supervisor Dr. Sandy Gray for his guidance, expert advice, encouragement and strong support throughout the course of the project. I would also like to thank Professor Mike Cole for his assistance and advice during the project, especially at the beginning, Dr. Adrian Linacre of the Forensic Science Unit, University of Strathclyde for his guidance and assistance with the DNA work and Dr Christine Carter of the Department of Immunology, University of Strathclyde, for her assistance and advice with the toxicology work and for allowing me use of the facilities in her Department for the toxicity work.

I am really grateful to the following: Charles Masilo at the Botswana Police Forensic Science Laboratory for helping me with the collection of the plant material; traditional practitioners, Messrs, Bolebano Bolebano, Molaodi Makgowe and Peterson Nthobatsang, for assisting with the location and identification of the plants for collection; Mrs Queen Turner at the Botswana National Herbarium for allowing me use of the herbarium specimens; Dr. Runner Majinda at the University of Botswana for his advice and assistance; Mr David Harris at the Royal Botanical Gardens for confirming the identity of the plants; Mr Joseph Kihanda for his advice and assistance with the computer work; the late Mrs Arvind Bhasin for recording the mass spectra; Dr. Mukhlesur Rahman and Ms. Alaine Martin for recording the NMR spectra; the technical staff in the University of Strathclyde Forensic Science Unit for their assistance at the beginning of the project; my colleagues in the Phytochemistry laboratories for their support and friendship and everybody at the Botswana Police Forensic Science Laboratory who contributed in one way or another to the success of this research.

This work would not have been possible had it not been for the financial support from the Botswana Government and the Commissioner of Botswana Police Service granting me the opportunity to pursue the work. Finally, let me thank my family for their love, prayers and support throughout the project.

Abstract

Traditional medicine is widely used in Botswana and in recent years a number of plant species have been submitted to the Botswana Police Forensic Science Laboratory by the Police as exhibits in cases of suspected poisoning by herbal medicine. The request would be for the forensic toxicologist to establish whether the plant material is toxic or not. In this study a selection of these plants are being investigated phytochemically and toxicologically. These include: *Jatropha erythropoda* Pax. (Euphorbiaceae), *Cassia italica* (Mill.) Lam. Ex. (Leguminosae), *Asclepias fruticosa* L. (Asclepiadaceae), *Albizzia brevifolia* Schinz (Leguminosae), *Argemone mexicana* L. (Papaveraceae) and *Enicostemma axillare* L. (Gentianaceae). A DNA-based test capable of identifying the species from powdered fragments of the plant material has also been developed in this project.

Albizzia brevifolia, Enicostemma axillare and *Jatropha erythropoda* have not been investigated before; neither phytochemically nor toxicologically.

Dichloromethane, methanol and water extracts of each of the plants were tested for cytotoxicity against a panel of four cell lines – three human and one murine cell line. While all extracts exhibited some degree of cytotoxicity, extracts from *A. fruticosa* were found to be the most toxic with LD_{50} values for the crude extracts of 1.3 –3.4 µg/ml.

Phytochemical investigation of the extracts revealed the presence of a variety of secondary metabolites from the plants. *A. brevifolia* yielded terpenoids, phenolics, phenolic glycosides, a component of procyanidins, a lignan glycoside and sugars. *E. axillare* yielded terpenoids, a secoiridoid, and sugars, whereas *A. mexicana* yielded alkaloids. Investigation of *C. italica* and *J. erythropoda* revealed the presence of terpenoids, flavonoids, glycosides and sugars and that of *A. fruticosa* the presence of cardenolide glycosides.

Among the compounds isolated and tested for toxicity, sanguinarine, an alkaloid from *A. mexicana,* was found to be the most toxic with an LD_{50} value of 0.22 – 1.4 µg/ml. The

compound expresses toxicity by inhibiting Na/K ATPases and by intercalating with DNA bases and thus interfering with the replication process. Swertiamarin, the secoiridoid isolated from *E. axillare*, constituted about 10% of the dichloromethane extract of this plant, which showed significant toxicity. The plant also yielded swertiamarin as about 60% of the methanol extractive, which in contrast did not show any toxicity. Swertiamarin itself did not show toxicity at the levels tested, an indication that it is not responsible for the toxicity exhibited by the dichloromethane extract. However, secoirridoids such as swertiamarin might transform *in vivo* to toxic alkaloids.

The phenolic compounds (and their glycosides), isolated from *A. brevifolia*, exhibited very weak or no toxicity, whereas the terpenoid, betulinic acid, showed some cytotoxicity. Another terpenoid, which was isolated from the plant, lupeol, is reported to be cytotoxic. The extracts of the plant showed significant toxicity, especially the methanol extract. The toxicity exhibited by betulinic acid could not account for the toxicity displayed by the extracts, particularly the methanol extract. This toxicity is perhaps due to other compound(s) that were not isolated or to synergistic activity.

A DNA-based test has been developed for species identification using allele specific amplicons that show polymorphisms in the length of DNA sequences between two conserved primers. This is going to allow the species identification in cases where only small amounts of plant material are available, sometimes in mixture form. This will particularly be useful where there are no unique chemical markers to be used for identification. The test, is Polymerase Chain Reaction-based (PCR) and therefore very sensitive. Once the species is known more of it can be collected from the source or the wild to allow detailed toxicological and phytochemical work.

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CHAPTER 1:

INTRODUCTION

1.0 INTRODUCTION

1.1 Overview

Until recently, the use of traditional medicine was a practice common to poor or developing countries, where it was practiced as an alternative to orthodox medicine; or was the only form of therapy as modern health facilities could not adequately reach everybody. However, interest in herbal medicine is on the increase all over the world, as evidenced by the booming sales of herbal products in Europe, United States of America and China. In the United States alone the market for herbal supplements is now approaching $4 billion (BMJ 2000, editorial). In addition, researchers are involved in rigorous screening of plants used in traditional medicine in an attempt to discover new drugs, although "big Pharma" (large multinational companies) such as Merck are abandoning natural product research, their involvement being through the financing of small companies and universities. An example is the backing of Instituto Nacional de Biodiversidad (INBIO, Costa Rica) by Merck.

According to Hedberg and Staugard (1989), up to the beginning of the last decade, traditional medicine was used by more than two thirds of the rural population in Botswana. As is the case worldwide, herbal medicine is increasingly gaining popularity with the urban population. In general, people have different reasons for using herbal medicine. Some individuals who have lost faith in modern medicine use herbal medicine as their first choice where others are firm believers in phytotherapy and would only resort to modern medicine when herbal treatments have failed.

Taking as an indicator the cases which have been handled by the Botswana Police Forensic Science Laboratory, it is apparent that the outbreak of HIV/AIDS has led to more people turning to traditional healing since modern medicine has failed to identify a cure. The widespread use of traditional medicine nowadays has led to such herbs being sold in open markets in urban areas in Botswana and other parts of the world.

Herbal medicine is dispensed in various forms. It may be given to users as pieces of roots, leaves, bark or seeds, as a powder of each of these, or a mixture, or a mixture of different plants, or in a ready-made liquid form. Little information on the exact doses to be taken is given. Usually these materials are boiled in a liquid medium, mostly water but sometimes milk, allowed to cool and the liquid is taken by the mugful, rarely in spoonful quantities (personal communication with traditional healers). An alternative to boiling would be to let the plant material stand in the liquid for a period of time, for example steeping overnight, before being ingested. While with modern medicine there is always an indication of adverse effects, due to licensing requirements, such information is normally not available to users of traditional medicine, mainly because the healers may not be aware of these. As herbal medicine becomes more popular, potential toxicity problems should be investigated thoroughly. There are limited data on the pharmacology, toxicology, quality and active principles of most herbal medicines.

In Southern Africa (and supposedly in most African countries) possible toxicity of herbal medicines has become a significant problem for forensic science services (personal experience). Police in this part of the world often find themselves handling cases that involve alleged poisoning by herbal medicines. This has become a challenge for forensic toxicologists who have a duty to assist the Criminal Justice Systems in resolving such matters. Not only in the interest of the state but also to assist the herbalists who often face criminal charges against allegations of deliberately poisoning clients.

1.2 Reasons for this Study

As already stated, in Botswana, a number of these medicinal plants have been implicated in cases of fatal poisoning. Though such prescriptions are often obtained willingly and are given in good faith, the tendency nowadays is for the relatives of the deceased to blame the traditional healer and to enlist police assistance in charging the individual with poisoning. Therefore the forensic scientist is expected to establish the toxicity, if any, of the culprit plant material and the possibility of it being the cause of death or discomfort. As an indication of the scope of the problem, figures from the Forensic Pathologist's

office for the Southern part of Botswana show that in 1999, 17 % of the 58 cases handled by this pathologist were due to traditional medicine poisoning. In 2000 this figure was 28% of 47 cases and in 2001, 8% of 37 cases. The human organs affected are normally the heart (pericarditis and pericardial effusion), the lungs (pleuritis and pleural effusion) and the brain.

In the majority of cases the toxicologist has to work with only a few grams of the powdered plant material recovered from the victim. This powder is usually a mixture of several plants/parts and other non-botanical materials, such as animal waxes, petroleum jelly, and inorganic chemicals. The toxicologist has to determine the toxic levels of the material without any knowledge of the active components of the plant material or the identity of the plant(s). In a few instances the original plants can be identified but there are no documented data on the toxicity of most of the plants used.

At recent meetings of forensic scientists in the Southern African region it has become evident that the problem of solving cases involving traditional medicine is universal to the whole region. Indeed in some of the laboratories they refuse to work on such cases as they consider them to be a waste of time. This frustrates law enforcement agencies, the criminal justice system and the relatives of the deceased who would want such cases to be resolved in one way or another.

Therefore detailed data on active constituents of medicinally used plants are required to establish whether they are toxic, and if so, the level at which toxicity is manifested. These details are vital in forensic work because for toxicity (lethal or otherwise) to be attributed to a substance, the amounts involved should be stated. In addition, during the course of such research, methods of isolation, purification, identification and toxicity testing would be developed that can be used in a forensic science setting for routine casework in African countries. Most of the work currently carried out by plant researchers is focusing on identifying new medicines from plants and the toxicity of the extracts and the isolated compounds is poorly researched.

A large number of issues need to be considered when dealing with the toxicity of herbal medicines. Whereas modern medical practitioners use purified ingredients, herbal medicines are in most cases taken as mixtures and thus the possibility of synergic activity is high. Interactions with other drugs i.e. prescriptions from doctors is also possible since in most cases these herbs are used simultaneously with other drugs. Chronic toxicity is also bound to occur as much as it does with other substances. Another issue to be considered is that of possible cross contamination due to poor handling (quality control) especially in third world countries where only minimal forms of quality control exist.

1.3 What Are Plant Active Principles

Plant products are divided into primary and secondary metabolites. Carbohydrates, proteins, and lipids are considered primary metabolites, as they are important for the fundamental survival of the plant. In addition to a plant's primary metabolites, a plant also produces secondary metabolites, which are compounds with no apparent function in the primary metabolism of the plant (Harborne, 1988). The importance of secondary metabolites to the plant is still under debate but it is thought that some are produced by the plant to defend itself against predators. For instance, some terpenes inhibit the growth of other competing plants and others are insecticidal. Secondary metabolic products have an extensive history as therapeutic agents. Plants produce hundreds of these. It is estimated that about 23% of the currently employed drug prototypes for orthodox medicines is of plant origin (Sneader, 1996). The most common secondary metabolites occur in the following groups: terpenoids (monoterpenoids - essential oils and iridoids, sesquiterpenoids, diterpenoids and triterpenoids - steroids, saponins, cardiac glycosides), phenolic compounds (simple phenols, phenylpropanoids, coumarins, flavonoids, tannins, quoinones) and alkaloids. (Cannell, 1998; Kaufman, *et al.*, 1999). Examples of each of these groups include, respectively, menthol (**1**), loganin (**2**), santonin (**3**), phytol (**4**), stigmasterol (**5**), lemmatoxin (**6**), oleandrin (**7**), Catechol (**8**), myristicin (**9**), umbelliferone (**10**), kaempferol (**11**)), procyanidin B-1 (**12**), emodin (**13**) and codeine (**14**).

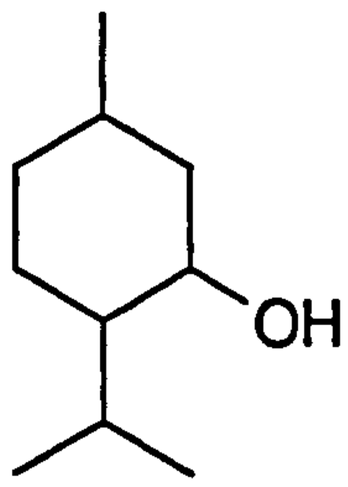
Some of these are known to be toxic and the incidence of toxicity is not limited to certain groups of compounds. The following are a few examples, but, as Paracelsus observed centuries ago, "all substances are poisons; there is none which is not a poison; the right dose differentiates a poison and a remedy" (Paracelsus (1493-1541). Some, however, exhibit outstanding toxic properties in relation to others and are mentioned here to demonstrate the potential hazard posed by some plant products. Polyacetylenes (e.g. **15**), which are hydrocarbons, from the water dropwort, *Oenanthe crocota* (Kaufman *et al.*, 1998) are known to be toxic. Cytotoxic lactones have been isolated from the *Prodacarpus spp.* (Bruneton, 1995) and some iridoids are also known to be toxic. Among the triterpenes, saponins such as lemmatoxin (**6**) and cardiac glycosides are well known to be very toxic, especially the latter. The toxicity of cardiac glycosides from *Digitalis spp.* is well known (Calixto, 2000). The use of cardiac glycosides for cardiovascular problems is a good example of the thin line that sometimes exists between the therapeutic and toxic effect of a drug; at a certain concentration they exhibit therapeutic properties but a slight increase in the dose results in life threatening toxicity. Alkaloids are the most commonly known class of plant toxins, even though not all are highly toxic. However, according to Harborne (1988), most alkaloids are liable to show some toxic effects if ingested in any quantity over an extended period of time.

Zoapatonol (**16**), a diterpenoid derived from the Mexican plant *Montanosa tomentosa* is an example of plant drugs that exhibit other side effects other than toxicity; it has been used as an abortifacient (Kaufman *et al.*, 1998; Phytochemical Dictionary, 1999).

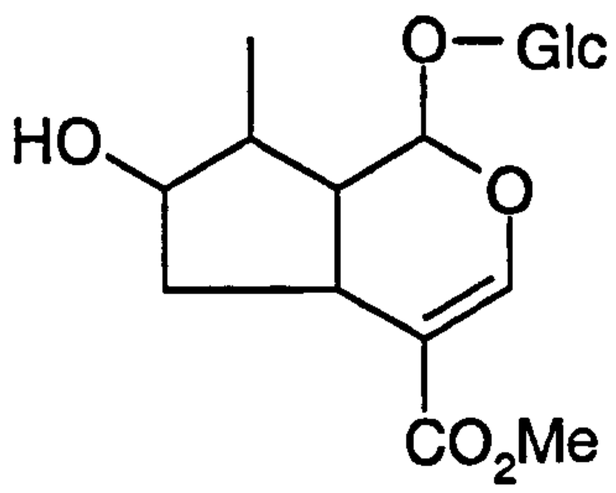
1.4 Forensic Toxicology

This is a field of Forensic Science that deals with the study of poisons to the elucidation of questions that occur in judicial proceedings (Moffat, *et al.*, 1986). The work mainly involves the detection of poisonous materials (substances harmful to living organisms) in body fluids and organs in both the living and the dead depending on the nature of the investigation.

Experience has shown that the types of analysis dealt with by Forensic Science laboratories in developing countries in Southern Africa differ in many aspects from the type of toxicology cases dealt with by laboratories in Europe and America. While most laboratories in the latter deal mainly with the detection of drugs of abuse/recreational drugs in biological specimens, laboratories in the former have to deal with the detection of a variety of materials, with drugs being a small portion of the work. Materials handled include, *inter alia*, pesticides, other organic and inorganic substances and mainly poisoning by herbal medicine.



1



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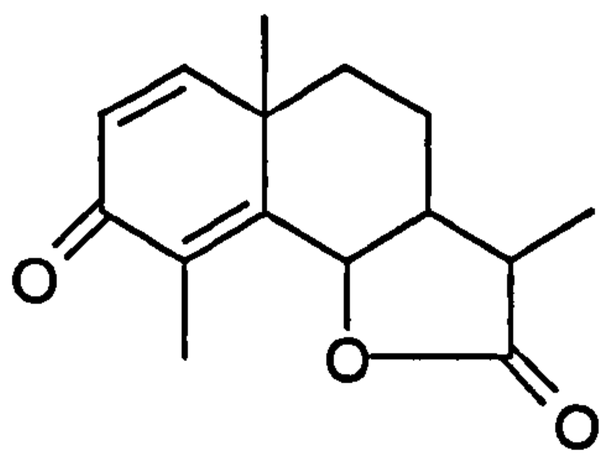

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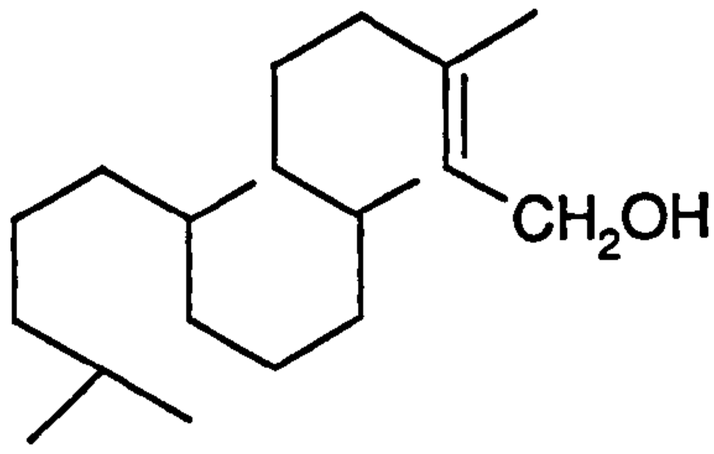

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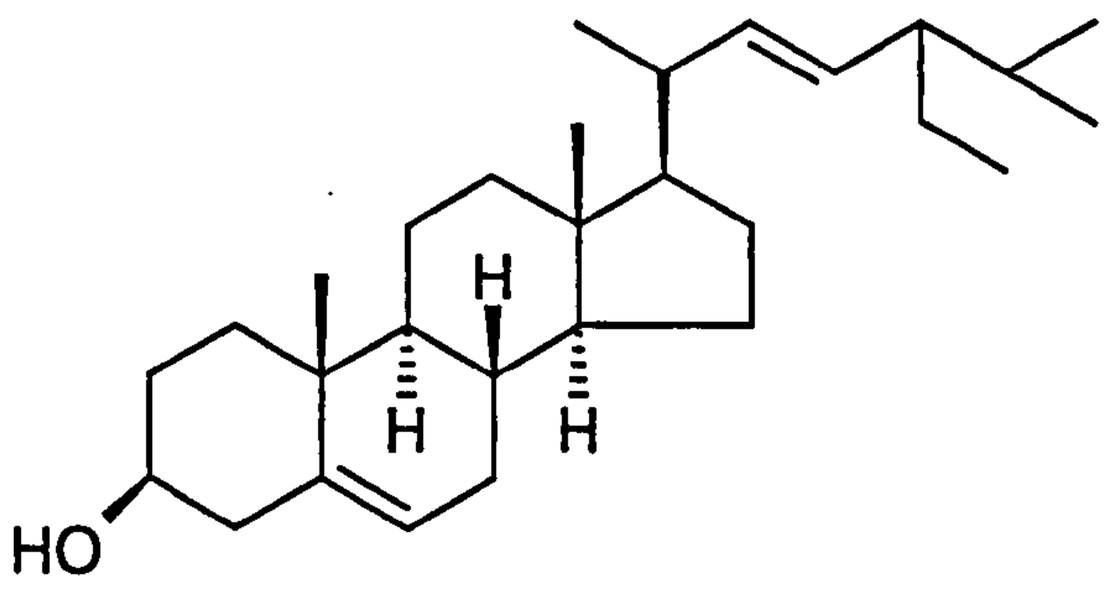

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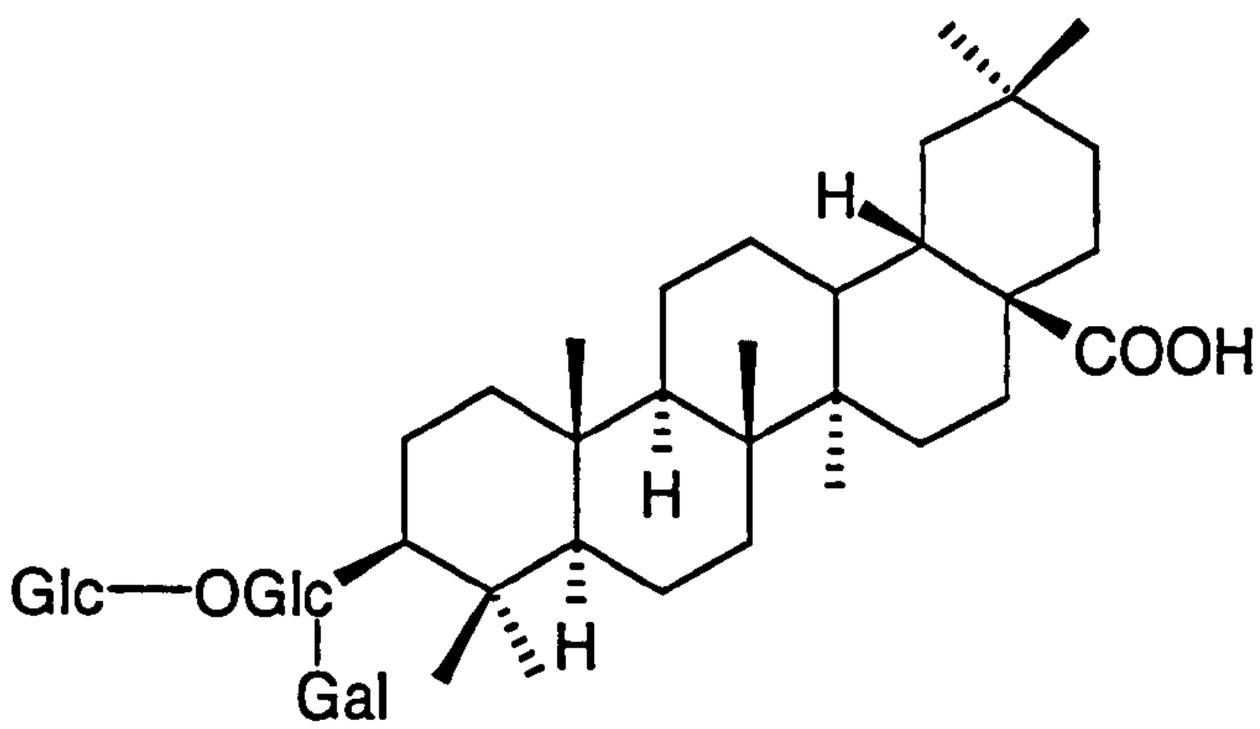

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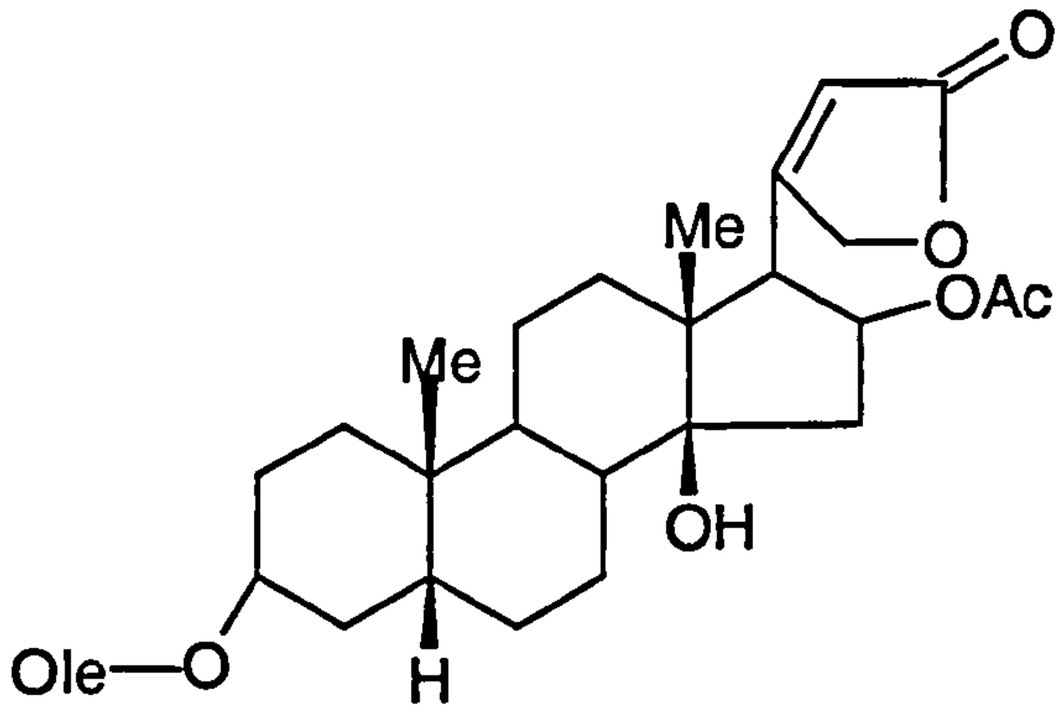
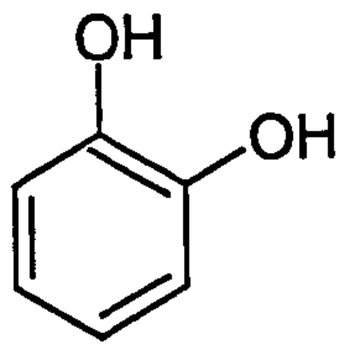


7

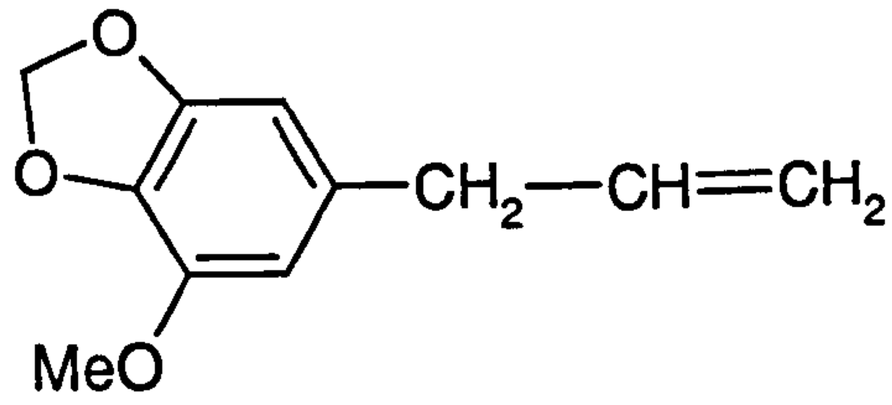
gal = galactoside

glc =glucoside

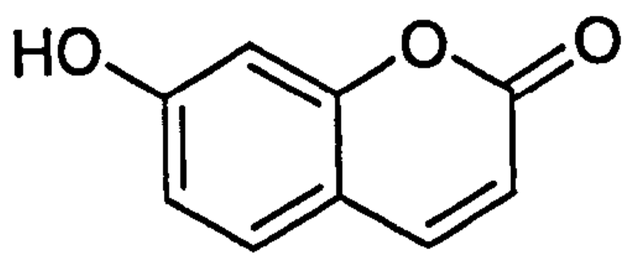
ole = oleandroside


OH
OH

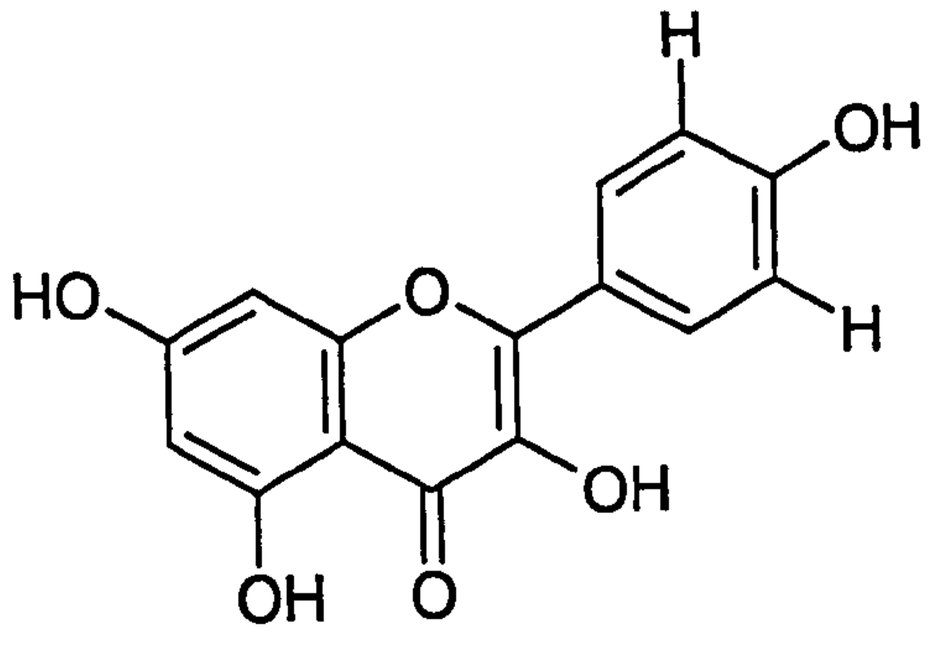
8


O
O
CH2—CH=CH2
MeO

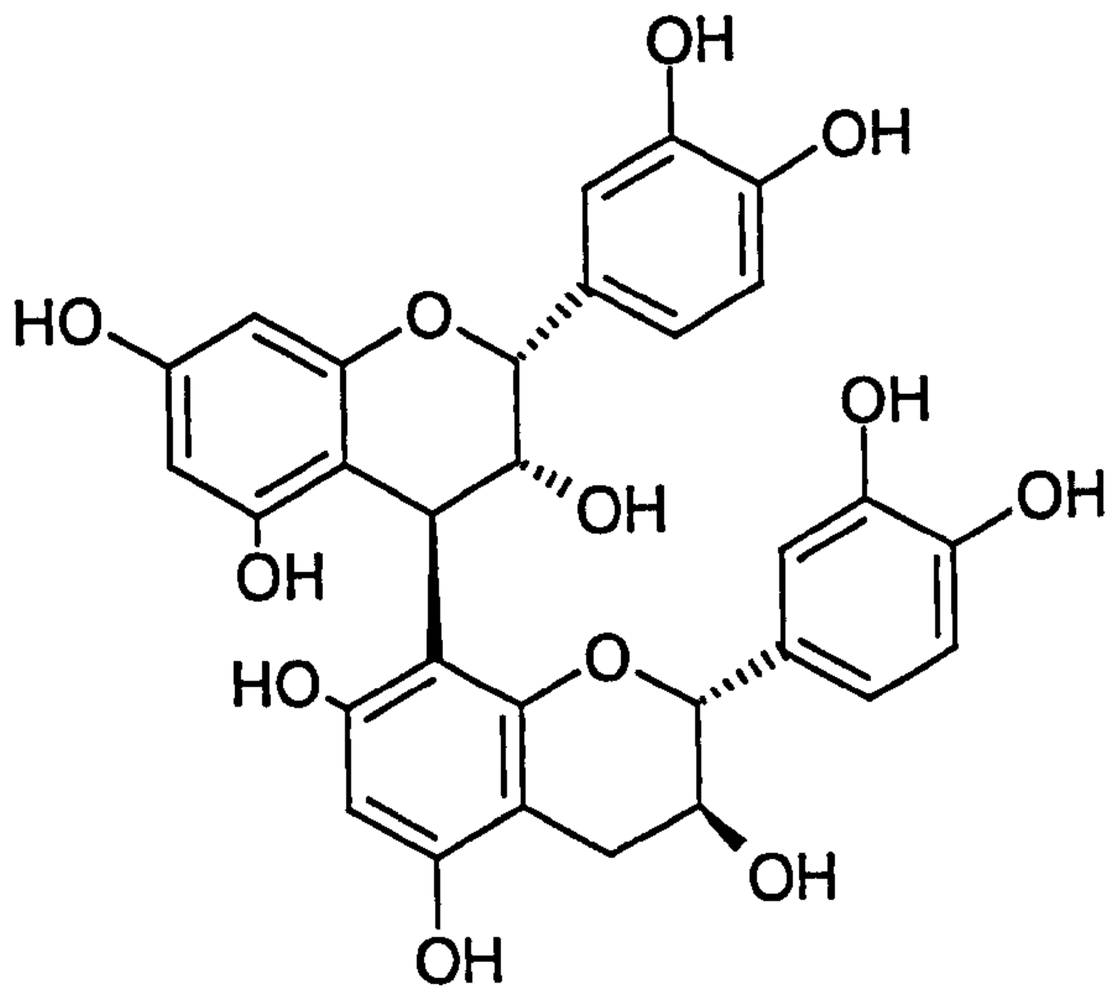
9


HO
O
O

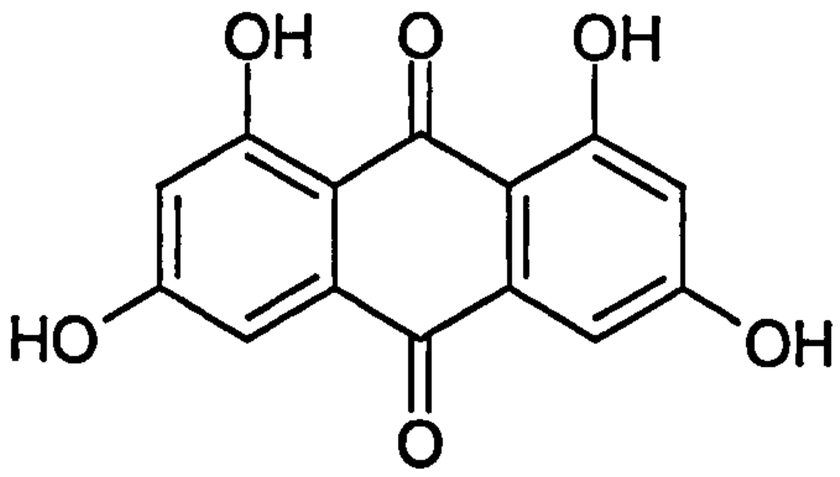
10


H
OH
HO
O
H
OH
OH
O

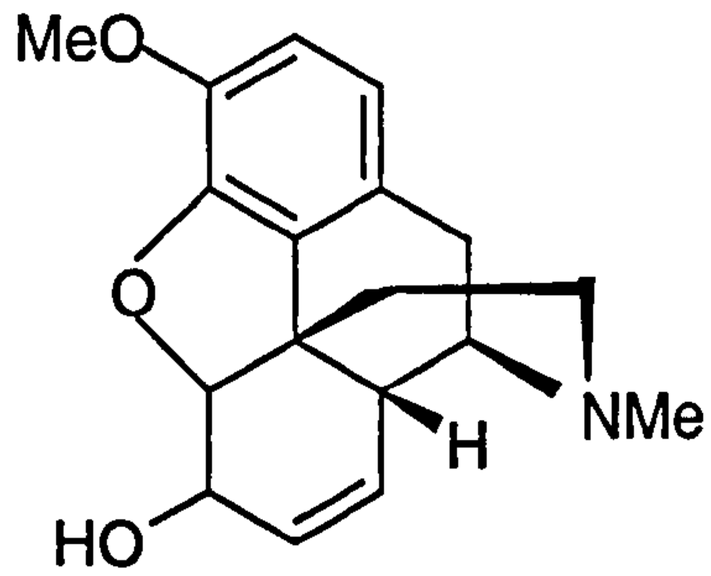
11


OH
OH
HO
O
OH
OH
OH
HO
O
OH
OH

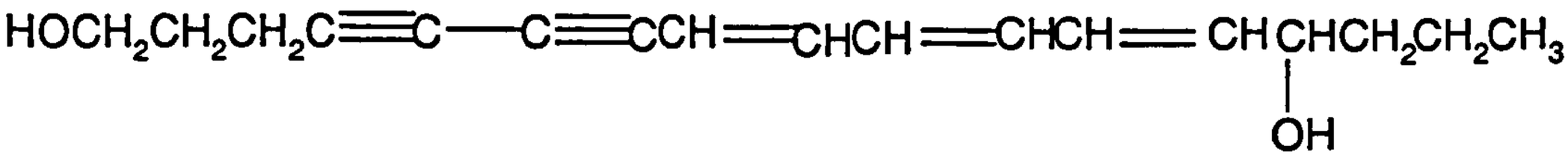
12


OH
O
OH
HO
OH
O

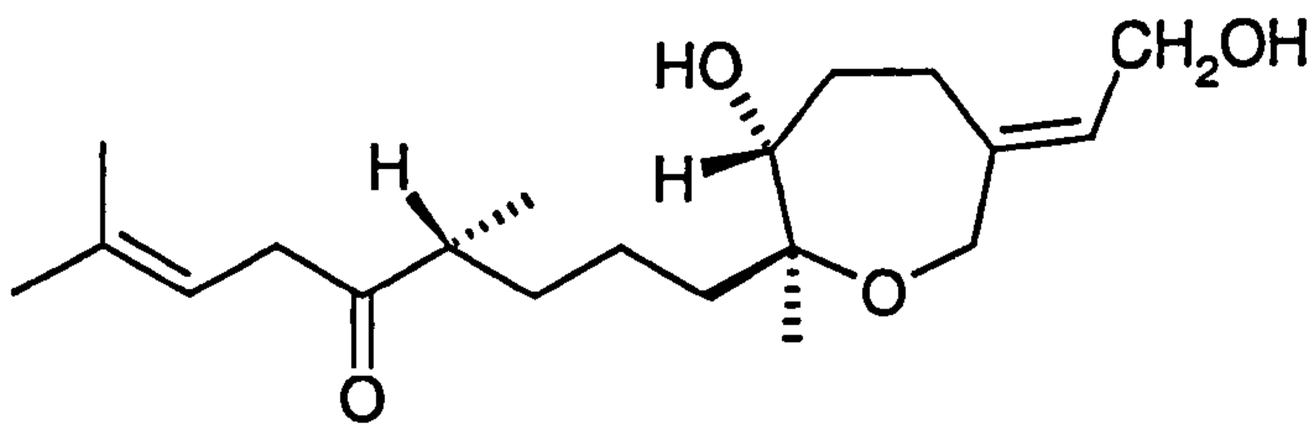
13


MeO
O
H
NMe
HO

14


HOCH2CH2CH2C≡C—C≡CCH=CHCH=CHCH=CHCHCH2CH2CH3
OH

15


HO
CH2OH
H
H
O
O

16

1.5 Objective of this study

The objective of this study was to carry out phytochemical, toxicity studies and DNA analysis on some of the plants that have been encountered in cases of suspected poisoning by traditional medicine at the Botswana Police Forensic Science Laboratory. This involved:

- Collecting the plant material and taxonomically identifying the species to be investigated
- Toxicity testing of the crude extracts
- Phytochemical investigation of the extracts – extraction, isolation and identifying the secondary metabolites from the plants
- Toxicity testing of isolated and purified compounds
- Method development
- Investigation of case samples from Botswana
- Development of a DNA-based test for species identification

Six plant species from different genera were investigated in this study. The following section outlines background information on each of the species.

1.6 Description, previous phytochemical and toxicological studies on the plants

1.6.1 *Jatropha erythropoda* Pax.

Picture 1.1: *Jatropha erythropoda*

J. erythropoda belongs to the order Geraniales and the family Euphorbiaceae which comprises 300 genera and about 600 species most of which are trees and few are herbs. The genus *Jatropha* comprises 175 species. *J. erythropoda* is a herb growing to about 30cm above the ground, with serrated green leaves, pinkish white flowers, brownish black seeds, has tubers underground (about 20cm from the top) mostly roundish. It is found in the Southern, Central and Northern parts of Botswana. This plant is known as

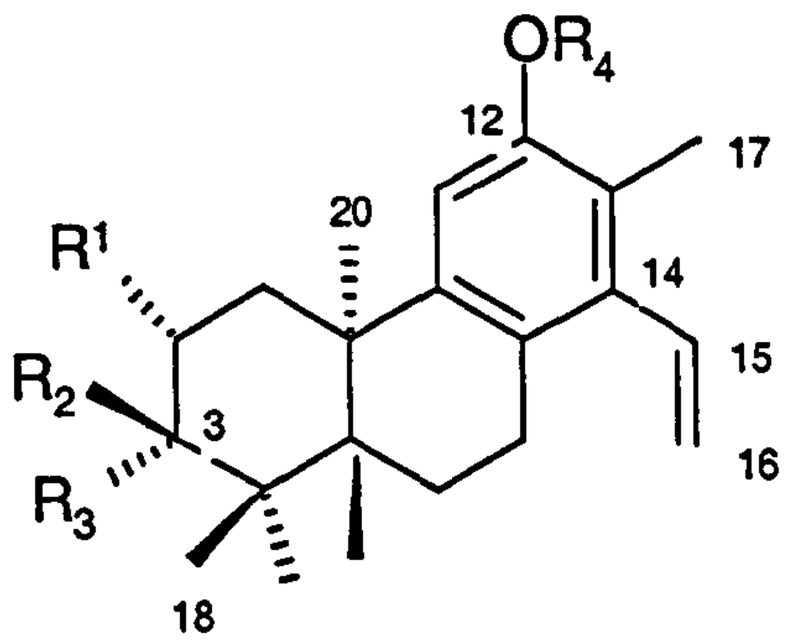
Thotamadi (a name that refers to the blood-like sap exuded by the tubers) in Botswana and is medicinally used for the treatment of sexually transmitted diseases. The tubers are boiled in water and a cup of the liquid is taken 3 times a day. Other species in the genus *Jatropha* are reported to have medicinal use, for example *Jatropha curcas* is used throughout most parts of Africa (Watt and Breyer-Brandwijk, 1962) including Somalia (Samuelsson *et al.*, 1992). It is also used in Panama (Gupta *et al.*, 1996), India and Brazil (Ghandi, *et al.*, 1995).

1.6.1.1 Phytochemical

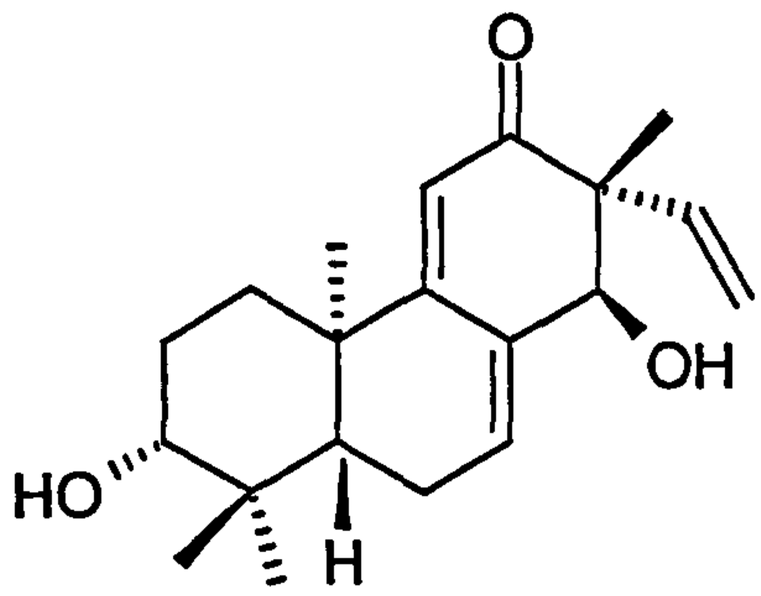
Some members of the genus *Jatropha* are reported to produce unsaturated fatty acids, fatty acid epoxides, triterpenoids, anthraquinones, phenolics, flavonoids, cyclicpeptides and sometimes alkaloids (aporphine, indole, quinoline or tropane), (Trease and Evans, 1983). Ahmad *et al.* (1992) reported the isolation of two imidazole and one piperidine alkalods from *J. gossypifolia*. Most of the work that has been carried out is on the species *Jatropha curcas L.* and the studies show that the species produces mainly terpenoids and lignans. Examples of compounds that have been isolated from the genus are provided in Table 1.1.

Table 1.1: Examples of secondary metabolites isolated from the genus *Jatropha*

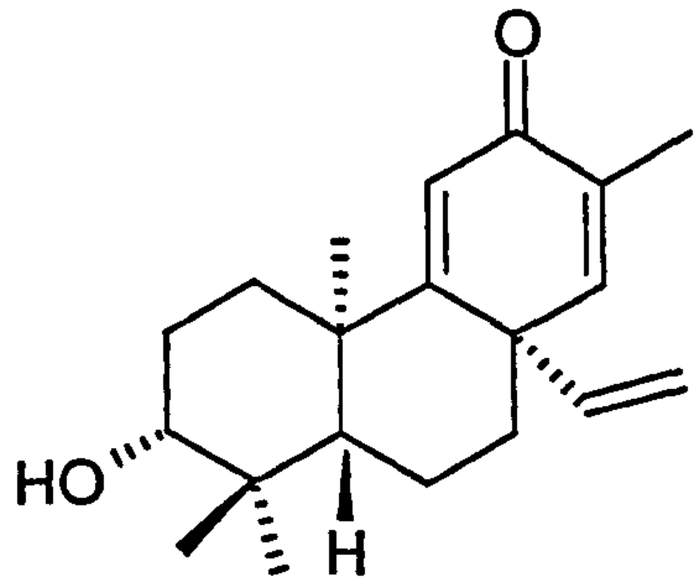
Compounds	Source (Reference)
Diterpenes:	*J. divaricata* (Denton *et al.*, 2001)



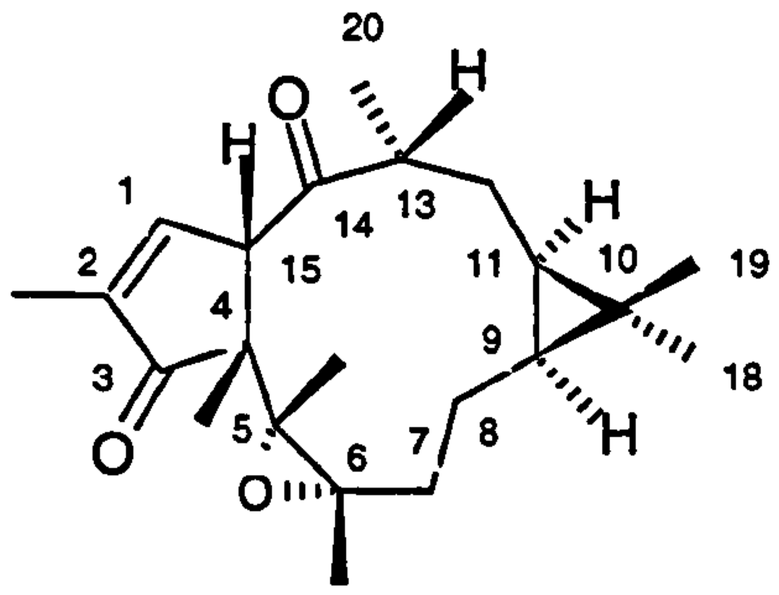
	R_1	R_2	R_3	R_4
Spruceanol (**17**)	H	H	OH	H
Cleistanthol (**18**)	OH	H	OH	H



ent-3β, 14α-hydroxypimara-7-9(11),15-trien-12-one (**19**)

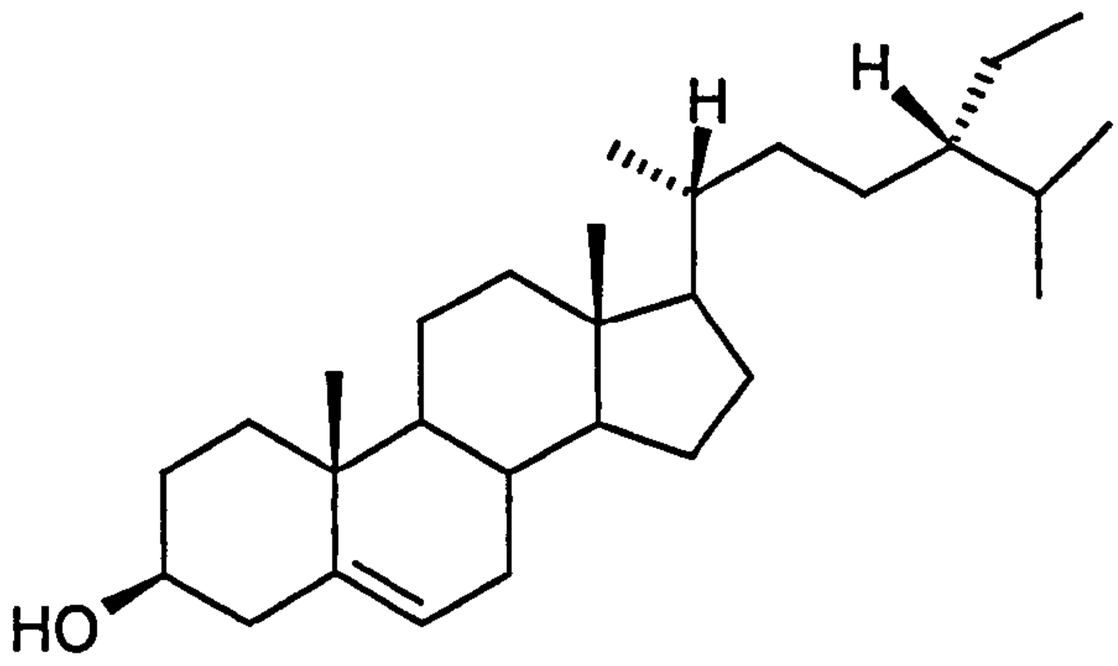


ent-15(13→8)*abeo*-8α(ethenyl)-pimarane (**20**)



Jatrowedione (**21**) *J. weddlliana* (Brum *et al.*, 1998)

Ttriterpenes:

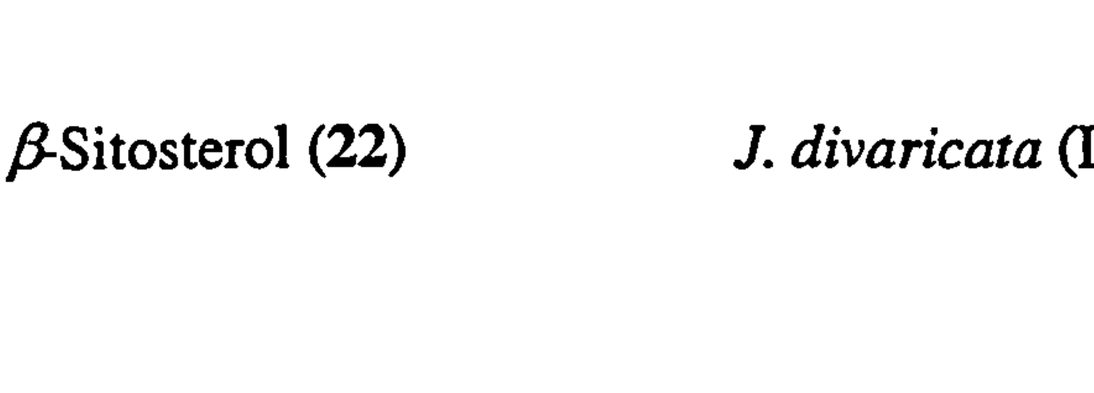


β-Sitosterol (**22**) *J. divaricata* (Denton *et al.*, 2001)

Flavonoids:

5-hydroxy, 7,4'-dimethoxyflavone (**23**) *J. podagrica* (Odebiyi, 1982)

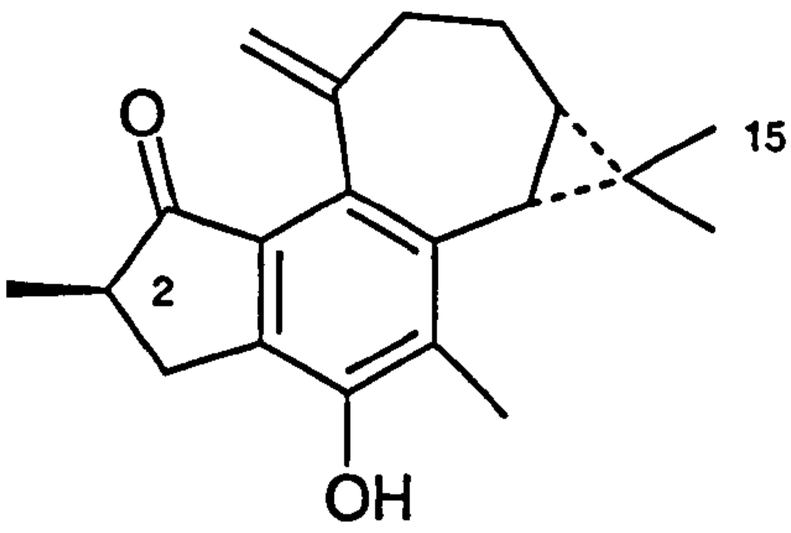
Lignans:



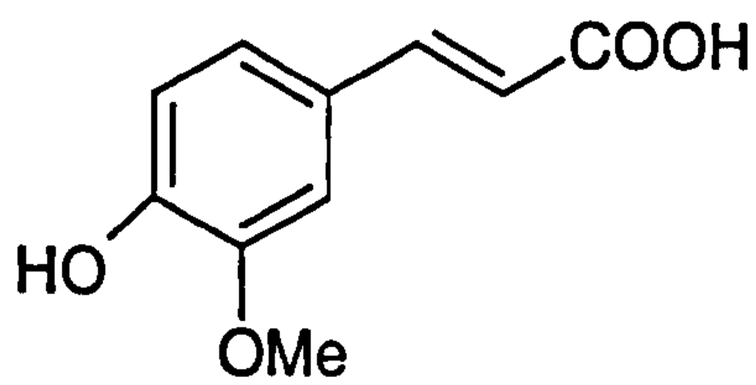
Jatrodien (**24**) *J. gossypifolia* (Das *et al.*, 1996)

Other phenolics:

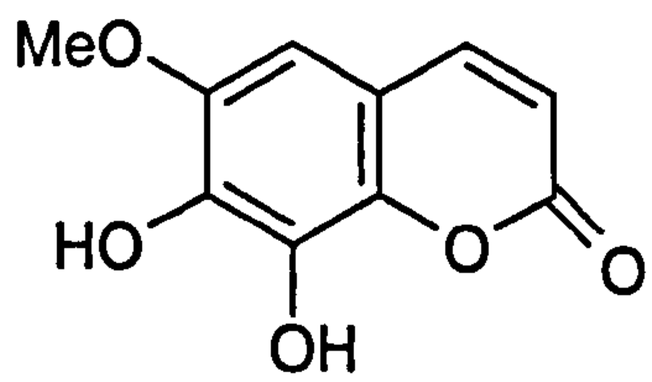
J. gossypifolia (Das and Kashinatham, 1997)



Jatrophone B (**25**)

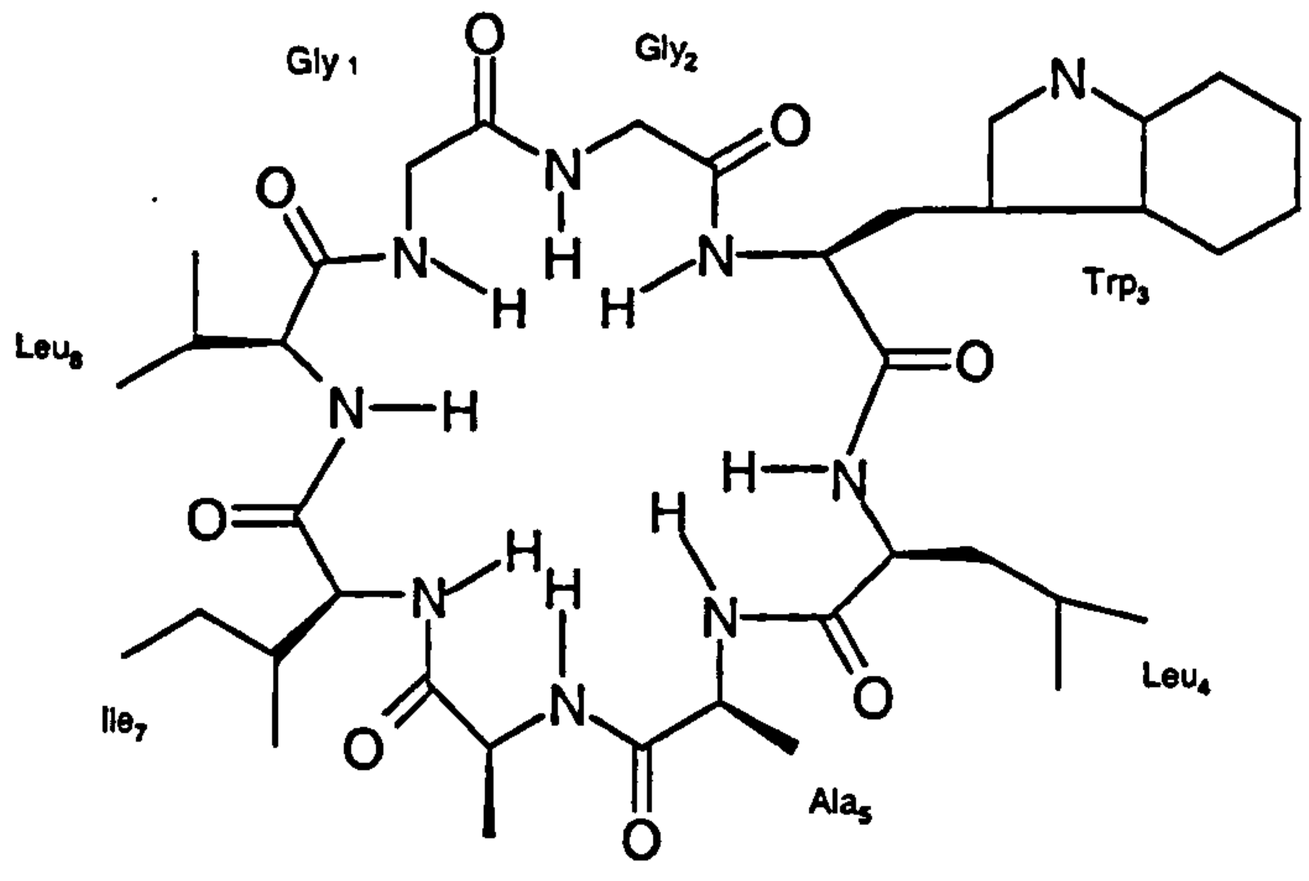


Ferulic acid (**26**)



Fraxetin (**27**)

Cyclicpeptides:



Cyclogossine B (**28**) *J. gossypifolia* (Auvin-Guette *et al.*, 1997)

Glc = glucose

Ole = oleandrose

To date there is no report on any phytochemical study on *J. erythropoda.*

1.6.1.2 Toxicological

Many of the species of this genus are reported to contain irritant or piscidal substances, for example the bark of *Jatropha curcas* according to Watt and Breyer-Brandwijk (1962) has been used as fish poison, the fish being stupefied. The seed has been reported to have caused poisoning in Children in South Africa and even in adults where the seed would have been taken for medicinal reasons or as an abortifacient (Watt and Brandwijk, 1962). Oluwole and Bolarinwa (1997) reported that *J. curcas* extracts in their study caused anaemia in rats and that the LD_{50} in the acute toxicity test in mice was 25.19 mg/kg of body weight. Ghandi *et al.* (1995) studied the toxicity of *J. curcas* oil and reported that its extract was seen to be toxic to rats (acute LD_{50} 6 ml/kg body weight). In mice it was found to have a dermaly toxic and lethal effect. Extracts from the plant were found to be toxic against snails that act as hosts for schistosomes by Rug and Ruppel, (2000). El Badwi *et al.* (1995) compared the toxicity of *Ricinus communis* and *J. curcas* on brown Hissex chicks. They found that *J. curcas* exhibited toxic effects even though *R. communis* was found to be the more toxic of the two. *R. communis* produces ricin, which is very toxic. It has recently been reported that ricin was allegedly being produced for potential use in terrorist attacks. The substance was allegedly used in 1978 to kill the Bulgarian journalist, Georgi Markov in London (Timbrell, 1989); a tiny metal pellet was recovered from a wound on the victim's leg, seemingly inflicted accidentally by an umbrella. It was through the metal pellet that the poison was supposedly introduced. In Botswana *Ricinus communis* has been implicated in fatal cases (personal knowledge). Goonasekera *et al.* (1993) studied pregnancy-terminating effects of *J. curcas* fruits in rats and found that pregnancy interruption occurred at an early stage. The animals were also seen to lose weight during the period and marked toxicity was observed with some extracts given over a period of 10 days.

The seed of *Jatropha multifida L.* is also reported to produce similar toxic effects as that of *J. Curcas* (Watt and Breyer-Brandwijk, 1962). Levin *et al.* (2000) reported poisoning

of children after ingestion of J. multifida seeds in 2000 and Singh (2000) reported that dilute aqueous solutions of *J. gossypifolia* were effective in killing fish with an LC_{50} of 4.34 g/L in 96 hours.

No information has been found on the toxicity of *J. erythropoda* during the literature search.

1.6.2 *Cassia Italica* (Mill.) Lam. Ex.

Picture 1.2: *Cassia Italica* (Mill.) Lam. Ex.

This plant belongs to the order Rosales and the family Leguminosae (subfamily, Caesalpinaceae), which comprise 600 genera and about 12 000 species. The subfamily Caesalpinaceae contains 133 genera (Trease and Evans, 1983) and the genus *Cassia* about 600 species (El-Sayed *et al.,* 1992) It is a creeping plant with green leaves, bright yellow flowers, flattish fruits – green when immature and black roots. It grows to about

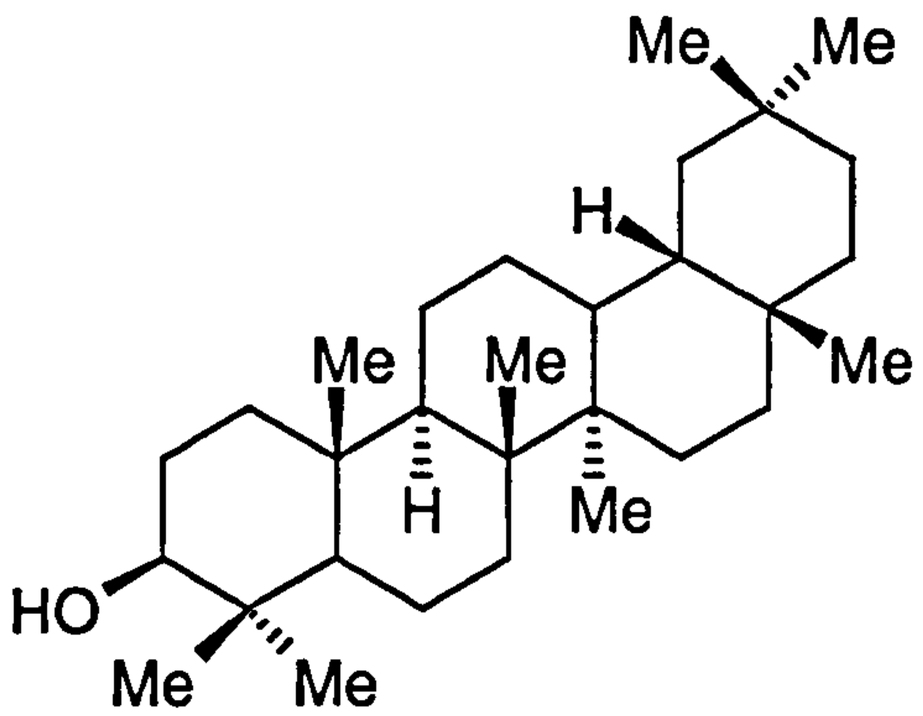
10 cm high. It is found abundantly in sandy areas with relatively thick growth. It is found in most parts of East Central, Eastern, Central and North Botswana. Many of the species of *Cassia* such as *C. senna, C. tora* and *C. occidentalis* are used in traditional medicine for the treatment of round worm infection, constipation, fever, pleurisy, oedema, ringworm and pustular or eruptive skin conditions (Bakhiet and Adam, 1996; Watt and Breyer-Brandwijk, 1962). In the Sudan pounded leaves of some of the species are used for healing fresh wounds, the seeds for ring worm, the roots as diuretics and fruits for purgation (Galal *et al.*, 1985). The plant is reportedly used for ailments such as liver problems, constipation, bloody diarrhoea, and syphilis in Botswana (Hedberg and Staugard, 1989). It is locally known as *Sebete* in Botswana.

1.6.2.1 Phytochemical

A variety of secondary metabolites including terpenoids, sterols, anthraquinones, alkaloids, flavonoids, and tannins have been isolated from this plant and other species belonging to the genera. A summary of the compounds isolated from *C. italica* is provided at Table 1.2.

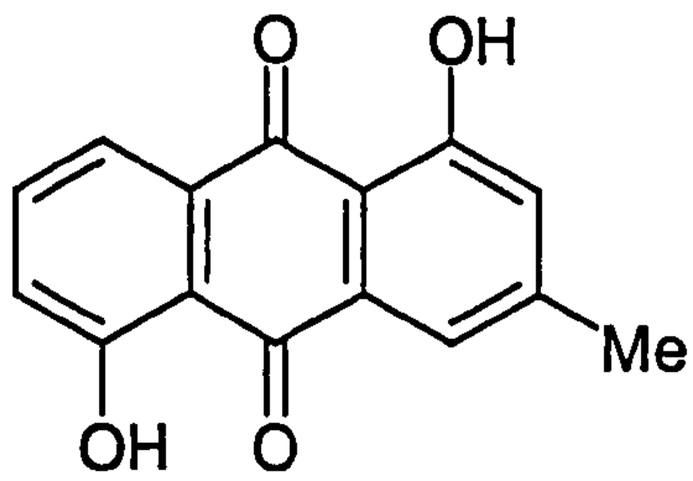
Table 1.2: Secondary metabolites isolated from *C. italica*

Compounds isolated	**Reference**
Lipids from seeds	Osman and Fiad, (1975)
Terpenoids:	
β-sitosterol (**22**)	
Stigmasterol (**5**)	

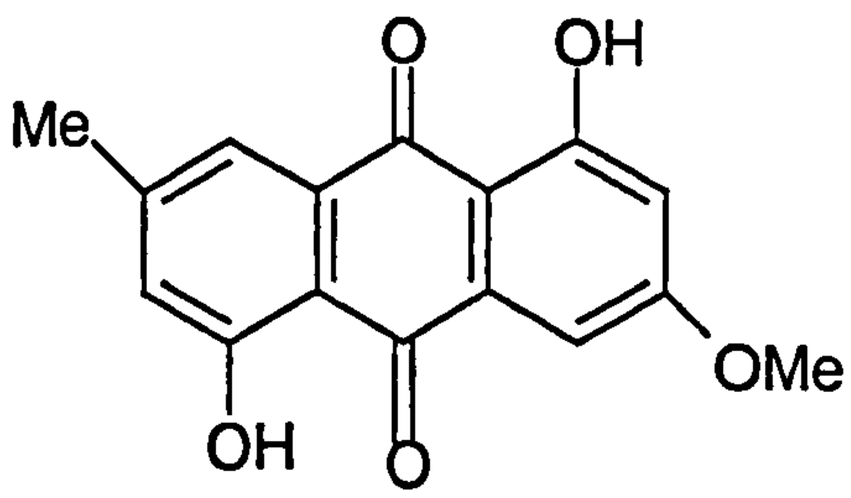


β-amyrin (**29**)

Anthraquinone derivatives: Kazmi *et al.*, (1994)



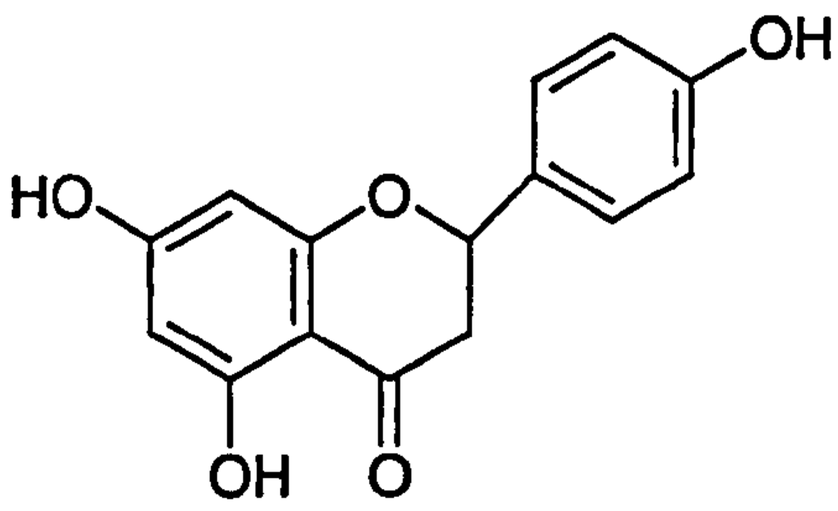
1,5-dihydroxy-3methylanthraquinone (**30**)



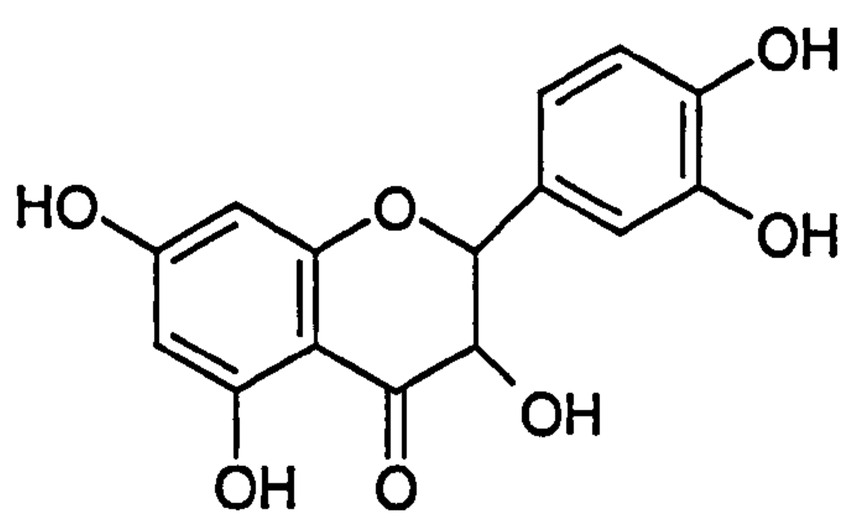
1,5-dihydroxy-3-methoxy-7-methylanthraquinone (**31**)

Emodin (**13**) El-Sayed *et al.*, (1991)

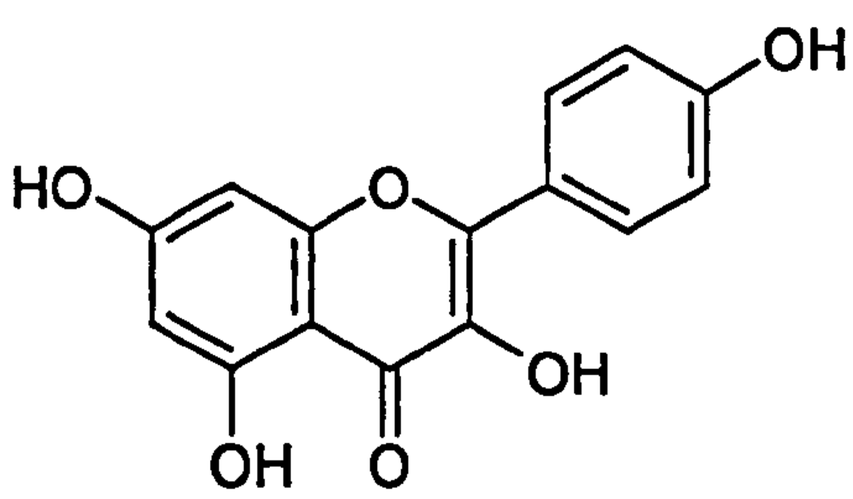
Flavonoids: Al-Sayed *et al.*, (1991)



apigenin (**32**)



quercetin (**33**)



kaempferol (**11**)

7-glucosides of apigenin, kaempferol, apigenin (**34**)

3-rutinosides of kaempferol and quercetin (**35**)

3-rutinoside-7- rhamnoside isorhamnetin (**36**)

tamarixetin 3-*0*-rutinoside7-*O*-rhamnoside (**37**)

1.6.2.2 Toxicological

The anthracene derivative active principles in various *Cassia* species are irritant purgatives, which stimulate the peristaltic movements of the large intestine. They may result in increased menstrual haemorrhage, possibly abortion (Watt and Breyer-Brandwijk, 1962). They are also used as wound healing agents. Assane *et al.* (1994) showed that *C.italica* leaves and pod extracts have dose related stimulation of intestinal contractions. Watt and Breyer-Brandwijk (1962), report toxicity of some species of the genus. These include *C. didymobotrya* Fres., the leaf of which is said to be very

poisonous, and is sometimes used as fish poison. It has reportedly caused poisoning in livestock. In Australia *C. leavigata* Willd. is regarded as being toxic to cattle. The toxicity of Sudanese *C. senna* and *C. occidentalis* to small ruminants has been reported (Bakhiet and Adam, 1996 quoting Suliman *et al.* (1982) and Sayed *et al.* (1983). These authors carried out toxicity studies of *C. italica* seeds on 7-day-old Bovans chicks. They found that 10% of *C. italica* seeds in the diet of the chicks resulted in decreases in weight and efficiency in feed utilisation. There was also evidence of enterohepatonephrotoxicity. Galal *et al.* (1985) studied the effects of *C. italica* on goats and sheep and found that the fresh shoots, fruits and leaves of this plant were toxic to Nubian goats and Desert sheep and that death occurred at different intervals after the commencement of oral dosage. The minimum dosage that caused death was 0.5 g/kg in both types of animals. At the end of their study they reported that the fruits and leaves of this plant were found to be equally toxic to the experimental animals. Ali *et al.* (1996) studied the effects of this plant on the Central Nervous System of mice at concentrations of 0.5 and 1g/kg body weight and they concluded that the crude extract of *C. italica* has CNS depressant properties manifested as antinociception and sedation.

1.6.3 *Asclepias fruticosa* L. *(Asclepiadaceae)*

Picture 1.3: *Asclepias fruticosa* L.

This plant belongs to the order Gentianales, and family Asclepiadaceae which comprises 130 genera and about 2000 species of shrubs and herbs. The genus Asclepias contains 120 species. (Trease and Evans, 1983). *Asclepias fruticosa* L. (previously *Gomphocarpus fruticosus,* Cheung *et al*. (1981)) is an erect shrublet, which grows to about 2 metres tall, has multiple long thin stems, narrow opposite green leaves, greenish-yellow flowers, and greenish pear shaped hairy seedpods. It is used medicinally for a number of ailments including sexually transmitted diseases in Botswana (personal communication). Watt and Breyer- Brandwijk, 1962 report that a number of *Asclepias* species are used for medicinal purposes. In South Africa the leaf of *Asclepias fruticosa L.* is used for intestinal troubles in children, finely ground leaves are used as snuff to cure headaches and tuberculosis. It

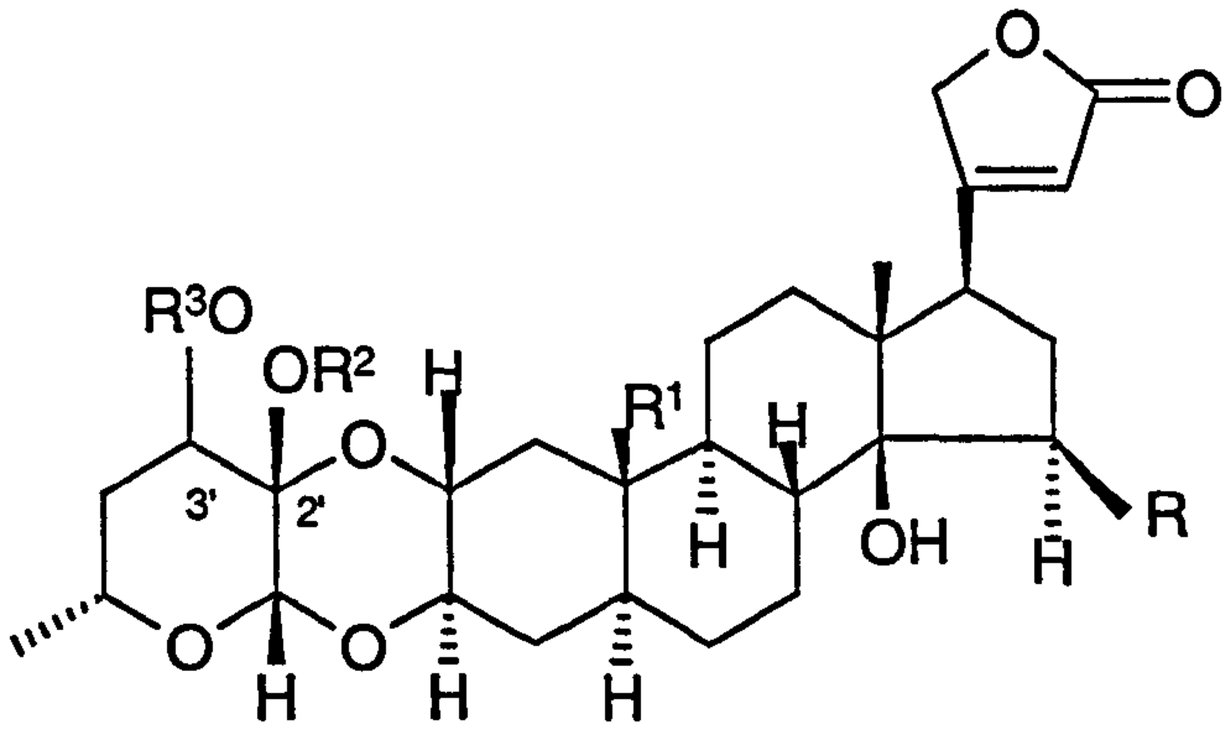
is also used as an emetic. Some tribes use the leaf and latex as a purgative enema. According to Hann and Fonseca (1991) some species of the genus have been used in the treatment of certain cancerous states and others have been found to possess insecticide properties. Other species, which are used medicinally, are *A. physocarpa* and *A. crispa*. The roots and leaves of these plants are mainly used for this purpose.

1.6.3.1 Phytochemical

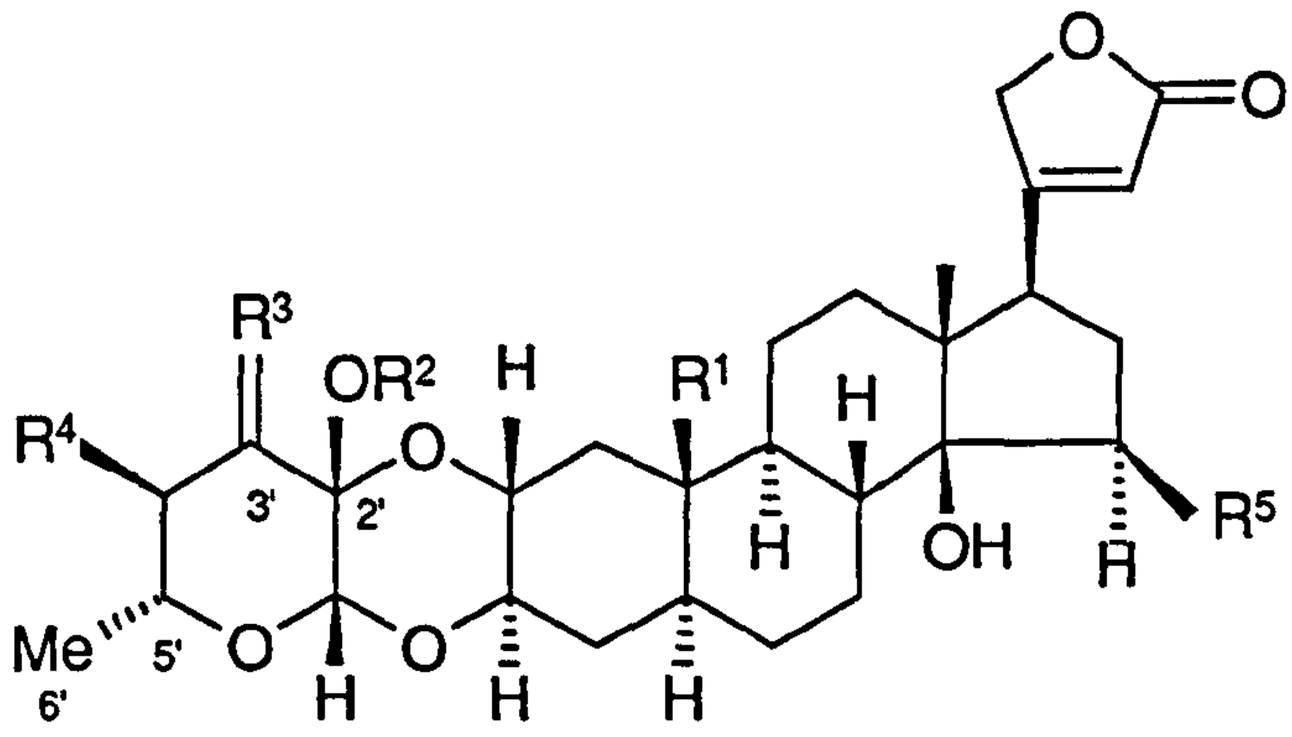
Plants belonging to the family Asclepiadaceae produce a latex, which is reported to be rich in triterpenes. Other secondary metabolites, which are reportedly produced by the family, include cardenolides, cardenolide glycoside, alkaloids of indole, phenanthraindolizidine and pyridine groups, cyanogenic glycosides, saponins, tannins and cyclitols (Trease and Evans, 1983). The family is however famous for the production of the cardenolides and cardenolide glycosides and most studies on the genus *Asclepias* have concentrated on the isolation and characterization of these as evidenced by the large number of compounds in Table 1.3, which summarises the compounds isolated from *Aclepias fruticosa*. Other genera that have been studied include *Sarcostemma, Calotropis* and *Pergularia* (Cheung *et al.* 1980). Pregnane and cardenolide glycosides have been isolated from *A. fruticosa* by a number of researchers (Warashima and Noro 1993, 1994, 1994; Cheung *et al.* 1980, 1981, 1983, 1988, Cheung and Watson ,1980; and Abe *et al.* 1994). Warashima and Noro (1994) isolated steroidal glycosides as well as the cardenolide glycosides. Hann and Fonseca (1991) isolated and identified cardenolides from the aerial parts of *A. linaria*. They isolated calactin, calotropin, proceroside, gomphoside, and desglucouzarin and 6'-*p*-coumaroyl desglucouzarin. Abe *et al.* (1994) isolated and determined the structure of 3'-epi-19-norafroside and 12β-hydroxycoroglaucigenin, along with known cardenolides and cardenolide glycosides from the stems of *A. curassavica.* El-Askary *et al.* (1995) reported the isolation and identification of cardenolides from the stems of *Gomphocarpus (Asclepias) sinaicus* Boiss. Cheung *et al.* in their 1988 publication reported the isolation of cardenolide glycosides from adult Monarch butterflies (*Danaus plexippus*) the larvae of which was reared on *A. fruticosa* leaves.

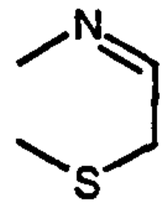
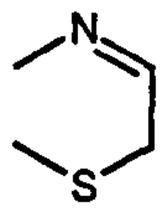
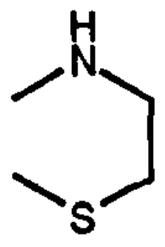
Table 1.3: A summary of compounds isolated from *A. fruticosa*

Compound	Reference

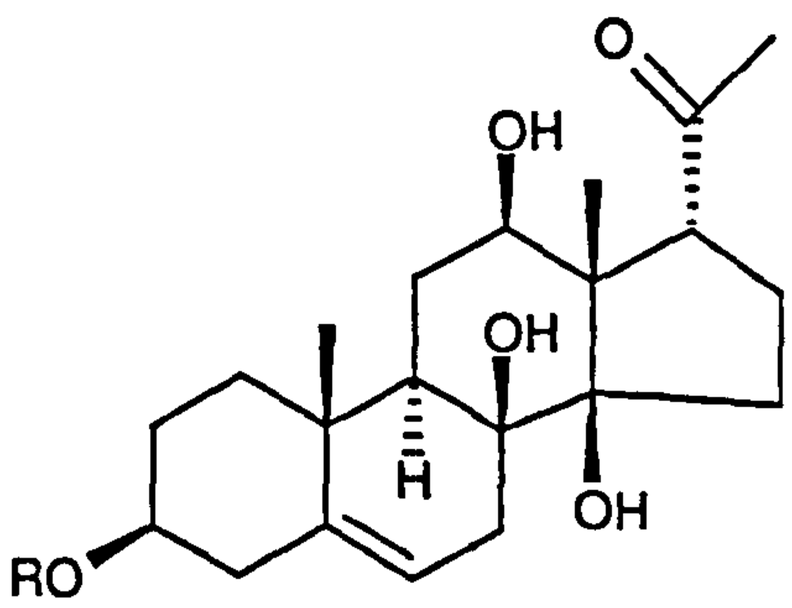


	R	R^1	R^2	R^3
Gomphoside (**38**)	H	OH	H	H (Cheung *et al.*, 1981, 1988)
Afroside (**39**)	OH	OH	H	H (Cheung *et al.*, 1981, 1988)

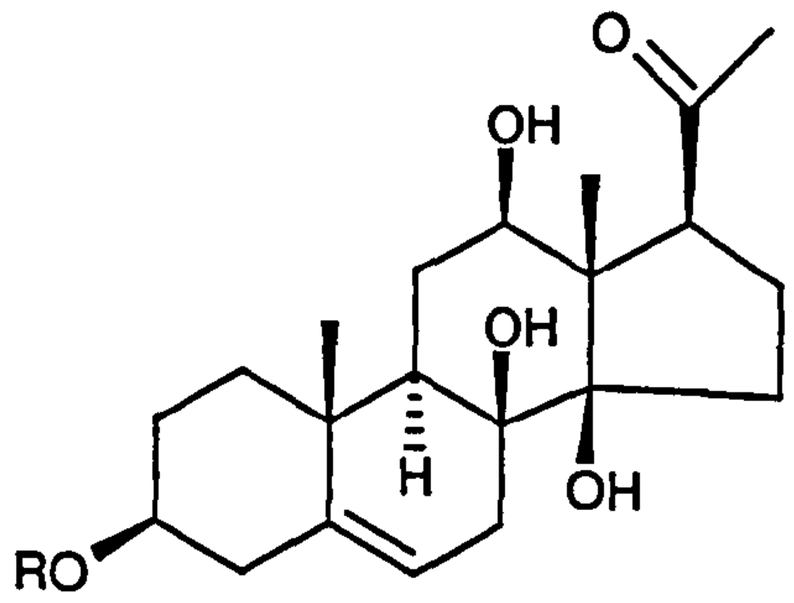


	R^1	R^2	R^3	R^4	R^5	
Calactin (**40**)	CHO	H	β-OH, α-H	H	H (Cheung *et al.*, 1983, 1988)	
Asclepin (**41**)	CHO	H	α-OAc, β-H	H	OH	(“)
Calotropin (**42**)	CHO	H	α-OH, β-H	H	OH	(“, 1988)
Calotoxin (**43**)	CHO	H	β-OH, α-H	OH	H	(“, 1988)
19-Deoxyusscharin (**44**)	Me	H		H	H	(“)
Uscharin (**45**)	CHO	H		H	H	(“)
Voruscharin (**46**)	CHO	H		H	H	(“)
3’-didehydrogomphoside(**47**)	Me	H	O	H	H	(“)
3’-didehydroafroside (**48**)	Me	H	O	H	OH	(“)
Uscharidin (**49**)	CHO	H	O	H	H	(“)
3’-Epigomphoside (**50**)	Me	H	α-OH, β-H	H	H	(“)
3’-Epiafroside (**51**)	Me	H	α-OH, β-H	H	OH	(“)
Calotropin (**52**)	CHO	H	α-OH, β-H	H	H	(“)
3’-Epigomphoside 3’-acetate (**53**)	Me	H	α-OAc, β-H	H	H	(“)
3’-Epiafroside 3’-acetate (**54**)	Me	H	α-OAc, β-H	H	OH	(“)
4’β-Hydroxygomphoside (**55**)	Me	H	β-OH, α-H	OH	H	(“)

Pregnane glycosides:

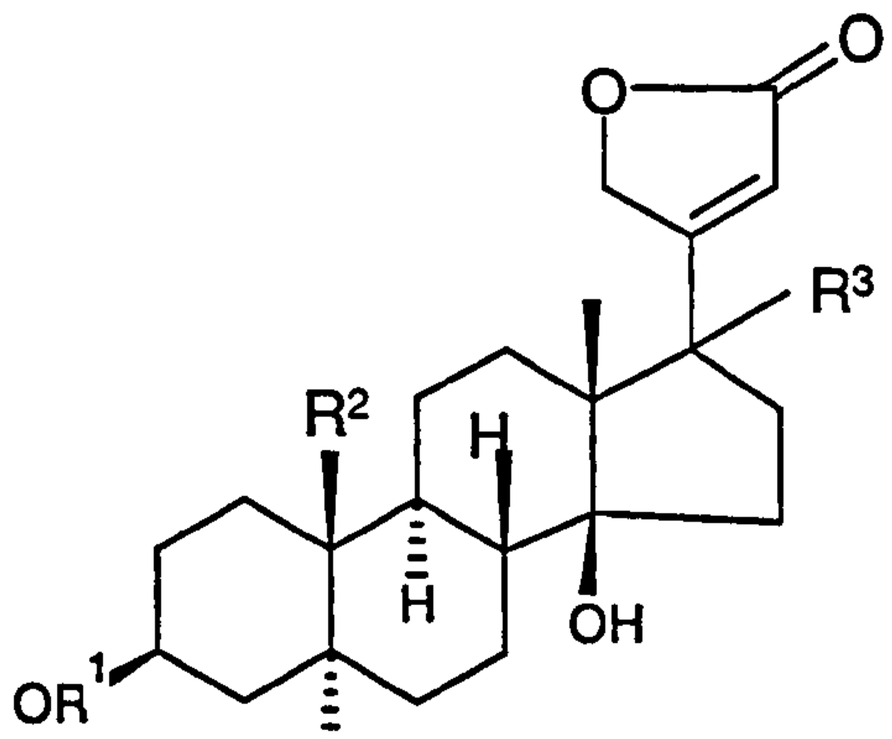


	R	
56	H	
57	dig(4→1)-oli (4→1)-dig (4→1)ole	(Warashima and Noro, 1994)
58	dig (4→1)-ole(4→1)-dig(4→1)ole	(")

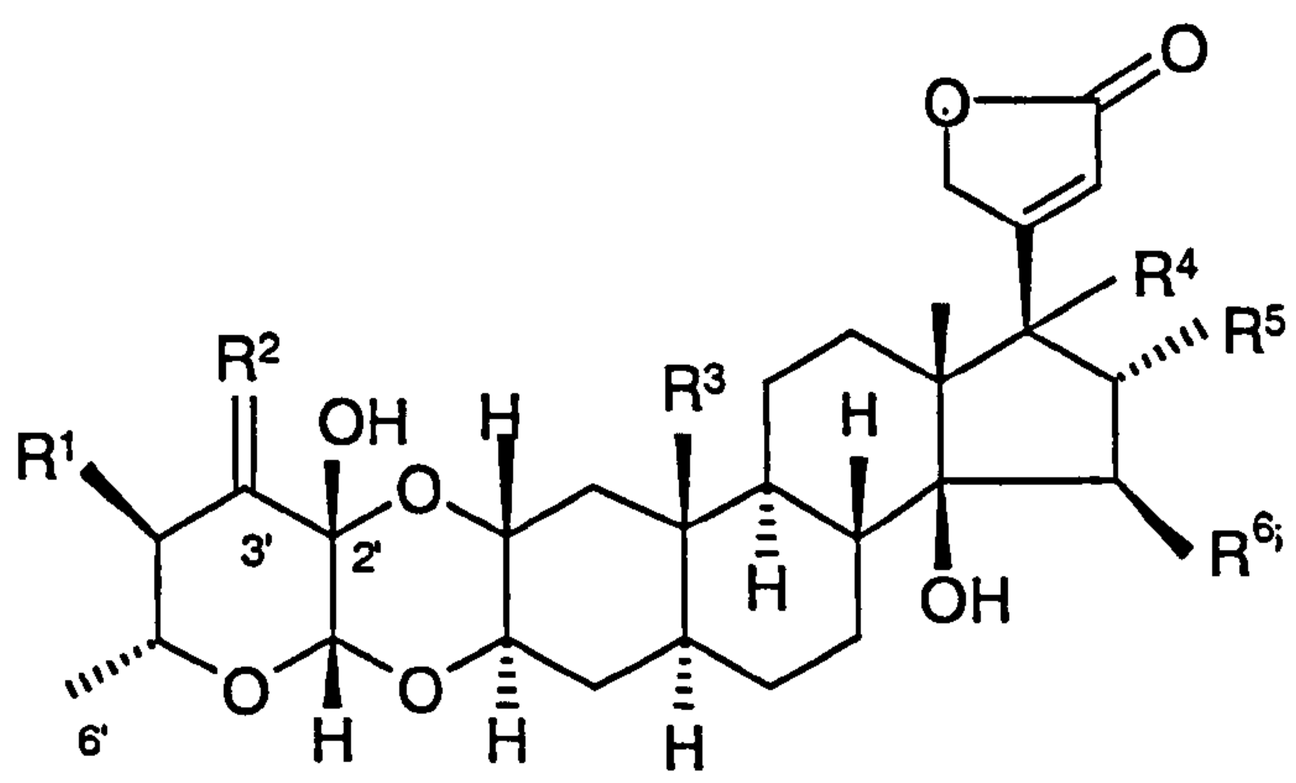


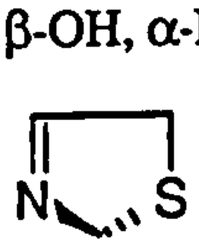
59	dig(4→1)-ole(4→1)-dig(4→1)ole	(")
60	dig(4→1)-ole(4→1)-cym(4→1)ole	(")

***Cardenolide glycosides*:**

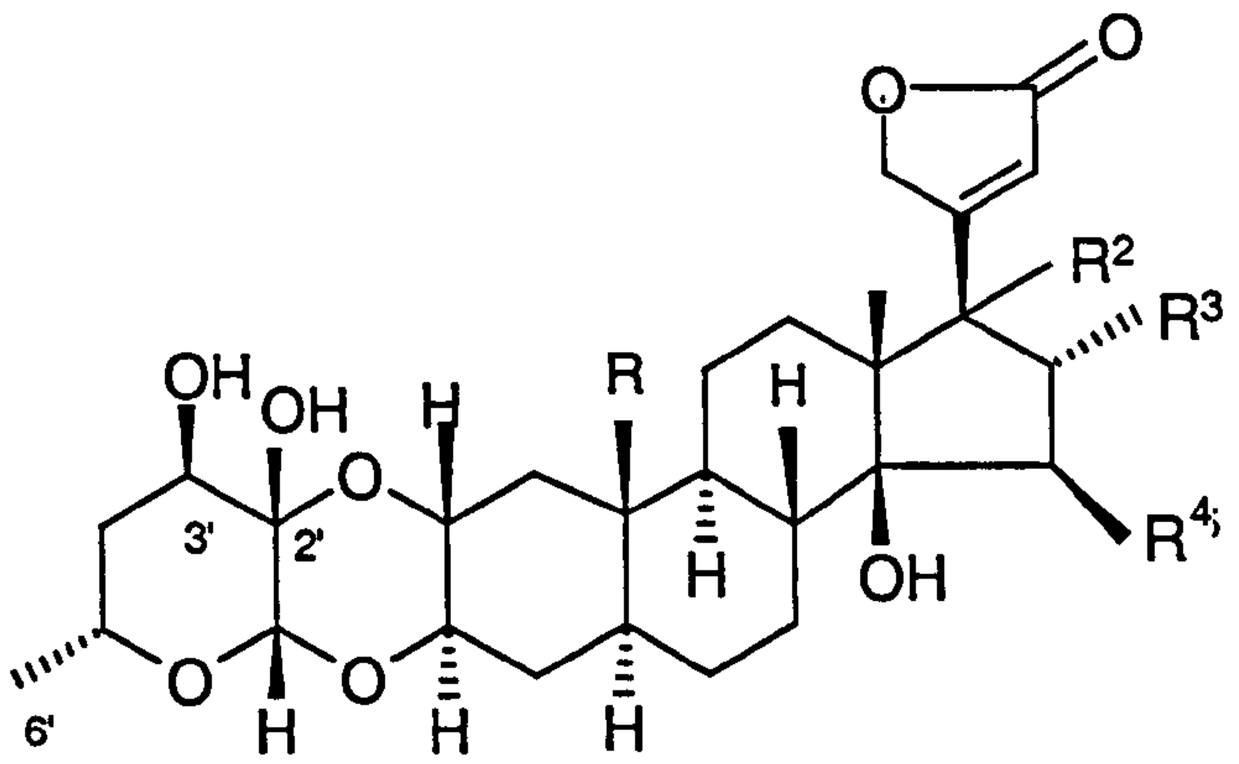


	R^1	R^2	R^3
61	H	Me	α-H (Warashima and Noro, 1994)
62	H	CH_2OH	α-H (")
63	β-6-deoxyallose	CHO	α-H (")
64	β-6-deoxyallose	CHO	β-OH (")
65	β-6-deoxyallose	Me	α-OH

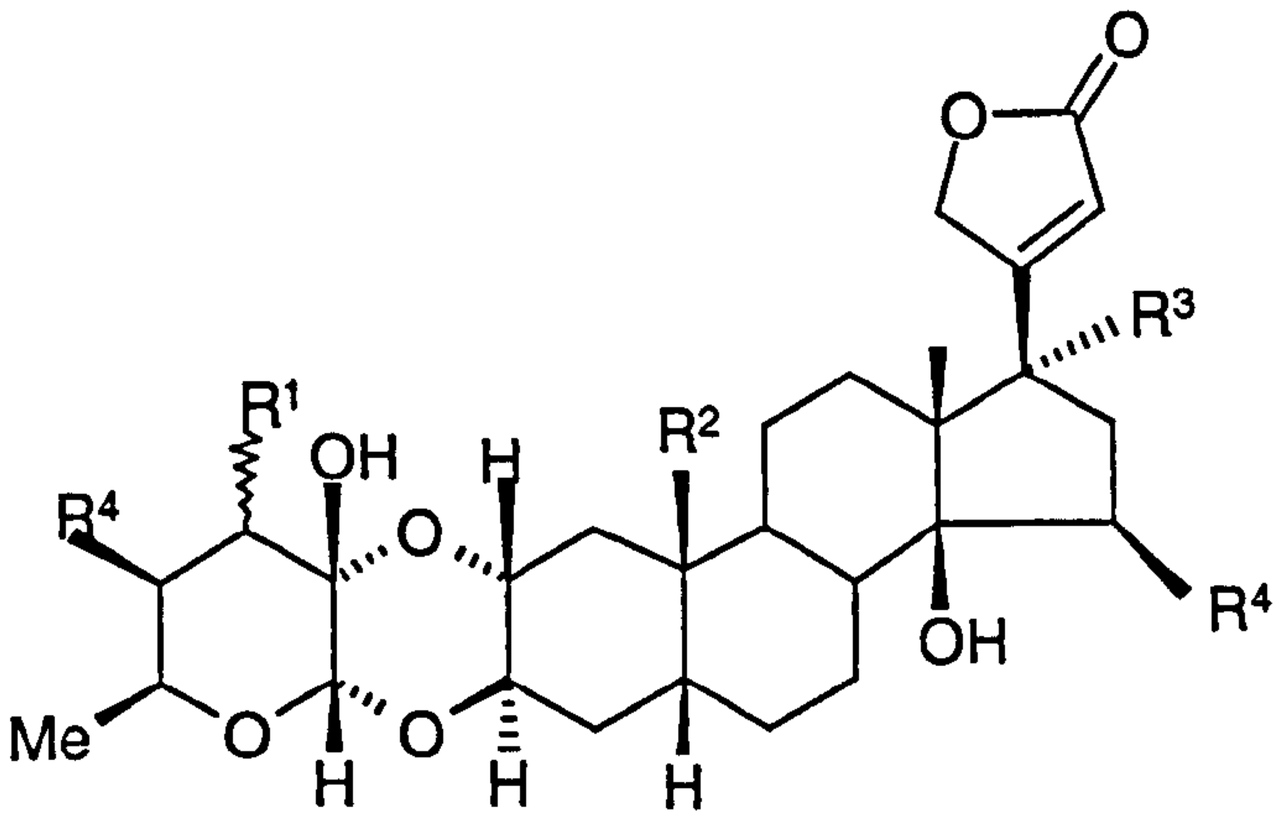


	R^1	R^2	R^3	R^4	R^5	R^6	
66	H		Me	α-H	H	OH	(Warashima & Noro, 1994)
67	OH	β-OH, α-H	Me	α-H	H	OH	(")
68	H	β-OH, α-H	Me	α-OH	H	H	(")
69	H	β-OH, α-H	CHO	α-OH	H	H	(")
70	H		CHO	β-OH	H	H	(")

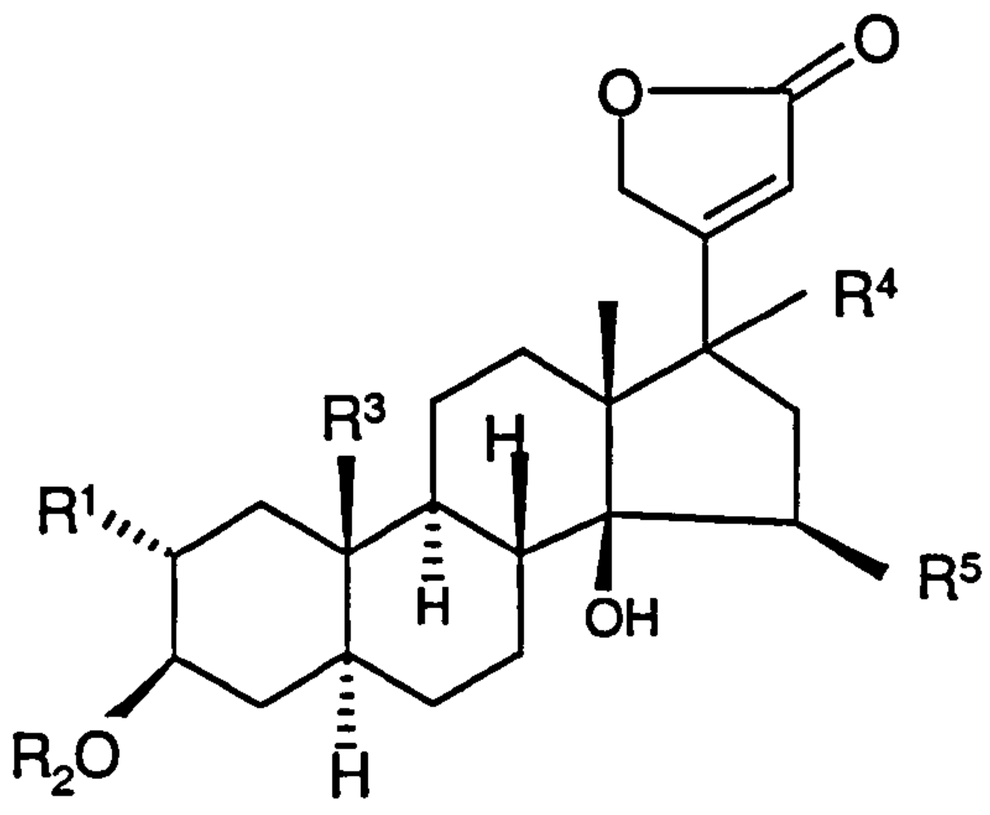
71	H	β-OH, α-H	CHO	β-OH	H	H	(“)
72	H	β-OH, α-H	Me	β-OH	H	OH	(“)
73	H	β-OH, α-H	Me	β-OH	H	OH	(“)
74	H		CHO	α-H	OH	H	(“)



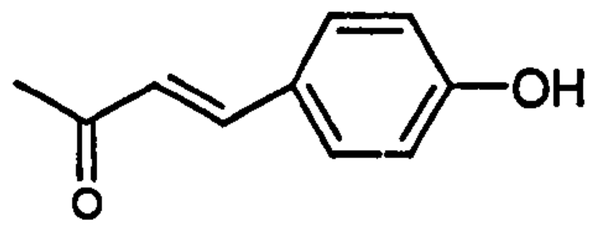
	R^1	R^2	R^3	R^4	
75	CHO	α-H	OH	H	(Warashima and Noro, 1994)
76	Me	β-OH	OAc	H	(“)
77	Me	α-OH	H	OH	(“)
78	Me	α-H	H	OH (Δ^5)	(“)



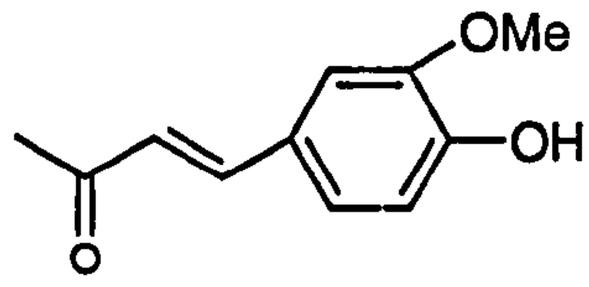
	R^1	R^2	R^3	R^4	
79	β-OH	CHO	OH	H	(Abe *et.al*, 1994)
80	β-OH	CH_3	OH	OH	(“)



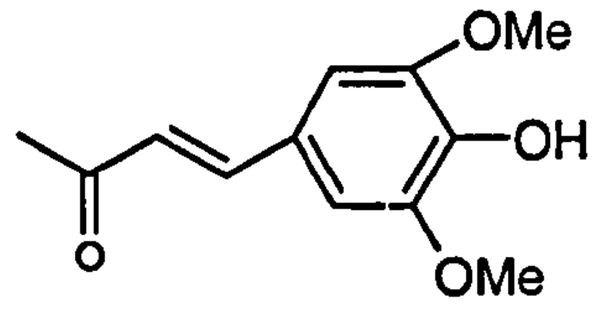
A:



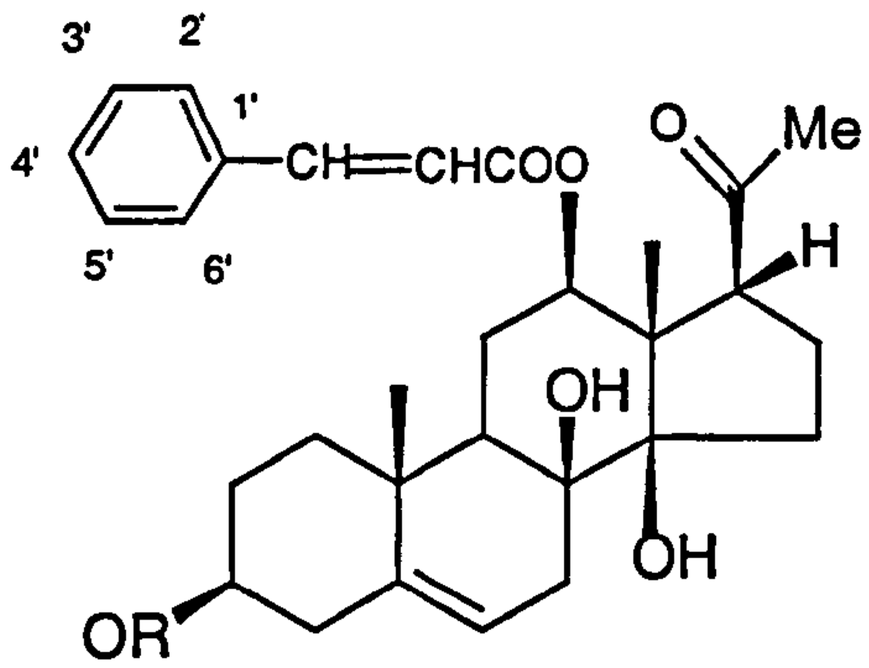
B:

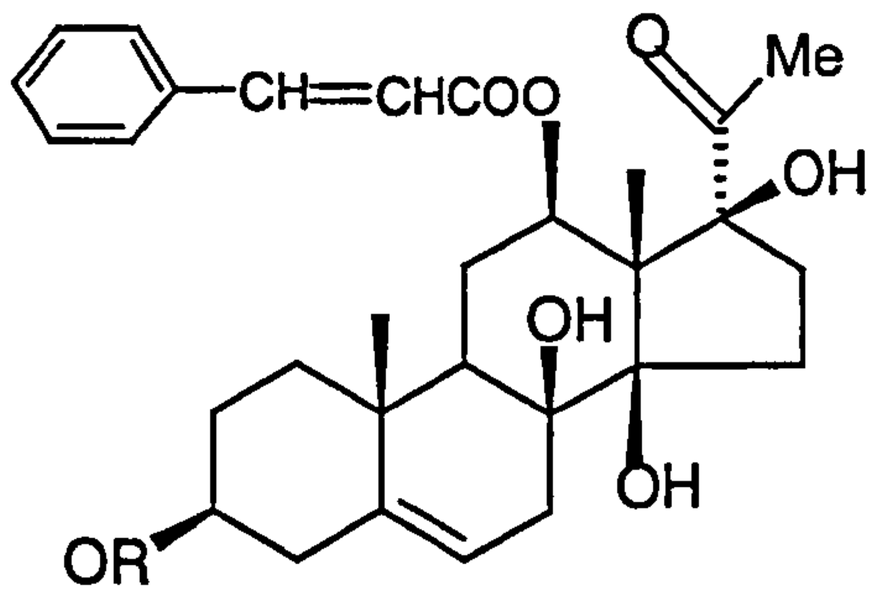


C:



	R1	R2	R3	R4	R5	(Warashima & Noro, 1994)
81	H	H	CH_2OH	β-OH	H	(“)
82	H	β-allose	Me	α-H	H	(“)
83	H	β-6-deoxyallose	CHO	α-OH	H	(“)
84	H	β-6-deoxyallose	Me	β-OH	H	(“)
85	H	β-glucose $\overset{6}{\text{——}}$ A	Me	α-H	H	(“)
86	H	β-glucose $\overset{6}{\text{——}}$ B	Me	α-H	H	(“)
87	H	β-glucose $\overset{6}{\text{——}}$ B	Me	α-H	H	(“)
88	H	H	Me	α-H	H	(“)





Aglycone:	R=H	Aglycone:	R=H	(Abe *et al*, 1994)
89	R=a	76	R=a	
90	R=b	77	R=c	
91	R=c	78	R=d	
92	R=d	79	R=e	
93	R=e	80	R=f	
94	R=f	81	R=g	

a: dig(4→1)-ole(4→1)-dig(4→1)-cym(4→1)-glc

b: dig(4→1)-ole(4→1)-dig(4→1)-ole(4→1)-glc

c: dig(4→1)-ole(4→1)-cym(4→1)-cym(4→1)-glc

d: cym(4→1)-ole(4→1)-dig(4→1)-cym(4→1)-glc

e: cym(4→1)-ole(4→1)-ole(4→1)-cym(4→1)-glc

f: cym(4→1)-ole(4→1)-ole(4→1)-ole(4→1)-glc

g: cym(4→1)-cym(4→1)-ole(4→1)-cym(4→1)-glc

---

dig = digitoxose oli = olivose

cym = cymarose all = allose

1.6.3.2 Toxicological

Plants of the genus *Asclepias* are reported to have been responsible for livestock poisoning (Watt and Breyer-Brandwijk, 1962, Cheung *et al.*, 1988). Some are used as arrow poisons (Trease and Evans, 1983). Their pharmacological activity is chiefly due to the cardenolide glycosides, which are inotropic and cytotoxic agents. They cause an increase in the force of contraction of the heart and inhibit the growth of primate tumour cells (Cheung *et al.*, 1988). The *Asclepias* compounds therefore exhibit pharmacological properties similar to those of the heart drugs from *Digitalis spp.*, which have a very low therapeutic index and therefore are highly toxic at relatively higher doses. In this project extracts of the leaf material were found to be highly cytotoxic. It has also been shown that insects feed on the leaves of these plants at the larval stage and the cardenolides are stored in the adult body as a defence substance (Abe *et al.*, 1991). These studies indicate that the compounds of medium and high polarity are stored unchanged in the animals. Watt and Breyer-Brandwijk (1962), report that fatal results were obtained when 40g of fresh flowers, leaves and stem of *A. fruticosa* were fed to rabbit. They go on to state that the milky juice is also reportedly poisonous and that feeding experiments were used to confirm its toxicity.

1.6.4 *Albizzia brevifolia* Schinz (Leguminosae)

Picture 1.4: *Albizzia brevifolia*

Like *C. italica, A. brevifolia* belongs to the order Rosales and the family Leguminosae but to a different subfamily, namely, Mimosoideae, which comprise 40 genera. The genus Albizzia contains about 150 species (Rukunga, 1995). Most members of this subfamily are trees or shrubs (Trease and Evans, 1983). *A. brevifolia* is a small tree. In Botswana it is locally known as *Molalakgakala* in East Central Botswana. The plant can grow up to 4 – 5 metres tall, normally on rocky areas on hill slopes. It has small thin green leaves, green fruits (pods) that turn brown when dry, a woody stem, brown seeds (when mature), thick reddish brown roots. Medicinal use of the plant has been reported. It has been used to treat gonorrhoea in both sexes in Botswana (Hedberg and Staugart, 1989). The root is used for any intestinal troubles in Tanzania (Watt and Brandwijk, 1962). A number of other species from the genus *Albizzia* are said to be used medicinally. These include *A.*

amara Boiv., *A. angolensis, A. anthelmintica, A. antunesiana harms* and *A. lebbeck* Benth.

1.6.4.1 Phytochemical

Secondary metabolites that have been isolated from some species include triterpenes, saponins, flavonoids, spermine alkaloids, tannins and other miscellaneous phenolics. Rukunga, (1995) isolated a number of lupane and oleonane triterpenes from four species, *A. schimpera, A. gummifera, A. versicolor and A. adianthifolia.* Triterpene saponins are reported to have been isolated by a number of authors; Debella *et al.* (2000) from *A. gummifera*, Zou *et al.* (2000) from *A. julibrissin* and Pal *et.al.* (1994) from *A. lebbeck.* Flavonoids have also been isolated from the genus; El-Mousallany (1998) isolated two from the leaves of *A. Lebbeck* and Rukunga, (1995) from *A. versicolor* and flavanones from *A. adianthifolia.* Ma *et al.* (1997) isolated tannins from *A. lebbeck.* A summary of some of the compounds that have been isolated from this genus is provided in Table 1.4.

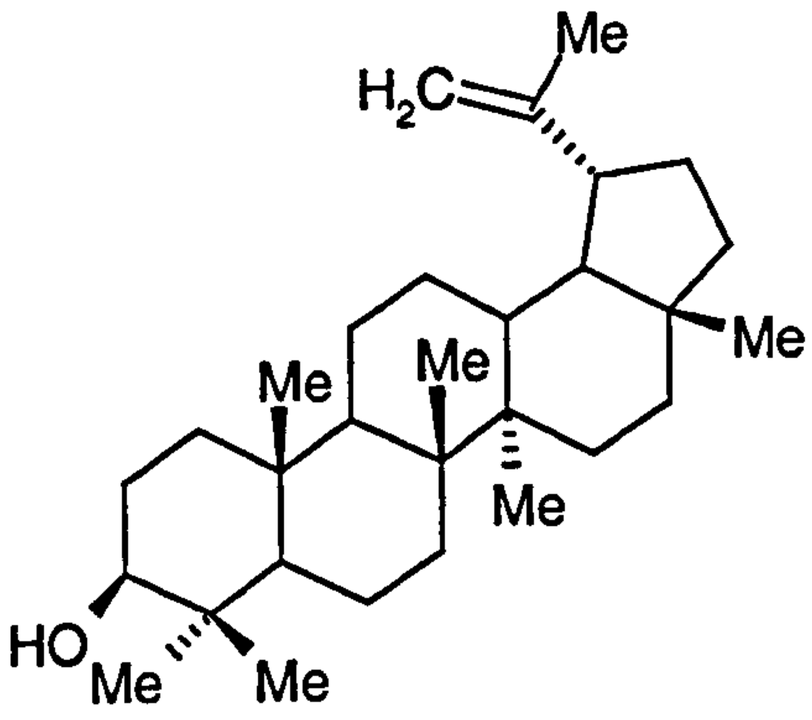
Table 1.4: Compounds isolated from *Albizzia spp.*

Compound	Source (Reference)
Triterpenes:	
Lupeol (**95**)	*A. schimpera, A. gummefera, A. Avesicolor, A. adianthifolia.* (Rukunga & Waterman, 2000)
Lupenone (**96**)	" (")
Oleanolic acid (**97**)	" (")
Hederagenin (**98**)	" (")
Vitalboside – A (**99**)	*A. gummefera* (")
β-sitosterol (**22**)	*A. adianthifolia, A. Avesicolor* (")
Acacic acid lactone (**99**)	*A. Avesicolor, A. adianthifolia* (")
Daucosterol (β-sitosterol glycoside,	*A. adianthifolia* (Rukunga 1996)

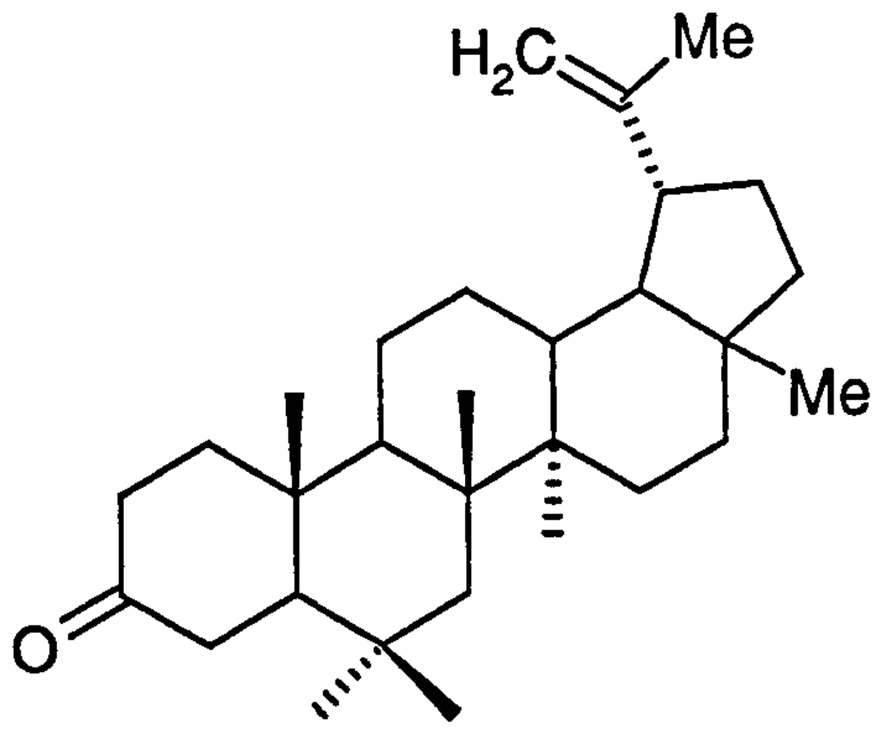
β-glucose, sugar moiety) (**100**)

β-amyrin (**29**)

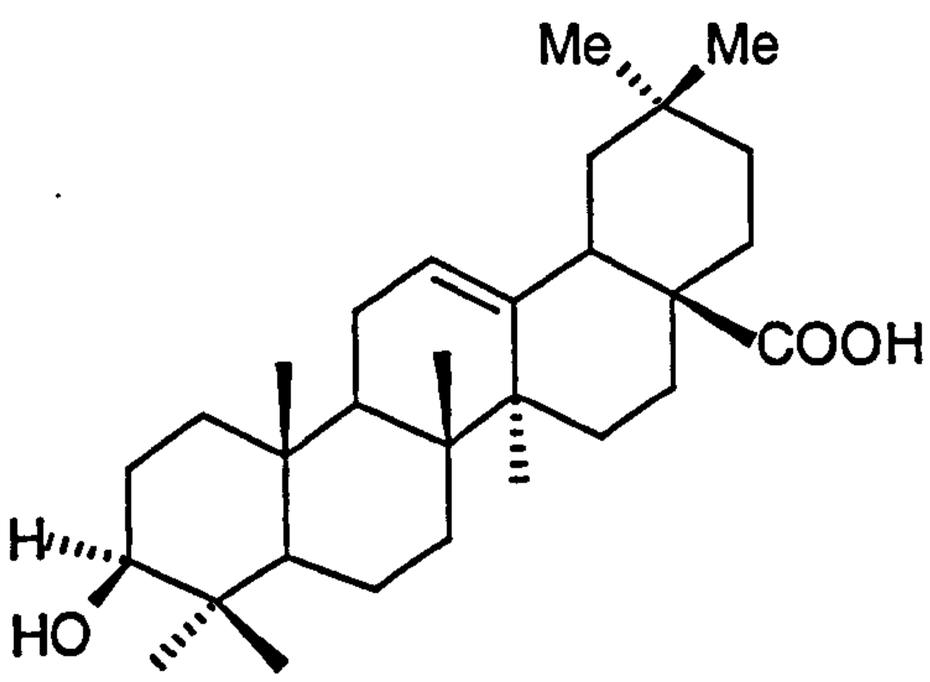
A. adianthifolia (Rukunga 1996)



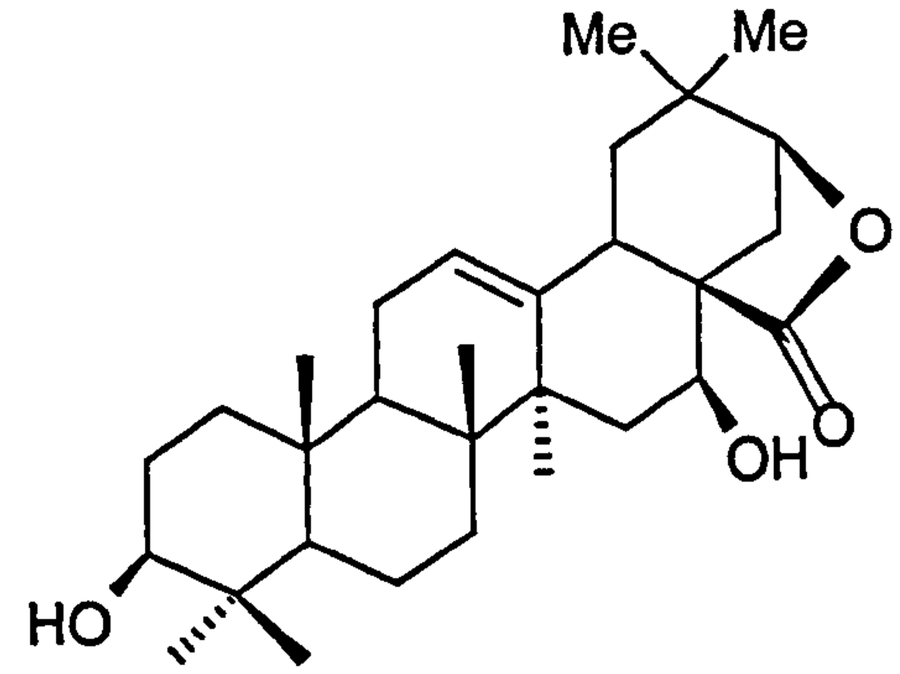
95



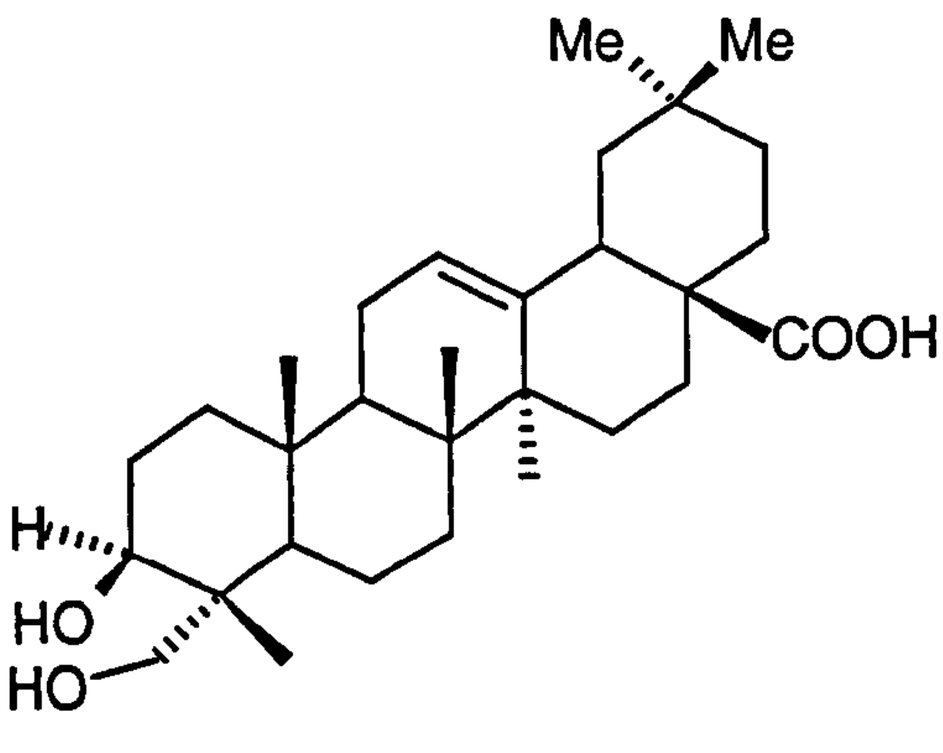
96



97



100



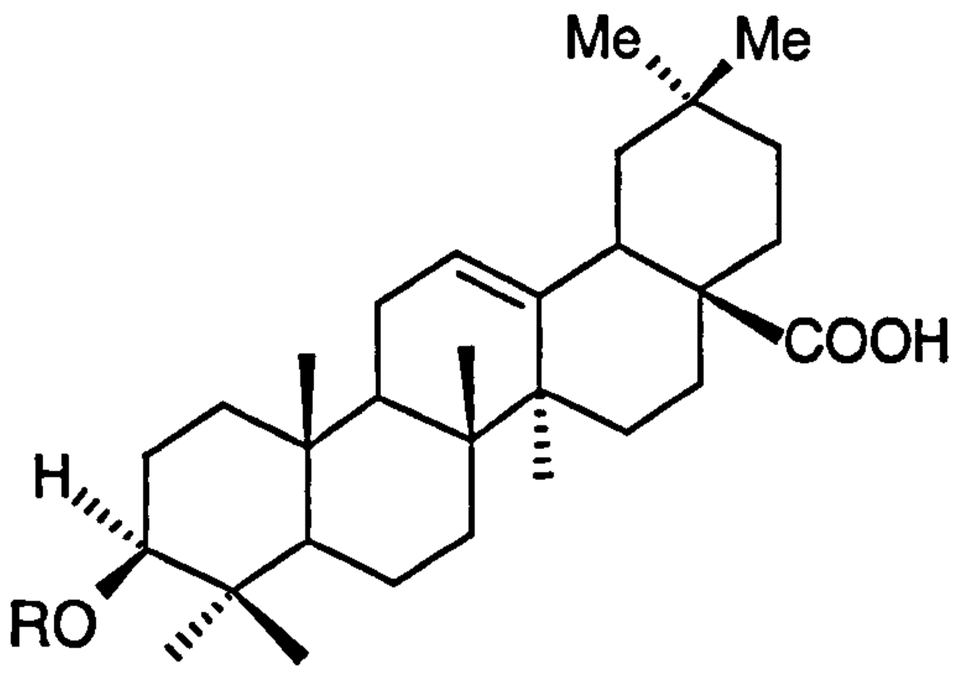
98

Saponins:

Acacic acid lactone 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (**102**) *A.lebbeck (*Pal *et al.*, 1994

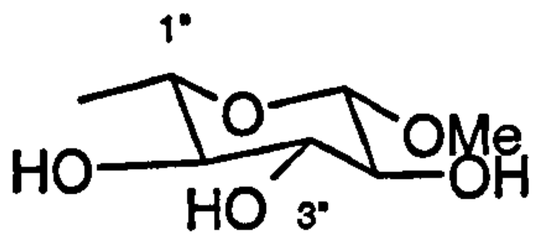
Acacic acid lactone 3-*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→6)]-β-D-glucopyranoside (**103**) (“)

Acacic acid lactone 3-*O*-β-D-xylopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→6)]-*O*-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranoside (**104**) (“)

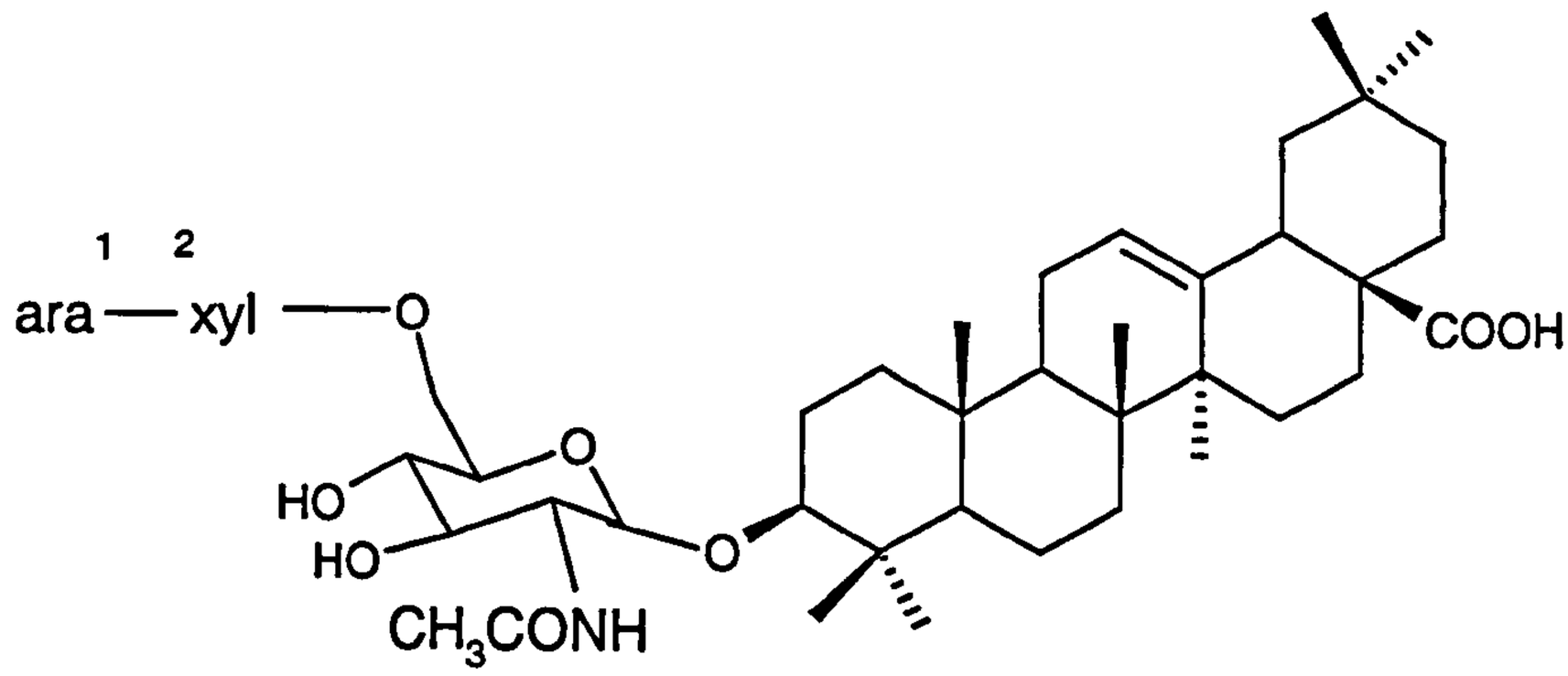


R = H (**99**)

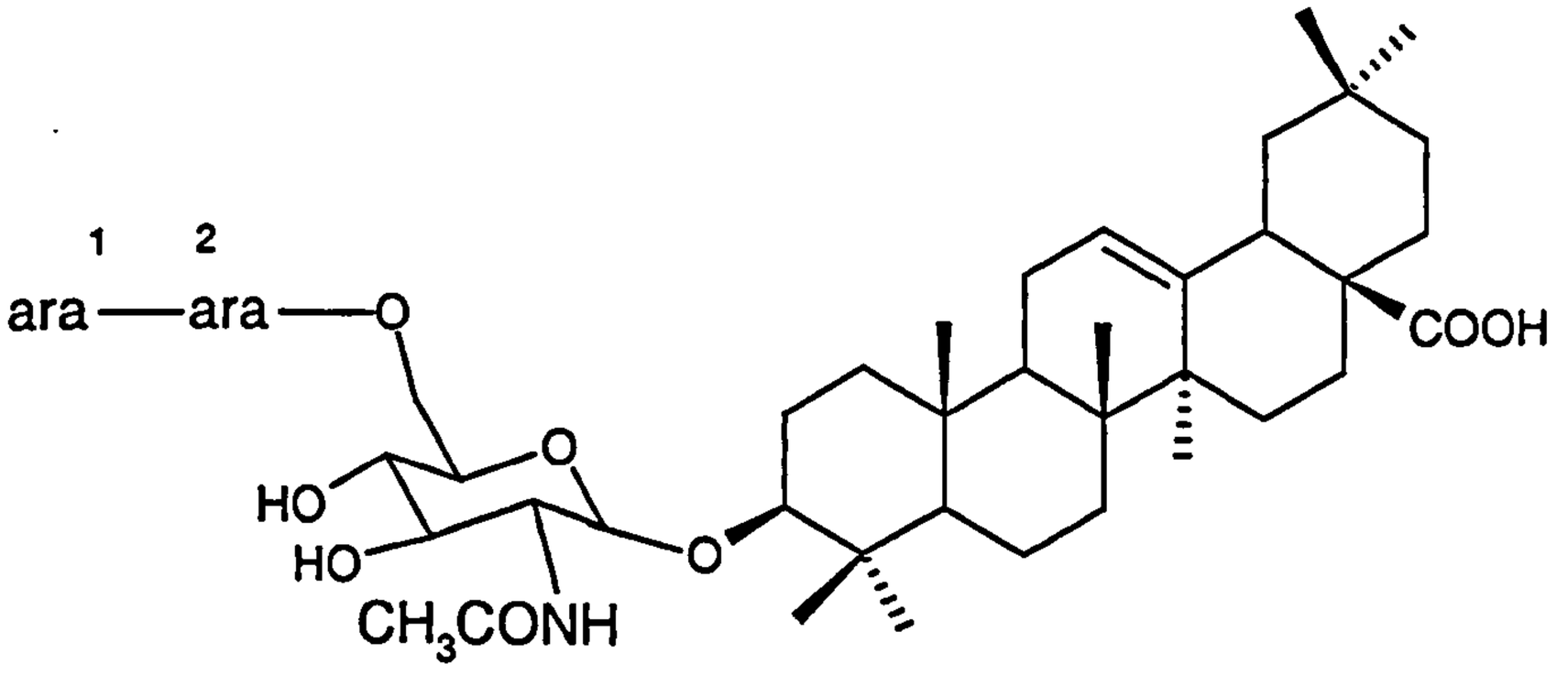
Vitalboside - 2'-*O*-methyglucuronate (**105**) R =



A. gummifera (Rukunga and Waterman, 2000)



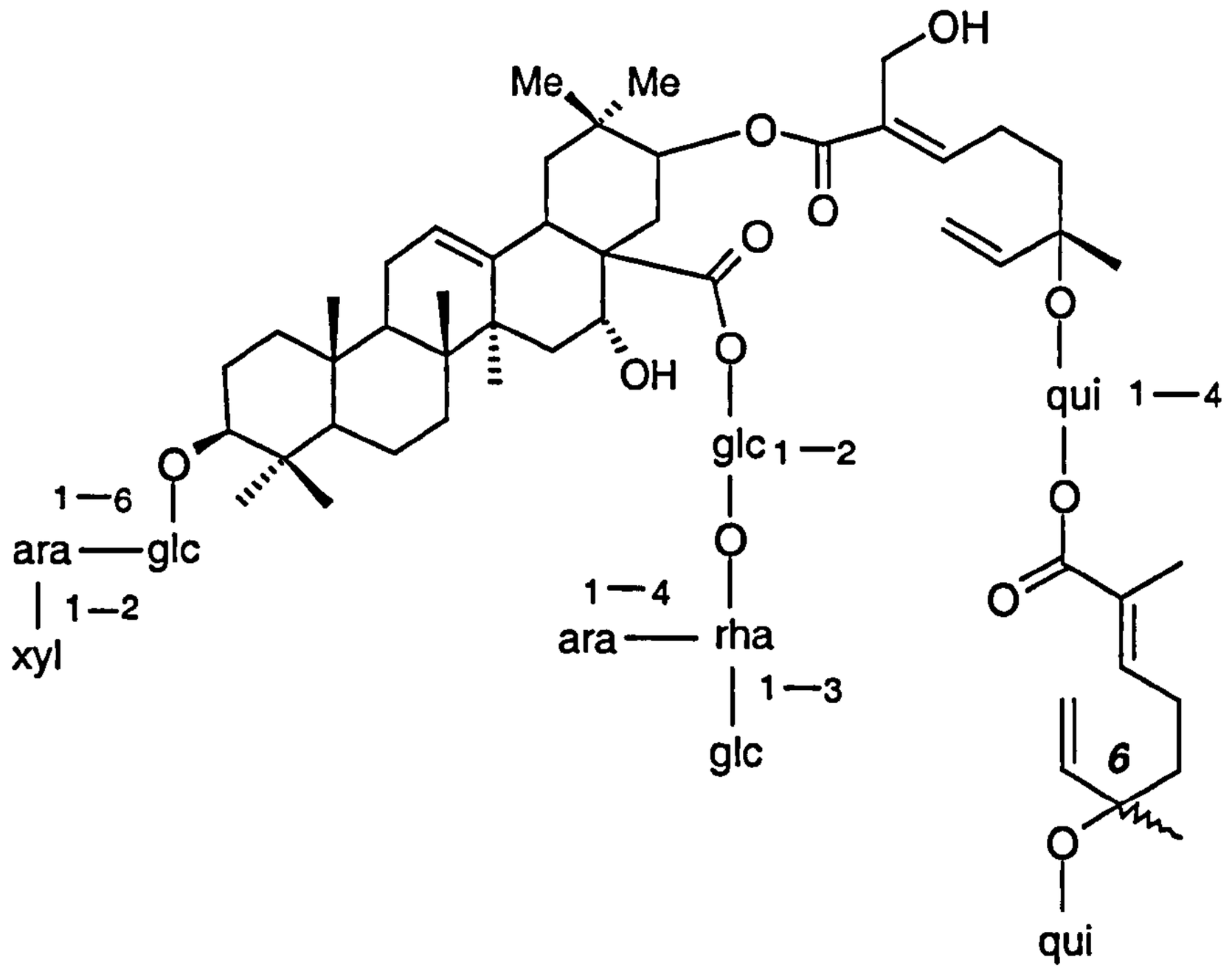
106 *A. subdimidiata* (Abdel-Kader, 2001)



107 *A. subdimidiata* (Abdel-Kader, 2001)

3-*O*-[β-D-xylopyranosyl-(1→2)-α-L-arabinopyl-ranosyl-(1→6)-β-D-glucopyranosyl]-21-*O*-{(6S)-2-*trans*-2-hydroxymethyl-6-methyl-6-*O*-[4-*O*-(6R)-2-*trans*-2,6-dimethyl-6-*O*-(β-D-quinovopyranosyl)-2,7-octadienoyl)-β-D-quinovopyranosyl]-2,7-octadienoyl}acacic acid-28-*O*-β-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl ester (**108**)

A. julibrissin (Zou *et al.*, 2000)



108 (6R)

109 (6S)

3-*O*-[β-D-xylopyranosyl-(1→2)-α-L-arabinopyl-ranosyl-(1→6)-β-D-glucopyranosyl]-21-*O*-{(6S)-*2-trans*-2-hydroxymethyl-6-methyl-6-*O*-[4-*O*-(6S)-2-*trans*-2,6-dimethyl-6-*O*-(β-D-quinovopyranosyl)-2,7-octadienoyl)-β-D-quinovopyranosyl]-2,7-octadienoyl}acacic acid-28-*O*-β-D-glucopyranosyl-(1→3)-[α-L-arabinofuranosyl-(1→4)]-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl ester (**109**)

*A.julibrissin (*Zou *et al.*, 2000)

Δ^5-stigmasterol-3-*O*-β-D-glucopyranoside (**110**)

A. gummifera (Debella *et al.*, 2000)

3-*O*-{β-D-glucopyranosyl(1⟶2)-[α-L-arabinopyranosyl(1→6]-β-D-glucopyranosyl}-oleanoic acid (**111**) *A. julibrissin (*Zou *et al.*, 2000

β-D-glucopyranosyl(1⟶2)-β-D-glucopyranosyl-(1⟶2)-[*O*-α-L-arabinopyranosyl(1→6]β-D-glucopyranosyloxy}-machaerinic acid γ-lactone (**112**)

*A.julibrissin (*Zou *et al.*, 2000)

3β-*O*-β-D-glucopyranosiduronic acid (1⟶2)- β-D-glucopyranosyloxy]-machaerinic acid γ-lactone (**113**

A. julibrissin (Zou *et al.*, 2000)

Flavonoids glycosides:

Kaempferol 3-*O*-α-rhamnosyl(1⟶6)-β-glucopyranosyl(1⟶6)- β-galactopyranoside (**114**)

A. lebbeck (El-Mousallamy, 1998)

quercetin 3-*O*-α-rhamnosyl(1⟶6)-β-glucopyranosyl(1⟶6)-β-galactopyranoside(**115**)

A. lebbeck (El-Mousallamy, 1998)

Tannins:

(-)-epichatechin (**115**)

A. lebbeck(Ma *et al.*, 1997)

procyanidin B-5 (**116**)

(“)

prpcyanidin B-2 (**117**)

(“)

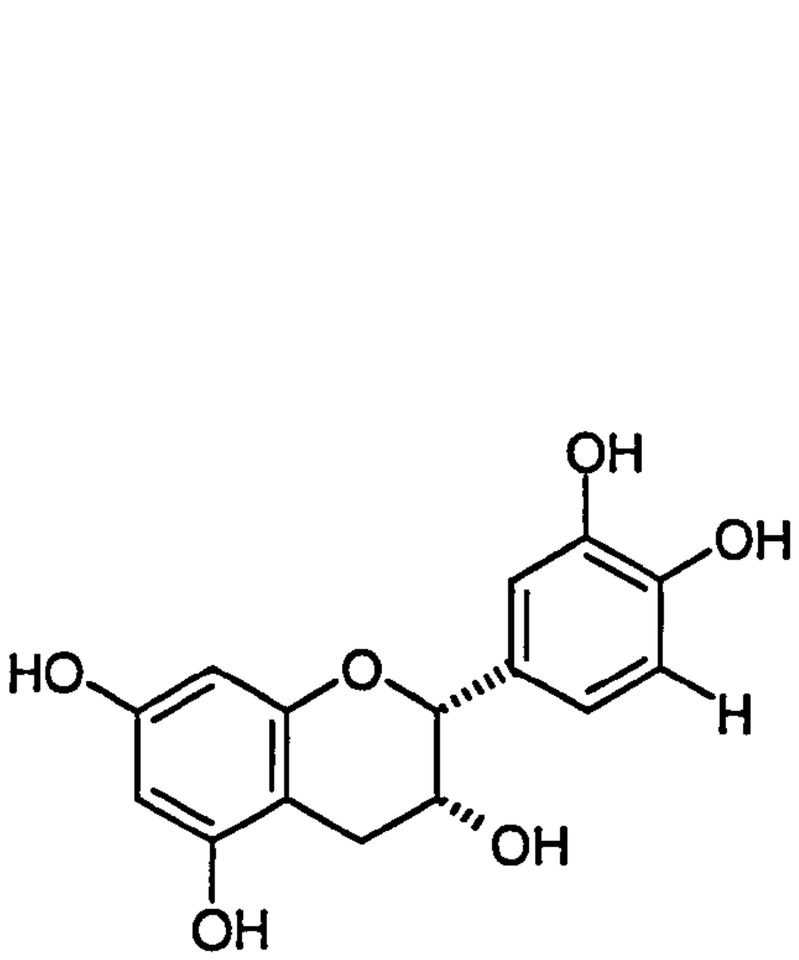
Procyanidin C-1 (**118**)

(“)

Gentisic acid 5-*O*-[5-*O*-syringoyl-β-D-apiofuranosyl(1⟶2)- β-

Glucopyranosie (albizinin) (**119**)

A. lebbeck (Ma *et al.*, 1997)



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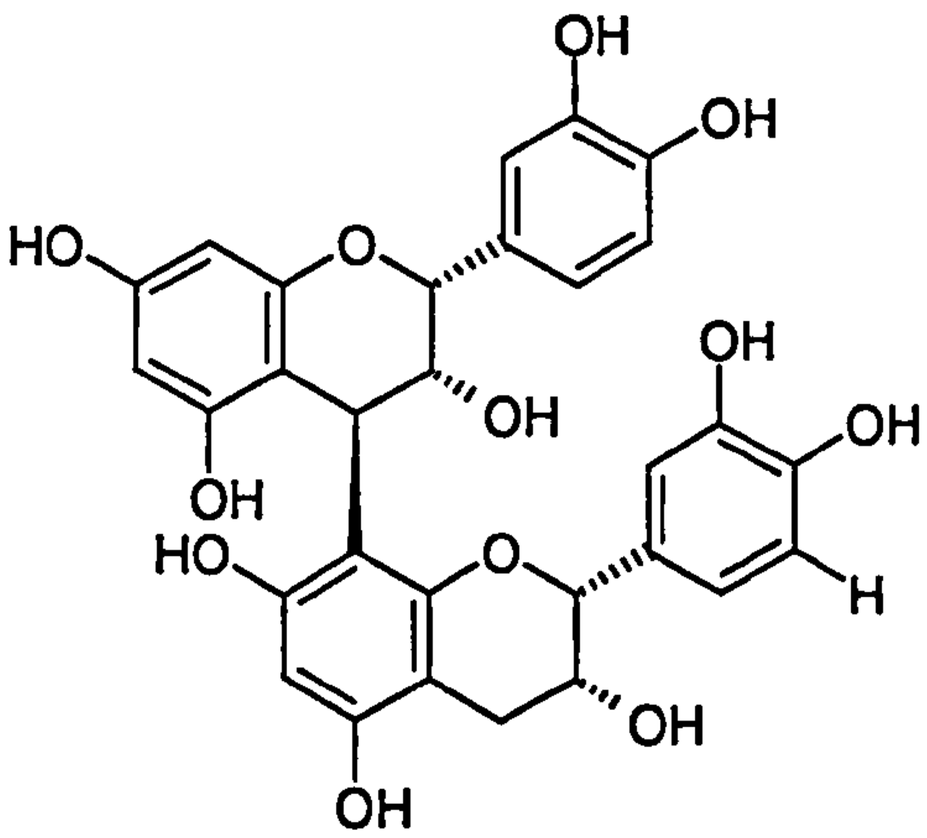

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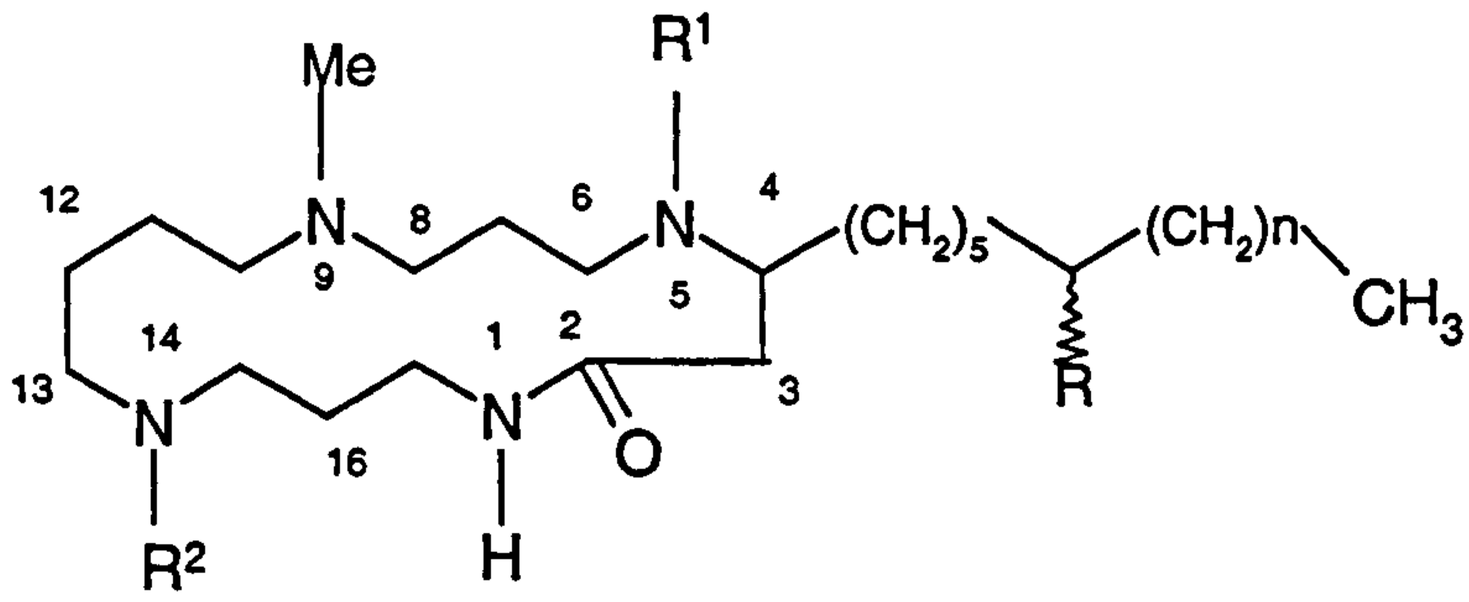
***Alkaloids*:**



A. schimperana (Rukunga & Waterman, 1996)

	n	R	R_1	R_2
Budmunchiamine-A (**120**)	4	H	Me	Me
6ξ'-Hydroxybudmunchiamine-C (**121**)	6	OH	Me	Me
5-Nomethylbudmunchiamine-K (**122**)	8	H	H	Me
6ξ-Hydroxy-5-nomethylbudmunchiamine-K (**123**)	8	OH	H	Me
14-Nomethylbudmuchiamine-K (**124**)	8	H	Me	H

ara = arabinose xyl = xylose rha = rhamnose

dex = deoxyallose qui = quinose

No reports of phytochemical work have been found yet on *A. brevifolia* during the literature search.

1.6.4.2 Toxicological

Watt and Breyer-Brandwijk (1962) report toxicity of some species of the genus. For example, a decoction of the bark of *A. anthelmintica* is said to be toxic to earthworms. *A. gummifera* C.A. Sm. is used as fish poison and the bark of *A. maranguensis* Taub. is also said to be toxic. Soldan *et al.*, (1996), reported poisoning of sheep and goats by *A. versicolor* in Malawi. Animals were thought to have been poisoned when they ingested ripe dry pods of the plant. 75% of the clinical cases are reported to have made a full and rapid recovery. The authors conducted a series of experiments in which animals dosed with 6.4g/kg or more of dry pods died with typical clinical signs. Gummow *et al.*, (1992) also carried out an experiment in which sheep were fed *A. versicolor* pod material and all showed signs of poisoning but recovered after treatment with pyridoxine hydrochloride (a vitamin B_6). The untreated sheep died 2 hours after receiving the pod material.

Some of the saponins isolated from *A. julibrissin* are reported to have exhibited cytotoxic activity. The two diastereomeric saponins (**108** and **109**), isolated by Zou *et al.*, (2000) showed cytotoxicity to KB cell line *in vitro* and Ikeda *et al.*, (1997) also isolated cytotoxic glycosides from the species. Mar *et al.* (1991) isolated some budmunchiamines from *A. amara* and they found that some of them were cytotoxic. Rukunga (1996) found that some of the ones he isolated also exhibit cytotoxicity properties.

No information has been found on the toxicity of *Albizzia brevifolia* during literature search.

1.6.5 *Argemone mexicana* L.

Picture 1.5: *Argemone mexicana* L.

This plant belongs to the order Papaverales and family Papaveraceae that comprises 42 genera and about 650 species. The genus *Argemone*, a genus of prickly herbs, includes about 12 species (Rahman and Ilyas, 1961). *A. mexicana* (English Mexican poppy, prickly poppy, Rahman and Ilyas, 1961; Pahwa and Chartterjee, 1989), in India is known as pivla dhatura or satyanashi (Bose *et al.*, 1963). It is widely spread throughout tropical and subtropical countries (Watt and Breyer-Brandwijk, 1962) including Botswana. The plant grows to about 50cm tall, it is prickly all over, has yellow flowers, has thorny greyish green prickly seed pods, which become brown when matured and the leaves are

serrated and prickly, greyish green in colour. The seeds are brownish black in colour and are very small (less than 1mm in diameter) and resemble mustard seeds. The plant is abundant in most parts of Eastern, Central and Northern Botswana as a weed in fields and along roadsides.

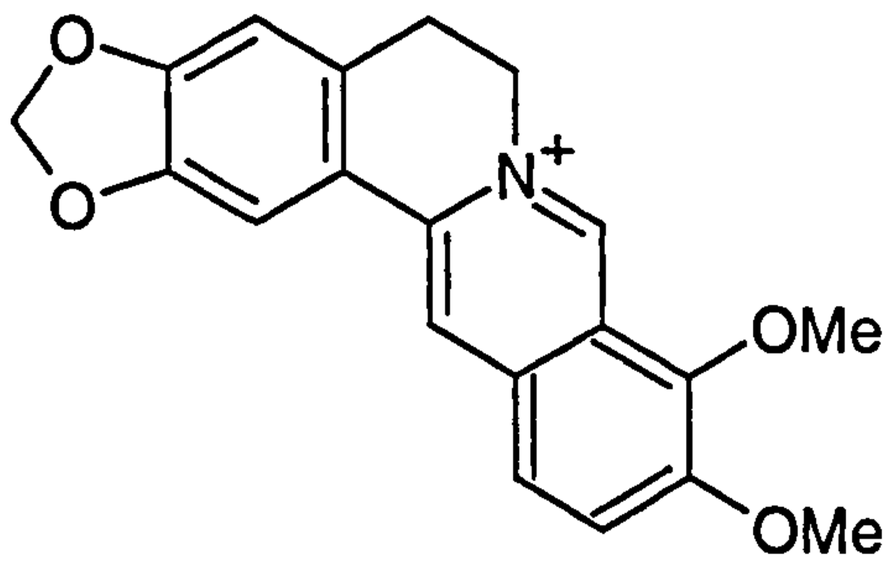
The use of the plant for medicinal purpose is well known. In South Africa the yellow juice and leaf are used for their narcotic and anodyne properties to dress wounds and as an application to warts. Sometimes it is used to repel termites, the seed oil as a purgative. The aerial parts of this plant are widely used in traditional medicine for their analgesic properties (DeFeo, 1972; Watt and Breyer-Brandwijk, 1962). The yellow milky juice is used to treat dropsy, jaundice, skin diseases, indolent or syphilitic ulcers, eye conditions, respiratory disorders and constipation in India (Bose *et al.*, 1963)

1.6.5.1 Phytochemical

The family Papaveraceae is known to be rich in alkaloids. Chemical investigations of various parts of *Argemone mexicana* have been reported to yield, isoquinoline alkaloids (Sakar, 1926; Dalvi, 1985; Takken, 1993), flavonoids from the flowers (Rahman and Ilyas, 1962; Krishnamurti *et al.*, 1965) and from the seeds (Bhardwaji, *et al.*, 1981; Harbone and Williams, 1982), alcohols (Sangwan and Malik, 1998), sugars (Saraf, *et al.*, 1994), fatty acids (Gunstone *et al.*, 1977; Takken, 1993), free amino acids (Dada and Bandyopadhyay, 1986), and aliphatic compounds (Dinda and Barnejee, 1987). Protopine alkaloids are widely distributed in the families Berberidaceae, Fumariaceae, Papaveraceae, Ranunculaceae and Rutaceae (Brossi, 1988). Berberine (**125**) and protopine (**126**) were first isolated from *A. mexicana* by Schlotterbeck in 1902. Santos and Adkilen confirmed the presence of these alkaloids in 1932. The isoquinolone alkaloid, oxyhydrastine (**127**) is reported to have been isolated from this plant by Hassain *et al.* in 1983. Pathak, *et al.* (1985) reported the isolation of nor-sanguinarine in addition to berberine, protopine, and β-sitosterol (**22**) from the roots and 3-methoxyquercetin (**128**) and vanillic acid (**129**) from the flowers. Other alkaloids that have been isolated from *A. mexicana* are sanguinarine (**130**) allocryptopine (**131**), coptisine (**132**),

chelerythrine (**133**), dihydrochelerythrine (**134**), norchelerythrine (**135**), scoulerine (**136**) and the derivatives of protopine alkaloids having a ketonic group on C-13, namely, 13-oxoprotopine (**137**), 13-oxocryptopine (**138**), 13-oxomuramine (**139**) and 13-oxoallocryptopine (**140**) (Manske, 1954, 1968, 1975). A nematicidal triglyceride was isolated by Saleh *et al.* (1987) from the crude petroleum ether extract of the plant.

Other types of compounds isolated from the plant are flavonoids. Isorhamnetin-3-glucoside (**141**), isorhamnetin-3,7-diglucoside (**142**) and 4',5,7-trihydroxy-3-methoxyflavonol have been isolated from the plant. The fatty acids that have been isolated include: linoleic, oleic, palmitic, ricinoleic, stearic, linolenic and palmitoleic. The free amino acids isolated include: glycine, histidine, serine, proline, alanine, tyrosine, tryptophan, methionine, leucine, aspartic acid and glutamic acid.



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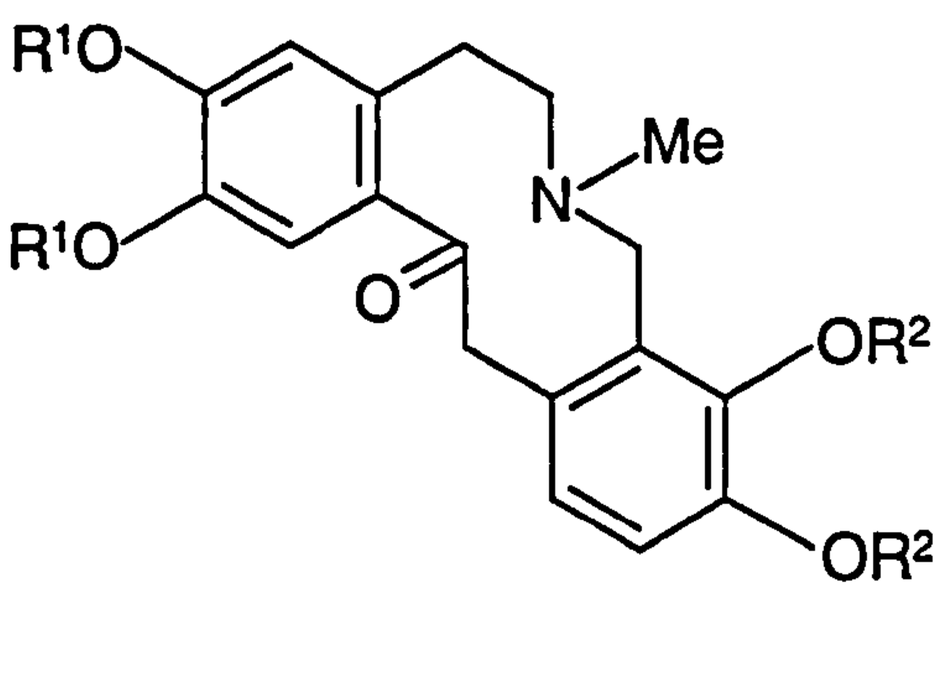
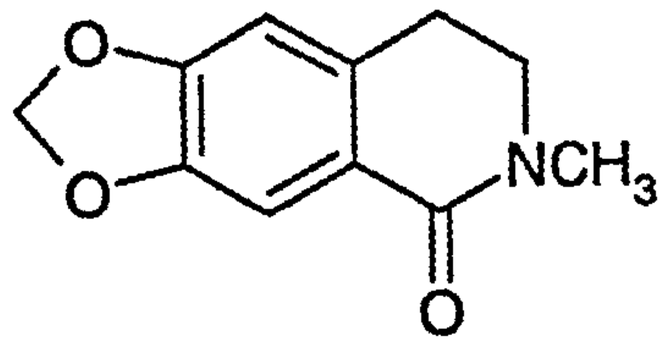


126	Protopine	$R^1 + R^1 = R^2 + R^2 = CH_2$
131	Allocryptopine	$R^1 + R^1 = CH_2$, $R^2 = Me$



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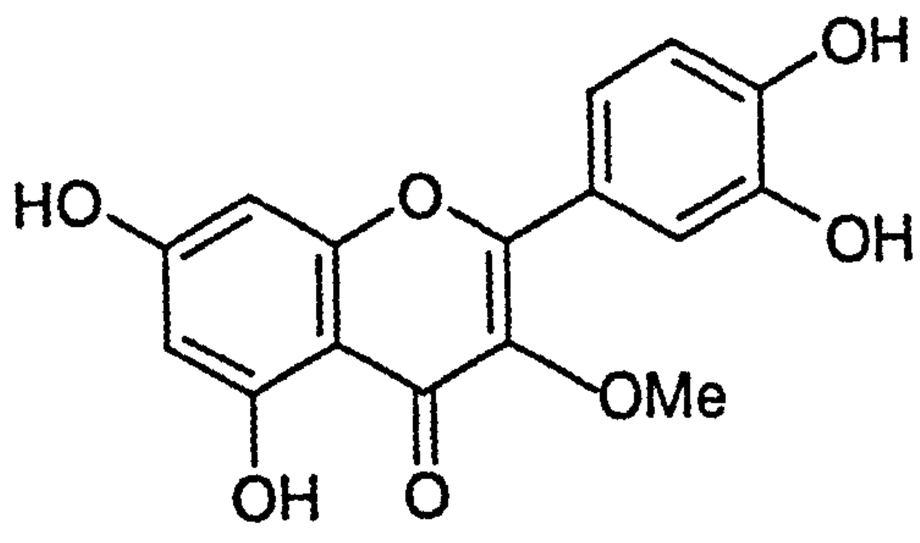

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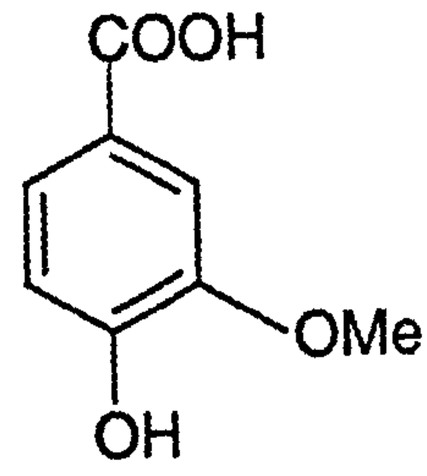

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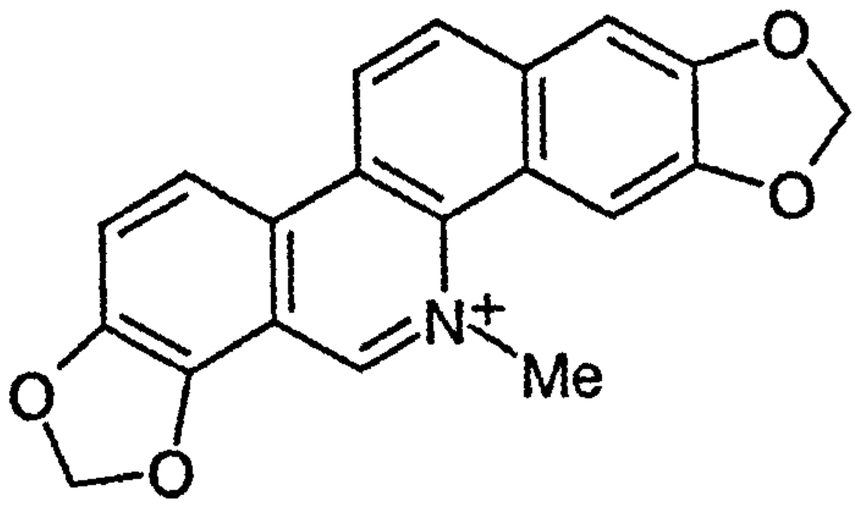

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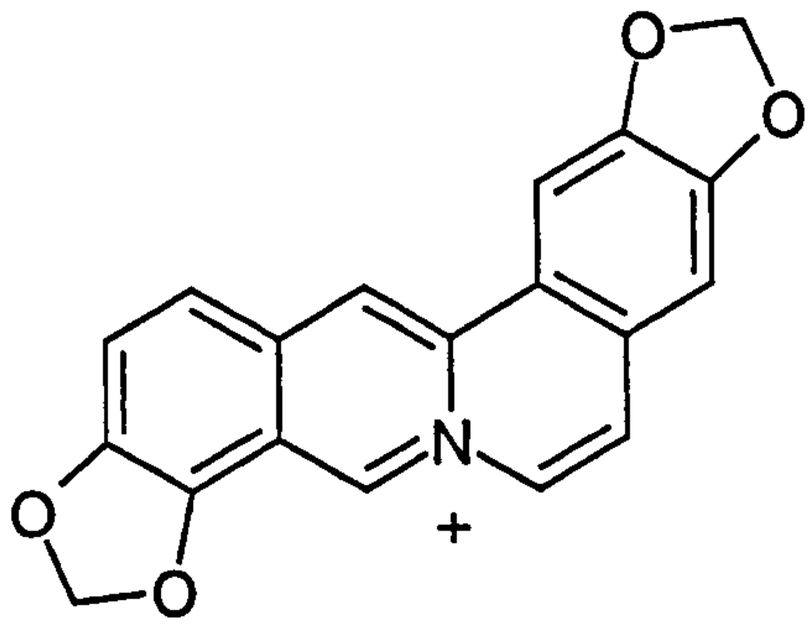

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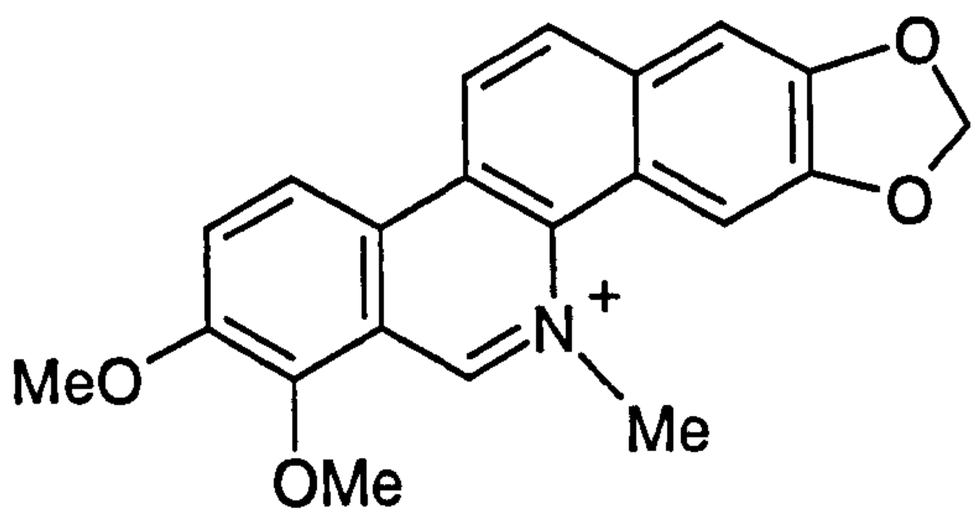

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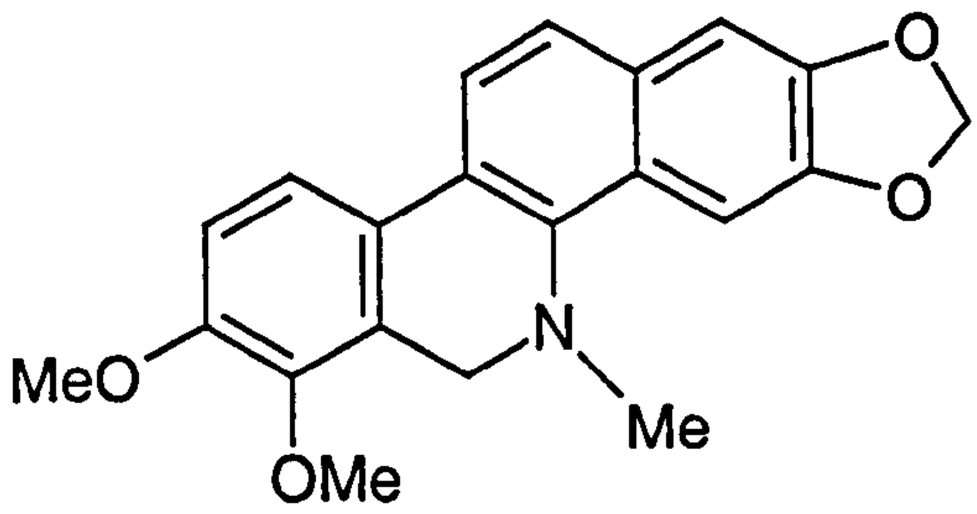

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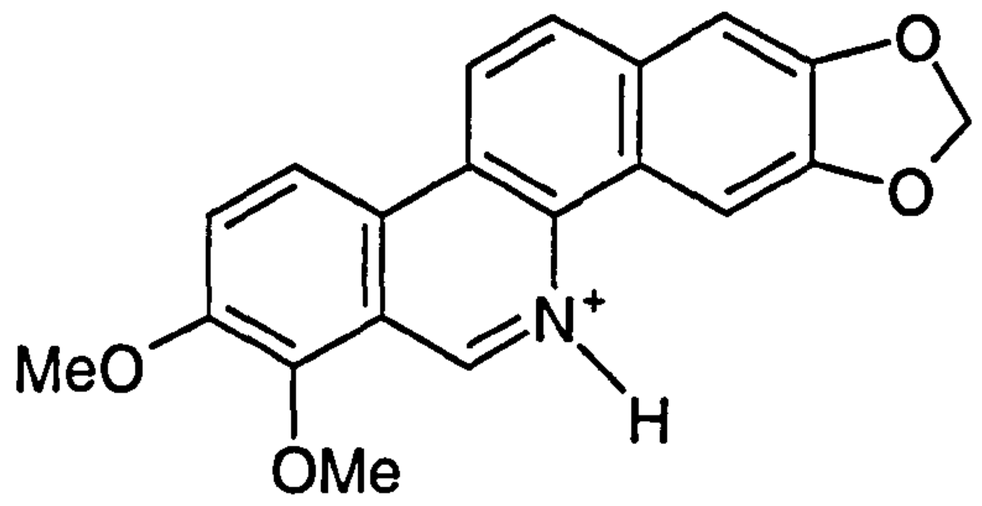

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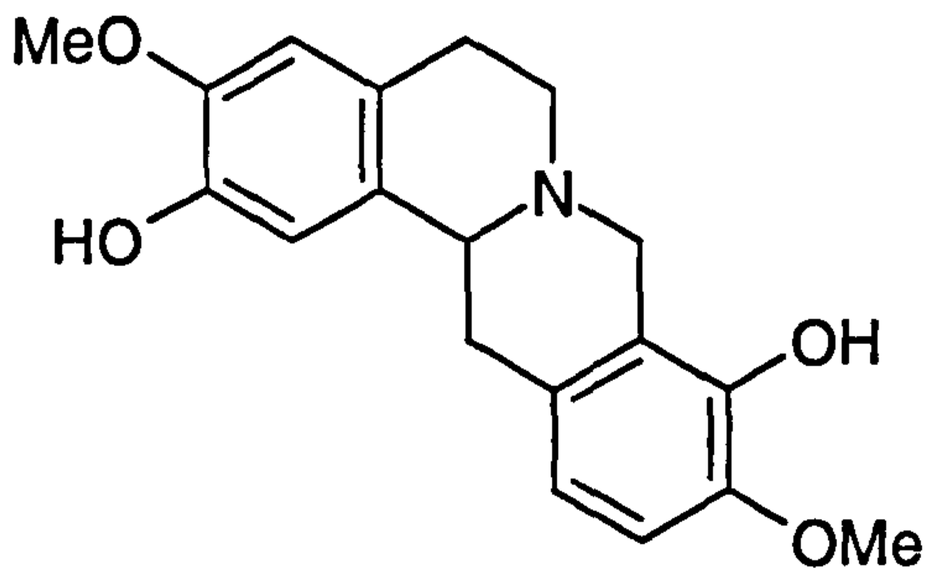

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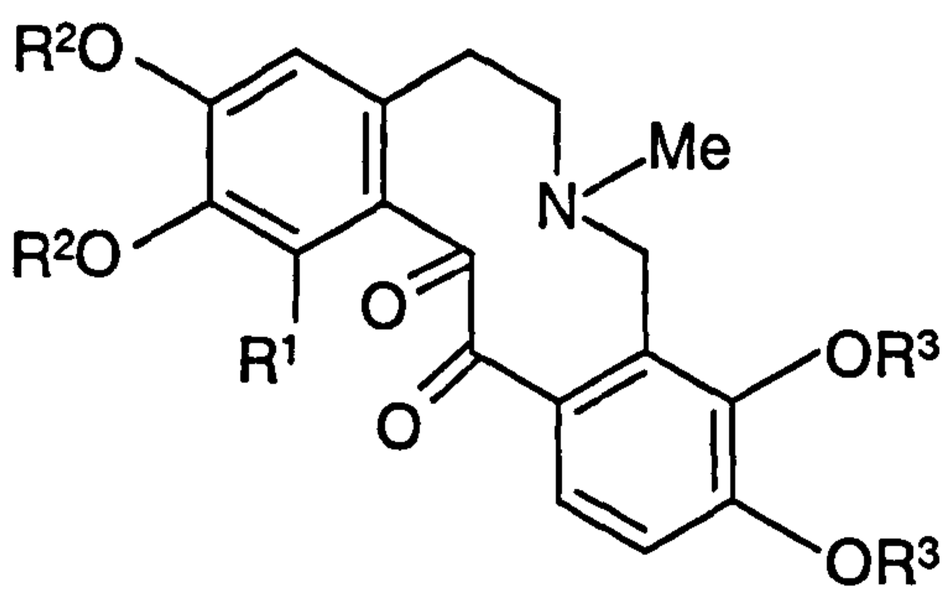
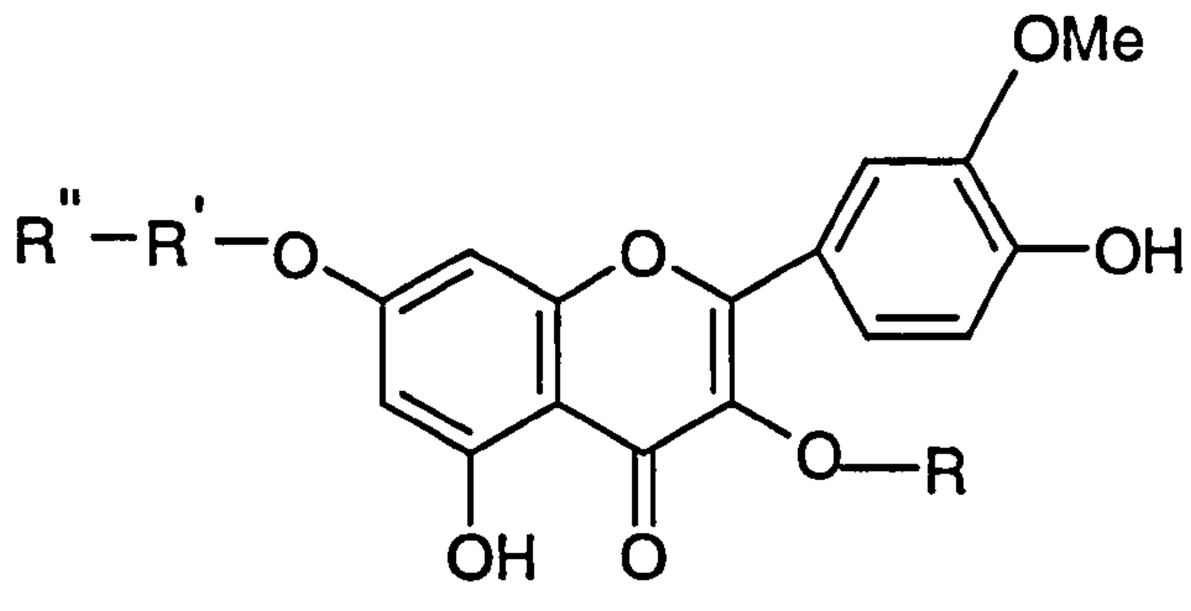


137	13-oxoprotopine	$R^1 = H$, $R^2 + R^2 = R^3 + R^3 = CH_2$
138	13-oxocryptopine	$R^1 = H$, $R^2 = Me$, $R^3 + R^3 = CH_2$
139	13-oxomuramine	$R^1 = H$, $R^2 = R^3 = Me$
140	13-oxoallocryptopine	$R^1 = H$, $R^2 + R^2 = CH_2$, $R^3 = Me$



141	Isorhamnetin-3-glucoside	R = Glucose residue, R'-R" = H
142	Isorhamnetin-3,7-diglucoside	R = R'-R" = Glucose residue

1.6.5.2 Toxicological

There are a number of reports on the contamination of other materials such as grains and seed oils (e.g. mustard seeds) with *Argemone mexicana* seeds. Such contamination has been reported to have contributed to several outbreaks of poisoning in humans called epidemic dropsy in India (Sakar, 1926; Singh *et al.*, 1983; Sohrab, *et al.*, 1961), South Africa (Meaker, 1950), Mauritius and Fiji (Das and Khanna, 1997). Shenolikar *et.al.* (1962) detected the presence of sanguinarine in the blood and urine of patients suffering from epidemic dropsy. As a result of these reports of poisoning a lot of research has been carried out to investigate the chemistry of the oil obtained from the seeds of *A. mexicana.* Bui (1974) found that seeds grown in Vietman contained 52.8% oil and 0.43% sanguinarine and those grown in the USSR produced 32.84% oil and contained allocryptopine. Hatman and his colleagues (1972) detected *A. mexicana* seeds in mustard seeds using paper chromatography. Others (Bose, 1972; Mohiuddin and Saidi (1973), Bose and Roy (1977) also developed methods for the detection and estimation of *A. mexicana* oil in edible oils. Sakar (1948) and Hakim *et al.* (1961) reported that the isoquinoline alkaloids detected in the seeds of *A. mexicana* include dihydrosanguinarine as the major component (87%) and smaller amounts of sanguinarine, berberine and protopine. Sakar in this paper also demonstrated that sanguinarine is poisonous to rats. Pahwa and Chartterjee (1989) investigated the toxicity of *A. mexicana* seeds in roof rats. They observed signs of poisoning, which included sedation, passiveness, sluggishness,

feeble or no muscular jerks, abdominal contractions, increased defecation, and death (14 out of 16 rats died).

Reports on the toxicity of the individual alkaloids suggest that sanguinarine is responsible for most of the toxicity (see section 3.4.3 for discussion on mode of toxicity). According to Manske (1975), sanguinarine has a strong antibacterial effect upon gram positive bacteria and that its LD_{50} for mice was found to be 19.4 mg/kg. He also reports that on intra-abdominal administration to mice the LD_{50} for berberine was determined to be 0.275mg/10g, the main symptoms being loss of motility and diminished respiration. Intravenous administration to dogs and cats resulted in a temporary decrease in blood pressure and stimulation of respiration immediately after injection. Small doses of protopine were found to retard heart activity, decrease blood pressure and have a sedative effect in experimental animals. Large doses of protopine were found to produce excitation and convulsions. The triglyceride isolated by Saleh (1987) and his colleagues was found to have nematicidal activity against the plant parasitic nematode, *Meloidogyne incognita*. Thus, most of the studies outlined above concentrated on the toxicity of the seed oil, contamination by which is normally accidental. The present study will focus mainly on investigating the toxicity of the aerial parts (leaves and stems) and roots because of their extensive use as medicine.

1.6.6 *Enicostemma axillare* (Lam.) A. Rayal *subsp. Axillare*

Picture 1.6: *Enicostemma axillare*

E. axillare belongs to the order Gentianales, family Gentianaceae which comprises 80 genera and about 1000 species. Many of the species are herbs and a few are shrubs. The genus *Enicostemma* consits of 3 or 4 species (Trease and Evans, 1983). Ghosal and Jaiswal, (1980) reported the genus to be monospecific with *E. hyssopifolium* (Willd.) Verd (synonymous with *E. litorole*, Ghosal *et.al*, 1974) being the only species to the genus, however now it is known that there are more than one species belonging to the genus. *E. axillare* is a small herb which grows to about 30cm with green grouped thin leaves, white flowers and very short roots. It grows in Central and South Central regions of Botswana.

Plants from this family have long been used in tradition medicine, for example, Gentian root from *Gentian lutea* and Indian gentian from *Swertia chirata*. In India *E.*

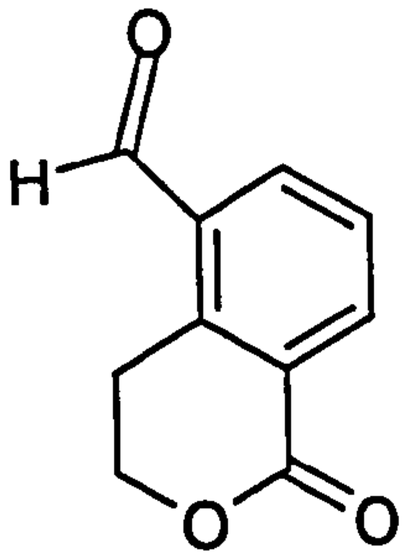
hyssopifolium is used for cardiac dropsy, rheumatism, some mental disorders (Ghosal *et.al*, 1980). The plant is also reported to have glucose lowering properties (in diabetic rats) (Maroo *et al.*, 2002; Murali *et al.*, 2002). In Botswana *E. axillare* is used to treat a variety of ailments and it is, not surprisingly, locally known as "*makgonatsotlhe*" which means "cure all". Some members of the family, on account of their bitter principles, are used in liqueurs.

1.6.6.1 Phytochemical

A wide variety of secondary metabolites are reported to be produced by the members of the family including alkaloids (Trease and Evans, 1983), iridoid glycosides and secoiridoid glucosides (Ghosal *et al.*, 1974, 1980; Ma *et al.*, 1994; Hamburger, 1990) (Wolfender, 1991), flavones (Trease and Evans, 1983), flavonoids (Ghosal *et al.*, 1980; Murali *et al.*, 2002), xanthones and their glycosides (Wolfender *et. al*, 1991, 1993), phenolic acids, tannins, and the trisaccharide, gentianose, (Trease and Evans, 1983). Table 1.5 provides a summary of some of the compounds isolated from Gentianaceae species.

Table 1.5: compounds isolated from Gentianaceae family

Compound	Source (Reference)
Iridoids:	
Erythrocentaurin (**143**)	*E. hyssopifolium, Swertia lawii* (Ghosal *et al.*, 1974)
Rhodenthoside A (**144**)	*Gentiana rhodentha* (Ma *et al.*, 1994)
Sweroside (**145**)	"
Swertiamarin (**146**)	"
Kingiside (**147**)	"



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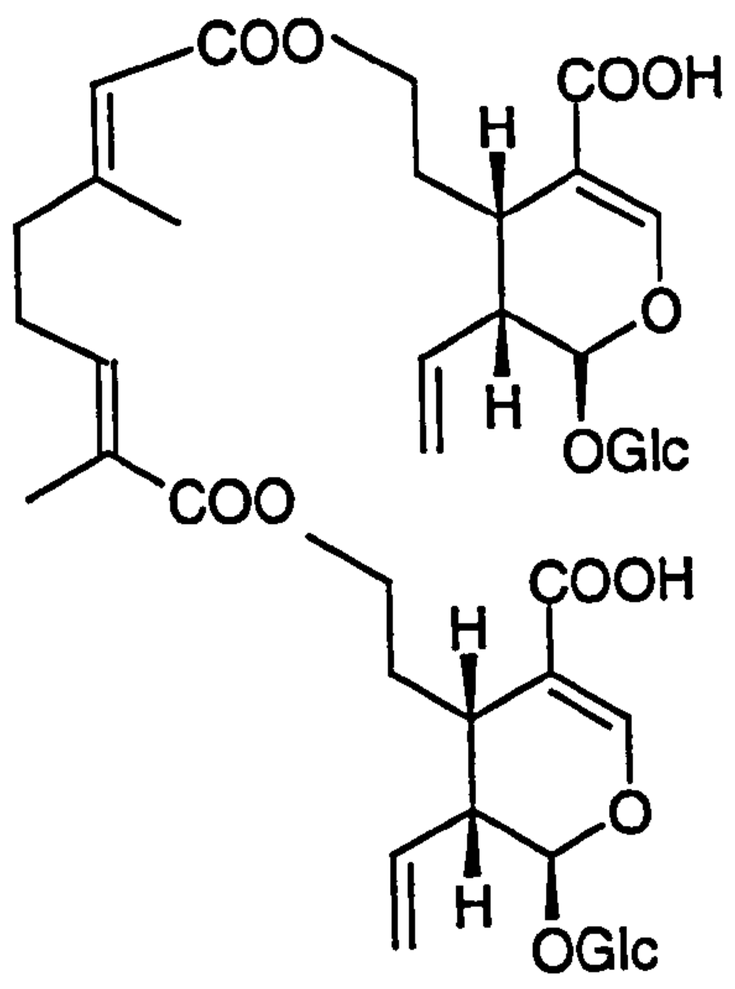

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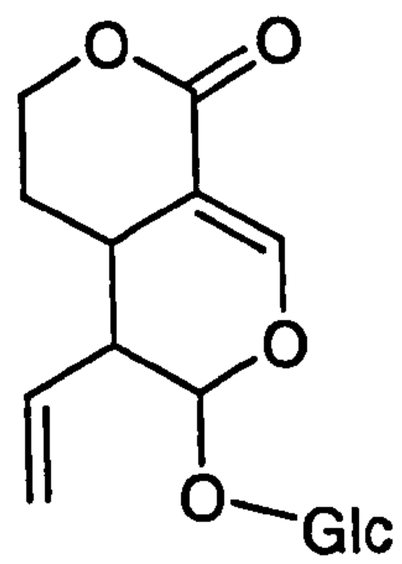

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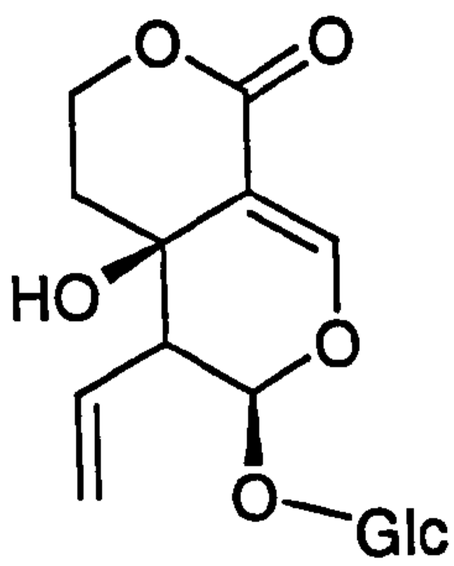

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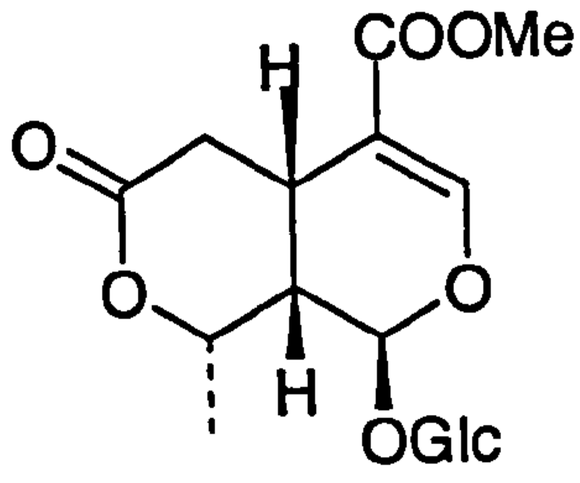

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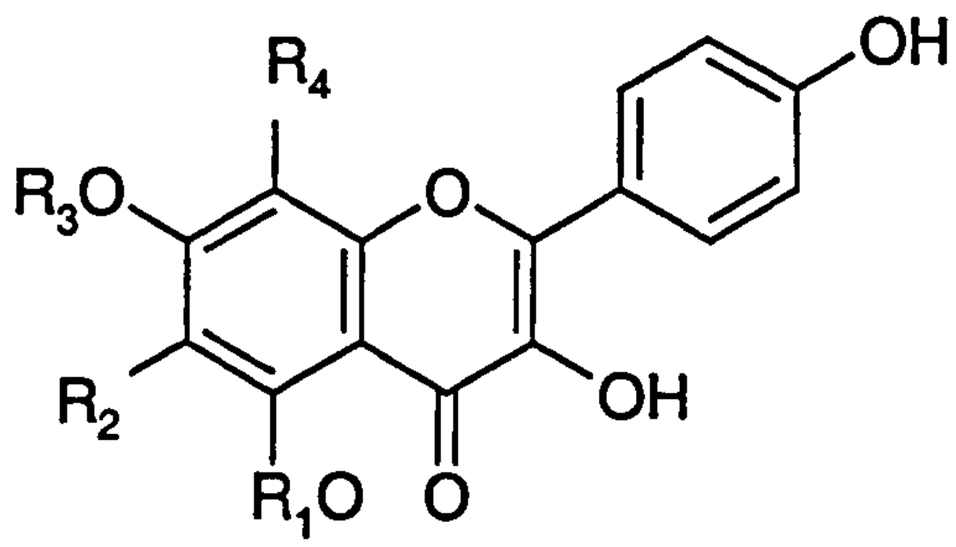
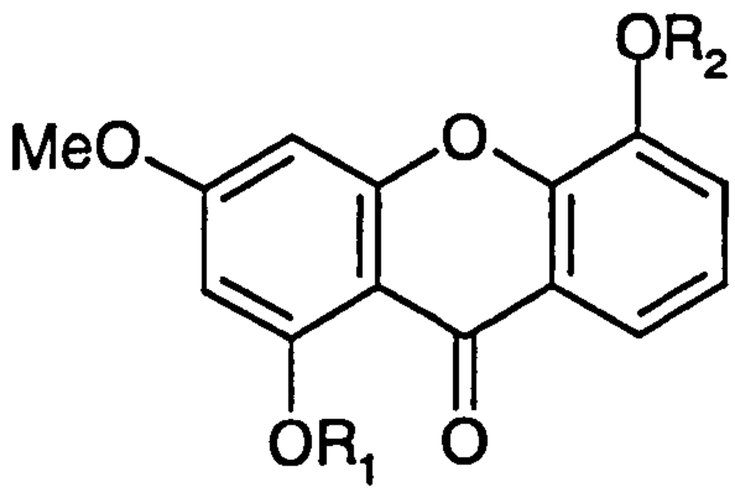
Flavonoids:

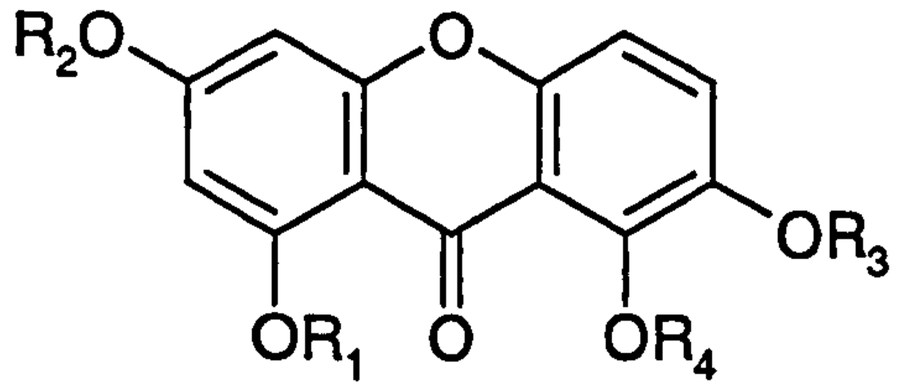


E.hyssopifolium (Ghosal *et al.*, 1980)

	R1	R2	R3	R4
Apegenin (**148**)	H	H	H	H
Genkwanin (**149**)	H	H	Me	H
Isovitexin (**150**)	H	glc	H	H
Swertisin (**151**)	H	glc	Me	H
Saponarin (**152**)	H	glc	glc	H
5-*O*-glucosylswertisin (**153**)	glc	glc	Me	H
5-*O*-glucosylisoswertisin) (**154**)	glc	H	Me	glc

Xanthones:



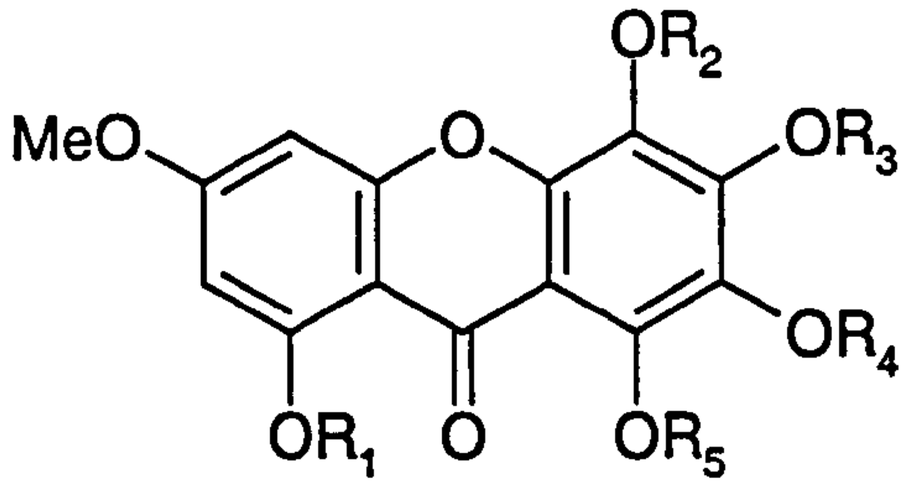


Chironia krebsii (Wolfender, 1991)

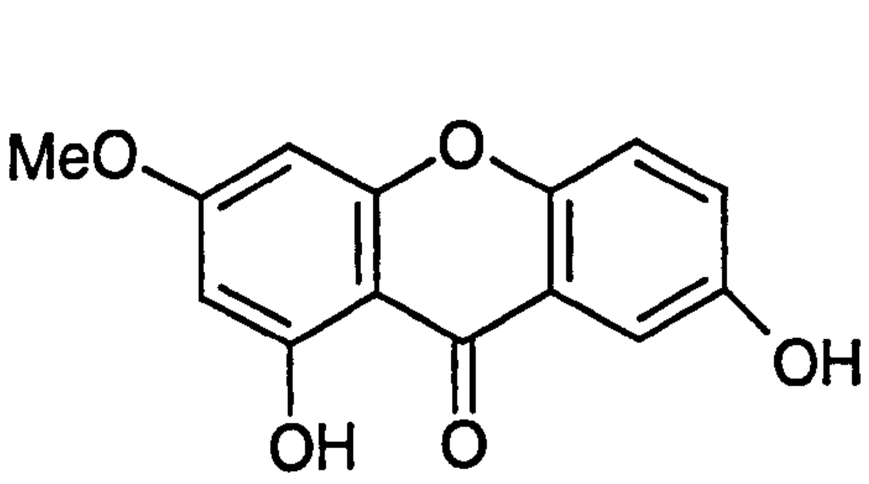
	R_1	R_2
155	H	Me
156	H	H
157	Glc	H

	R_1	R_2	R_3	R_4
161	H	Me	H	H
162	H	Me	Me	Me
163	H	H	H	H

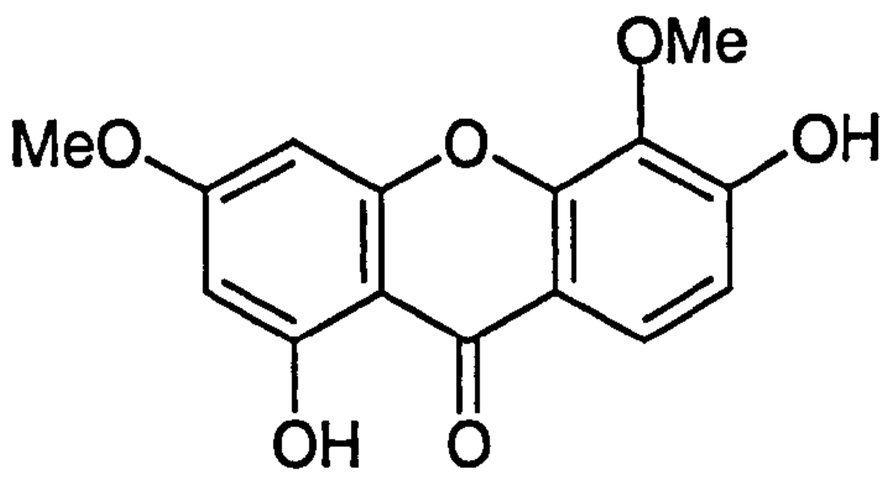
158	Prim	Me			
159	Prim	H			
160	H	Prim			
164	Prim	Me	H	H	



	R_1	R_2	R_3	R_4	R_5
165	H	Me	H	Me	H
166	H	Me	H	Me	Me
167	H	Me	Me	Me	H
168	H	Me	Me	Me	Me
169	Prim	Me	Me	Me	Me



170



171

Prim = primeverose (= β-D-xyllopyranosyl(1→6)-β-D-glucopyranoside

No phytochemical work has been reported so far on *E. axillare*.

1.6.6.2 Toxicological

Other than antitumour activity (cytotoxicity) of *E. hyssopifolium* methanol extracts (Kavimani and Mansenthikumar, 2000), there are no reports of Gentianaceae species being toxic. These authors reported a significant enhancement of mean survival time of *E. hyssopifolium* methanol extract treated tumour bearing mice as compared to the controls.

To date no toxicological work has been carried out on *E. axillare.*

1.7 Toxicity testing

As already stated, the fact that all chemicals are "poisons" has been recognised for centuries. The aim of toxicological studies is therefore to determine the degree and nature of toxicity of a substance. The brine shrimp lethality bioassay has been used in the past by a number of researchers to estimate cytotoxicity of biologically active plant extracts and compounds isolated from them. In this study toxicity evaluation of the plants was carried out *in vitro*, using established human and murine cell lines. These cells were exposed to crude extracts of the plant material and isolated compounds at varying concentrations and percentage suppression in cell viability compared to controls determined. The culture of animal cells and tissues is now a widely used technique. Use of *in vitro* assay systems for potential anticancer agents has been in common practice since the beginnings of clinical cancer chemotherapy in 1946 (Wilson, 1992).

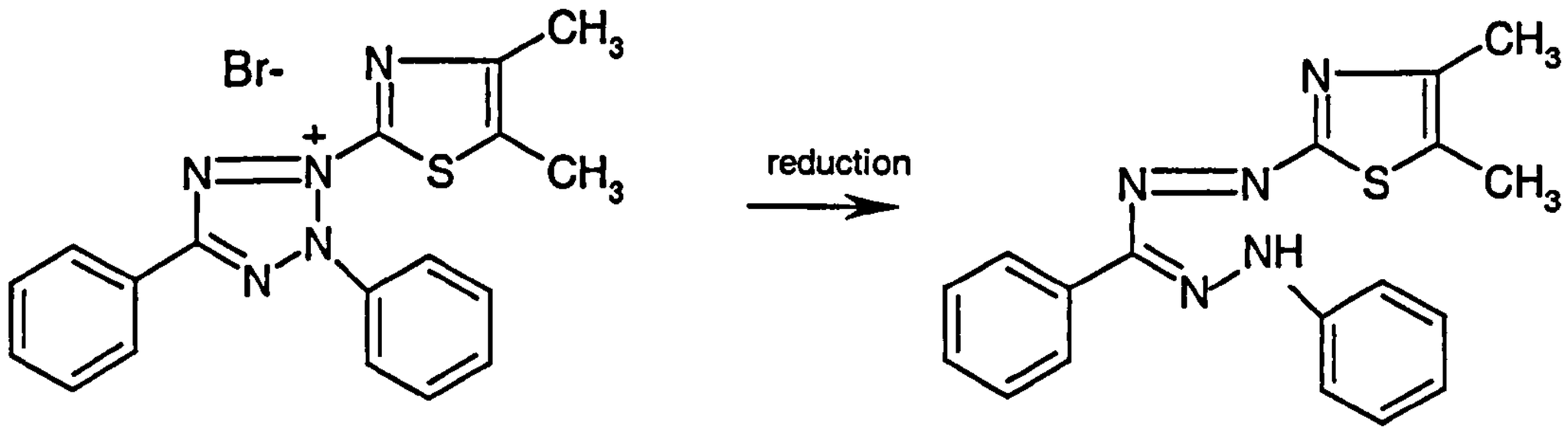
The ideal substrate for toxicity or any biological activity investigation would of course be the target organism but for obvious reasons in the case of toxicity to humans, this is not feasible. The second best mode of testing would be the use of animals to simulate what could happen in the human body, but where such facilities are not available and/or the moral implications of animal experimentation inhibit this, then cell and tissue cultures are the second best thing. Compared to *in vivo* testing, cell cultures have an economic advantage being less expensive to maintain than animals and that only small amounts of test samples are used, the latter being an important consideration in forensic work where normally small amounts of material are available. There is also an increasing realization of limitations of animal models in relation to human metabolism, as increasing numbers of metabolic differences between species come to be identified (Freshney, 1992).

Cytotoxicity was determined by measuring cell viability. Cytotoxicity assays measure drug-induced alterations in metabolic pathways or structural integrity, which may or may not be related directly to cell death.

The aims of this part of the project were to:

- determine which of the plant extracts and therefore plant is toxic
- reveal potentially toxic compounds
- identify the range of activity of the extracts and compounds
- to determine whether the effect is cell specific
- identify the toxic concentration range

The MTT assay was used to determine cell viability in order to determine the approximate toxicity levels of samples. This is a widely used technique involving the use of a tetrazolium salt, 3-(4,5-dimethlythiazol-2-yl)-2-5-diphenyltetrazolium bromide (**172**) as a colorimetric indicator of cell viability. The assay measures the activity of various dehydrogenase enzymes in the mitochondria of living (viable) cells. The enzymes reduce the salt to produce a blue formazan product (**173**).



172 **173**

The blue crystals are seen at the bottom of the wells. The amount of formazan product produced is proportional to the levels of the dehydrogenase enzymes and therefore the number of living cells. The formazan crystals are dissolved in DMSO and absorbance readings obtained at 620nm using an ELISA reader (Multiskan MCC/340 P Version 2.20). The assay has the advantage of not using radioisotopes and it allows many samples to be processed in a short period of time.

The cell lines used in this study (see section 2.3, Materials and Methods) were chosen because of their fast replication rate, especially the melanoma and stomach cancer. This allowed the processing of a considerable number of experiments in a relatively short time. The lung cancer and murine cell lines multiplied relatively slower compared to the other two.

1.8 DNA analysis

A large portion of materials submitted for analysis in cases where poisoning by herbal preparations is suspected is in mixture form (i.e. a mixture of different plants), usually in small amounts. Only in rare occasions are whole plants or parts of plants with adequate features to allow botanical identification available. Establishing the identity of the species involved would make it easy for the toxicologist as more material could be collected from the wild to allow detailed phytochemical and toxicological studies. Screening the mixtures and detecting certain groups of secondary metabolites alone is not enough as some of these are common to a number of species, genera and families. The best that could be established from such chemical screens would be the identity of the possible family of the plants from which the plant material could have come from. The development of a DNA based method that can be used to assist in establishing species constituent of an herbal mixture or to discriminate between different plant species (or even cultivars) is thus necessary.

A DNA based test can be produced and used that is specific to a species known. DNA within members of the same species may show variation based upon geographical isolation. Two seed stocks of *E. axillare*, for example grown in different geographical regions, although of the same species and therefore sharing most of their DNA, may show slight genetic variations. These genetic variations, or polymorphisms, can be detected provided a relevant method is used. Botanical samples encountered may be at trace levels or of powdered form as is usually the case with forensic samples. The effect of environmental factors on the phenotype is avoided and, as is common with forensic samples, the phenotype may not be recognisable. The advent of the polymerase chain reaction (PCR) (Siaki *et al.*, 1988) has greatly increased the number of genetic markers that can be identified from such materials. Particular sections of the DNA can be analysed based upon genetic information from closely related species. Little if any DNA sequence information was known for the six plant species prior to this study.

The cytochrome b gene is the most wide spread DNA locus used in taxonomy (Bartlett and Davidson (1992). This is a valuable marker where little intra-species variation is detected. The marker may not be sufficiently polymorphic to discriminate between members of closely related plant species and different cultivars within the same species. Additionally the test is labour intensive requiring the amplification of the product, the purification of the product and the complete DNA sequencing of the product. A simpler test based upon simple length polymorphisms that can be performed in one reaction is more applicable to this study.

In eukaryotic organisms DNA is found mostly in the nucleus, some in the mitochondria and in the case of plants, also in chloroplasts. The DNA contained in the mitochondria and chloroplasts is less than that found in the nucleus. However, since each cell contains many chloroplasts and mitochondria, this form of DNA forms a significant portion of the total DNA of an organism. Chloroplast DNA (cpDNA) occurs as a circular loop. Encoded on the DNA are a number of tRNA genes. These regions of the DNA occupied by genes are called coding regions. The regions not occupied by genes are called non-coding regions and these are found either between genes (intergenic spacers) or separating parts of a gene (introns). DNA coding regions are highly conserved between different green plants (Taberlet *et al.*, 1991; Fangan *et al.*, 1994) and would therefore show little or no polymorphism at all.

Non-coding DNA forms the vast majority of DNA for eukaryotic organisms and displays the highest frequency of mutations (Saiki, *et al.*, 1988). It is these non-coding regions that are exploited to discriminate between species either interspecifically or intraspecifically. These regions show a high degree of polymorphism between closely related species of plants (Linacre *et al.*, 2000). The differences being due to size and sequence differences within the introns and intergenic spacers. Length polymorphisms can readily be detected by fluorescent labelling of the primer. Because gene sequences surrounding introns and intergenic spacers are highly conserved, primer sequences identified in one organism can be used in a distantly related organism. This cross-transferability of primer sites allows

amplification of polymorphic unknown introns from botanical samples with no prior knowledge of the organism being tested, as is the case in this study.

Primers have been synthesised for the purpose of binding to parts of the coding sequences, which would flank non-coding regions and therefore allow amplification of these more variable and thus informative regions. Six universal primers have been designed for the amplification of three non-coding regions of chloroplast DNA (Taberlet *et al.*, 1991). These are listed in the table below. These primers have been shown to amplify chloroplast DNA over a wide taxonomical range.

Table 1.6: Universal primers for the amplification of the region between tRNA T and tRNA Phe of chloroplast DNA

Name	Sequence 5' – 3'
Forward A primer	5'CATTACAAATGCGATGCTCT3'
Forward B primer	5'TCTACCGATTTCGCCATATC3'
Forward C Primer	5'CGAAATCGGTAGACGCTACG3'[1]
Forward D Primer	5'GGGGATAGAGGGACTTGAAC3'
Forward E Primer	5'GGTTCAAGTCCCTCTATCCC3'
Forward F Primer	5'ATTTGAACTGGTGACACGAG3'[1]

In this study two loci in the tRNA gene complex of chloroplast DNA and ITS (Internal Transcribed Spacer) locus of ribosomal DNA were used to discriminate between the six plant species that are being investigated in this project. All six are from different genera.

The tRNA genes are highly conserved between different green plants. The region chosen is between the tRNA Leu and tRNA Phe genes. This region consists of an intron within the tRNA Leu gene which is amplified by primers C and D, and an intergenic spacer separating the two genes, which is amplified by primers E and F. The length of the intron

and intergenic spacer is variable between different species. The tRNA gene complex map is shown at Figure 1.1 and the primers used in this study are outlined at Table 1.7.

The other locus targeted, the ITS region, is found on ribosomal RNA. Because all cells require a lot of rRNA, rather than having one gene constantly encoding for this, most eukaryotes have evolved multiple copies of this gene sequence producing large copies of this locus per cell. The gene sequences are tandemly repeated along the chromosome (Lee *et al.*, 2000). A map of the gene complex is shown at Figure 1.2. The gene complex consists of a series of coding regions separated by non-coding regions (i.e. the ITS's). As in cpDNA the gene sequences, i.e. the coding regions, are highly conserved, as they evolve slowly and are therefore unlikely to distinguish between species. The non-coding regions on the other hand have been found to be highly polymorphic both in sequence and length between different species and to a lesser extent within species (White *et al.*, 1990). Lee *et al.*, (1990) successfully used the ITS1 locus to identify members of the genera *Panaeolus* and *Psilocybe*. This region was amplified by the forward and reverse ITS primers (Table1.7).

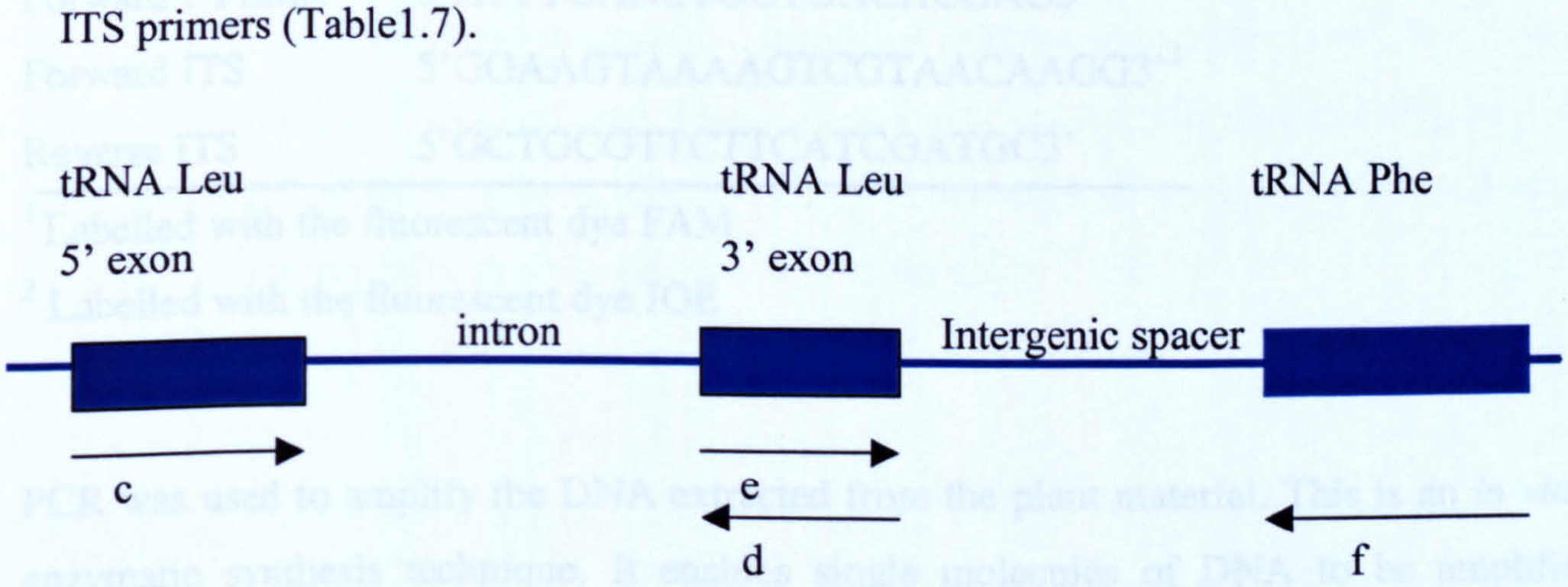


Figure 1.1: Map of the tRNA gene complex targeted in this study

NTS	ETS	16-18S	ITS-1	5.8S	ITS-2	26-28S

Figure 1.2: rRNA Gene Complex. NTS is a non-transcribed spacer; ETS is an external transcribed spacer; 16-18S, 5.8S and 26-28S are coding sequences for the ribosomal RNA; and ITS1 and ITS2 are internal transcribed spacers. The whole gene complex is many kilobases long and is repeated many times one after the other along the chromosome.

Table 1.7: Primers used for the amplification of the intron within the tRNA Leu gene, the intergenic spacer between tRNA Leu and tRNA Phe, and the ITS locus

Name	Sequence 5' – 3'
Forward C Primer	5'CGAAATCGGTAGACGCTACG3'[1]
Forward D Primer	5'GGGGATAGAGGGACTTGAAC3'
Forward E Primer	5'GGTTCAAGTCCCTCTATCCC3'
Forward F Primer	5'ATTTGAACTGGTGACACGAG3'[1]
Forward ITS	5'GGAAGTAAAAGTCGTAACAAGG3'[2]
Reverse ITS	5'GCTGCGTTCTTCATCGATGC3'

[1] Labelled with the fluorescent dye FAM

[2] Labelled with the fluorescent dye JOE

PCR was used to amplify the DNA extracted from the plant material. This is an *in vitro* enzymatic synthesis technique. It enables single molecules of DNA to be amplified millions of times, producing exact copies of the original. This allows analysis of DNA from only a few cells. To carry out PCR, short single strands of DNA of known base sequence (about 20 nucleotides) are needed. These are the primers and as described above they flank the targeted region of DNA for amplification. Thus the primers have to have sequences that are complementary to the flanking sequence of each strand of target DNA. Other components that are necessary for PCR are the nucleotides, deoxynucleotide triphosphates of the four DNA bases (dATP, dCTP, dGTP and dTTP), DNA polymerase and a buffer containing Mg^{2+} ions. Mg^{2+} ions are essential for the activation of the

enzyme, *Taq* (*Thermus aquaticus*) DNA polymerase which synthesises DNA by attaching deoxynucleotide triphosphates (dNTPs) to the 3' end of the primer. The nucleotide that the enzyme attaches will be the one that is complementary to the base in adjacent position on the template strand. *Taq DNA* polymerase is extracted and purified from the thermophilic bacterium, *Thermus aquaticus*. It can survive extended incubation at 95°C and thus is not affected by denaturation (Saiki, *et al.*, 1988).

They are three stages in a PCR amplification cycle; denaturation, annealing and extension, (Figure 1.3). A programmable thermal cycler is used to control the temperature for the different stages. During denaturation the reaction mixture is heated at 90°-95°C to break the hydrogen bonds that hold the base pairs together and separate the DNA double-stranded helix into individual strands. The second stage is annealing, which involves lowering the temperature of the reaction mixture to 40°-60°C to allow the primers to bind to the ends of each strand of the target DNA via complementary base pairing. The third stage is extension, during which the temperature is raised again to 70°-75°C to allow *Taq* DNA polymerase to extend the primers to complete the DNA double strand. The number of DNA strands doubles every time the cycle is repeated (Figure 1.4). Assuming 100% efficiency (which is normally not achieved in practice), at the end of 25 cycles, 2^{25}-fold amplification would have been achieved.

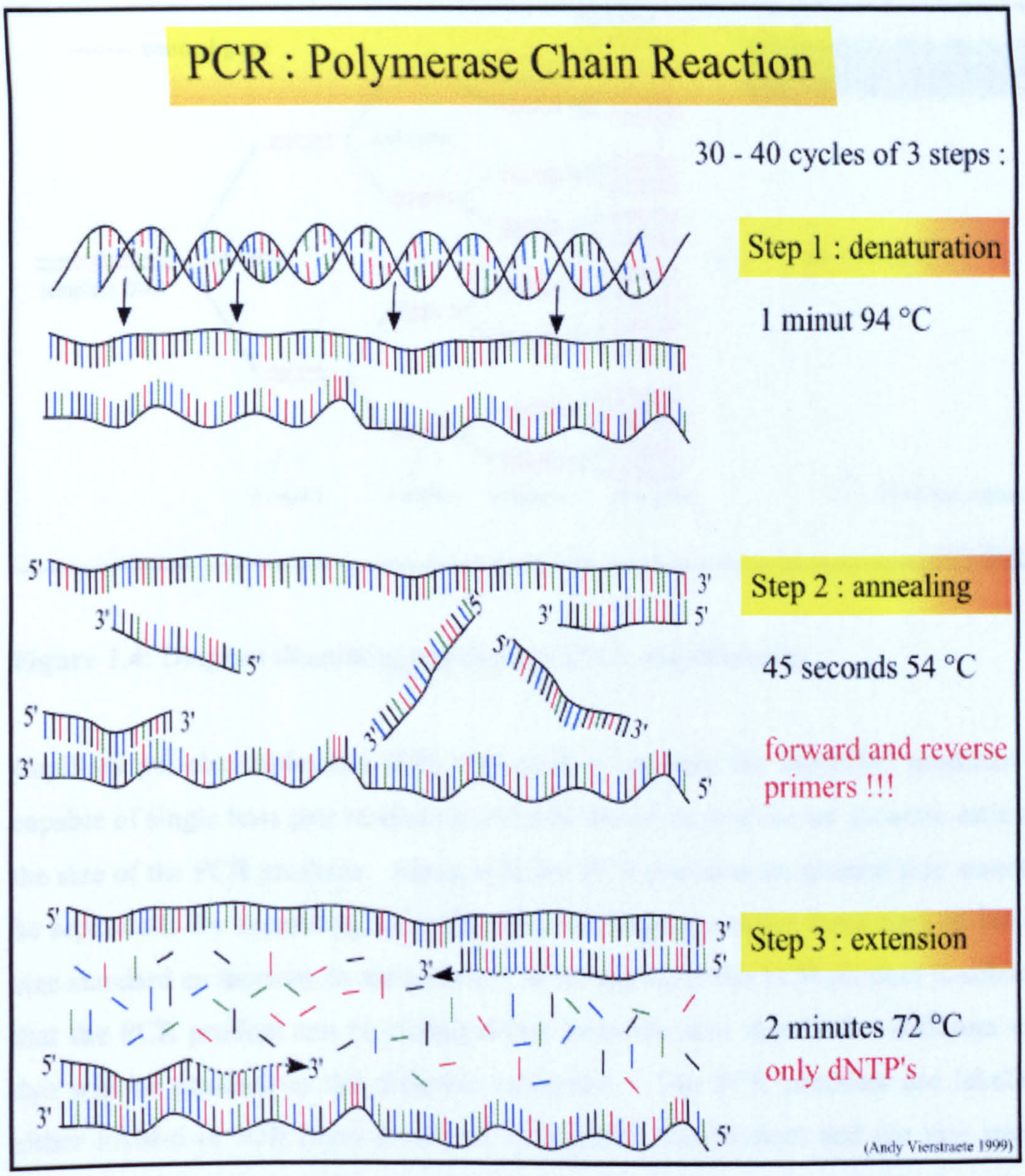


Figure 1.3: Diagram Illustrating the PCR process

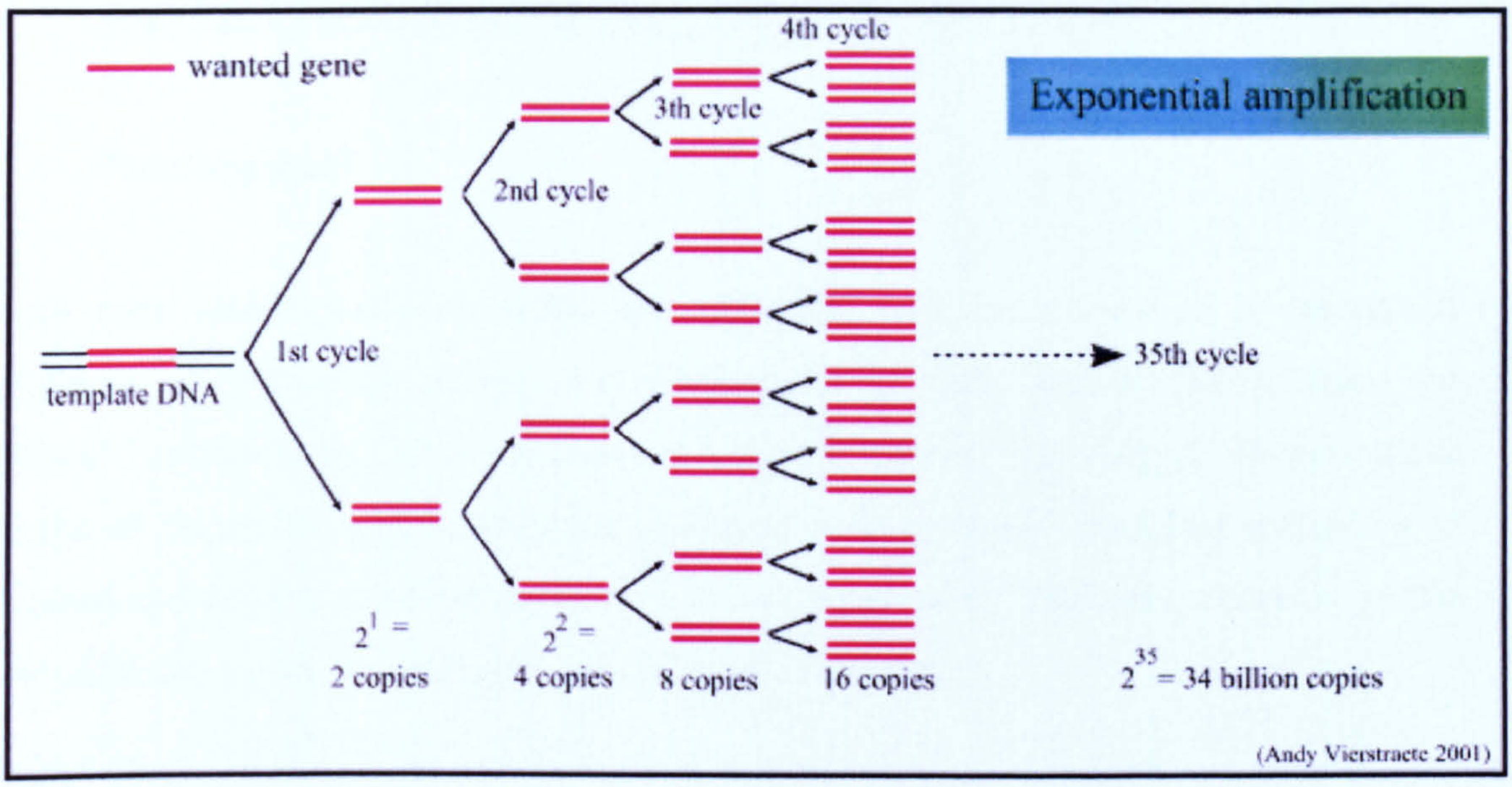


Figure 1.4: Diagram illustrating exponential DNA amplification

Capillary gel electrophoresis (CE) was used to separate the amplified products. CE is capable of single base pair resolution and will therefore produce an accurate estimation of the size of the PCR products. Along with the PCR products an internal size standard will be separated. By separating the unknown PCR fragment at the same time as the internal size standard an increase in the accuracy of the sizing of the PCR product is achieved. So that the PCR product can be distinguished from the size standard, a different coloured dye will be attached to the different molecules. The PCR products are labelled with either FAM-6 or JOE (dyes produced by Applied Biosystems) and the size standard is labelled with ROX in all the experiments. The PCR products are separated on a capillary with a fixed argon laser at the detector end. The laser passes through the capillary and a charged couple device (CCD) camera measures the amount of light reflected from the dyes attached to the molecules. The amount of light, and the time taken from injection to detection, is measured by the associated software to produce a size of the PCR product and the amount of product.

CHAPTER 2:

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 Plant Material

Plants were provisionally identified for collection with the assistance of traditional healers (using vernacular names) and photocopies of herbarium specimens from the National Herbarium in Gaborone Botswana. The collection was carried out during the months of December and January (rainy season in Botswana). The plant material was collected and dried at room temperature in an old warehouse. The areas where the plants were collected are shown in Figure 2.1 (Map of Botswana).

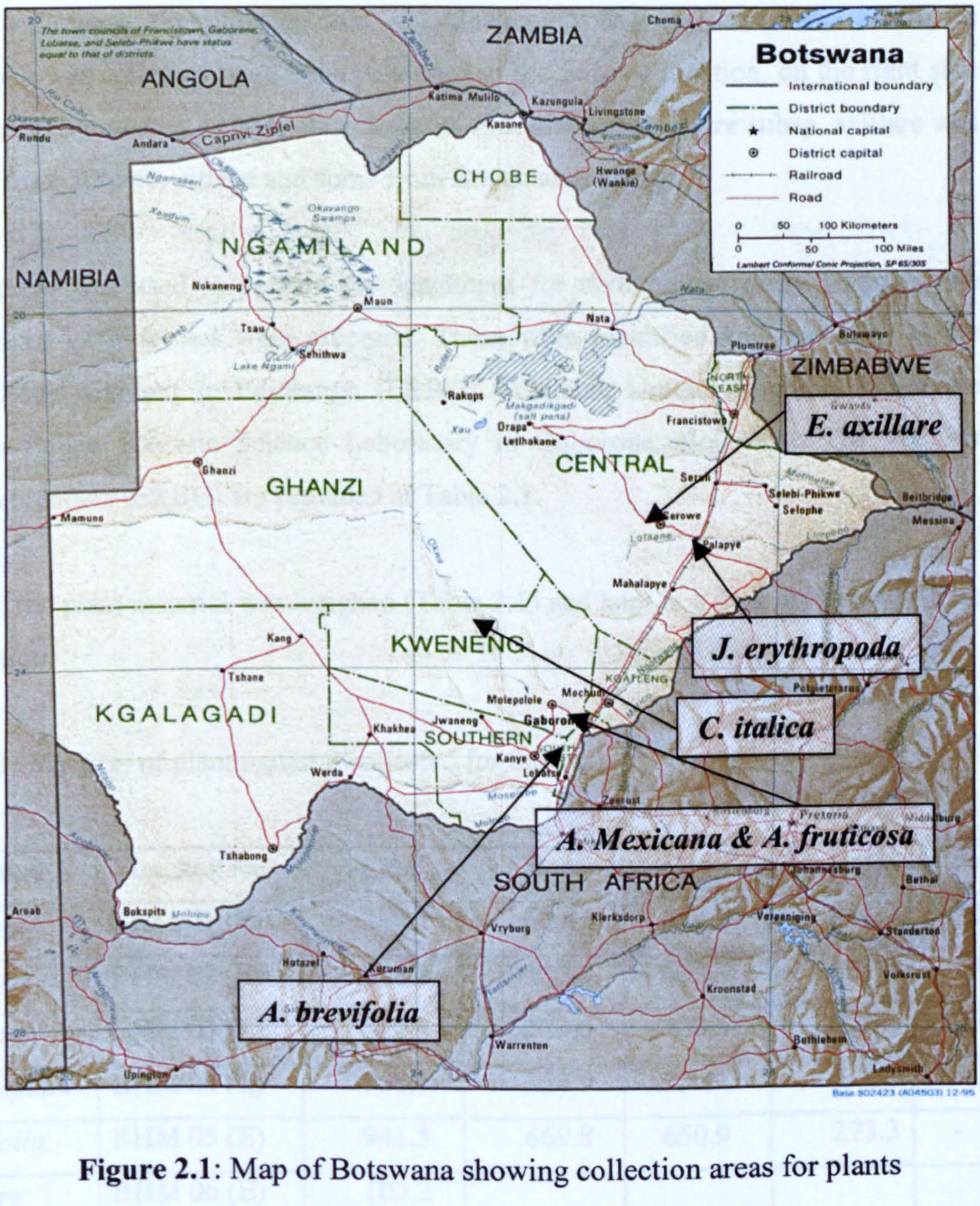


Figure 2.1: Map of Botswana showing collection areas for plants

Albizzia brevifolia was collected from about 4 km west of Otse village in the South East District near Balete farm. *Argemone mexicana* L. was collected from Mogoditsane fields (West of Mogoditshane village), in a once cultivated field consisting of loam soil. *Asclepias fruticosa* L. was collected from the same general location as *A. mexicana.* *Cassia italica* (Mil) Lam. ex was collected from Letlhakeng area (Eastern side of Serinare settlement along Molepolole – Letlhakeng road), on sandy soil. *Jatropha erythropoda* was collected from Palapye village at the Serowe junction, on the right side of the road to Serowe on a sandy area. Some of *Enicostemma axillare* subsp. axillare was collected from Serowe, village and some from Boataname village.

A plant press was used to prepare the specimens for taxonomical identification. Two specimens of each species were prepared. These were identified and deposited at the Royal Botanic Gardens in Edinburgh (ERBG), Scotland, United Kingdom and at the Botswana Police Forensic Science Laboratory in Gaborone, Botswana. The voucher specimen numbers (ERBG) are provided at Table 2.1.

Once dry, the plant material was weighed (Table 2.1) and kept in a dark dry place prior to investigation.

Table 2.1: Mass (g) of plant material collected for the study

Plant Name	Voucher No.	Leaf	Root	Stem	Fruit	Flower
A. mexicana	BHM 01 (E)	384.5	60.4	167.3	144.3	7.5
A. fruticosa	BHM 02 (E)	608.2	180.4	663.3	107.2	19.5
C. italica	BHM 03 (E)	882.8	289.6	308.5	42.7	26.0
J. erythropoda	BHM 04 (E)	308.7	320.9	33.4		
A. brevifolia	BHM 05 (E)	941.5	669.8	650.9	273.3	-
E. axillare	BHM 06 (E)	103.2				

2.2 Phytochemical Experimental work

2.2.1 Extraction

The plant material was powdered, either separately (i.e. leaf, root etc.) or as a whole (*E. axillare).*

Testing Thermostability of the compounds

Before subjecting the plant material to Soxhlet extraction, it was necessary to establish that the compounds of interest were heat stable. To do this, 0.5g of the powdered plant material was refluxed in 45ml of methanol for 24 hours. The extract was filtered and was concentrated under reduced pressure then evaluated by TLC against a cold methanol extract. Extracts were found to be heat stable.

Extraction

Sequential extraction was carried out in a Soxhlet apparatus using solvents of increasing polarity, starting with petroleum ether (60°C-80°C) or cyclohexane, then dichloromethane or ethyl acetate and ending with methanol. Each extraction stage was carried out to exhaustion. Cold extraction of each of the plant species was carried out in water and 70% ethanol for 24 hours. The extracts were filtered and the solvent removed under reduced pressure in a Rotary Evaporator. Extracts were stored in at -20°C prior to analysis. Each sample was re-dissolved in an appropriate solvent prior to analysis.

2.2.2 Fractionation and Isolation of compounds

2.2.2.1 Thin Layer Chromatography

TLC was used for screening the plant material for the presence of secondary metabolites, determining the eluting system for column chromatography and monitoring fractions during column chromatography. Pre-coated silica gel 60 PF_{254} aluminium backed plates (Merck) were used for this purpose. Different solvent systems were used to develop chromatograms. In general for petroleum ether and dichloromethane extracts combinations of petroleum ether and chloroform/dichloromethane/ethyl acetate were found to be suitable and combinations of chloroform/ethyl acetate for methanol extracts.

Plates were examined under UV light (254nm or 366nm) and where appropriate sprayed with vanillin-sulfuric acid reagent for non-alkaloid compounds or Dragendorff reagent for alkaloids as required. The vanillin-sulfuric acid reagent was prepared by adding 0.5mg vanillin to 50ml concentrated H_2SO_4 (95-99%). Dragendorff reagent was prepared according to the method in Wagner and Bladt (1984) from 2 solutions. The first comprised 0.17g bismuth subnitrate and 2g tartaric acid in 8ml of water. The second solution comprised 1.6g potassium iodide in 4ml water. The two solutions were mixed to make the stock solution. To spray, 0.5ml of the stock solution was mixed with 1g tartaric acid in 5ml water. The reagent was stored in a refrigerator to extend its shelf life. It's sensitivity was tested by spotting onto a TLC plate fixed volumes (10 µl) of different concentrations of codeine starting with 0.1 mg/ml down to 0.016 mg/ml and the minimum detection limit was found to be 310ng. Alkaloids were detected as dark spots (quenching), under UV light (254), and as orange spots against a yellow background after treating with Dragendorff reagent.

Other compounds were detected either as quenching or florescent spots under UV light and various colours were obtained after spraying with Vanillin-Sulfuric acid reagent and heating for 1-2 minutes with a hair dryer. For example, terpenoids, which are generally

not UV active showed as bright purple spots after reagent treatment and condensed tannin components showed as bright red to brown spots.

2.2.2.2 Preparative Thin Layer Chromatography

This technique was used where further purification of compounds was required. TLC plates of 0.5mm thickness were prepared by vigorously mixing 40g of silica gel (Silica gel 60 PF_{254} containing gypsum) with 80ml of distilled water i.e. a ratio of 1:2 and applying the paste as a thin coating to glass plates using a TLC applicator. The plates were allowed to air dry for a minimum of 30 minutes then activated in an oven at 76°C over night.

The sample was applied as a narrow streak 2.5cm from the bottom. The plate was allowed to dry and developed in a suitable system. Multiple developments were sometimes necessary to improve resolution. After drying the plate was visualised under UV light or sprayed with a suitable reagent where necessary and the compounds marked for collection by scraping with a spatula. The compounds were washed from the silica gel by elution through a cotton wool (washed) stoppered Pasteur pipette for small amounts or through a sintered glass funnel with petroleum ether, chloroform/ethyl acetate and methanol.

2.2.2.3 Gel Permeation Chromatography

Lipophilic Sephadex (LH-20 Sigma) was used to remove chlorophyll from the extracts and sometimes for preliminary fractionation. Fractionation is achieved due to differences in molecular size. Chlorophylls were effectively removed by this process. The Sephadex was introduced into the column as slurry tapping carefully to avoid air bubbles being trapped in the column. The sample was applied as a concentrated solution in the relevant solvent. The column was eluted with the system, with gradual increase of polarity where necessary. The column was finally washed down with methanol, dried and re-used.

2.2.2.4 Vacuum Liquid Chromatography

This technique was used as described by Pelletier *et al.*, (1986). A sintered glass Buchner filter funnel (generally, i.d. 7cm and column height 5cm) was used with an attachment to a water pump. Silica gel (Merck TLC grade) was loaded into the funnel with tapping to allow it to settle down evenly. Vacuum was applied and the silica gel was compressed to a hard layer with a glass stopper. The least polar solvent was allowed to run through the column to check whether the column was homogenously and tightly packed by ensuring that the solvent front moved down as an even horizontal band.

The sample was pre-adsorbed onto silica gel, dried, ground into a fine powder and loaded on top of the column as a tight layer. The column was eluted starting with the least polar solvent and gradually increasing the proportion of the more polar component by increments of 1-5%. The column was allowed to dry completely between fractions to improve resolution and separation.

2.2.2.5 Biotage Flash Chromatography

This was used to separate mixtures of 2-3 compounds. The technique employs gas pressure (N_2) to push down the eluent and thus separation can be achieved in a short period of time and with relatively small amounts of solvent used. The equipment, manufactured by Biotage, Inc. U.S.A. and U.K., consists of a 7.0 x 4.0 cm 40S Biotage column pre-packed with KP-Sil normal phase silica gel (Part No. FKO – 1107-17024, particle size: 32 –63 μm, 60 A, a stainless steel solvent reservoir and a sample inlet at the top of the column. The sample is introduced by a syringe to form a thin film on top of the column. The appropriate solvent system is poured into the reservoir, which is connected to the gas supply and then all parts are sealed airtight. Gas pressure is applied to push the solvent through the column at a flow rate of 10-25 ml/minute. The flow rate depends on the gas pressure and therefore can be regulated by adjusting the pressure at the cylinder. The drawback with the technique is that only small amounts of the sample can be

introduced to the column at a time and therefore the process has to be repeated several times for all the material to be separated.

2.2.2.6 Column Chromatography

The column was packed using the wet packing technique. Silica gel (particle size, 0.063-0.200 mm) was made into slurry using the least polar solvent of the eluting system. This was stirred until all the air bubbles were eliminated and introduced into a sintered glass column 1/3 full with the eluting system or the least polar solvent. Air bubbles were eliminated by tapping. Excess solvent was allowed to run through and the column allowed to settle. The sample was introduced to the top either as a concentrated solution or if not soluble in the least polar solvent (or eluting system) as a paste after pre-adsorbing onto silica gel. Elution was carried out either isocratically or as a gradient.

2.2.2.7 Analytical HPLC

This was used to develop a solvent system for LC-MS analysis of extracts and to obtain profiles of the extracts. The instrument used was equipped with a photodiode array detector and therefore UV spectra of the constituents were obtained during the separation. The instrument was a Thermo Separation Products AS 3000 HPLC. The column was a 150 x 4.60 mm C_{18} Sphereclone 5μ ODS (2) column. A gradient of acetonitrile and water was used for the separation, starting at 20% acetonitrile and ramping it to 100% in 25 minutes. The wavelength scan was from 200 to 360 nm.

2.2.2.8 Semi-preparative HPLC

This was employed to achieve further fractionation and purification of fractions obtained from either column chromatography or VLC. The column used was Hypersil Silica gel HS C-8 column (250mm x 10mm). Mixtures of methanol/water or acetonitrile/water sometimes with small (%) amounts of acetic acid were used as eluting systems at different flow rates. Samples were prepared in 50:50 organic: water as 2mg/ml solutions

and introduced into the column through a 2ml sample loop. Multiple injections were carried out and fractions were collected directly after passing through the detector. The computer was used to operate the HPLC and to monitor chromatograms. Separation was carried out using Gilson model 305 and 302 pumps and a Shimadzu SPD-6AV UV detector.

2.2.3 Identification of the compounds

2.2.3.1 Melting Points

A Reichert hotstage melting point apparatus was used for this purpose. All the melting points are uncorrected.

2.2.3.2 Optical Rotation

Specific rotations $[\alpha]_D$, were measured at the sodium-D line on Perkin Elmer 241 and 341 Polarimeters.

2.2.3.3 Ultraviolet-visible Spectroscopy

Spectra were obtained using a UNISCAN UV 300 spectrophotometer with Vision 3.2 software. Measurements were made between 200 to 400 nm.

2.2.3.4 Fourier Transform Infrared Spectrometry

FTIR spectra of the compounds were obtained using an ATI Mattson Genesis Series FTIR 1 with Winfirst Software. The number of scans was set at 16, the resolution at 4 and the signal gain at 1. Transmittance values were obtained from 4000 cm^{-1} to 500 cm^{-1}. To ascertain that the instrument was working properly, a spectrum of polystyrene film was obtained before analysing the samples. The samples were dissolved in a few drops of spectrophotomety grade chloroform and smaller drops of the solution

were deposited on freshly made potassium bromide (KBr) disk. This was repeated until enough sample was transferred onto the disk. An IR spectrum was obtained. Blanks were obtained from a freshly made KBr disk of the same dimension as the sample disk. Where this method did not work, the sample was mixed with KBr, ground to a fine powder, a disk made and a spectrum obtained. The compounds were recovered by grinding the disk to a powder, transferring to a Pasteur pipette plugged with glass wool and eluting with methanol. The methanol was evaporated off using nitrogen gas.

2.2.3.5 Mass Spectroscopy

High (sometimes low for low purity samples) resolution electron impact mass spectra were obtained from a JEOL 505HA spectrometer using direct probe at elevated temperature (110 - 160°C) at 70 eV.

2.2.3.6 Nuclear Magnetic Resonance Spectrometry

NMR spectroscopy is a valuable tool in natural products research and in this project it was used extensively to elucidate the structures of the isolated compounds and in some instances to evaluate extracts or mixtures for the present groups of secondary metabolites. One-dimensional (1-D) and two-dimensional (2-D) ^{1}H and ^{13}C NMR experiments were carried out on a JEOL-270 (270MHz) and a Bruker AMX-400 (400MHz) NMR spectrometers. The tubes used were Willmad 50 NMR tubes. The samples were dissolved in deuterated pyridine, chloroform or methanol depending on their solubilities. The solvents were considered as internal standards and their peaks were used for calibration.

2.2.3.6.1 ^{1}H NMR

This was always the initial experiment to be carried out. It provided information as to the nature of protons present in the molecule, their chemical shifts, multiplicities (coupling information) and estimated numbers from the integration. The information obtained was

used for initial evaluation of extracts to determine the groups of secondary metabolites present. In some instances the spectra obtained were used to examine mixtures of compounds (e.g. AHPE05 and AHPE06, p.111), at times to assess the purity of the compounds isolated and most importantly for structure elucidation of the isolated compounds.

2.2.3.6.2 ^{13}C NMR

This experiment provided information as to the number of carbon atoms present and type of carbon atoms present in a molecule. The spectra obtained were either Broad Band Decoupled or J-modulated. In Broad Band Doupled spectra the ^{1}H nuclei are irradiated during the ^{13}C acquisition so that all protons are fully decoupled from the ^{13}C nuclei. When this is done each distinct ^{13}C environment in the molecule gives rise to a separate single signal.

The J-modulated experiment, which is also known as the Attached Proton Test (APT) helps distinguish between different types of carbon atoms according to their proton attachments (C, CH, CH_2 and CH_3). In this experiment a 90° pulse is applied to the ^{13}C nuclei, the during the subsequent delay interval after the pulse, the magnetisation vectors of the ^{13}C nuclei of C, CH, CH_2 and CH_3 do not rotate at the same time but rotate at different angular velocities. The value of the delay time is kept at 1/J seconds which results in quaternary and CH_2 carbon atoms giving positive signals and CH and CH_3 giving rise to signals with negative amplitude. The decoupler is off during 1/J delay period to allow J-splitting and therefore provide coupling information. The decoupler is switched on at the beginning of the second delay interval. The obvious advantage of this experiment over the Broad Band Decoupled is that with this technique it is possible, in one experiment, to distinguish between C/CH_2 carbons and CH/CH_3 carbons. The other advantage is that it simplifies crowded regions of spectra, a useful attribute when dealing with secondary metabolites with crowded aliphatic regions such as terpenoids. However the experiment suffers from reduced sensitivity as compared to Broad Band Decoupled

spectra especially for quaternary carbons, which in some instances are not seen. An example of a J-modulated spectrum is Figure 3.21 on page 106.

2.2.3.6.3 Distortionless Enhancement by Polarization Transfer (DEPT) ^{13}C spectra

This experiment is a variation of the J-modulated experiment. It was sometimes necessary to carry out this experiment to distinguish CH_2 and quaternary carbon signals. Quaternary carbons are absent in the spectra obtained (e.g. Figure 3.52, p. 164). The experiment is more sensitive than the J-modulated carbon spectra and would therefore be useful when small amounts of material are being investigated. It also helps simplify crowded regions of spectra.

2.2.3.6.4 ^{1}H-^{1}H COSY (Correlation SpectroscopY)

This experiment gives information as to the proton-proton couplings in a molecule and with a suitable pulse sequence it is possible to reveal all the coupling relationships in one experiment. The proton shifts are plotted on both frequency axes and the correlations are displayed as cross peaks with the diagonal corresponding to the ordinary ^{1}H spectrum. COSY- 45, COSY90 (e.g. Figure 3.27) and COSY LR (Long Range COSY) were sometimes necessary to improve the information obtained. COSY-45 helps decrease the extension of the diagonal peaks and therefore reduces the problems associated with overlap. COSY LR (e.g. Figure 3.40, p 146) helps reveal couplings that may be more than 3 bonds away.

2.2.3.6.5 HMBC (Heteronuclear Multiple Bond Connectivity experiment)

The spectra obtained from this experiment give information on heteronuclear shift correlations via long-range couplings ($^{2}J_{CH}$ and $^{3}J_{CH}$). One bond, $^{1}J_{CH}$, direct correlation and $^{4}J_{CH}$ correlations are normally not seen in this experiment though possible. The proton spectrum is displayed on one axis and the carbon on the other. The correlations are shown as cross peaks (e.g. Figure 3.42, p.148).

2.2.3.6.6 HCCOBI (H-C Correlation using a BIRD pulse)

The spectra obtained reveal ^{1}J direct ^{1}H-^{13}C coupling. Correlations showing which proton(s) are connected to which carbons are displayed as cross peaks. One disadvantage of the experiment is that sometimes; direct correlations for methylene protons are weak or missing (e.g. Figure 3.41, p147).

2.2.3.6.7 Nuclear Overhauser Enhacement Spectroscopy (^{1}H-^{1}H NOESY)

In this experiment Nuclear Overhauser effect (NOE) is observed 2-dimensionally. The experiment measures "through space" interaction between nuclei which arises from dipolar coupling instead of scalar coupling through bonds, and is noticeable over short distances. In a NOESY experiment, nuclei are excited at the same time and therefore, the NOE between all protons can be observed simultaneously. The experiment is useful in determining the structure and stereochemistry of organic compounds. Spectra have the ^{1}H NMR spectrum on both of the frequency axes. The NOE interactions of spatially close protons in a molecule can be seen as cross peaks off a diagonal as in the COSY spectrum (e.g. Figure 3.43, 149).

2.2.3.6.8 Homonuclear Hartmann-Hann (HOHAHA) or Total Correlation Spectroscopy (TOCSY)

This experiment is useful in determining networks of mutually coupled protons (Nakanashi, 1990). The magnetisation of the first proton in the spin system is transferred to the next and so forth in a relay manner, until the network is blocked by a quaternary carbon or another atom; magnetisation can no longer be relayed to the protons on the side. Thus each network of protons can be detected by tracing the cross peaks from certain specific protons. The relay distance is dependent on the mixing time. The spectrum generated resembles a COSY spectrum. In this study, the TOCSY experiment was used to assist in the assignment of the disaccharide protons of the lignan glycoside AB Fn41-50 (Figure 3.43, p149).

2.3 Toxicological Investigation

In vitro acute toxicity was carried out on:

- The Soxhlet extracts of the six plants species
- Cold water and 70% ethanol extracts of the plants
- 70% cold ethanol extracts of actual case samples submitted to the BPFSL for analysis
- Isolated pure compounds

These samples were tested against a panel of four cell lines, namely:

- SCL 4° -a stomach cancer cell line
- NCI H460 – a lung cancer cell line
- C8161 – a melanoma cell line
- J774- a murine macrophage cell line

Cell lines NCI H460 and C8161 were obtained from Karen Fraser and SCL 4°and J774 were from Professor Stimson both from the Department of Immunology, University of Strathclyde.

The method employed was that described by Carter *et. al.*, (2000) with a few modifications. Cells were cultured in RPMI-1640, supplemented with heat inactivated 10% foetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine. All the cells were maintained in an incubator at 37°C in humidified air containing 5% CO_2. All work was carried out asceptically.

When ready, cells were seeded at a concentration of 1-2 x 10^5 cells/ well of a 96 well tissue culture plate. Each concentration was replicated 6 times (n=6). Trypan blue dye was used to determine the percentage of life cells before treatment and this was always 100%.

The cells were treated with:

- Two-fold serial dilutions of extracts or pure compounds
- Medium alone (control)
- Solvent alone (control)

Methanol was the preferred solvent but where not suitable, dimethyl sulfoxide(DMSO) was used instead. Samples were dissolved in 0.2ml of the solvent and then made up to 1ml with medium. This ensured that the solvent concentration was always minimal (1%) so that it had no adverse effects on the cells. The sample solution was filter sterilized before being added to the cells. 100µl of cells in medium was pipetted into each well, 90µl of medium was added and the 10µl of sample added accordingly. This was mixed gently (to avoid mechanical cell damage) by shaking and incubated for 24 hours.

After 24 hours, the plates were viewed under the microscope to record visual observations and check for contamination. The medium was then removed gently and then 150µl of fresh medium and 50µl of sterile solution MTT (1mg/ml medium) added. The plates were incubated for 3-4 hours; the well contents were carefully removed. At this stage blue formazan crystals were visible at the bottom of wells, which had contained live cells. 100µl of DMSO was added to dissolve any crystals present. The plate was shaken on a shaker for 5 minutes and absorbance determined.

The percent suppression in cell viability for the test sample was determined by comparing the absorbance of the test sample with the mean control value. The mean suppression and standard errors are shown on the dose-response graphs (Figures 3.1-3.19). These graphs were used to determine the $LD_{50,}$ which is the concentration at which 50% cell death (suppression) occurred compared with solvent control. Since only 10µl of sample was pippeted in each well of a total volume of 200 µl, the actual concentration of the test sample was further diluted 20X. Therefore the dose-response curves presented are of the actual drug concentration.

The Student t-test was used to determine whether there was any significant difference between treatments and controls and also between two cell lines. To test whether there was any cell specificity among the four cell lines, the one-way ANOVA test was applied.

2.4 DNA work

DNA extraction was carried out using MicroLysis™ 20 X (Microzone, Lewes, UK). This is a proprietary solution from Microzone Ltd., Lewes, U.K., which is able to extract, among other things, chloroplast DNA from plant cells in one step.

Reddy Mix, a PCR master-mix, from AB Gene Ltd. (Epsom, UK) was used for amplification of chloroplast DNA. This product contains the enzyme (*Taq* polymarase for the addition of the dNTPs to the primer), buffer (containing Mg^{2+} ions essential for the activation of the enzyme), dNTPs (deoxynucleoside triphosphates for the four DNA bases) and other essential reagents required to support successful DNA amplification.

Microlysis and ReddyMix PCR Master-Mix were shown to successfully extract and amplify DNA from *Cannabis sativa* (Harper *et al.*, 2000). The two reagents simplify sample preparation and thus reducing the chance for operation error and contamination. The primers used are listed at Table 1.7. Amplification was carried out on a Perkin Elmer 2400 Thermal Cycler.

Separation was achieved by Capillary Gel Electrophoresis on a PRISM 310 Genetic Analyser (Applied Biosystems, CA, USA) with a computer attachment for data acquisition and processing. In this technique, the double stranded DNA is denatured to allow injection onto the column. Forward C primer and Forward F primer were labelled with the fluorescent dye, FAM (Table 1.7) and Forward ITS primer was labelled with fluorescent dye, JOE (Table 1.7). This was to allow detection of the DNA products. A DNA size standard labelled with a different dye, ROX, was also included.

Experimental procedure

Approximately 1mg of dried plant material was added to a sterile 0.2mL tube. To this tube 20µl of 1x microLysis was added. The tube was placed in a Geneamp 2400 PCR machine (Applied Biosystems, Ca, USA) and treated using the following cycling

conditions: 65°C 5 min, 96°C for 2 min, 65°C for 4 min, 96°C for 1 min, 65°C for 1 min, 25 °C hold until required.

Amplification of the chloroplast DNA region was performed in 25 µl containing 12.5µl 2x Reddy mix, 100 pmol forward primer, 100 pmol reverse primer, 1µl of microLysis DNA extract. Amplifications were performed using the following conditions: 95 °C 1 minute followed by 30 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 30s; followed by 60 °C for 30 min.

For separation on a PRISM 310 Genetic Analyser (Applied Biosystems, CA, USA) 1µl of the amplification mix was added to 19µl de-ionised formamide and 1µl Rox 500 size standard (Applied Biosystems, CA, USA). The samples were heated for 2 min at 65 °C, before placing on ice for 2 min and then placing in the CE machine.

The sizes of the amplification products was determined using the Local Southern computer programme and Genescan® (Applied Biosystems, CA, USA).

CHAPTER 3

RESULTS AND DISCUSSIONS

3.0 RESULTS AND DISCUSSION

3.1 Toxicity of Extracts

Extracts were evaluated for cytotoxicity properties as outlined at Section 2.3. The results are shown and discussed below. LD_{50} values of below 1mg/ml indicate expression of toxicity.

3.1.1 *Albizzia brevifolia*

The toxicity of this plant is unknown. Cytotoxicity tests were carried out on the dichloromethane, methanol and water extracts of this plant. Dose-response curves and LD_{50} values (Table 3.1) are shown below.

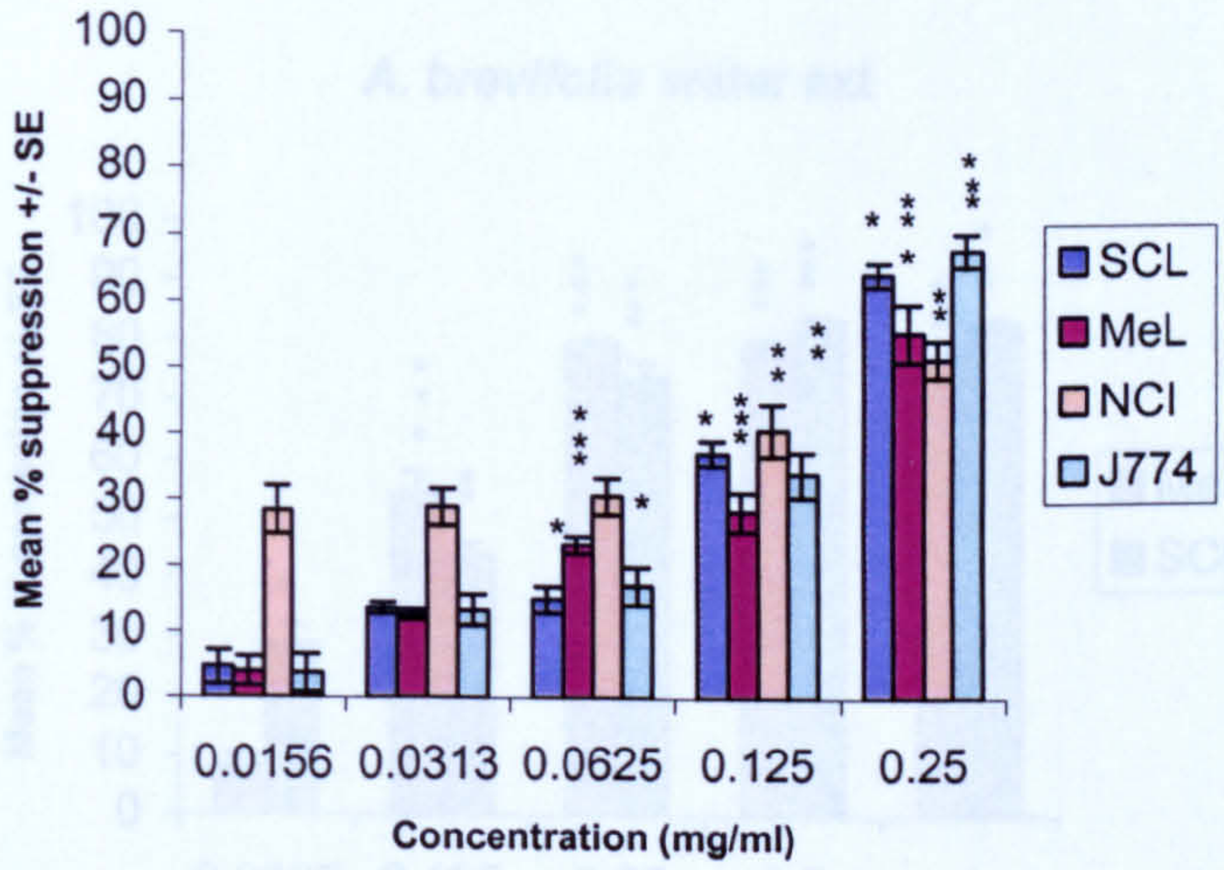


Figure 3.1: *A. brevifolia* DCM extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

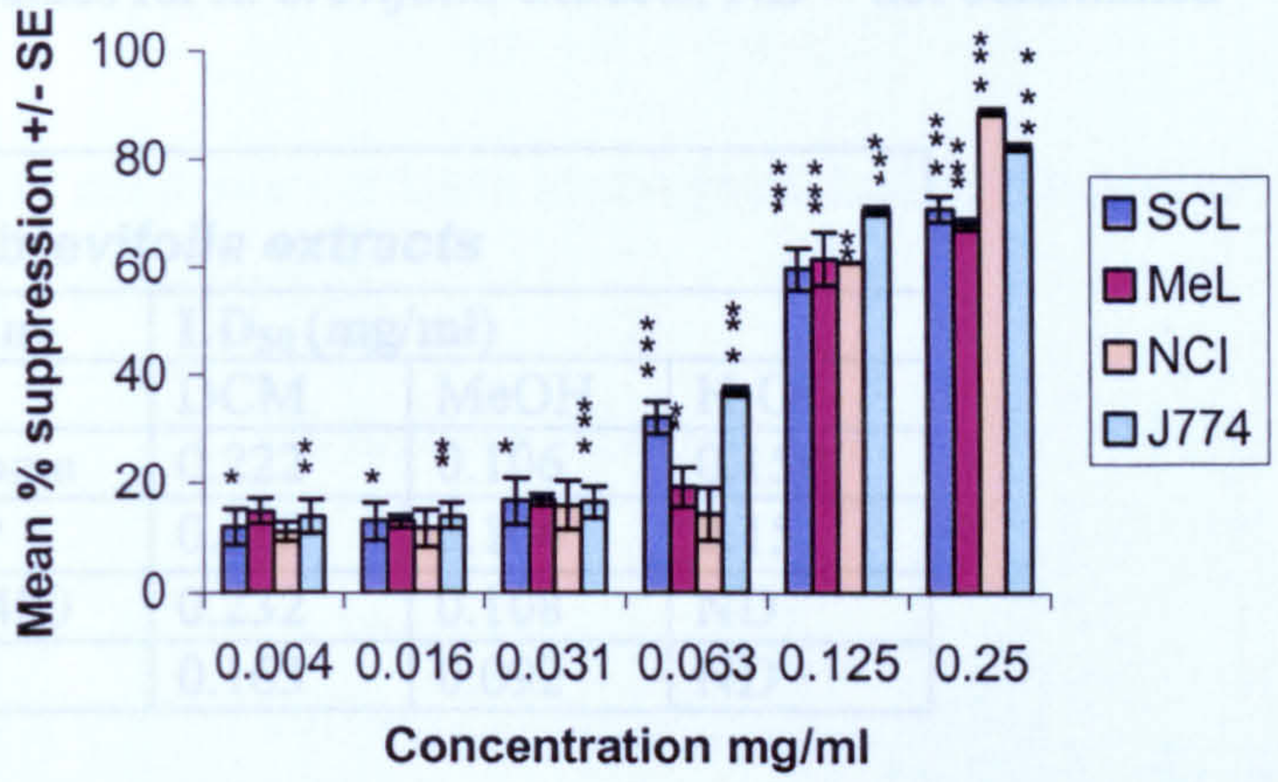


Figure 3.2: *A. brevifolia* MeOH extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs. solvent control

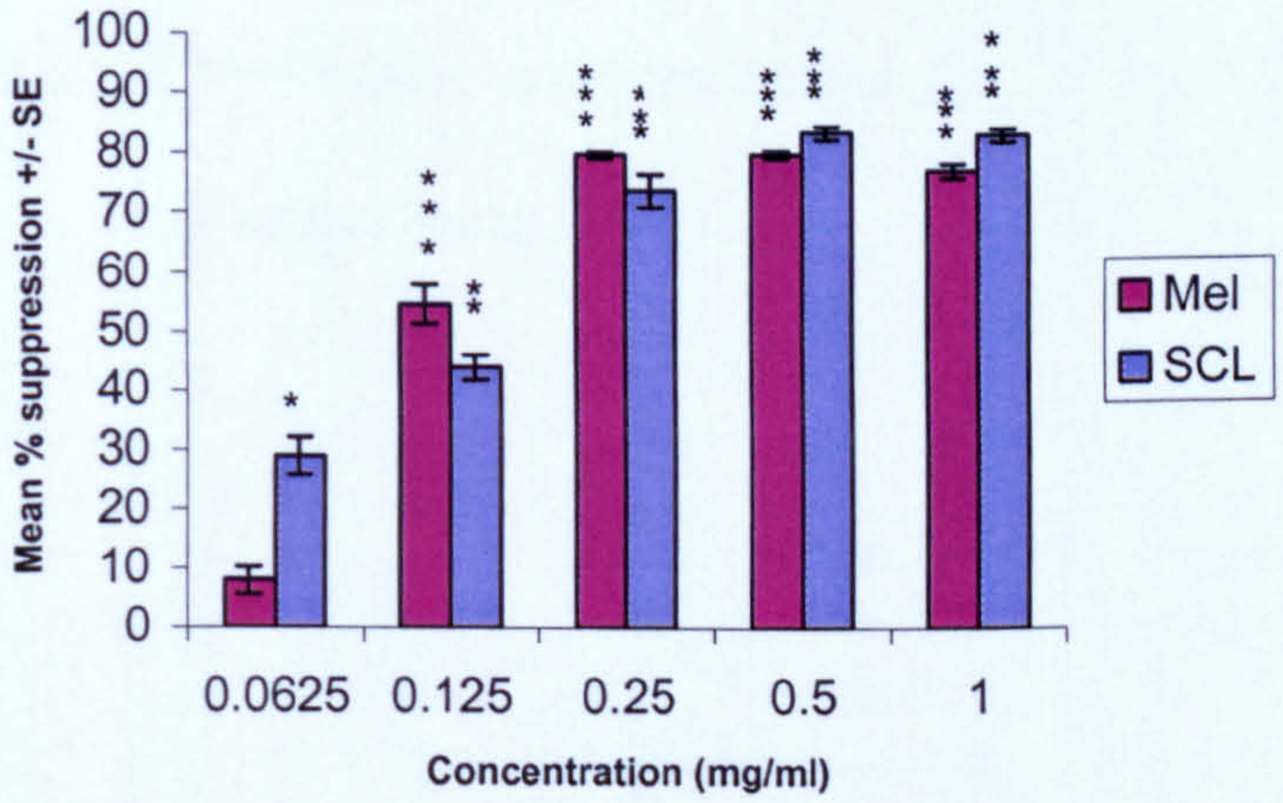


Figure 3.3: *A. brevifolia* water extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

Table 3.1: LD_{50} values for *A. brevifolia* extracts; ND = not determined

A. brevifolia extracts			
Cell Line	**LD_{50} (mg/ml)**		
	DCM	MeOH	H_2O
Melanoma	0.222	0.106	0.150
SCL 4°	0.189	0.104	0.151
NCI H480	0.232	0.108	ND
J774	0.163	0.092	ND

All the extracts from this plant were toxic to the cell lines. There was no significant difference in the response of the different cell lines to any of the extracts. Even though the NCI cell line appeared to be the most affected by the DCM extract and J774 to be the most affected by the methanol extract, the overall differences were found to be statistically nonsignificant. Overall, the MeOH extract was the most toxic followed by the aqueous extract.

3.1.2 *Enicostemma axillare*

The toxicity of this plant has not been investigated before. Dichloromethane, methanol and water extracts were evaluated.

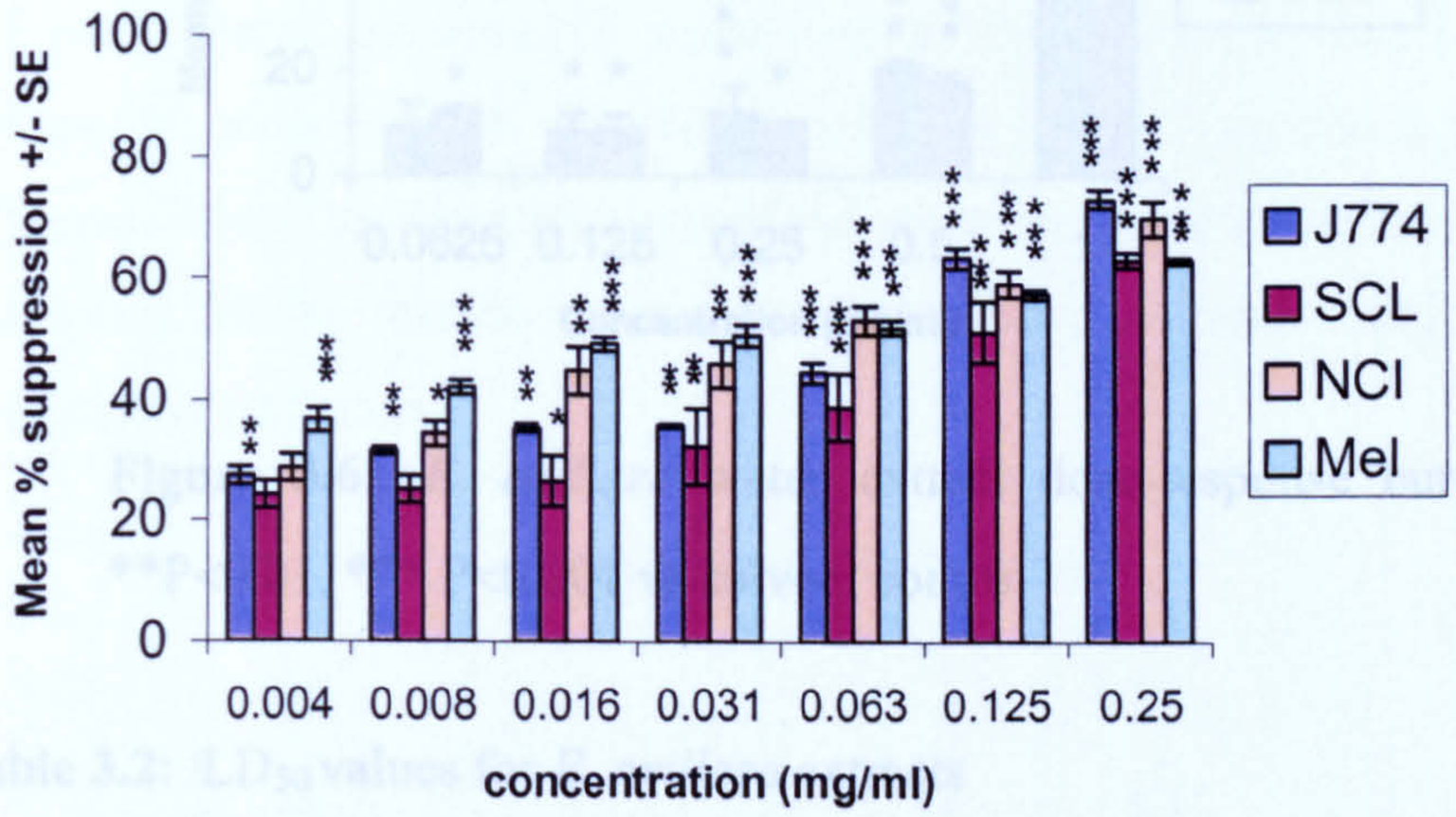


Figure 3.4: . *E. axillare* DCM extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

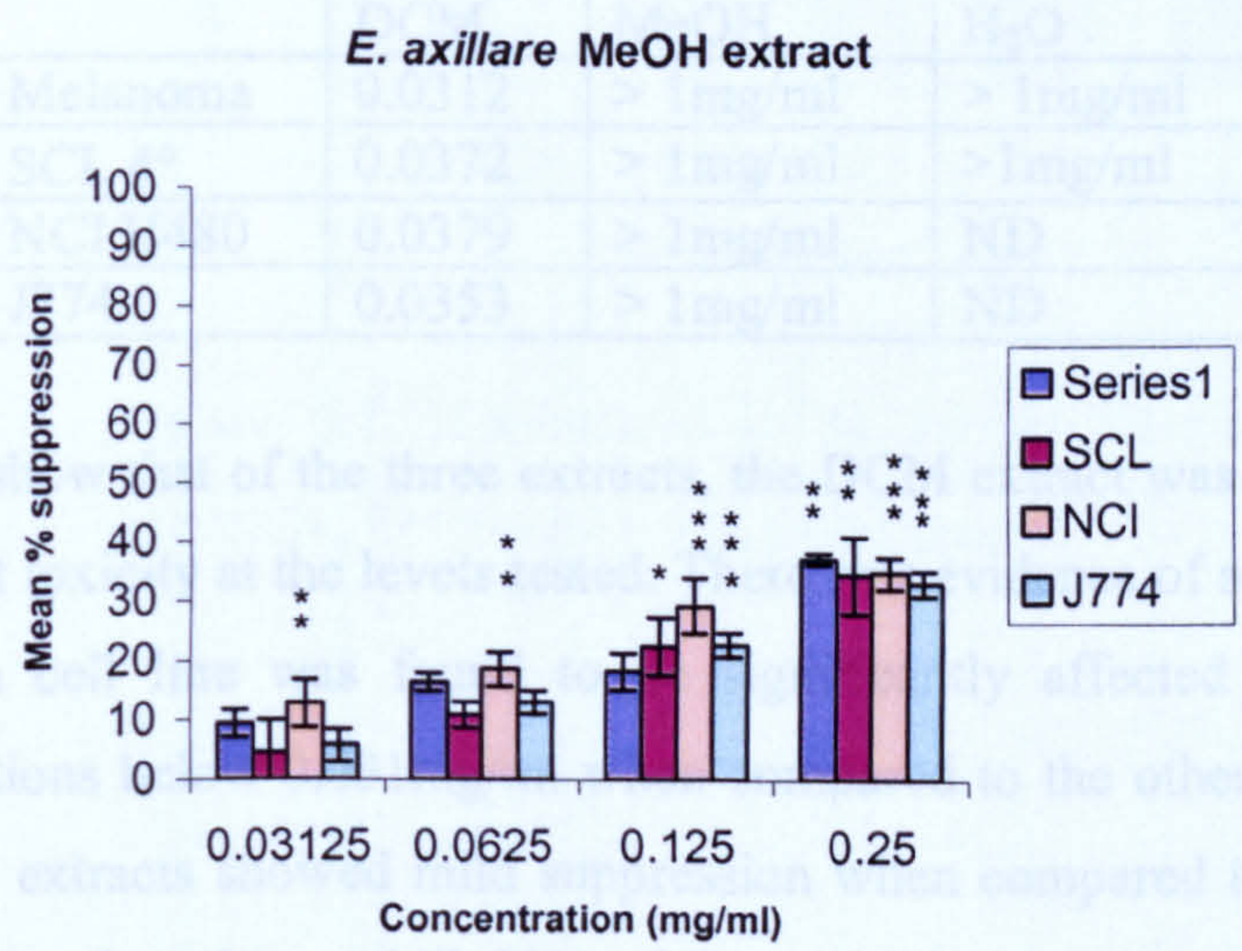


Figure 3.5: . *E. axillare* MeOH extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

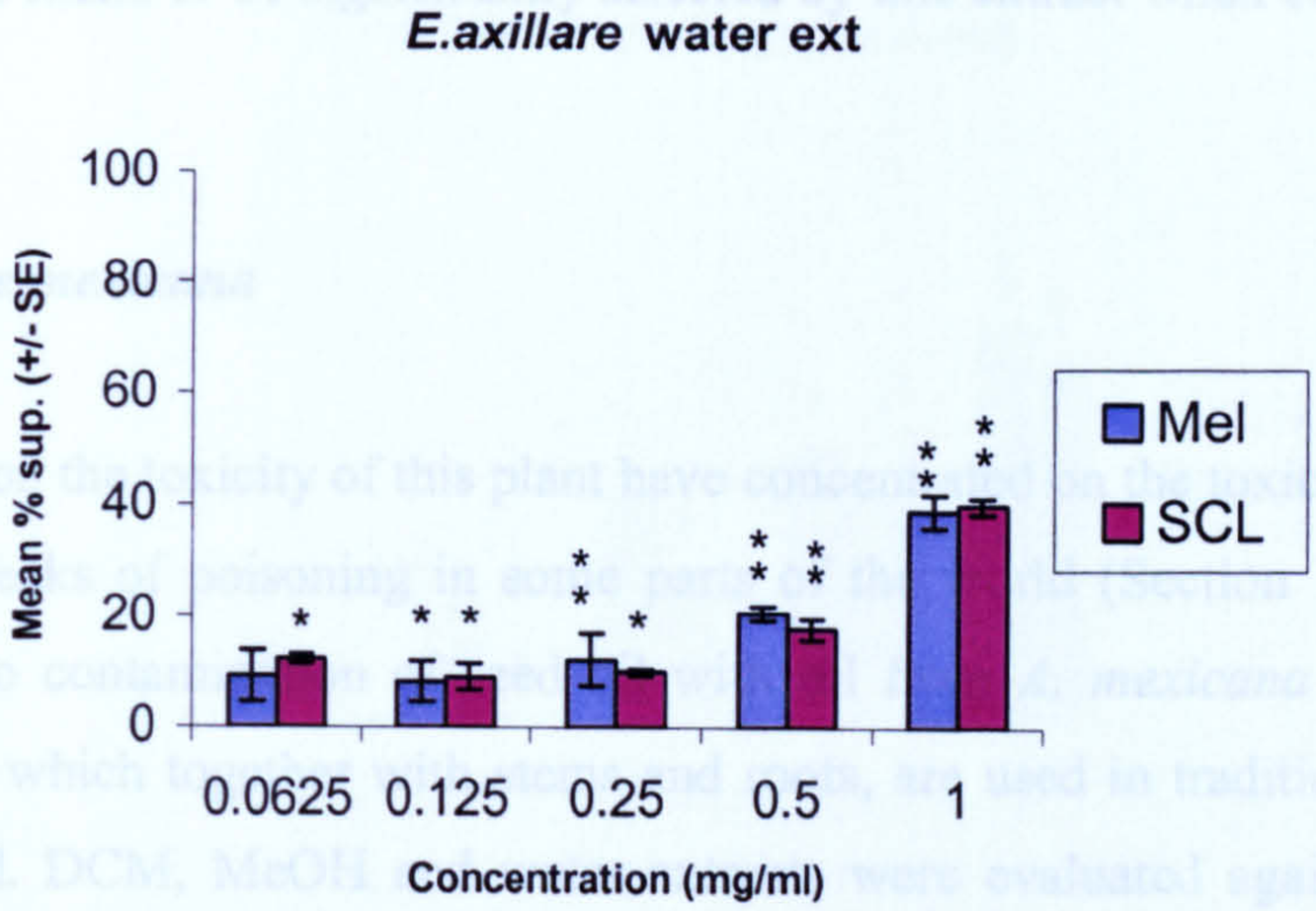


Figure 3.6:. *E. axillare* water extract dose-response curve; *P<0.05, **P<0.01, *** P<0.001 vs solvent control

Table 3.2: LD_{50} values for *E. axillare* extracts

E. axillare extracts			
Cell Line	**LD_{50} (mg/ml)**		
	DCM	MeOH	H_2O
Melanoma	0.0312	> 1mg/ml	> 1mg/ml
SCL 4°	0.0372	> 1mg/ml	>1mg/ml
NCI H480	0.0379	> 1mg/ml	ND
J774	0.0353	> 1mg/ml	ND

The data show that of the three extracts, the DCM extract was the only one that showed significant toxicity at the levels tested. There was evidence of some cell specificity as the melanoma cell line was found to be significantly affected by the DCM extract at concentrations below 0.031mg/ml when compared to the other cell lines. The methanol and water extracts showed mild suppression when compared to the controls (seen from the graph) and could not kill 50% of the cells at the concentrations tested. They both started showing significant toxicity to the cells around 0.125 mg/ml ($p<0.05$), except on NCI cell line on which toxicity of the methanol extract was significant from 0.078 mg/ml.

This cell line was found to be significantly affected by this extract when compared to the other cell lines.

3.1.3 *Argemone mexicana*

Previous studies on the toxicity of this plant have concentrated on the toxicity of its seeds because of outbreaks of poisoning in some parts of the world (Section 1.6.5.2) which were attributed to contamination of seed oil with oil from *A. mexicana* seeds. In this study the leaves, which together with stems and roots, are used in traditional medicine, were investigated. DCM, MeOH and water extracts were evaluated against melanoma and stomach cancer cell lines.

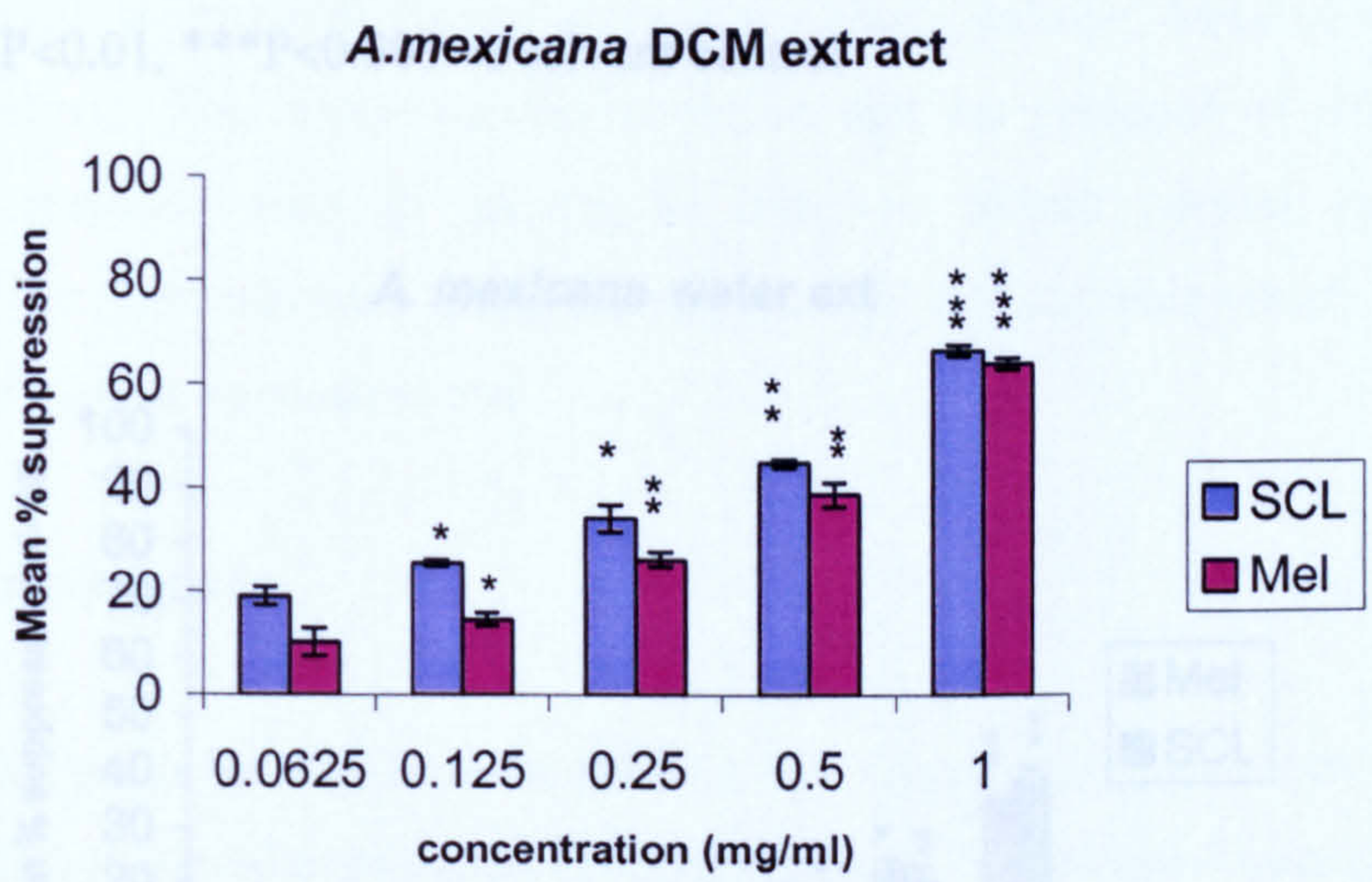


Figure 3.7: *A.mexicana* DCM extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

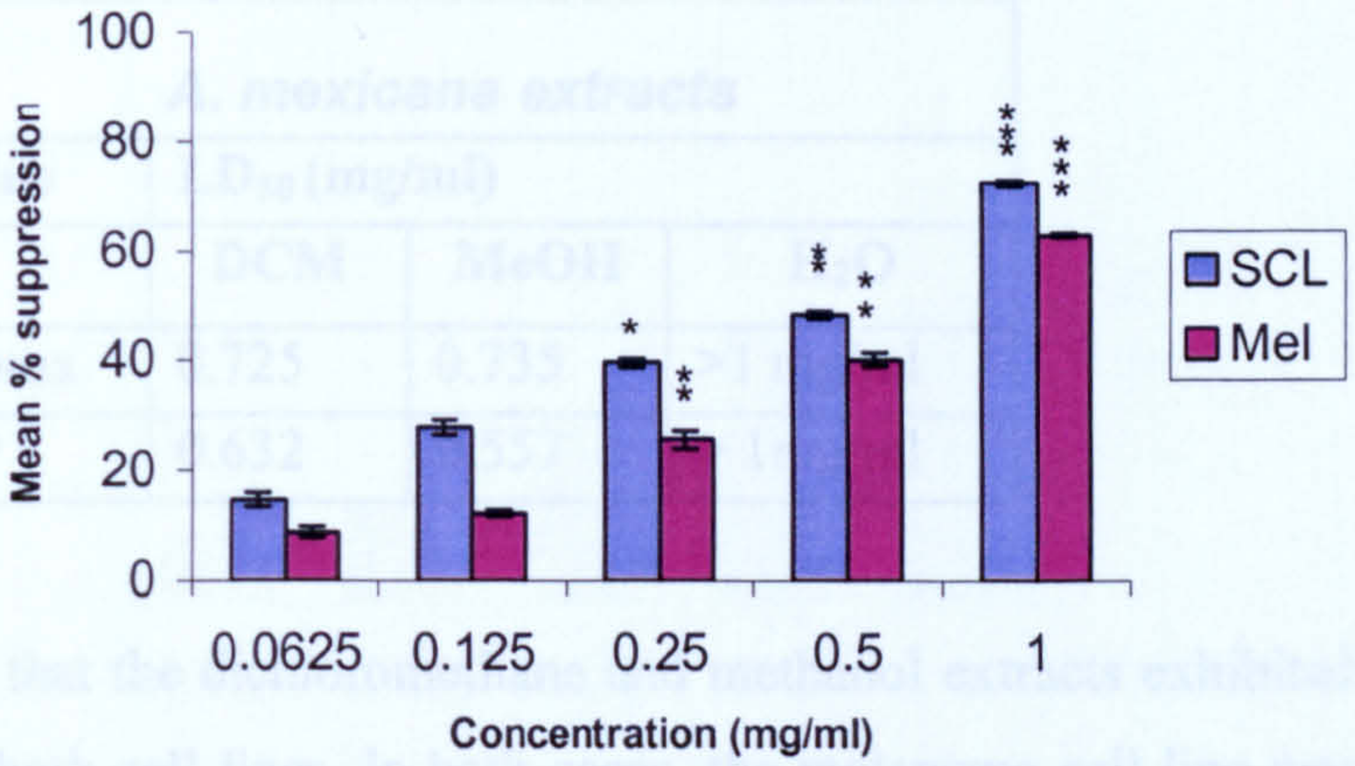


Figure 3.8: *A. mexicana* MeOH extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

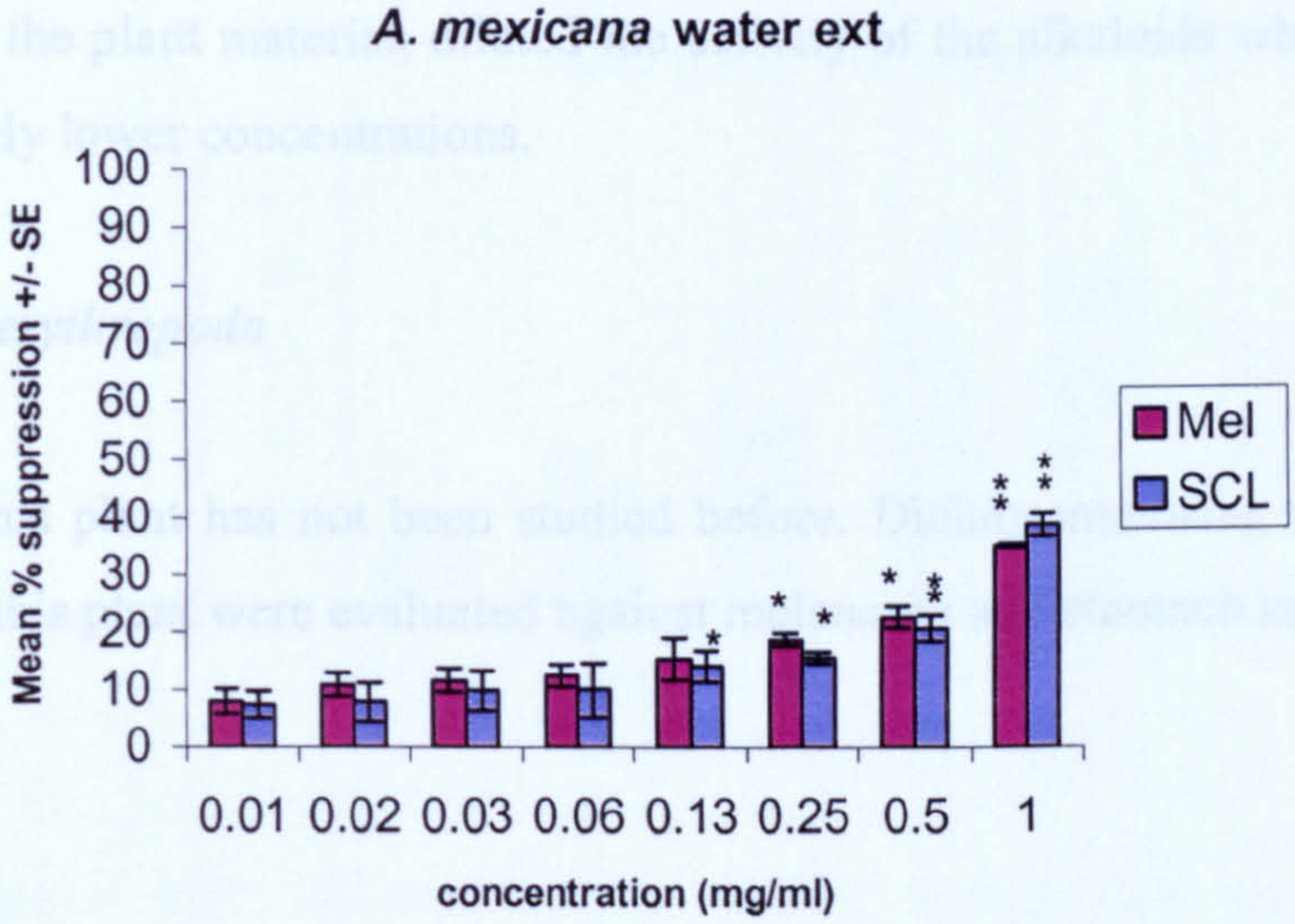


Figure 3.9: *A. mexicana* water extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

Table 3.3: LD_{50} values for *A. mexicana* extracts

A. mexicana extracts			
Cell Line	**LD_{50} (mg/ml)**		
	DCM	**MeOH**	**H_2O**
Melanoma	0.725	0.735	>1 mg/ml
SCL 4°	0.632	0.557	> 1mg/ml

The results show that the dichloromethane and methanol extracts exhibited more or less equal toxicity to both cell lines. In both cases, the melanoma cell line was significantly less affected than the stomach cancer line. The toxicity exhibited by the extracts is lower than would ordinarily be expected from a plant containing alkaloids, some of which are known to be cytotoxic. The reason for this could be that the presence of chlorophyll, which was not removed prior to testing, in order to mimic normal method of administration of the plant material, diluted the activity of the alkaloids which would be present in relatively lower concentrations.

3.1.4 *Jatropha erythropoda*

The toxicity of this plant has not been studied before. Dichloromethane, methanol and water extracts of this plant were evaluated against melanoma and stomach cancer lines.

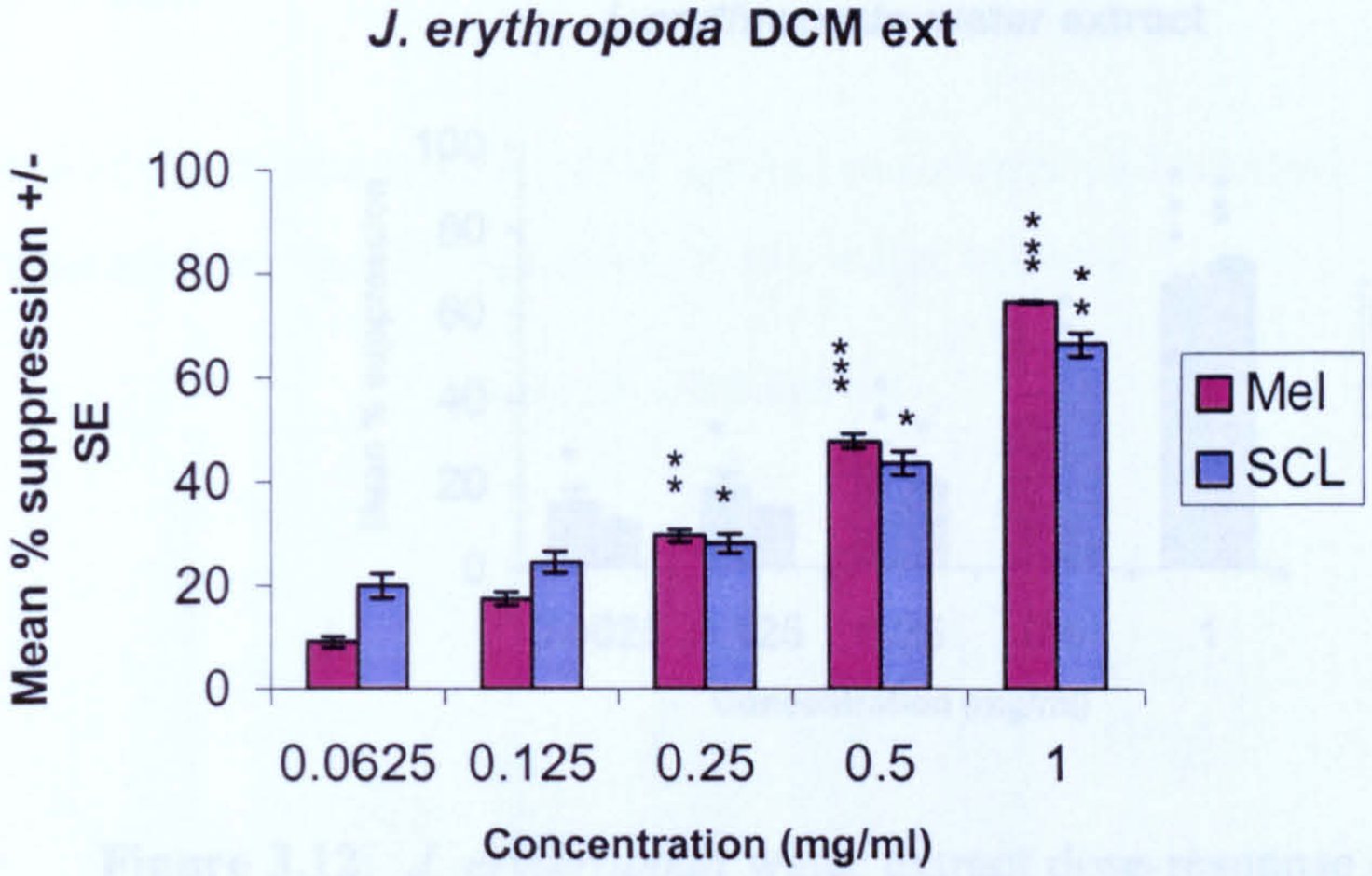


Figure 3.10: *J. erythropoda* DCM extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

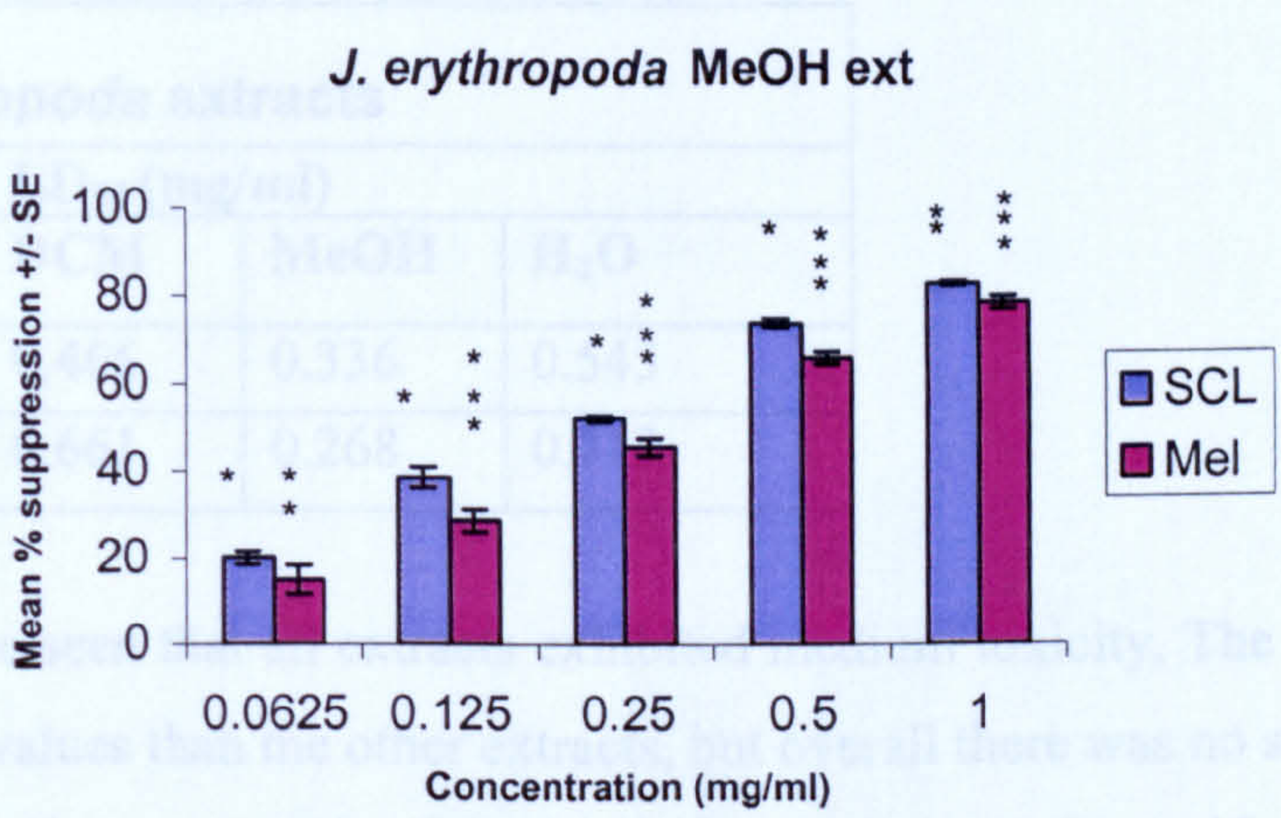


Figure 3.11: *J. erythropoda* MeOH extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

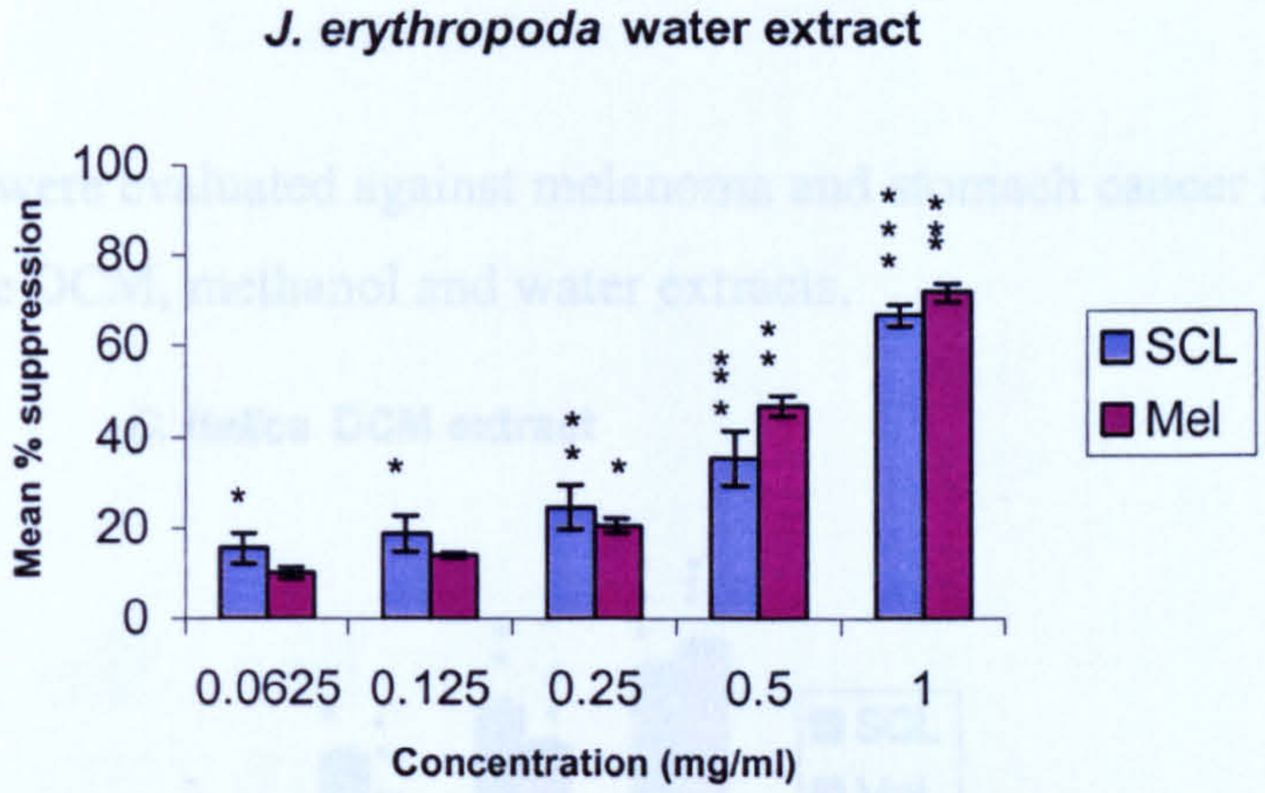


Figure 3.12: *J. erythropoda* water extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

Table 3.4: LD_{50} values for *J. erythropoda* extracts

***J. erythropoda* extracts**			
Cell Line	**LD_{50} (mg/ml)**		
	DCM	**MeOH**	**H_2O**
Melanoma	0.466	0.336	0.543
SCL 4°	0.661	0.268	0.713

From the results it can be seen that all extracts exhibited medium toxicity, The methanol extract had higher LD_{50} values than the other extracts, but overall there was no significant difference in the toxicity of the extracts to the two cell lines nor any cell specific effect.

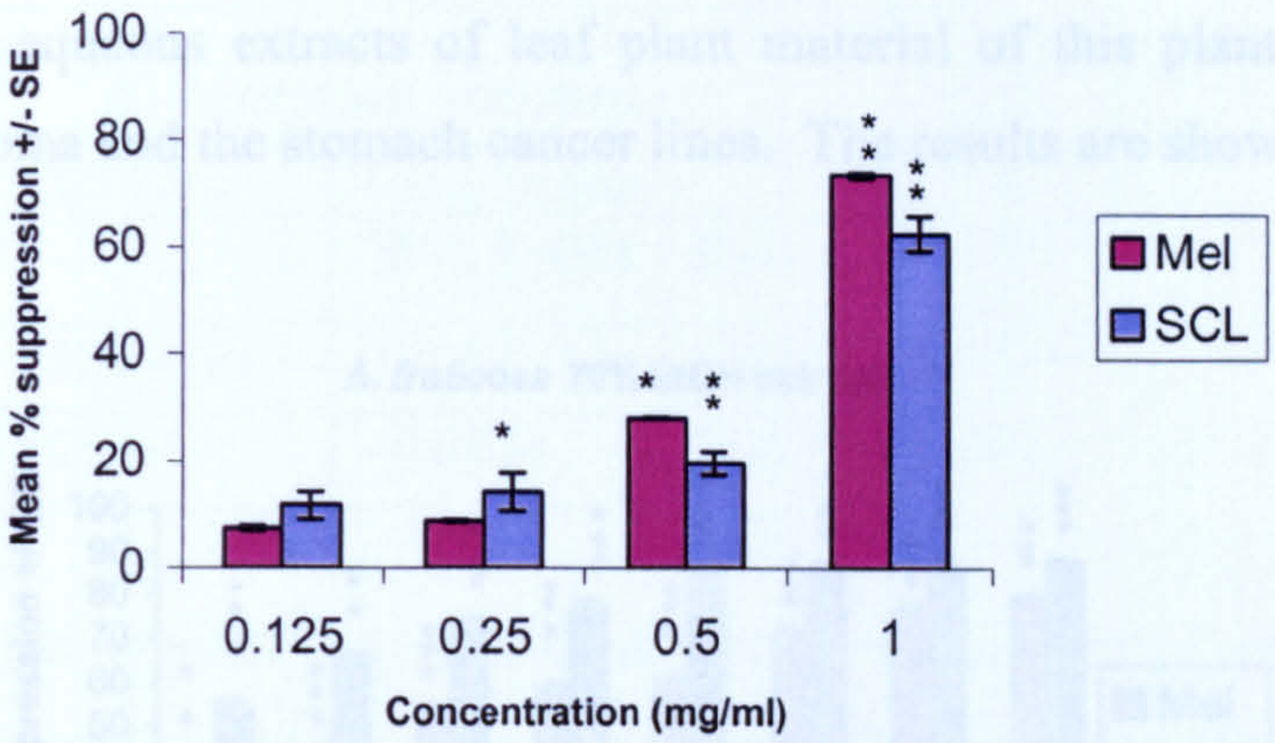


Figure 3.15: *C. italica* water extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

Table 3.5: LD_{50} values for *C. italica* extracts

***C. italica* extracts**			
Cell Line	**LD_{50} (mg/ml)**		
	DCM	**MeOH**	**H_2O**
Melanoma	0.481	1.085	0.734
SCL 4°	0.250	1.028	0.850

The results indicate that among the three extracts, the DCM extract was the most toxic, followed by the aqueous extract. The methanol extract showed the least toxicity; it was significantly less toxic than the DCM extract on the SCL cell line. There was no significant cell specific effect of the extracts on any of the cell lines.

3.1.6 *Asclepias fruticosa*

70% ethanol and aqueous extracts of leaf plant material of this plant were evaluated against the melanoma and the stomach cancer lines. The results are shown below.

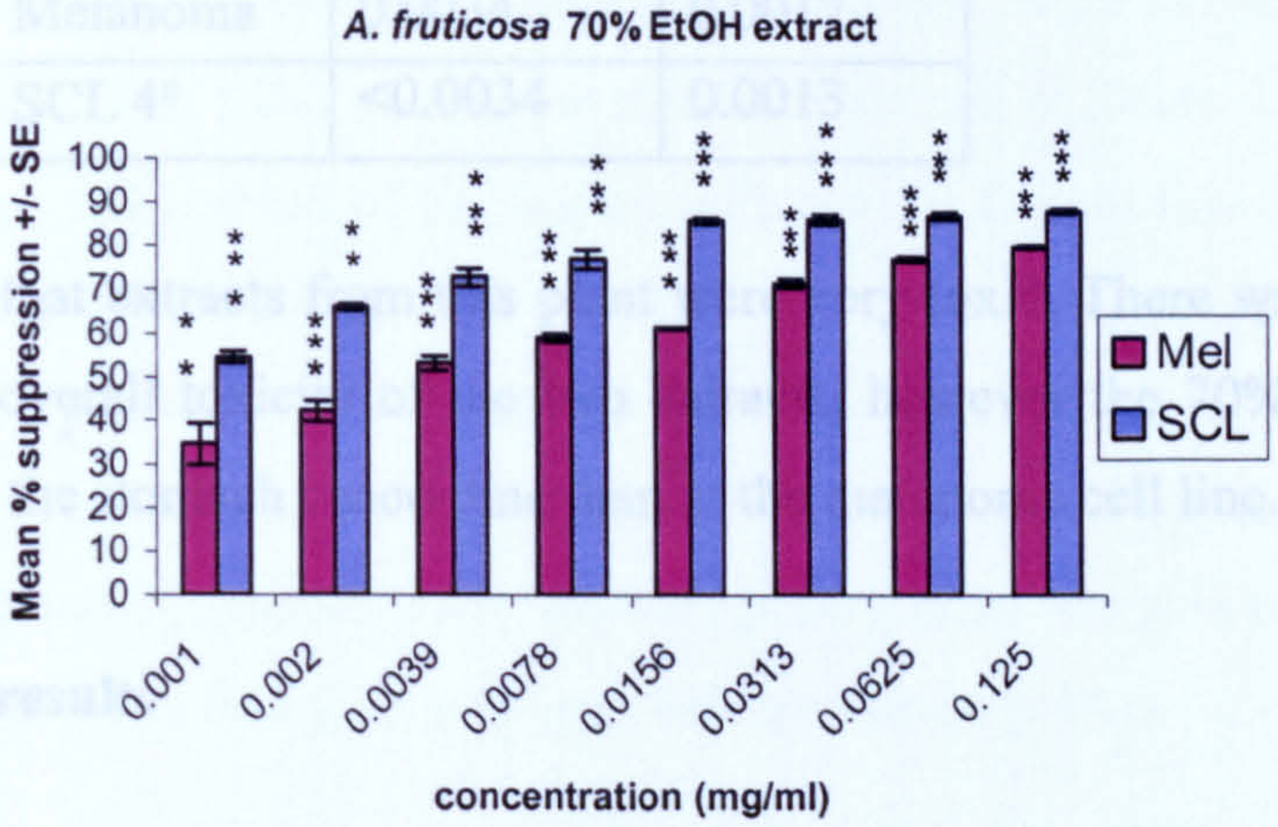


Figure 3.16: *A. fruticosa* 70% EtOH extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

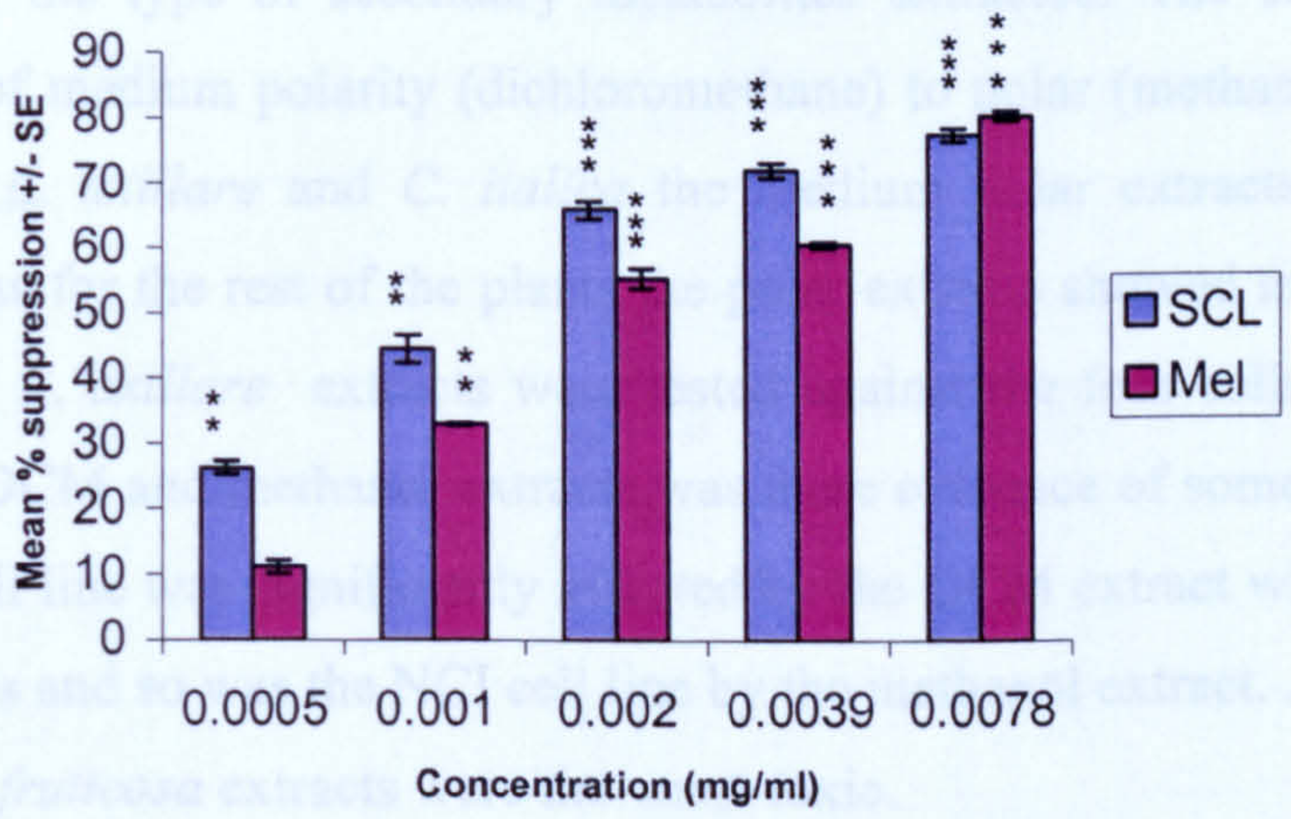


Figure 3.17: *A. fruticosa* water extract dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

Table 3.6: LD_{50} values for *A. fruticosa* extracts

***A. fruticosa* extracts**		
Cell Line	**LD_{50} (mg/ml)**	
	70% EtOH	**H_2O**
Melanoma	0.0034	0.0017
SCL 4°	<0.0034	0.0013

The results show that extracts from this plant were very toxic. There was no significant difference in the overall toxicity of the two extracts, however the 70% ethanol extract was more toxic to the stomach cancer line than to the melanoma cell line.

Summary of the results

In general, all the extracts expressed inhibitory effects on the mitochondrial activity of the cells, which is an indirect measurement of cell proliferation. Affected cells burst and were seen under the microscope floating in the medium. The effect was expressed to varying degrees by different extracts depending on which solvent was used for extraction and therefore on the type of secondary metabolites extracted. The solvents used for extraction were of medium polarity (dichloromethane) to polar (methanol, 70% ethanol and water). For *E. axillare* and *C. italica* the medium polar extracts were the most cytotoxic, whereas for the rest of the plants the polar extracts showed most cytotoxicity. *A. brevifolia* and *E. axillare* extracts were tested against the four cells lines, and only with *E. axillare* DCM and methanol extracts was there evidence of some cell specificity; the melanoma cell line was significantly affected by the DCM extract when compared to the other cell lines and so was the NCI cell line by the methanol extract. Among all of the plant extracts, *A. fruticosa* extracts were the most toxic.

3.2 Toxicity of case samples

These were actual herbal preparations from the Botswana Police Forensic Science Laboratory, which had been seized from victims (complainants) or accused herbal practitioners by police investigators as exhibits to be tested for toxicity. Twenty samples were tested against the melanoma and stomach cancer lines. Eight of these were found to be toxic to the cells. The LD_{50} values for these are shown at Table 3.7. Case number T93/98 (5) with an LD_{50} value of 24.6μg/ml, (Figure 3.18) was found to be the most toxic. This is clearly seen when its dose-response curve is compared with that of T35/00 (1) (Figure 3.19), which was also found to be toxic, but not as much. TLC examination of the extract indicated the presence of glycosides. The fact that these materials, which have actually been ingested by people, have shown toxicity is an indication that these toxic plant constituents actually find their way into vital processes *in vivo*.

Table 3.7: LD_{50} values for "Actual Case Samples"

Actual case samples LD50 (mg/ml)		
Case number	**Cell lines**	
	Melanoma	SCL 4°
T20/00	0.564	0.573
T24/00	0.200	0.231
T35/00 (1)	0.0648	0.0581
T35/00 (2)	0.226	0.202
T35/00 (3)	0.150	0.365
T35/00 (4)	0.110	0.130
T93/98 (1)	0.350	0.323
T93/98 (5)	0.0242	0.0246

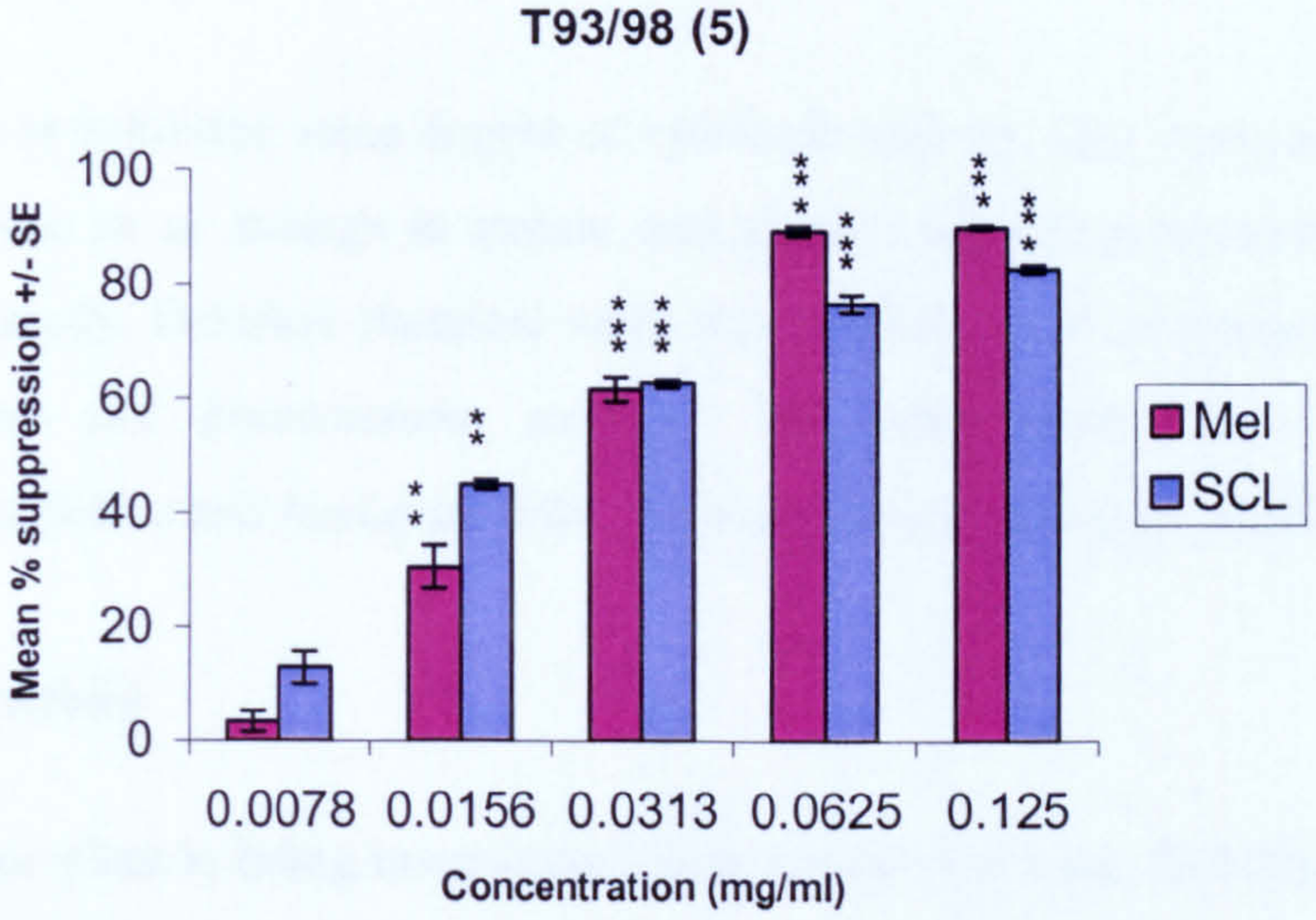


Figure 3.18: Case T93/98 (5) dose-response curve; *P<0.05, **P<0.01, ***P<0.001 vs solvent control

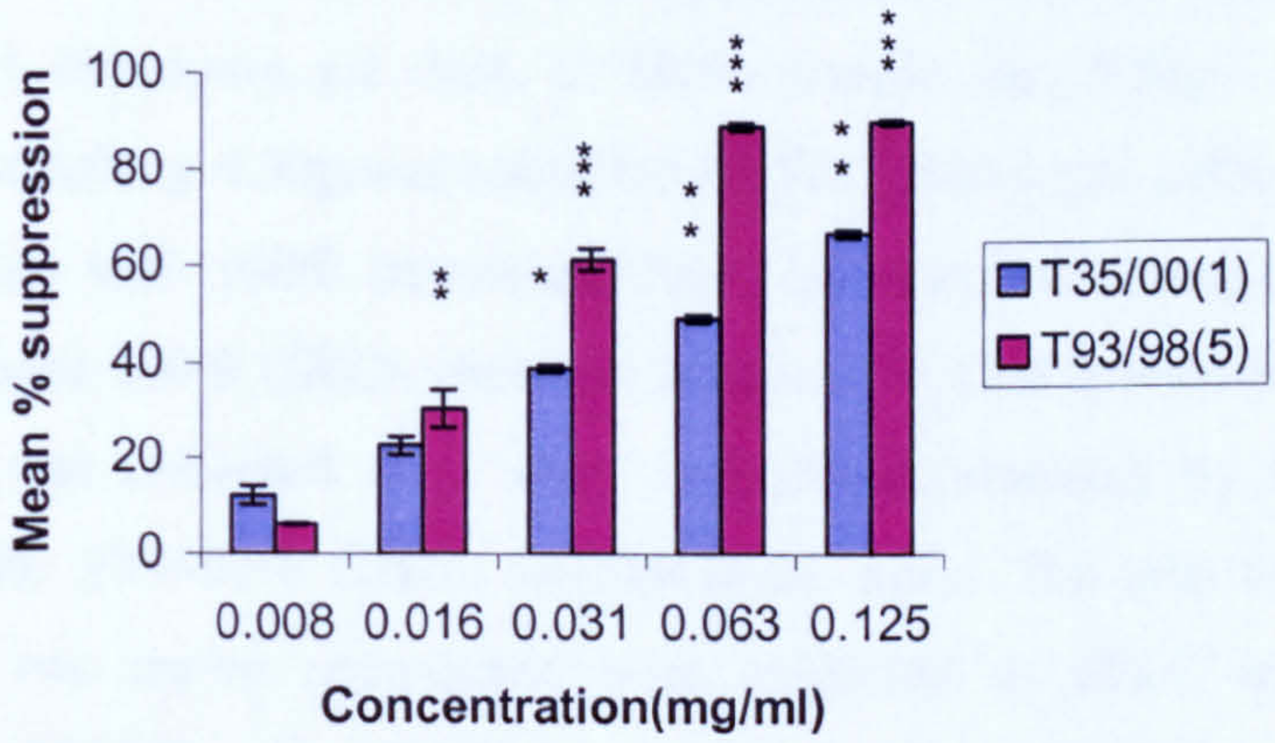


Figure 3.19: Dose-response graphs for cases T35/00 (1) and T93/98 (5) against the melanoma cell line

3.3 Phytochemical Work

Since all of the plants exhibited some degree of cytotoxic activity, they were subjected to chemical investigation in an attempt to isolate and identify the components responsible for the observed toxicity. Detailed chemical work was carried out on *Albizzia brevifolia, Argemone mexicana* and *Enicostemma axillare.* The other three plants, *Jatropha erythropoda, Cassia italica* and *Asclepias fruticosa* were only investigated analytically.

3.3.1 *Albizzia brevifolia*

The chemistry of this plant is being investigated here for the first time. 545.94g of young stem bark was extracted.

3.3.1.1 Petroleum ether extract

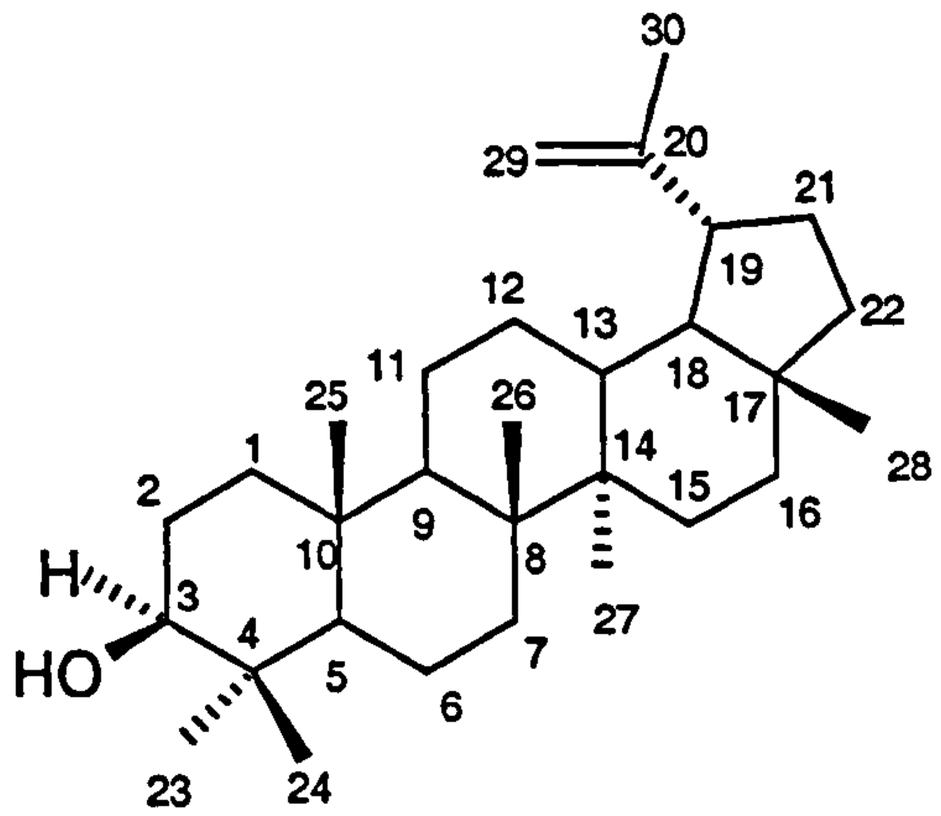
Chlorophyll was removed from the extract (5.96g) eluting with 30% $CHCl_3$ in petroleum ether (Sephadex LH-20 column, i.d. 3cm, L: 50cm, sample size, 0.58g – repeated). The remaining extract, weighing 4.90g was subjected to VLC (silica gel, column size L: 5cm i.d. 7cm) eluting first with 100% petroleum ether, and then increasing the polarity by increments of 2% until 100% $CHCl_3$, then 2% methanol in $CHCl_3$ until 6%. Compound AHPE01, 35 mg, was collected as a white crystalline substance by filtration from fractions eluted with 57%-67% $CHCl_3$ in petroleum ether. The rest of the fractions containing one or two major compounds were subjected to pTLC and compounds AHPE04, 6 mg, AHPE05 and AHPE06, 20 mg, were isolated as white crystals. Compounds AHPE05 and AHPE06 were isolated as a mixture.

3.3.1.1.1 Characterisation of AHPE04 as lupeol

The compound was obtained as a white crystalline solid, melting at 217-219°C. The FTIR spectrum showed a broad absorption band at 3300 cm^{-1} indicating the presence of a hydroxyl group. The mass spectrum showed a molecular ion of m/z 426 suggesting a molecular formula of $C_{30}H_{50}O$ expected for lupeol.

The ^{1}H spectrum (Figure 3.20) showed the presence of seven methyl signals and a signal characteristic of exomethylene protons at δ4.69 and 4.58 which accountant for the protons at C-29. The axial methine proton, H-3 was accounted for by the double of doublets signal at δ3.20, (J=11.6, 5.4 Hz). Another important signal was the doublet of triplets resonance at δ2.40 (J=11.4 5.2 Hz) due to H-19. The seven methyl singlets appeared at δ0.77, 0.81, 0.84, 0.95, 0.97, 1.04 and 1.69. the presence of the exomethylene protons and the deshielded methyl signal at δ1.69 suggested the existence of an isopropenyl moiety in the molecule.

The J-modulated ^{13}C NMR spectrum (Figure 3.21 and Table 3.8) showed thirty resonances among them the resonances at δ151.2 and 109.5 due to the carbon atoms C-20 and C-29 respectively. The signal at 79.2 was attributed to C-3. The seven methyl resonances were observed at 14.8, 15.5, 16.2, 16.3, 18.2, 19.5, and 28.2. The signal for C-19 was observed at 48.5. All this data agreed with the published data for lupeol (**95**), the structure of which is shown below.



Lupeol

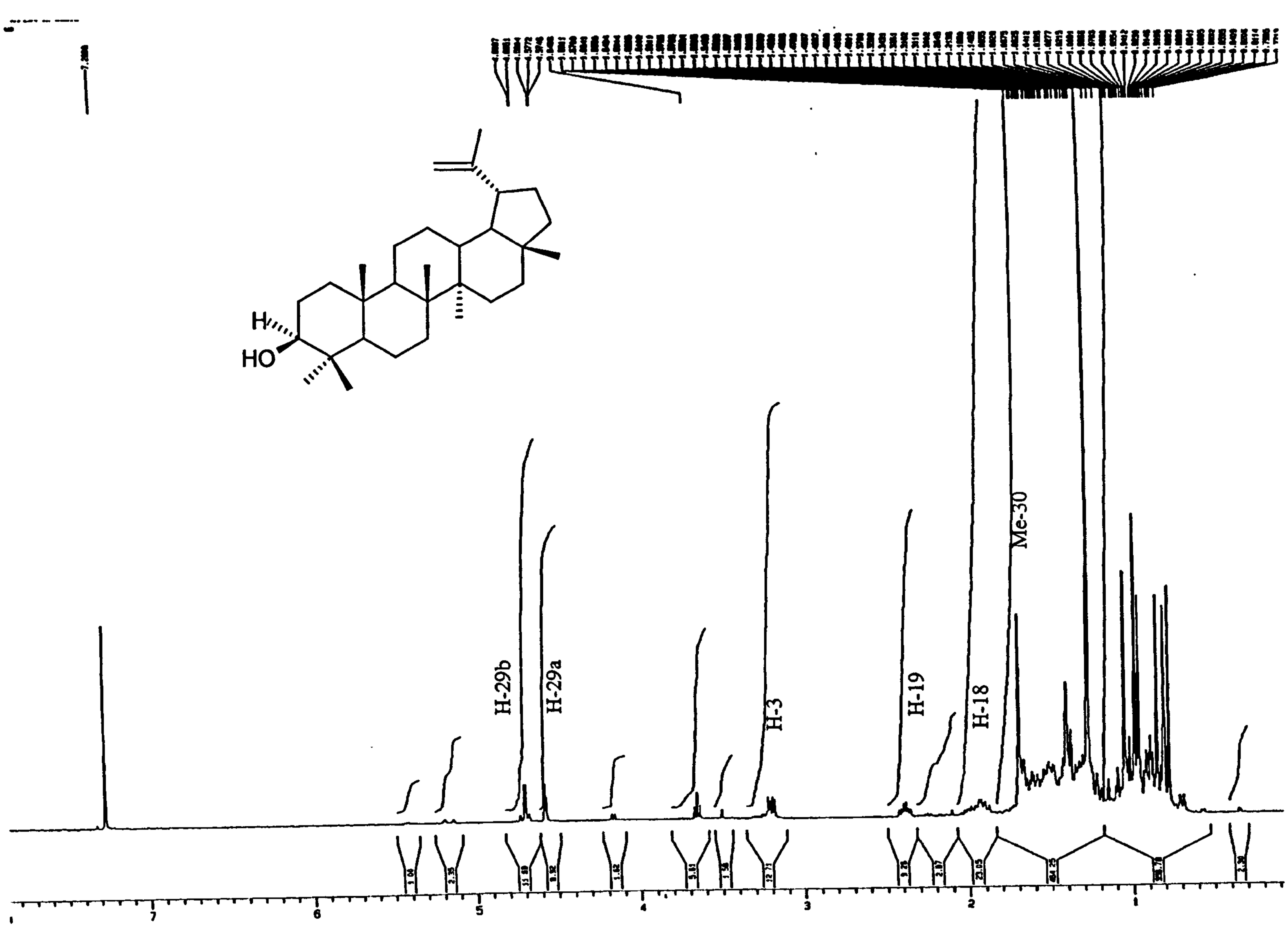


Figure 3.20: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of AHPE04

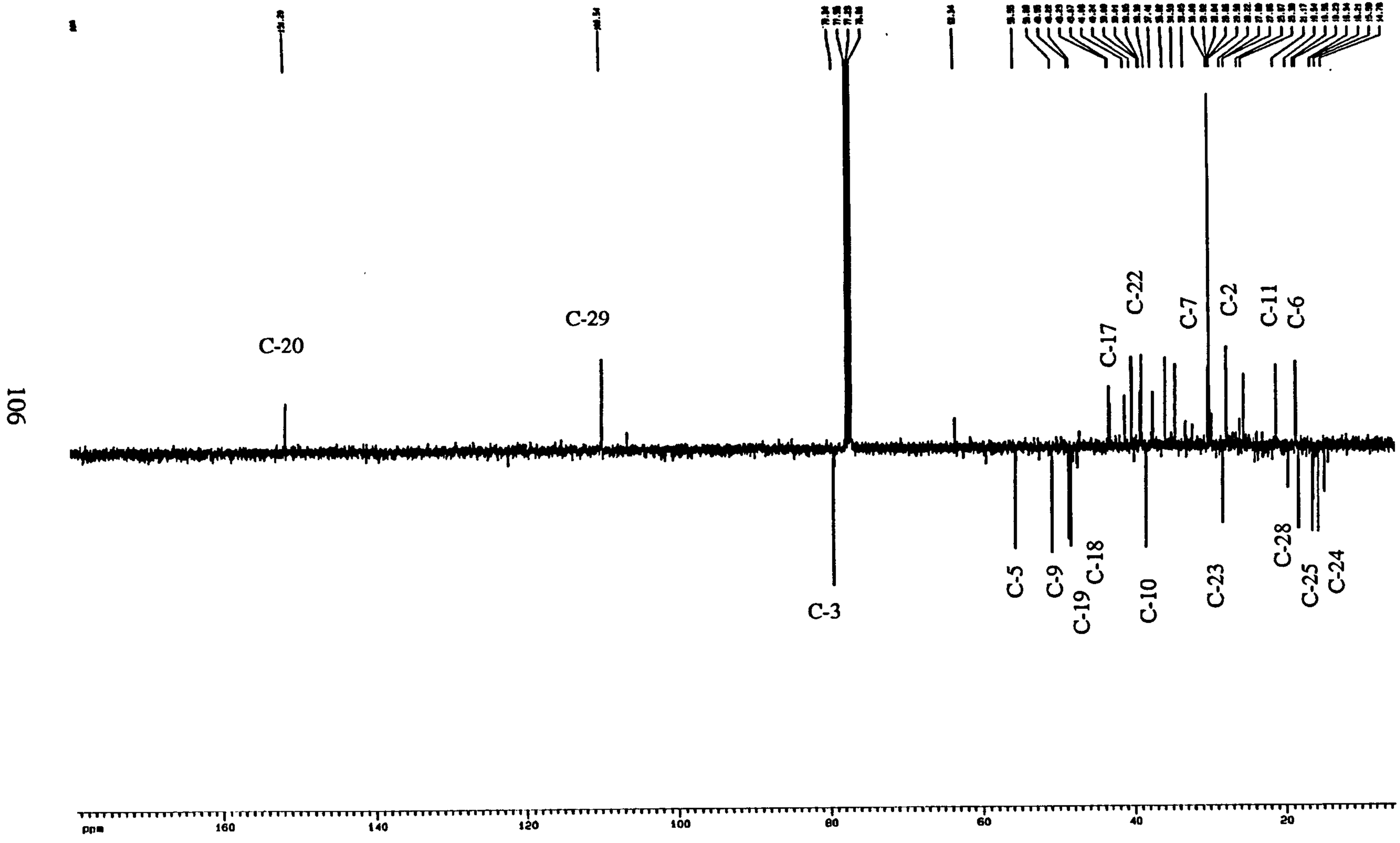


Figure 3.21: ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of AHPE04

Table 3.8: ^{13}C δ(ppm) NMR (400 MHz) for AHPE04

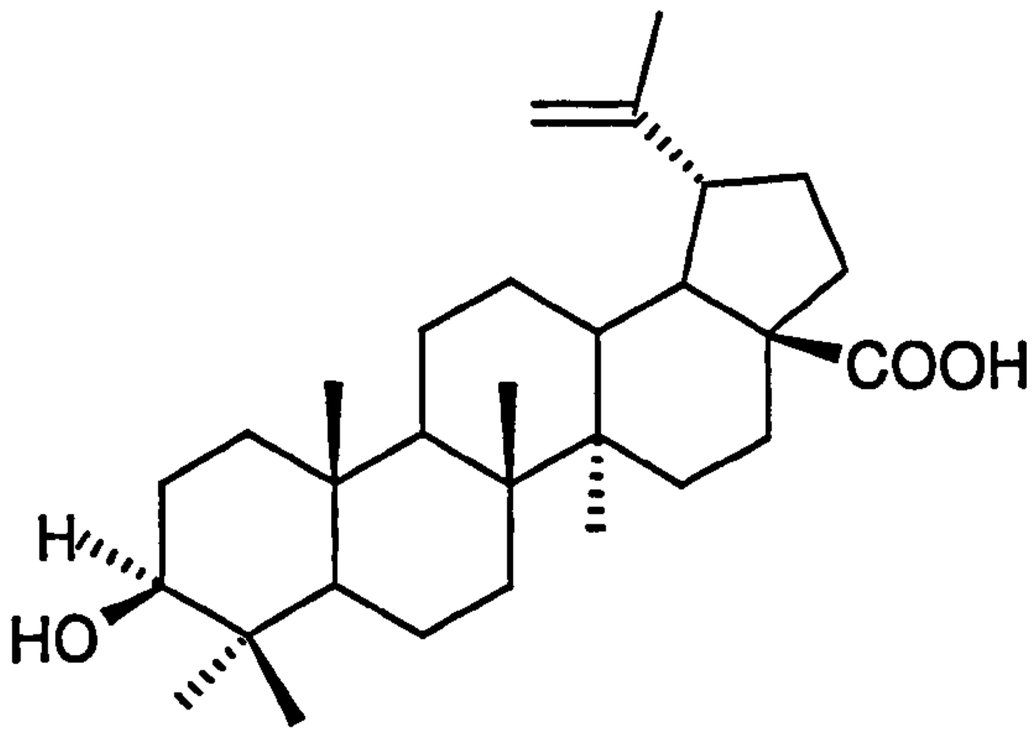
^{13}C	δ(ppm)	^{13}C	δ(ppm)
C-1	39.0	C-16	35.8
C-2	27.7	C-17	43.2
C-3	79.2	C-18	48.2
C-4	39.0	C-19	48.5
C-5	55.6	C-20	151.2
C-6	18.6	C-21	30.1
C-7	34.5	C-22	40.2
C-8	41.1	C-23	28.2
C-9	50.7	C-24	15.6
C-10	37.4	C-25	16.2
C-11	21.2	C-26	16.3
C-12	25.4	C-27	16.8
C-13	38.3	C-28	18.2
C-14	43.1	C-29	109.5
C-15	27.7	C-30	19.5

3.3.1.1.2 Characterization of compound AHPE01 as betulinic acid

This compound was isolated as a white crystalline solid melting at 314-315°C. The FTIR spectrum showed absorbances at 3400 and 1676 cm^{-1} indicating the presence of hydroxyl and carbonyl groups. High-resolution mass spectroscopy showed a molecular ion at m/z 456.36, corresponding to the molecular formula $C_{30}H_{48}O_3$.

The ^1H NMR spectrum (Figure 3.22) was similar to that of lupeol except that this one displayed the presence of six methyl signals instead of seven indicating oxidation at C-28. The characteristic signals for the exomethylene protons and the deshielded methyl at C-30 were present, again indicating the presence of an isopropenyl moiety. The signals at

δ 4.70 and 4.96 were assigned to the two exomethylene protons. The signal at δ1.82 was attributed to the allylic methlyl at C-30. In addition to these there were five other methyl singlets at δ 0.85, 1.03, 1.08, 1.10 and 1.25. Other important signals were the doublet of triplets at δ2.73 and doublet of doublets δ3.35 attributable to the proton at C-19 and the carbinolic proton attached to C-3 respectively. The signal at δ2.27 was assigned to the proton at C-18 and was more deshielded in this compound than in lupeol (δ1.92) thus evidencing the presence of more electron withdrawing functionality at C-28 which had a deshielding effect on the neighbouring carbon, C-17 which in turn deshielded C-18 and therefore the proton. These data suggested a pentacyclic triterpene of the lupane type and was found to be in agreement with literature values for betulinic acid (Tinto et.al., 1992,), the structure of which is shown below.



betulinic acid

3.3.1.1.3 Characterisation of AHPE05 and AHPE06 as Stigmasterol and β-sitosterol

These compounds were isolated as a white crystalline substance. ^{1}H NMR analysis showed this to be a mixture of two major compounds. HRSM spectrum showed the presence of ions at m/z 414 corresponding to the β-sitosterol (**22**) molecular formula ($C_{29}H_{50}O$) an ion at 412 corresponding to that of stigmasterol ($C_{29}H_{48}O$). The ^{1}H NMR spectrum (Figure 3.23) displayed a doublet at δ5.36 attributed to H-6. The signal was

integrated for 2 showing that there were two such protons, one attributed to each compound. Upfield to this were two signals at δ5.15 and δ5.05, which were assigned to the two methine protons at C-22 and C-23 of stigmasterol. Each signal was integrated for 1, thus showing that there was one of each. The signal at δ3.55, which was assigned to the axial proton at C-3 was integrated for 2 and is displayed in the spectrum clearly as two sets of signals for this proton. This again shows that there were two protons, one for each compound.

The mixture was subjected to further pTLC and was separated into the two compounds as shown by the ^{1}H NMR spectra in Figure 3.24 and 3.25. Figure 3.24 shows the major compound to be stigmasterol while Figure 3.25 shows the major compound to be β-sitosterol. Overall the data agree with that published for the two compounds (Aldrich). AHPE05 and AHPE06 were therefore resolved to be stigmasterol and β-sitosterol, respectively (**5** and **22**).

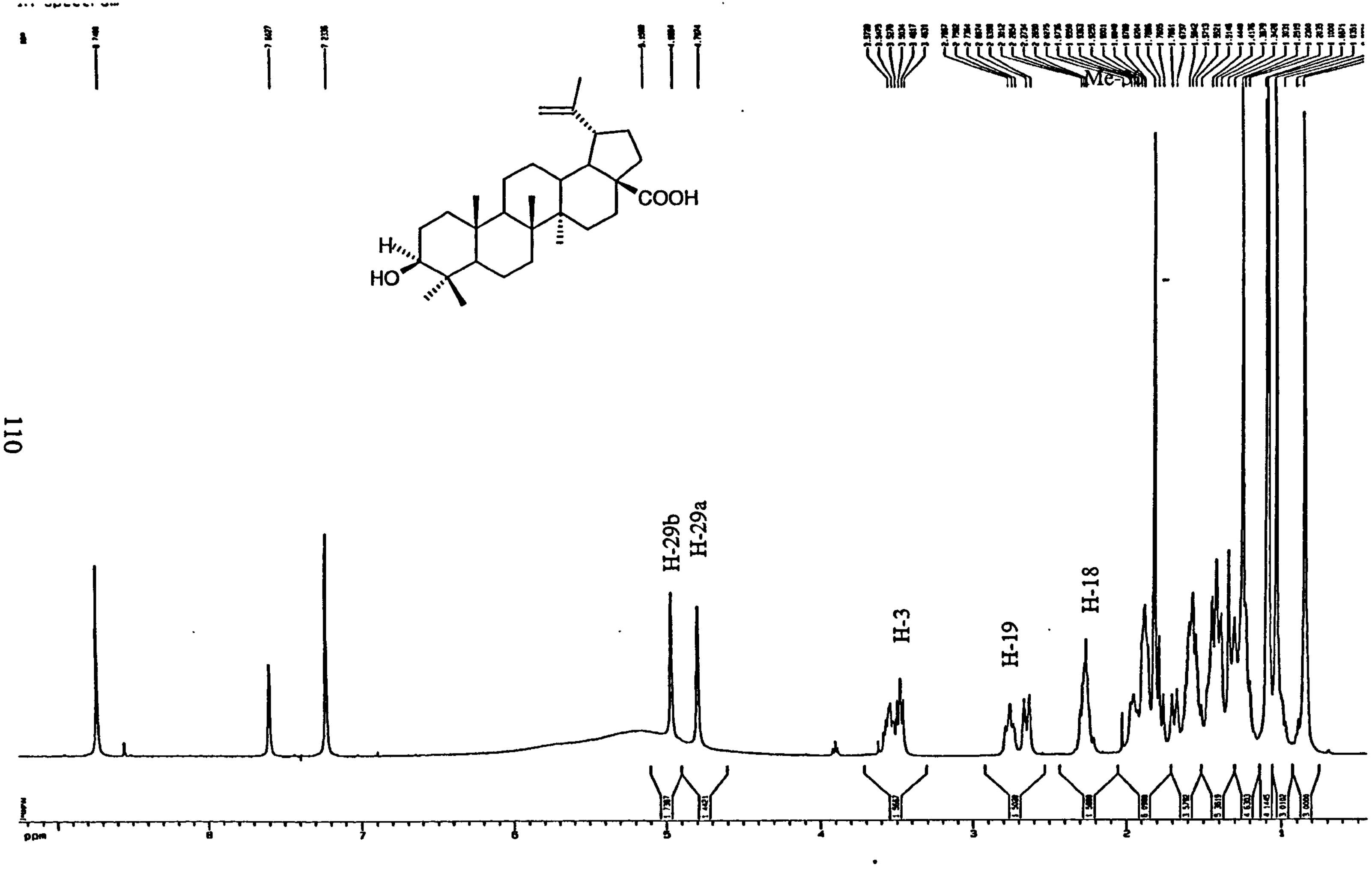


Figure 3.22: ^{1}H NMR spectrum (400 MHz, C_5D_5N) of AHPE01

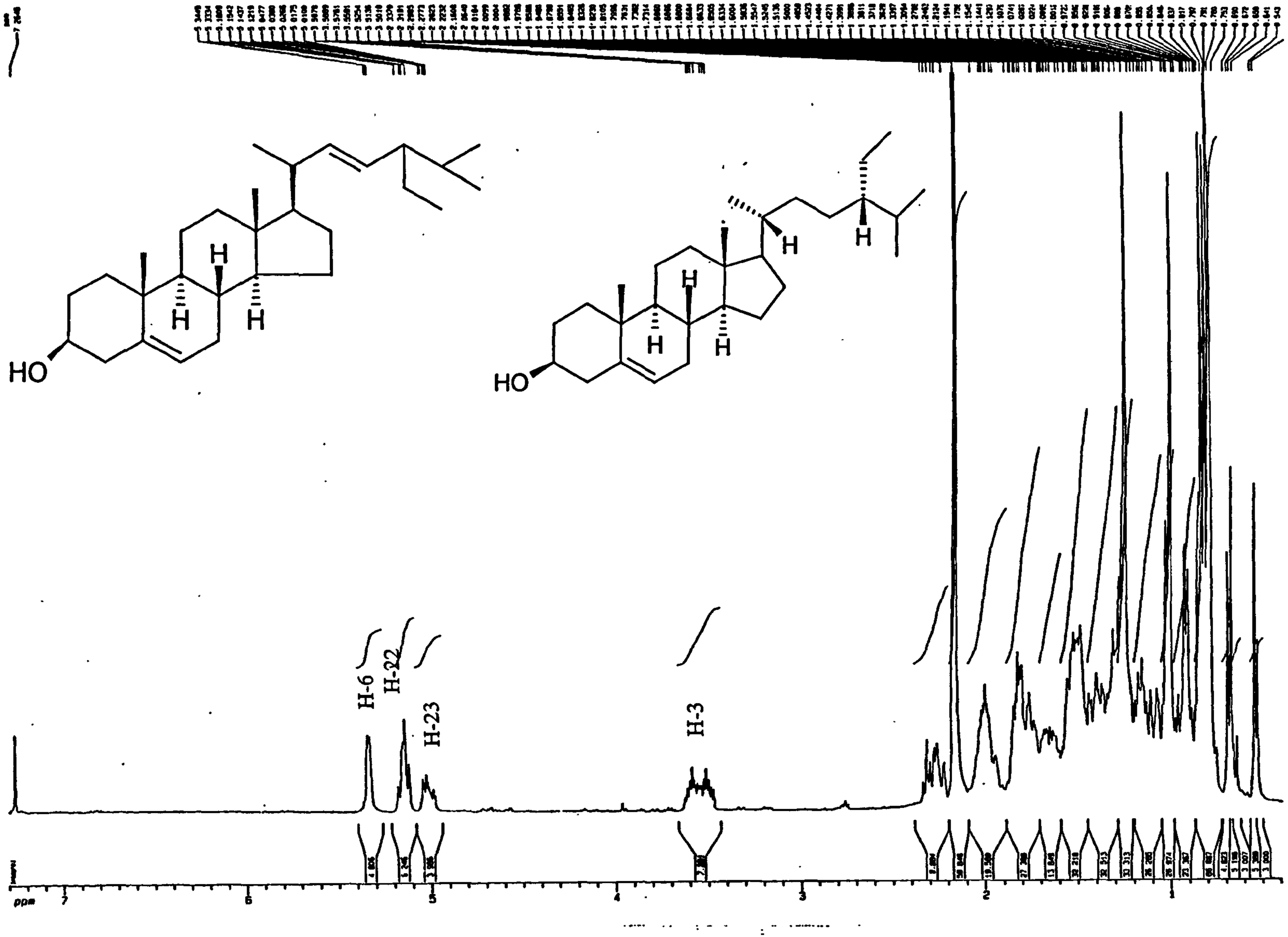


Figure 3.23: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of AHPE05 and 06 mixture

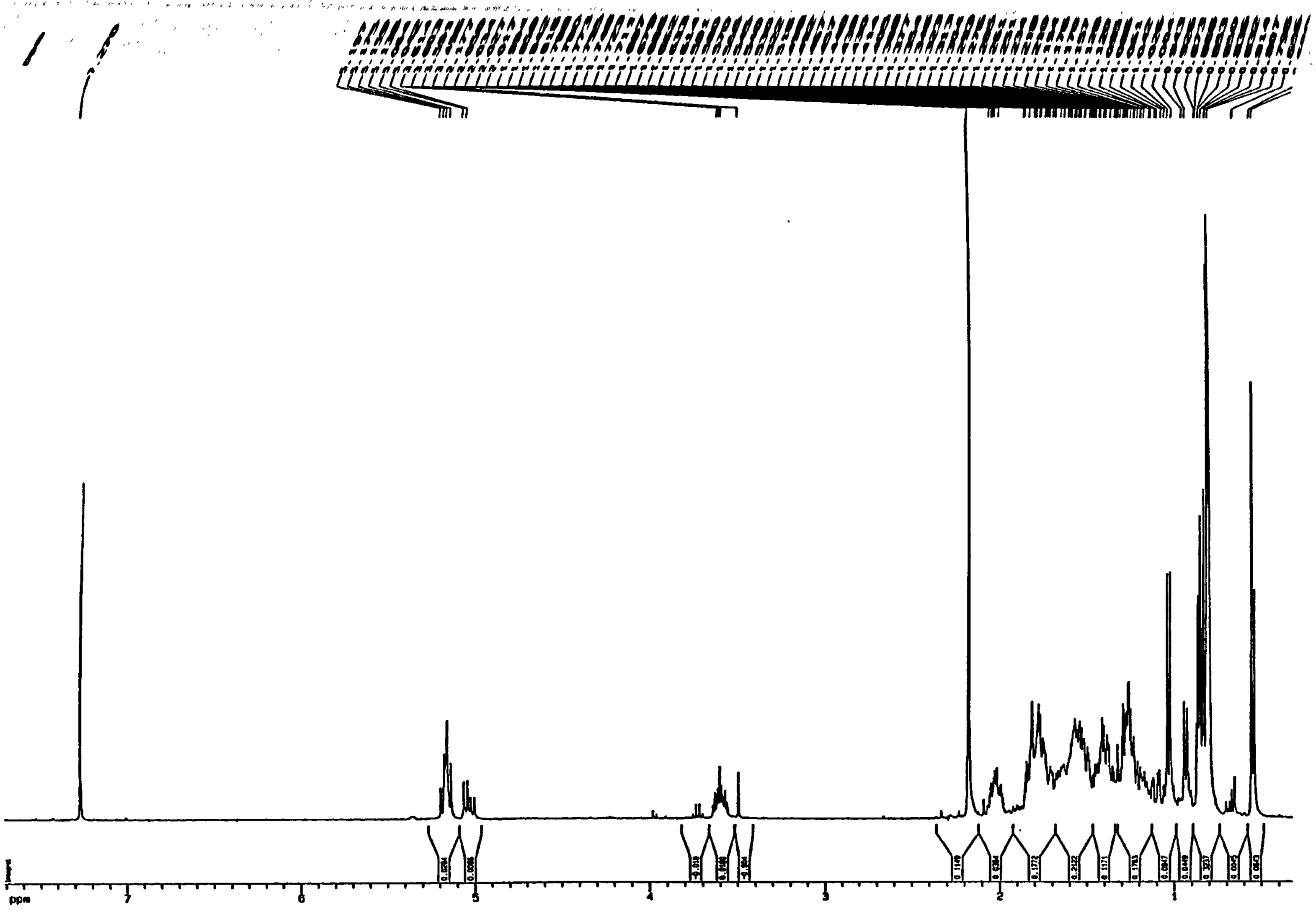


Figure 3.24: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of AHPE06

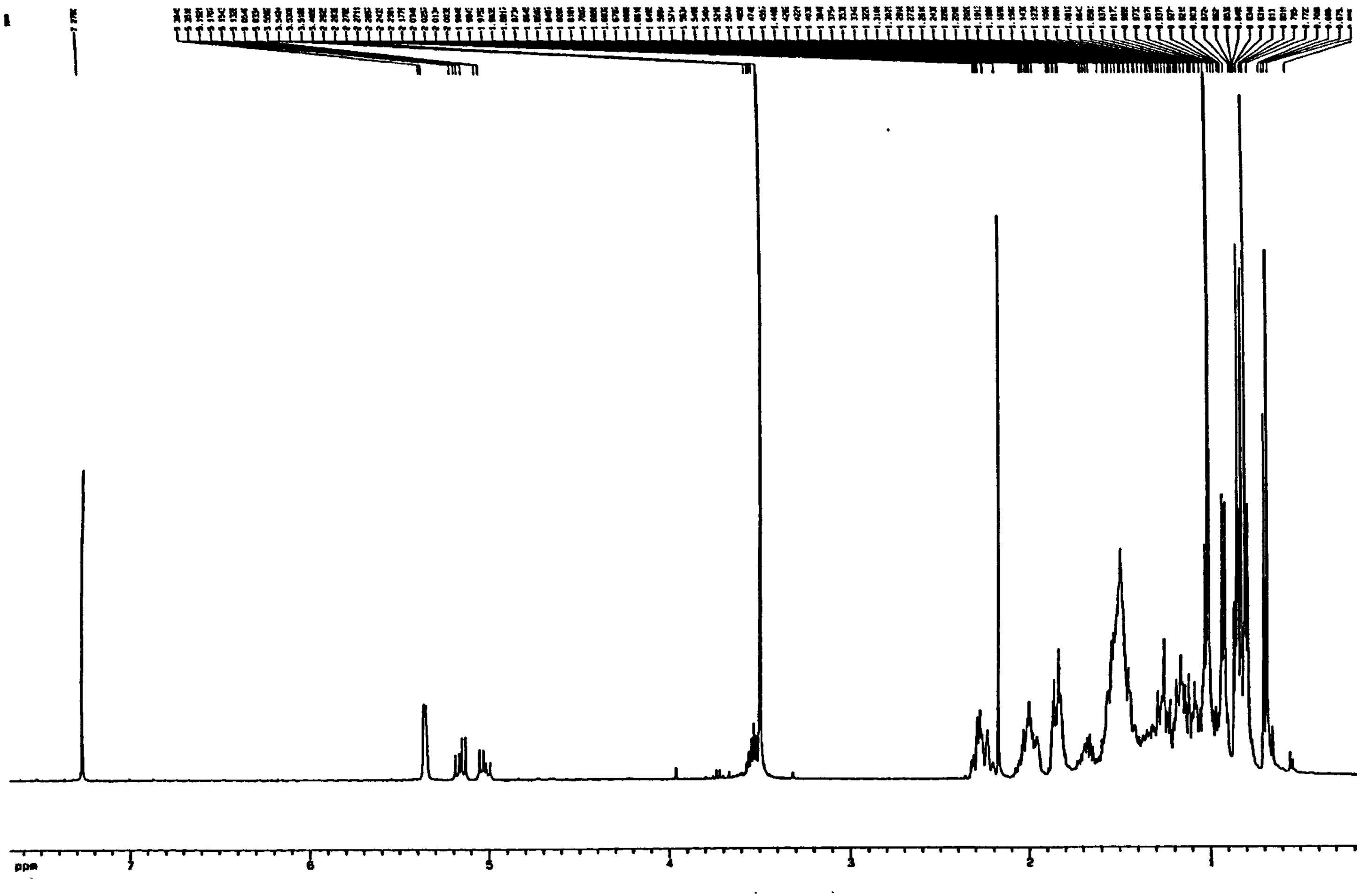


Figure 3.25: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of AHPE05

3.3.1.2 Examination of the Ethylacetate Extract

After chlorophyll removal (see section 2.2.2.3), the extract weighed 2.5g. This was subjected to VLC eluting first with 10% cyclohexane then increasing the polarity by increments of 30% $CHCl_3$ in cyclohexane to 100% $CHCl_3$. The polarity was then increased gradually by adding methanol to chloroform in increments of 2.5%. Fractions 1 to 7 were found to contain compounds similar to those in the petroleum ether extract but in minor amounts and were therefore not investigated further. The fraction eluted with 5% MeOH (8) showed one blue spot on TLC after spraying and heating. On standing the spot turned yellow. The ^1H NMR spectrum showed this to be a mixture of two compounds (AHCE 01 A and B). Fractions 9 to 10, eluted with 7.5% methanol were found to contain two major compounds that turned bright pink on TLC after spraying with vanillin-sulfuric acid reagent before heating and then turning yellow-green after heating and standing. These fractions were subjected to PTLC and the two compounds isolated as AB EtOAc Fn 10 and another compound (4mg) which could not be identified due to sample limitation.

The fraction eluted with 10-12% MeOH (11-12) was found to contain one major compound, which was UV active and turned bright red on spraying with vanillin-sulfuric acid reagent before heating and reddish brown on heating. This was easily recovered by more VLC and pTLC. It was coded AHSM01. Fraction 13, eluted with15% MeOH in chloroform contained AHSM01 and another slightly more polar compound which was also UV active and reacted the same way on TLC. Fractions 15 to17, eluted with 20% MeOH showed one major compound reacting the same way as those isolated from fractions 9-10. This was isolated by pTLC and coded AB EtOAc Fn 15/ AHRM04. These results are summarized in Table 3.9.

Table 3.9: Fractionation of Ethyl acetate extract of *A. brevifolia*

Fraction	System	Reaction On TLC	UV	R_f value (15%MeOH)	Code	Amount (mg)
1-7	Cyclo:$CHCl_3$	Purple	None	-	-	-
8	5%MeOH	Blue then yellow	Active	0.48	AHCE01 (A & B)	13
9-10	7.5% MeOH	Bright pink Yellow	Active (faint)	0.32 & 0.44	ABFn 10 -	10 4
11-12	12% MeOH	Bright red	Active	0.21	AHSM01	25
13	15% MeOH	"	Active	.18	-	9
15-17	20% MeOH	Bright pink	Active	.06	AHRM04/ ABFn15	17

Characterisation of AHCE01 as a mixture of A: 4-hydroxy-3-methoxycinnamaldehyde (ferulaldehyde) and B: 4-hydroxy-3-methoxybenzaldehyde (vanillin)

These were isolated as a yellowish solid. The FTIR spectrum showed a broad –OH absorption band at 3330 cm^{-1} and a carbonyl absorption band at 1645 cm^{-1}. HRMS showed an ion at m/z 178.1 corresponding to the molecular formula, $C_{10}H_{10}O_3$. the UV spectrum showed λ_{max} at 223 nm and 340 nm.

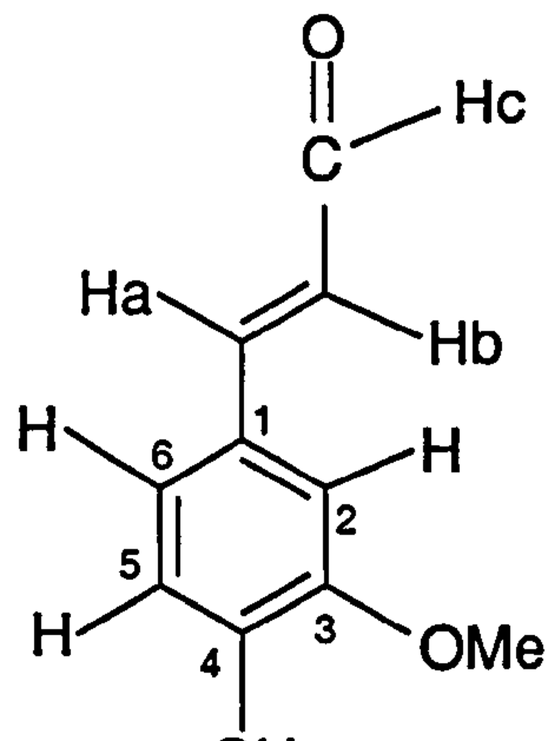
These compounds were characterized basing on the ^{1}H NMR, ^{1}H-^{1}H COSY and HMBC spectra.

3.3.1.2.1 Characterisation of AHCE01(A) as Ferulaldehyde

The ^{1}H NMR spectrum (Figure 3.26 and Table 3.10) showed the presence of three aromatic protons in an ABX spin system. There were also distinctive signals characteristic of two protons in a *trans* double bond. One of the protons (H*a* in the structure) showed ^{3}J coupling to the aldehyde carbon. In the ^{1}H spectrum the double of doublets at δ6.55 (J=15.8 Hz, 8.0 Hz) was assigned to the proton H*b* which showed *trans* coupling to Ha (J=15.8 Hz) and vicinal coupling to H*c* (J=8.0 Hz). H*a* appeared as a doublet at δ7.56 (J=15.5 Hz), thus showing *trans* coupling to H*b*. Hc showed a distinct aldehyde proton resonance at δ9.49 as a doublet (J=8.0 Hz) due to coupling to H*b*. The aromatic protons were accounted for by signal at δ6.74 (J=8.1 Hz) for H-5, which is *ortho* coupled to H-6. The signal at δ7.15 was assigned to H-6 as it displayed *ortho* and *meta* coupling to H-6 and H-2 respectively. H-2 appeared as a *meta* coupled doublet at δ7.09 (J=2.2). The singlet at δ3.86 was attributed to the methoxy group.

The COSY spectrum (Figure 3.27) showed correlations for the aldehyde proton and H*b*, and that between H*b* and H*a*. For the aromatic protons, H-6 and H-5 showed correlation. Examination of the HMBC (Figure 3.28) spectrum revealed information that further supported these assignments. H*a* showed ^{3}J coupling to the aldehyde proton, Hc, and to the aromatic protons at C-2 and C-6. This supports the placement of the aldehyde side chain between the two protons. H-5 showed ^{2}J coupling to C-6 and ^{3}J coupling to C-3. The methoxy displayed ^{3}J coupling to C-3.

All these data supported the characterization of this compound as ferulaldehyde and is in agreement with published data for the compound (Gibbons, 1994).



Feruladehyde

Table 3.10: ^{1}H and ^{13}C NMR (400 MHz) data for ferulaldehyde in CD_3OD

Posisition	^{1}H δ(ppm)	^{13}C δ(ppm)
C-1	-	126.8
C-2	7.09, d (J=2.2)	109.8
C-3	-	151.2
C-4	-	(?)
C-5	6.74,d (J=8.1)	118.0
C-6	7.15, dd (J=8.2, 2.0)	124.5
C-H*a*	7.56, d (J=15.5)	129.7
C-H*b*	6.55, dd (J=15.8, 8.0)	157.7
C-H*c*	9.49, d (8.0)	195.0
OMe	3.86, s	Not seen

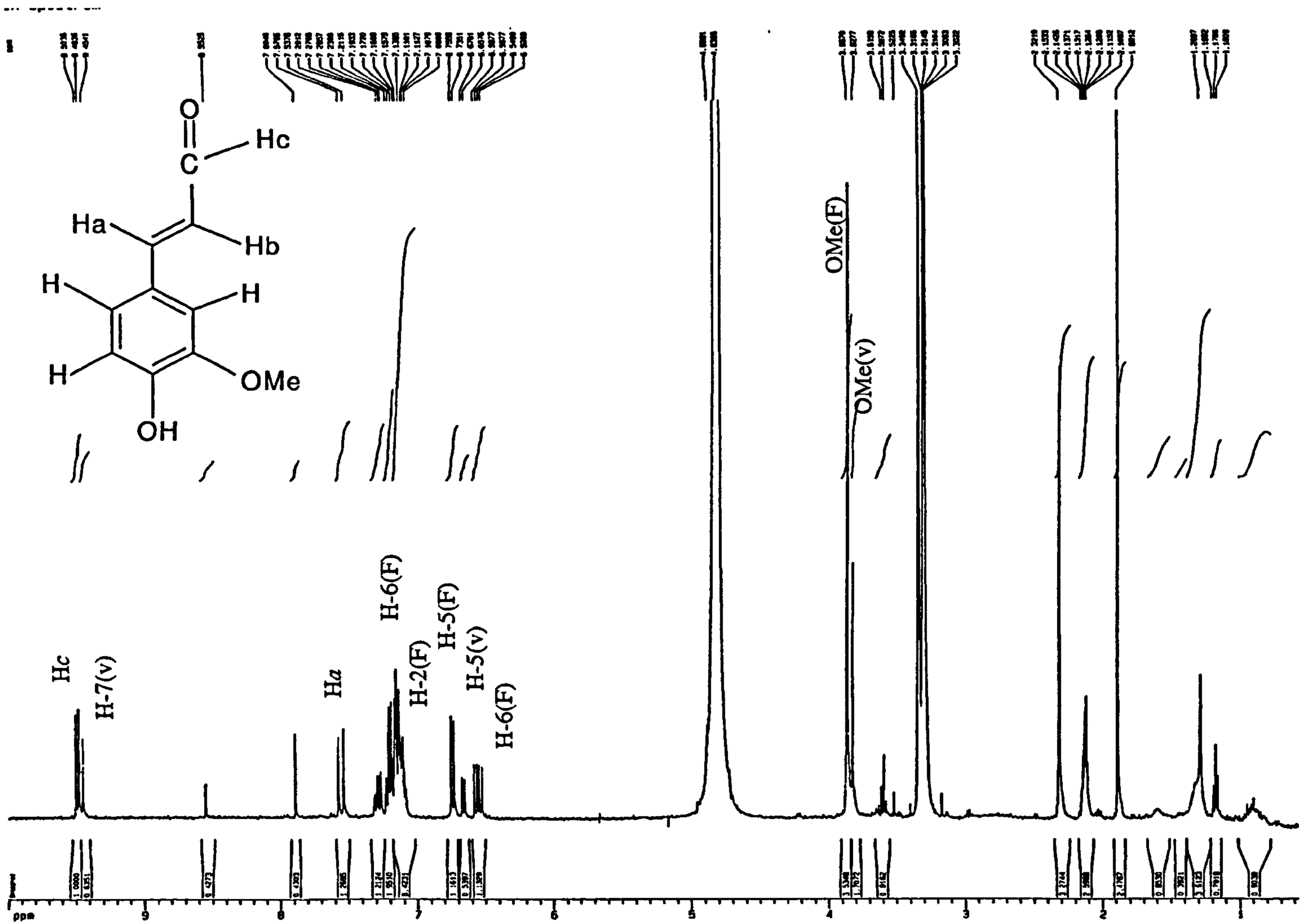


Figure 3.26: ^{1}H NMR spectrum (400 MHz, CD_3OD) of AHCE01

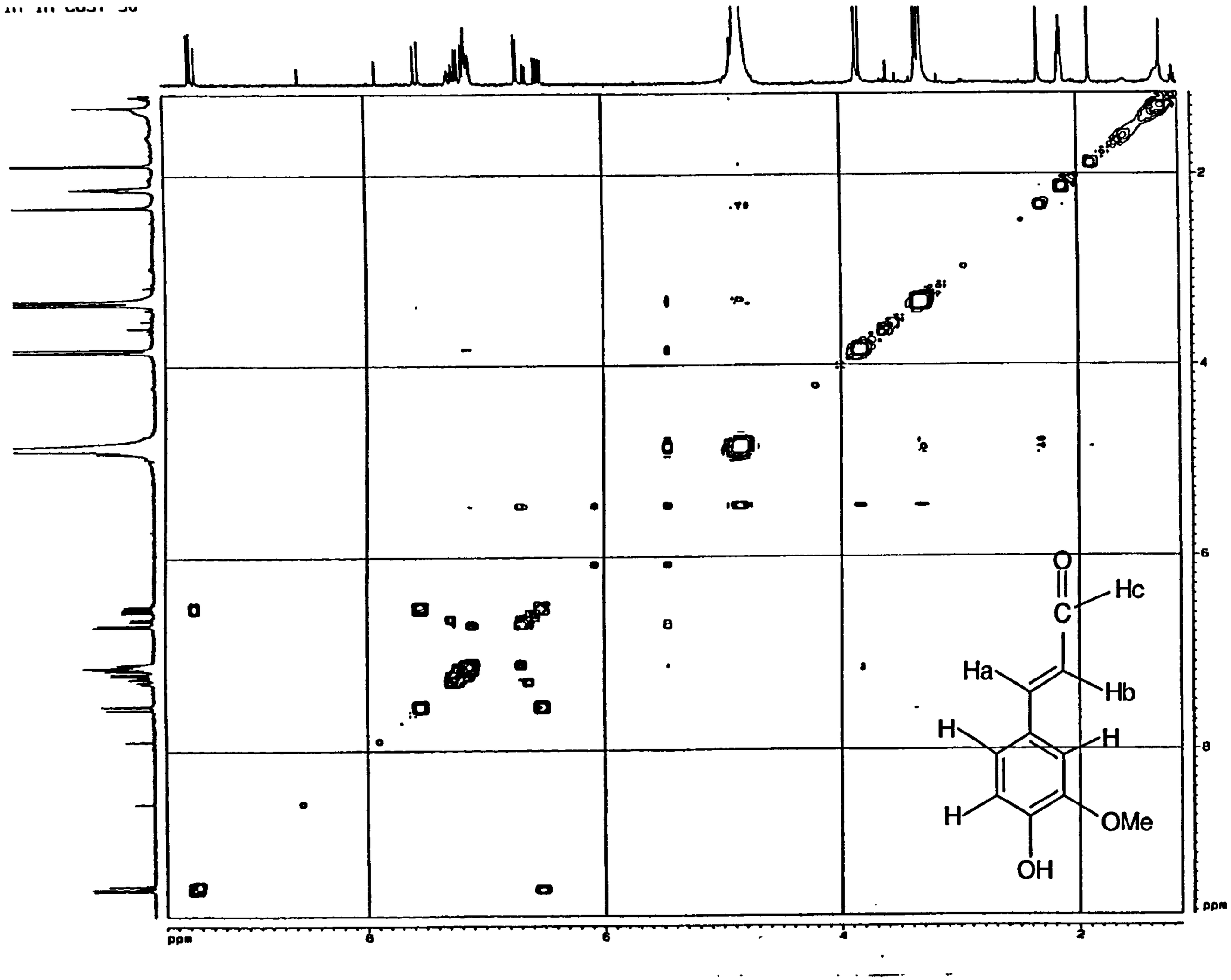
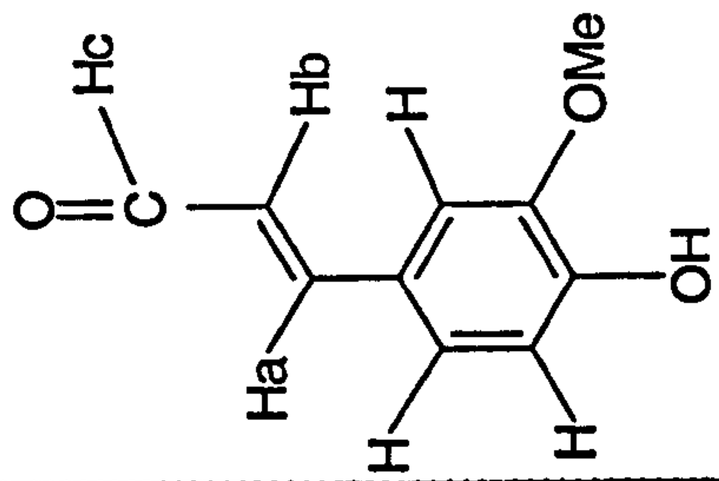


Figure 3.27: ^{1}H-^{1}H COSY90 NMR spectrum (400 MHz, CD_3OD) of AHCE01



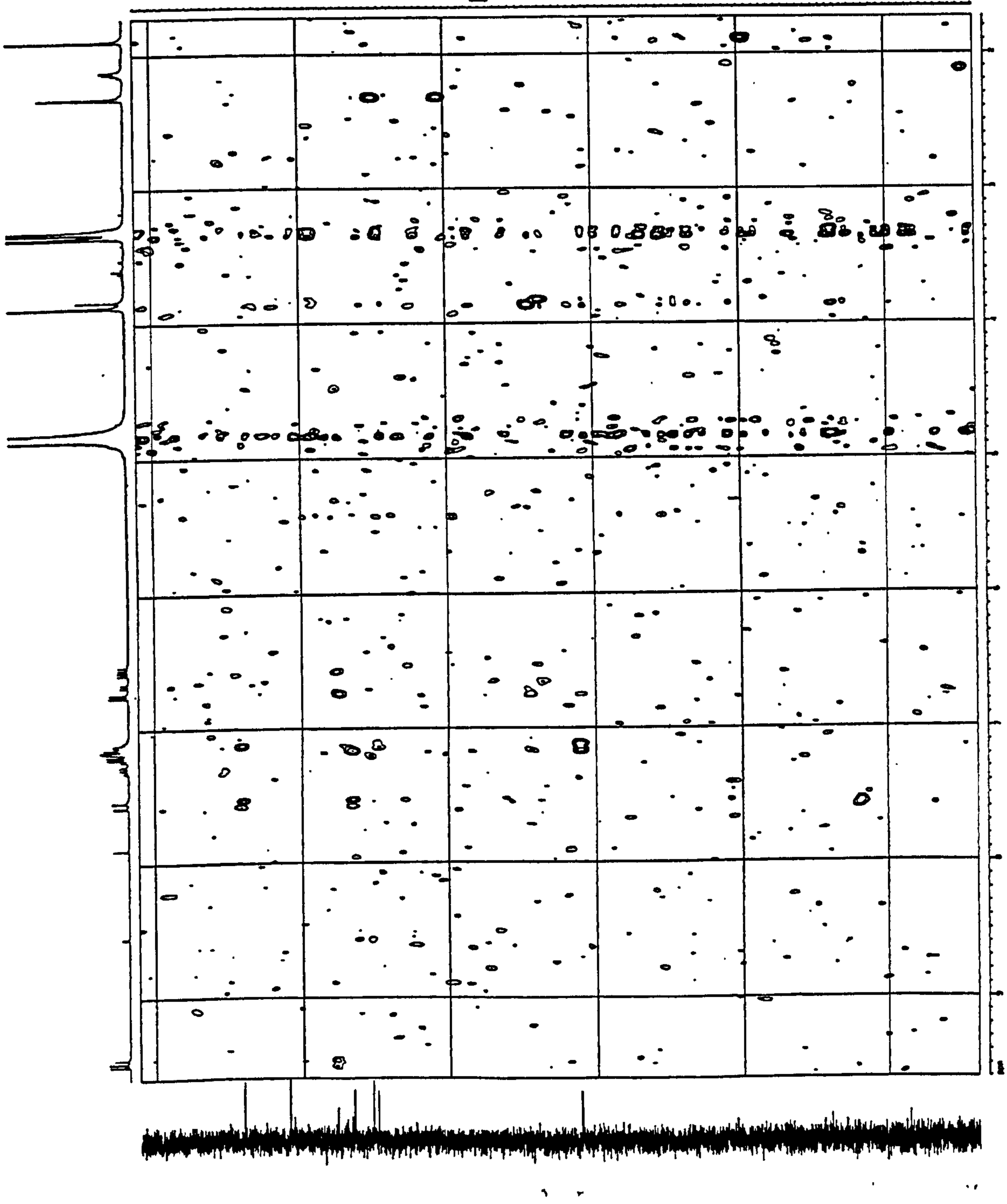


Figure 3.28: HMBC spectrum of AHCE01

3.3.1.2.2 Characterisation of AHCE01 (B) as Vanillin

This compound showed similar resonances to those of ferulaldehyde. The ^{1}H NMR spectrum (Figure 3.26 and Table 3.11) showed an aldehyde proton, which unlike in the ferulaldehyde appeared as a singlet at, δ7.45, thus showing no coupling to any other proton. The *trans* double bond resonances were also absent in the spectrum for this compound. There were three aromatic protons substituted as in the other compound i.e. *ortho/meta, ortho* and *meta.* The doublet at δ7.27 (J=2.0 Hz) was attributed to H-2 which is *meta* coupled to H-6 and that at δ6.56 to H-5 which is *ortho* coupled to H-6. H-6 appeared as a doublet of doublets at δ7.31 (J=8.1 Hz, 2.0 Hz) showing *ortho* coupling to H-5 and *meta* coupling to H-2. The methoxy appeared as a singlet at δ3.82. In the COSY spectrum (Figure 3.27), H-5 and H-6 showed correlation with each other. The HMBC spectrum revealed ^{3}J coupling of the aldehyde proton to C-6. These data supported the characterization of this compound as 4-hydroxy-3-methoxybenzaldehyde (vanillin).

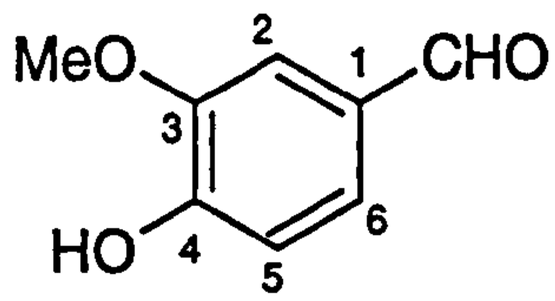


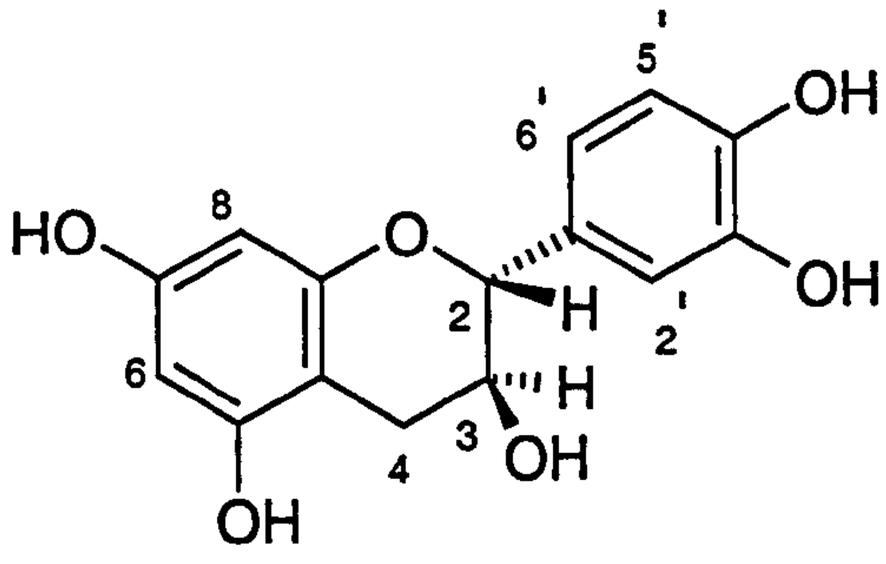
Table 3.11: ^{1}H NMR (400 MHz) data for vanillin in CD_3OD

Position	^{1}H δ(ppm)
C-1	-
C-2	7.27, d (J=2.0)
C-3	-
C-4	-
C-5	6.56, d (J=8.2)
C-6	7.31, dd (J=8.1, 2.0)
C-7	9.45, s
OMe	3.82, s

3.3.1.2.3 Characterisation of AHSM01 as (+)-catechin

This compound was isolated a yellow solid, (mp: 178-179°C, $[\alpha]_D$, methanol: +17). The FTIR spectrum showed a broad absorption band at 3325 cm^{-1} indicating the presence of hydroxyl functionality. The UV spectrum showed λ_{max} at 206, 230 (shoulder) and 280 nm, The MS spectrum showed a molecular ion peak of m/z 290 indicating a molecular formula of $C_{15}H_{14}O_5$. The reaction on TLC after spraying with vanillin-sulfuric acid reagent (reddish-brown) suggested a procyanidin (tannin component) and the molecular mass suggested either (+)-catechin or (-)-epicatechin.

These properties and comparison of the ^1H NMR spectrum (Figure 3.29 and Table 3.12) spectrum with literature values (Cai *et.al.*, 1991) helped in characterizing the compound. The ^1H NMR spectrum displayed a doublet at δ4.56, (J=7.4Hz) which was attributed to the proton at C-2. The double of doublets at δ2.51 (J-8.2 Hz, 16.1 Hz) and δ2.84 (J=5.4 Hz, 16.1 Hz), showing *trans* and *cis* coupling to the proton at C-3 were assigned to the two protons at C-4. The appearance of the signal at δ4.56 for H-2 as a doublet with a coupling constant of J=7.4Hz (axial-axial vicinal coupling) suggested a 2,3-trans configuration for ring C, thus ruling out (-)-epicatechin. The identification of this compound as (+)-catechin was further confirmed by signal assignments for the protons of ring A and B. The signal at δ5.85, d, (J=2.0 Hz) was assigned to H-6 which showed *meta* coupling to the doublet at δ5.92, (J=2.1Hz) assigned to the proton at C-8. For ring B, the doublet at δ6.83, (J=2.0Hz) was assigned to H-2' (*meta* coupled to H-6'), the later showing as a double of doublets at δ6.74, (J=8.2Hz, not determined). The coupling constant of J=8.2 Hz is due to *ortho* coupling with H-5' and the other to *meta* coupling with H-2'. H-5' appeared as a doublet at δ6.76, (J=8.1Hz) due to *ortho* coupling with H-6'. The protons in ring B displayed an ABX spin system and therefore supported the hydroxylation pattern to be 3',4' dihydroxylation for ring B. The structure of AHSM01 was thus confirmed to be 2,3-*trans*-(5,7,3',4'-tetrahydroxy)flavan-3-ol [(+)-catechin]. The structure of the compound is shown below.



(+)-catechin

Table 3.12: ^{1}H (4000MHz) NMR data for AHSM01 in CD_3OD

Position	δ ^{1}H (ppm)
2	4.56, d (7.4)
3	3.98, m
4	2.51, dd (8.2, 16.1
	2.84, dd (5.4, 16.1)
6	5.85, d (2.0)
8	5.92, d (2.1)
2'	6.83, d (2.0)
5,	6.76, d (8.4)
6'	6.74, dd (8.2, 2.0)

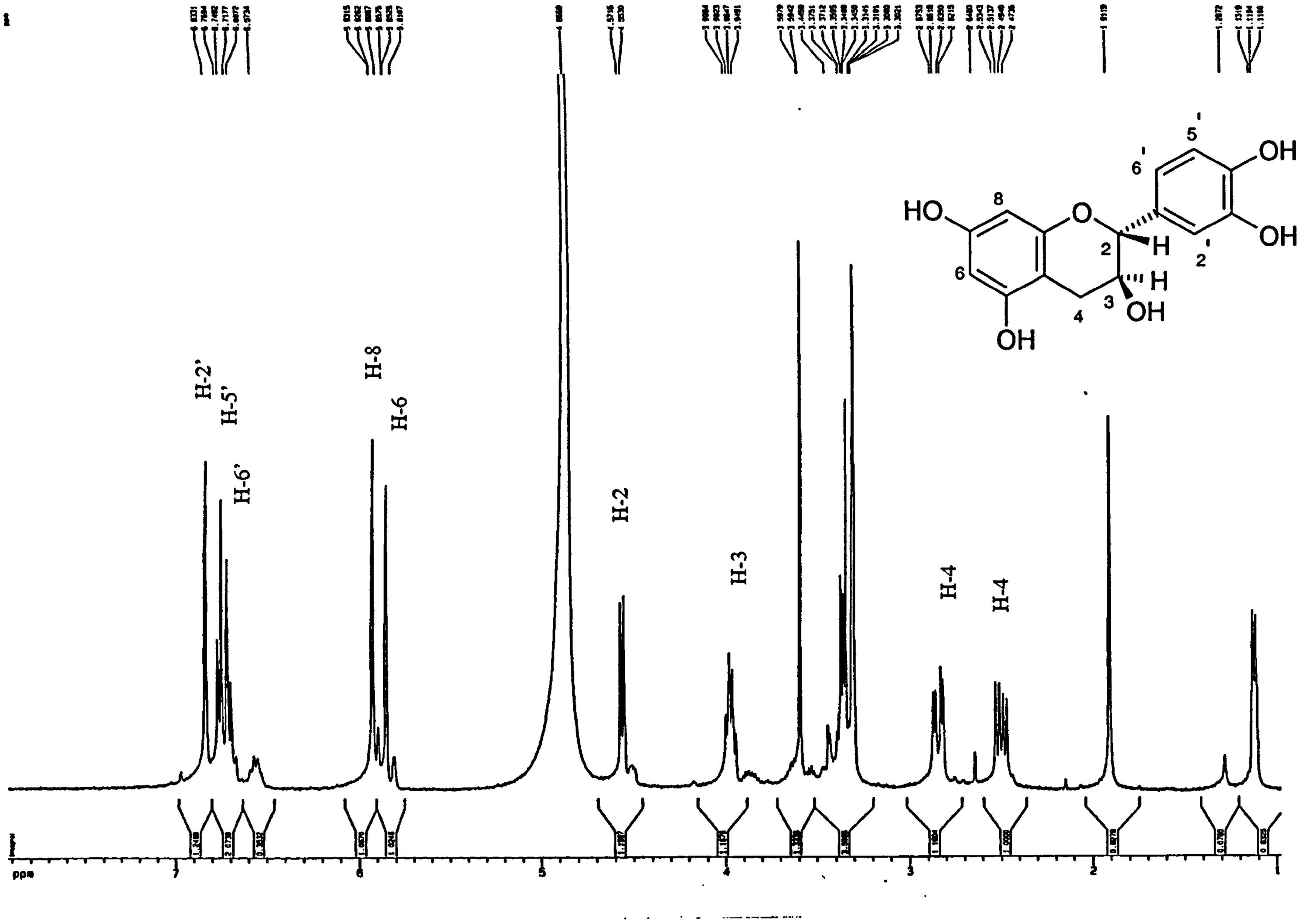


Figure 3.29: ^{1}H NMR spectrum (400 MHz, CD_3OD) of AHSM01

3.3.1.2.4 Characterisation of AHRM04 (AB EtOAc Fn 15/AHRM04) as 2-(3'-β-D-glucopyranosyl-4'-hydroxyphenyl)ethanol

This compound was isolated as a yellowish solid. The FTIR spectrum showed a broad absorption band at 3407 cm^{-1} due to –OH functionality. The UV spectrum showed λ_{max} at 278 nm and a shoulder at 225nm.

The structure of the compound was deduced using information provided by a series of NMR experiments; ^{1}H NMR, ^{13}C J-modulated, ^{1}H-^{1}H COSY, HC-COBI, HMBC and ^{1}H-^{1}H NOESY. The ^{1}H NMR spectrum (Figure 3.30 and Table 3.13) displayed signals due to 3 aromatic protons in an ABX spin system, signals characteristic of a sugar and two methylene resonances. This indicated the presence of a trisubstituted benzene ring with a two carbon side-chain and a sugar attachment. The ^{13}C J-modulated spectrum showed the presence of 14 carbon atoms. In the ^{1}H NMR spectrum, the triplet at δ2.70 was assigned to the methylene directly attached to the ring and the multiplet downfield to this at δ3.70 was assigned to the oxymethylene attached to the –OH group of the ethanol. The aromatic region showed a doublet at δ7.07, (J=1.4 Hz) indicating a *meta* coupled proton and was therefore assigned to H-2'. The group of signals upfield to this was integrated for two protons one of which was *ortho/meta* coupled and the other *ortho* coupled. Thus the double of doublets at δ6.76 (J=8.1 Hz, 1.8 Hz) was assigned to H-6' and that at δ6.8, doublet, (J=8.1 Hz) to H-5'. The ^{1}H-^{1}H COSY spectrum showed correlation between H-2' and H-6' and between the two methylenes that supports their placement next to each other.

The assignment of protons was also based on the ^{13}C J-modulated (Figure 3.31 and Table 3.13) and HCCOBI (Figure 3.32). The ^{13}C spectrum displayed 3 CH's, 3 quaternary carbons and 2 CH_2's together with the sugar carbons. The signals at δ39.6 and δ64.5 showed direct correlation with the two methylenes of the ethanol on the HCCOBI spectrum. These were therefore assigned to C-2 and C-1 respectively. The aromatic protons showed direct correlation to the signals at δ119.8 (C-2'), δ117.1 (C-5') and δ125.3 (C-6'). The quaternary carbon atoms were assigned basing on the information provided by the HMQC spectrum (Figure 3.33). The methylene protons at C-2 showed

strong ^{2}J to the quaternary carbon signal at δ132.20 and ^{3}J to C-2' and C-6'. The signal at δ132.2 was thus assigned to C-1'. These correlations supported the attachment of the side chain between C-2' and C-6'. The methylene protons at C-1 showed ^{3}J to the quaternary carbon C-1'. The other signal due to a quaternary carbon was that at δ146.7 which showed ^{3}J to H-5' and ^{2}J to H-2' whereas that at δ146.9 displayed ^{2}J to H-6' and H-2'. Therefore δ146.7 was assigned to C-3' and δ146.9 to C-4'. Other correlations that supported the assignments were those shown by H-5', showing ^{3}J to C-1' and H-2' showing ^{3}J to C-6'

The positioning of the sugar molecule was evidenced by the fact that the anomeric proton, H-1'' showed ^{3}J to C-3' (δ146.7) and a strong NOESY (Figure 3.34) correlation to H-2'. H-2' also showed NOESY correlation to the methylene protons at C-2, thus supporting its position between the sugar and the side-chain. NOESY correlations were also shown by H-6' to the methylene protons at C-2 which is most likely due to the conformation of the molecule. The two methylenes show NOESY correlation to each other. H-5' and H-6' also show NOESY correlation to each other.

The ^{13}C J-modulated displayed six carbon atoms for the sugar molecule including one CH_2 signal. By comparison with literature values the sugar moiety was identified as glucose. The coupling constant of 7.6Hz for the anomeric proton at δ4.74, doublet, to H-2'' showed that it is a β-D-glucose since the large coupling constant is due to axial-axial vicinal coupling of the two protons. The structure of the compound was therefore concluded to be as shown below. The arrows show NOESY correlations.

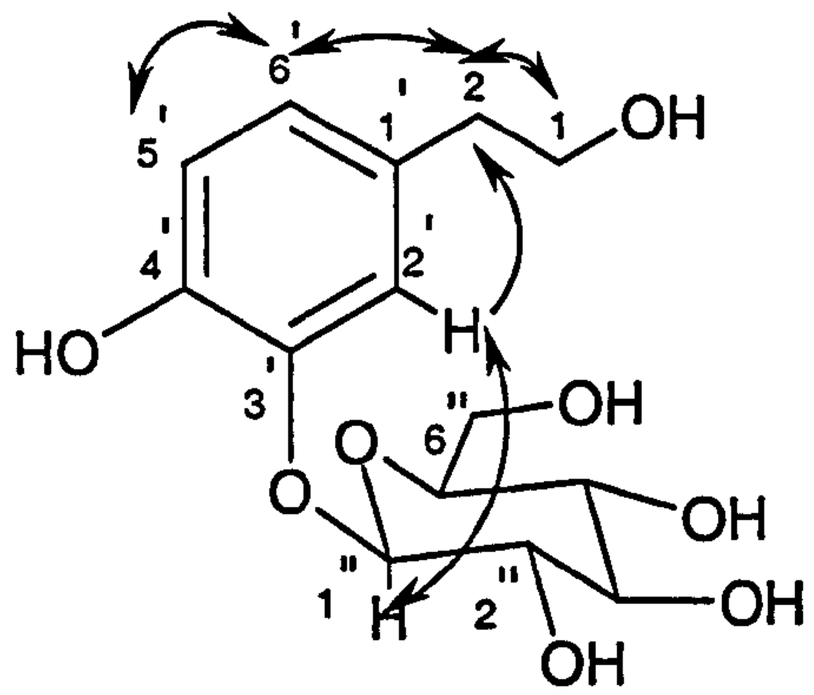


Table 3.13: ^{1}H and ^{13}C NMR (400MHz) data for 2-(3'-β-D-glucopyranosyl-4'-hydroxyphenyl)ethanol

Posisition	^{1}H δ(ppm)	^{13}C δ(ppm)
C-1	3.70, m	64.5
C-2	2.70, t (J=7.0, 14.1,7.0)	39.6
C-1'	-	132.2
C-2'	7.07,d (J=1.4)	119.8
C-3'	-	146.7
C-4'	-	146.9
C-5'	6.78, d (J=8.1)	117.1
C-6'	6.76, dd (J=8.1, 1.8)	125.3
Sugar moiety		
C-1''	4.74, d (J=7.6)	104.6
C-2''	3.47	75.1
C-3''	3.45	77.8
C-4''	3.41, m	71.5
C-5''	3.88	78.5
C-6''	3.78, 3.80	62.6

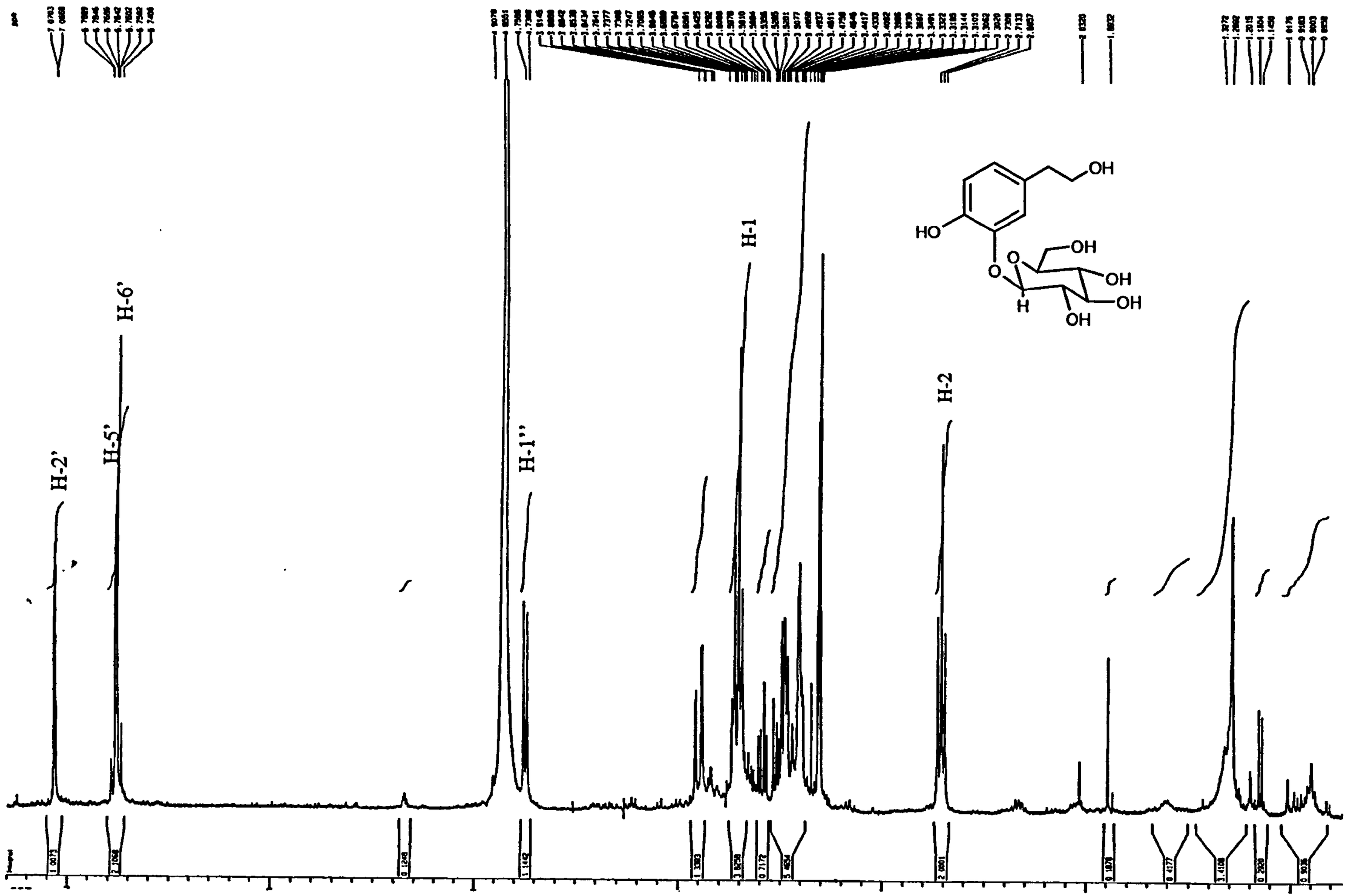


Figure 3.30: ^{1}H NMR spectrum (400 MHz, CD_3OD) of ABFn15

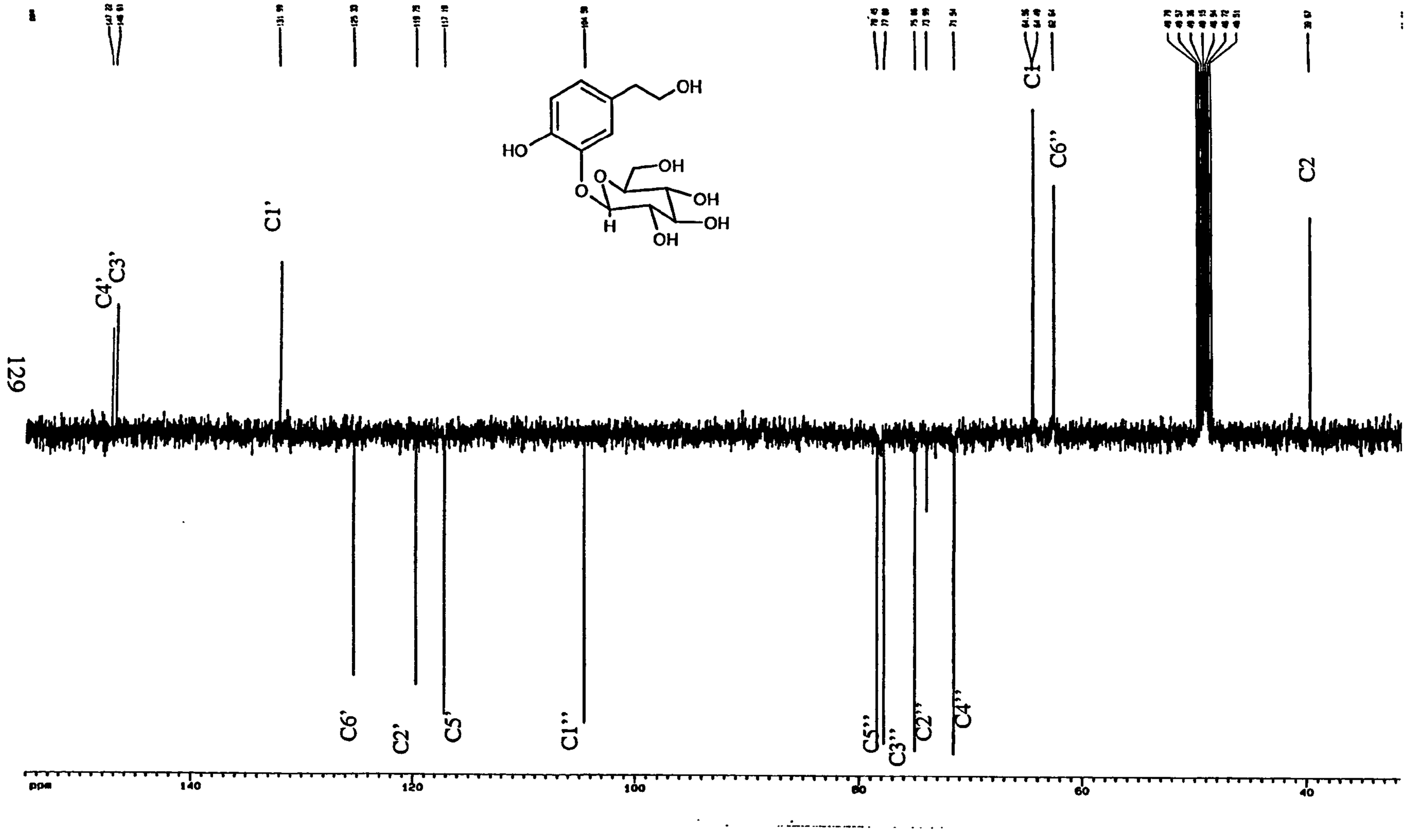


Figure 3.31: ^{13}C NMR spectrum (400 MHz, CD_3OD) of ABFn15

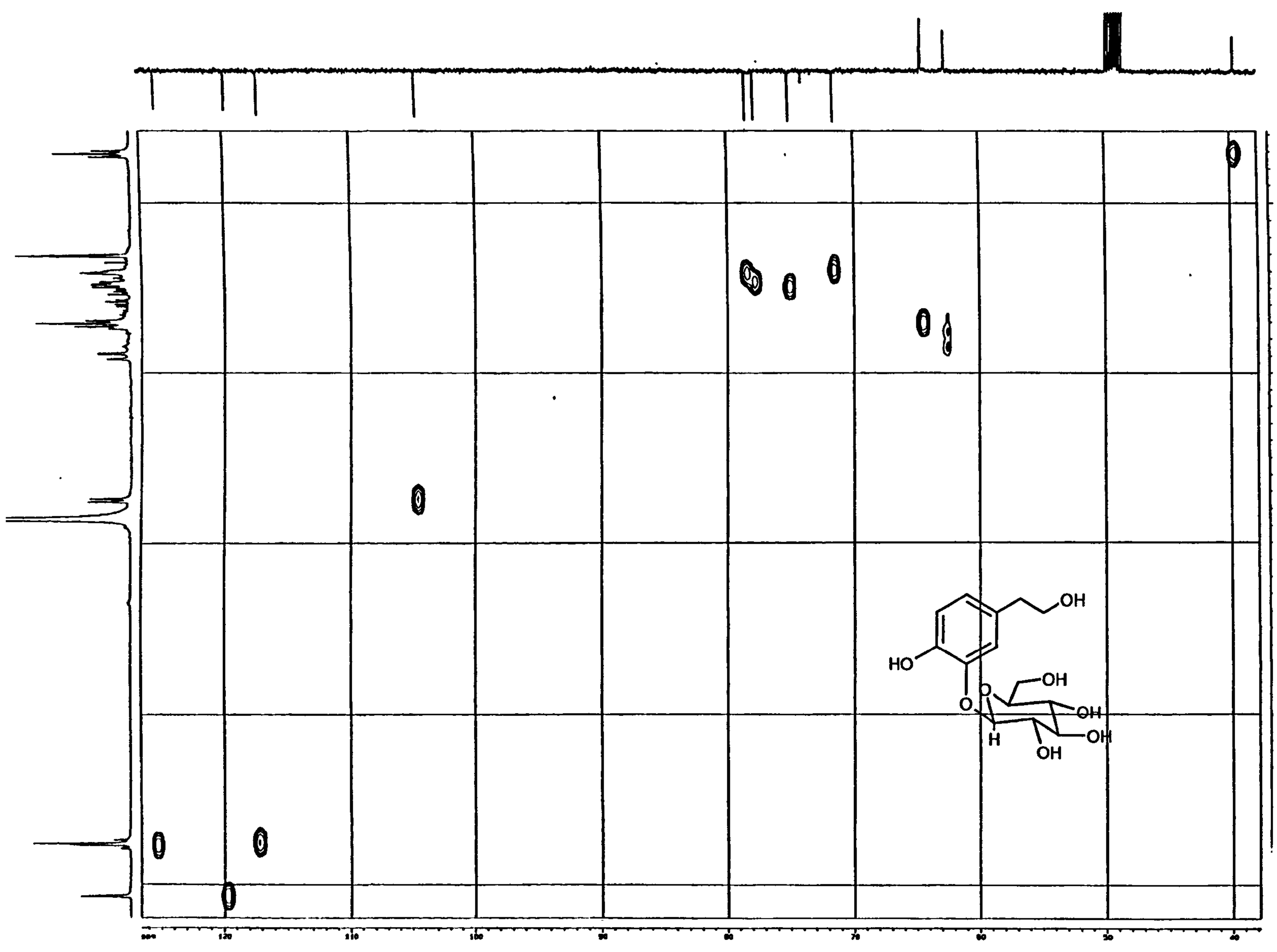


Figure 3.32: HC-COBI NMR spectrum (400 MHz, CD_3OD) of ABFn15

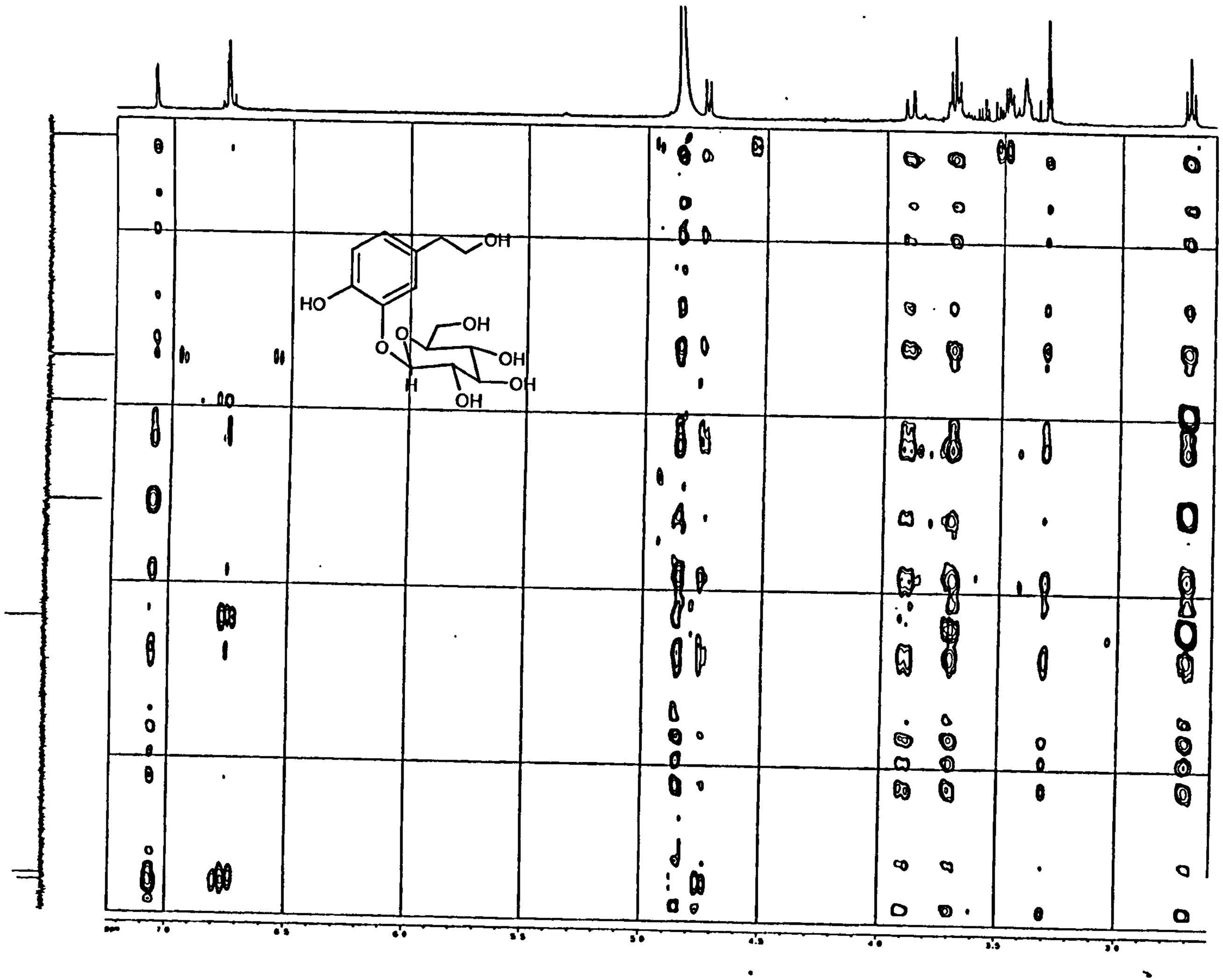


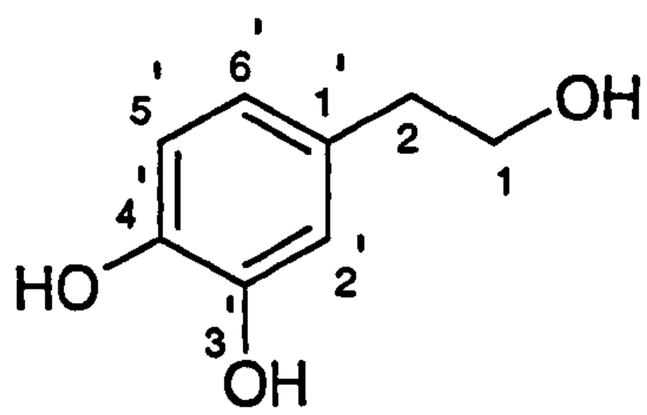
Figure 3.33: HMBC NMR spectrum of AB Fn 15

Figure 3.34: ^{1}H-^{1}H NOESY NMR spectrum (400 MHz, CD_3OD) of ABFn15

3.3.1.2.5 Characterisation of EtOAc (fr 10) as 2-(3',4'-dihydroxyphenyl)ethanol

The compound was isolated as a solid, reacting the same way as AHRM04 on TLC. However, this compound was less polar than AHRM04. The FTIR showed a broad band 3400 $cm^{-1}$and indicating the presence hydroxyl groups. HRMS spectrum displayed an ion at m/z 154.06274 corresponding to the molecular formula $C_8H_{10}O_3$. Other significant ions were at m/z 136.05, 123.04 and 105.032. The UV spectrum showed λ_{max} at 282 nm and a shoulder at 225 nm.

The ^1H NMR spectrum (Figure 3.35) and ^{13}C J-modulated spectrum (Figure 3.36) were similar to those of AHRM04, the only difference being that in this compound the resonances due to the sugar molecule were missing. The ^1H NMR spectrum showed the presence of the 3 aromatic protons and the 2 methylene signals. The ^{13}C J-modulated spectrum showed the 3 quaternary carbon atoms, the 3 aromatic CH's and the 2 methylene signals. The compound was therefore identified as the aglycone of AHRM04, 2-(3',4'-dihydroxyphenyl)ethanol.



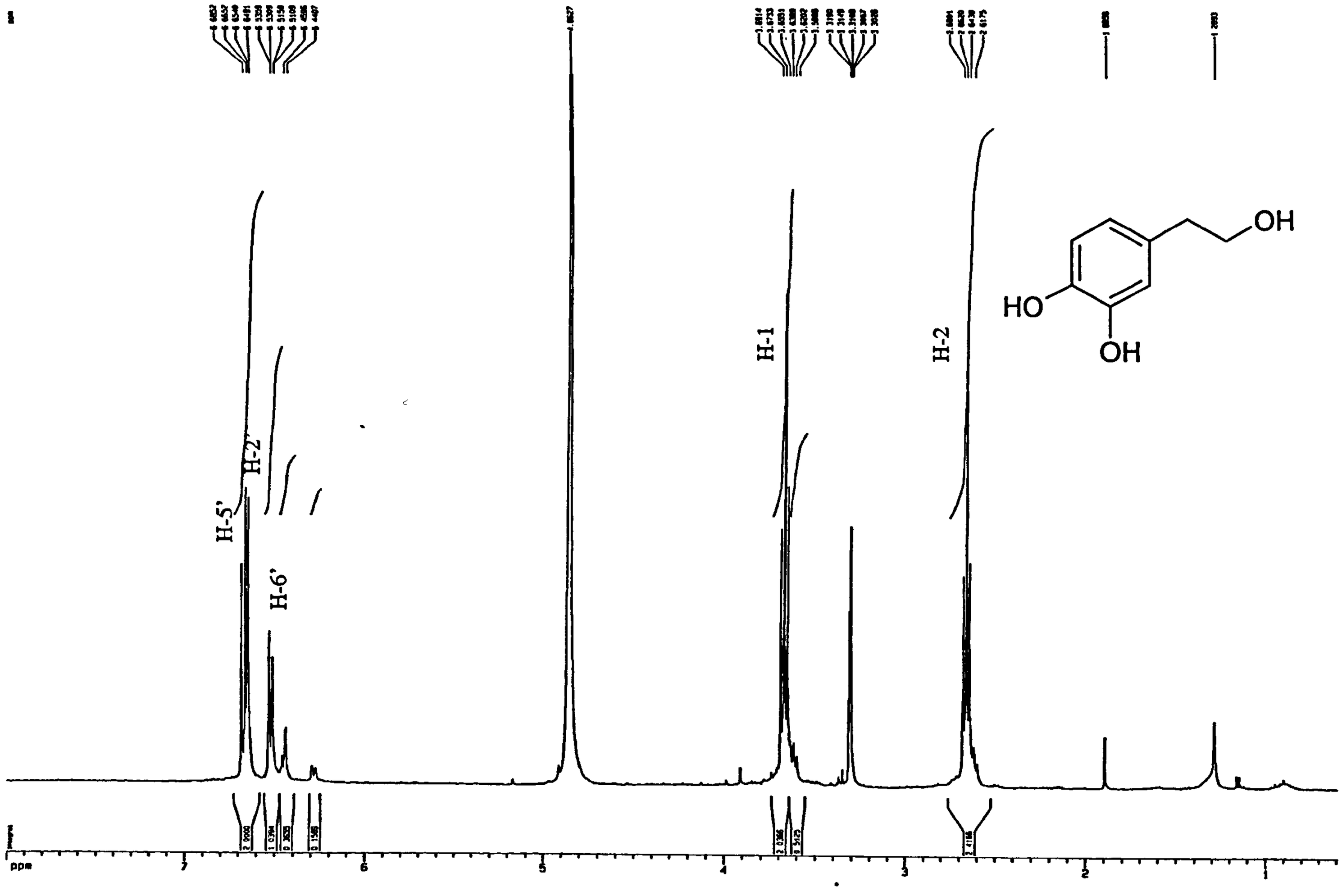


Figure 3.35: ^{1}H NMR spectrum (400 MHz, CD_3OD) of ABFn10

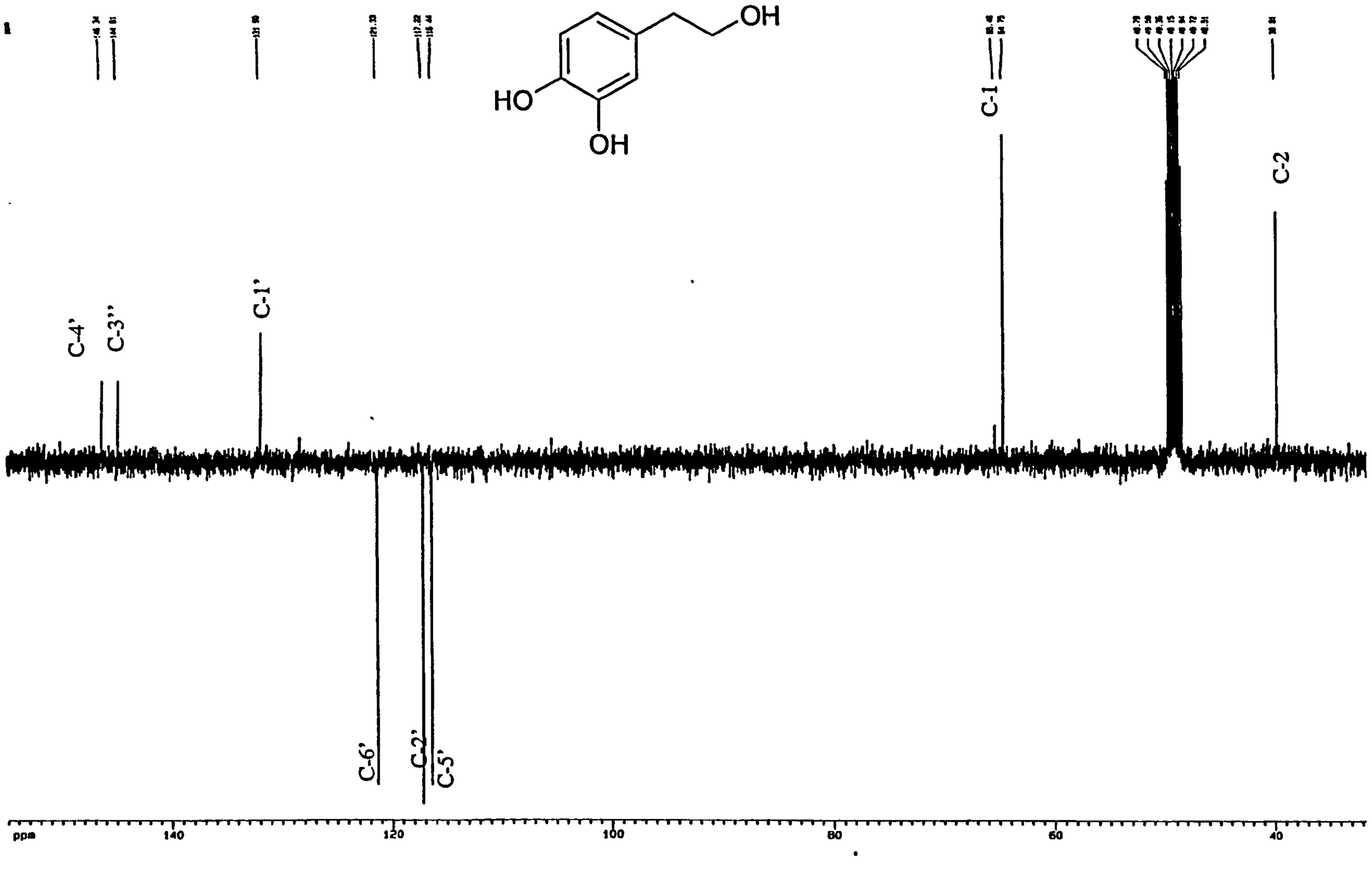
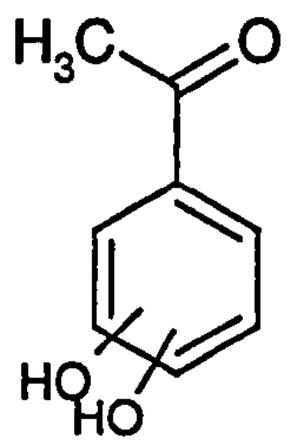


Figure 3.36: ^{13}C NMR spectrum (400 MHz, CD_3OD) of ABFn10

3.3.1.2.6 Attempted characterisation of AHSM03

This compound was isolated from the ethyl acetate fraction. It turned blue then purple on spraying with vanillin sulfuric acid reagent and heating. The FTIR spectrum showed a broad absorption band at 3347 cm^{-1} indicating the presence of –OH functionality and another absorption band at 1671 cm^{-1} suggesting the presence of a carbonyl functional group.

The ^{1}H NMR spectrum (Figure 3.37) displayed the presence of three aromatic protons in an ABX system. The signal for the most deshielded of the three appeared as a *meta* coupled triplet. There was another signal at δ1.56 for a methyl. The signal at δ2.09 is probably from acetone. The yield for the compound could not permit ^{13}C investigation. The signal at δ5.29 suggest the presence of two –OH groups. These data indicate that the compound is a simple phenolic with a methyl, which is not coupling to any other proton. The compound is therefore tentatively identified as a dihydroxy acetophenone.



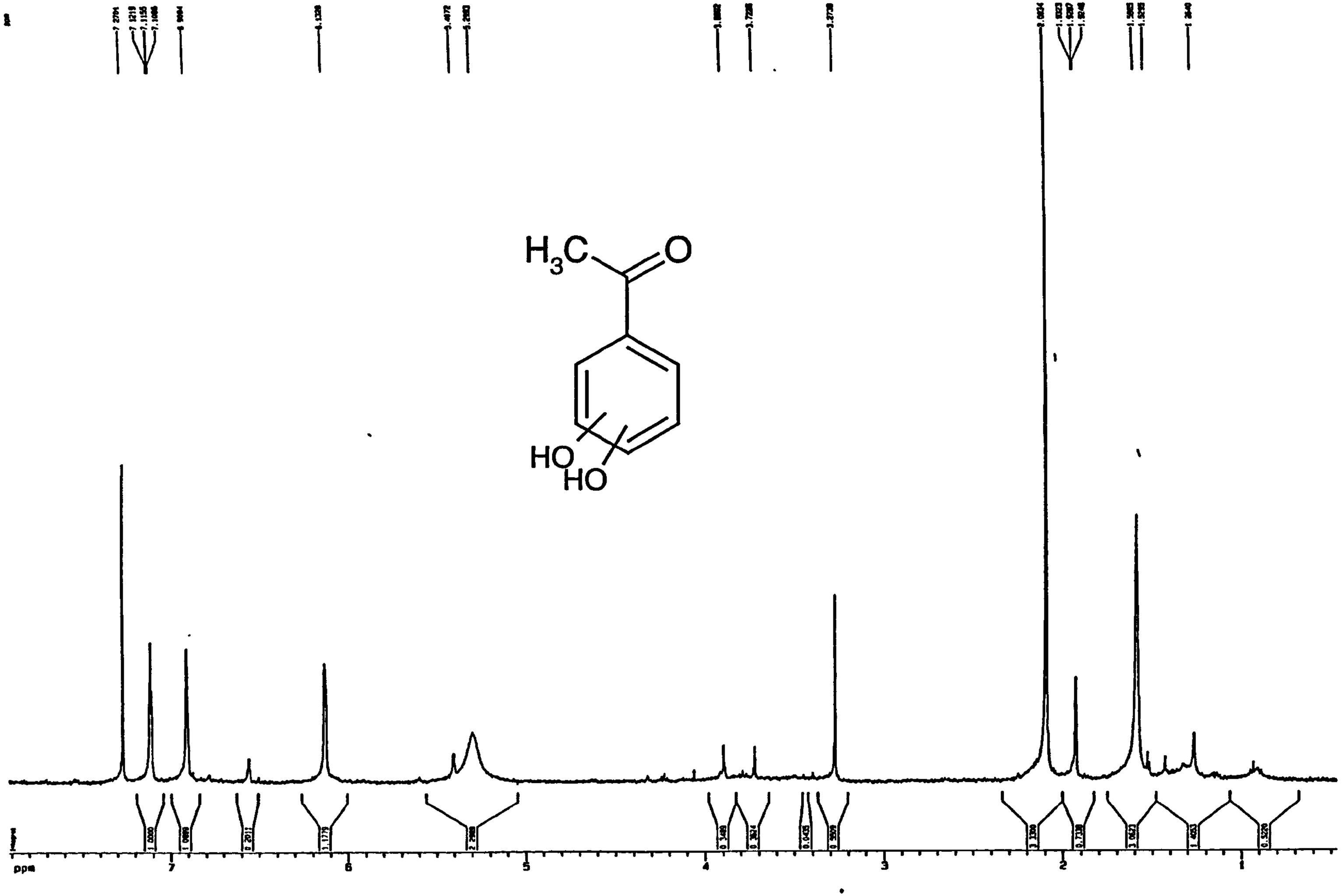


Figure 3.37: ^{1}H NMR spectrum of AHSM03 in $CDCl_3$

3.3.1.3 Methanol Extract

The extract weighed 44.54g. 10g of this was subjected to VLC. The column was eluted first with 50% cyclohexane; increasing proportions of $CHCl_3$ by 25% to 100% to increase the polarity. Methanol was then added to the system by increments of 2.5% to 100%. Fractions eluted with 12.5% MeOH, after further VLC and pTLC yielded more of compound AHSM01 and those eluted 22.5% MeOH yielded more of AHRM04 as a brown solid, 34mg. Fractions eluted with 25% methanol, after being subjected to further column chromatography yielded 18mg of one major compound which showed as a brownish black spot after spraying with vanillin-sulfuric acid reagent and heating. This compound was coded AB FN 41-50. The fractions eluted with 45% showed 1 major compound, which turned black on TLC after spraying with vanillin-sulfuric acid reagent. This was coded AB Fn 64xs.

3.3.1.3.1 Characterisation of ABFn 41-50 as (ξ)-pinoresinol 4-*O*-[α-L-rhamnosyl(1→2) -β-D-glucopyranoside]

This compound was isolated as a clear amorphous solid. The structure of the compound was elucidated based on information provided by NMR spectroscopy only. The experiments carried out were ^{1}H NMR, ^{13}C NMR (both broad band and J-modulated), ^{1}H-^{1}H COSY90, ^{1}H-^{1}H COSYLR, HC-COBI, HMBC, NOESY and HOHAHA (TOCSY), (see section 2.2.3.6).

The ^{1}H NMR spectrum (Figure 3.38 and Table 3.14) displayed resonances between δ3.0 and δ5.0 typical of sugars. The second spin system was observed between δ6.7 and δ7.7, typical of aromatic protons. There were signals for 6 aromatic protons in what appeared to be 1,3,4 trisubstituted benzene ring systems. This information suggested that the compound was a phenolic glycoside and the presence of a methyl doublet at δ1.78 (J=6.0Hz) and a doublet at δ5.65 (J=7.8 Hz) indicated that there were two sugars; rhamnose and glucose respectively. Further examination of the spectrum showed it to be in close agreement with that of a 2,6 diaryl-3,7-dioxabicyclo- [3:3:0]-octane lignan.

Lignans of this structural type have been isolated and characterised before by several authors including Pelter and Ward, (1976) and Anjaneyulu *et al.* (1974). The NMR resonances and therefore structures, in most of the compounds show an interesting symmetrical substitution pattern, asymmetry being observed where there are different substituents on the aryl groups. Though not completely symmetrical, this compound displayed a similar pattern with pairs of close chemical shifts being observed in both the proton and carbon spectra. An initial problem with the structure assignment was that the methylelene protons the lignan in positions 4 and 8 were "buried" within the sugar protons around δ3.9-4.5. This was later clarified by examination of the HMBC (Figure 3.42) and NOESY (Figure 3.43) spectra. The aromatic protons on each ring displayed an ABX spin system. There were signals for 2 methoxys associated with the benzene rings (this was supported by COSY (Figure 3.40) and NOESY spectra).

The ^{1}H NMR spectrum displayed 2 multiplets at δ3.17 and δ3.20, which were assigned to the two ring junction methines, H-1 and H-5 respectively. These assignments were supported by HC-COBI (Figure 3.41) and HMBC spectra. Thus H-1 showed ^{3}J to the carbon atom at δ136.6 assigned to the quaternary carbon of the glycosylated benzene ring and H-5 showed ^{3}J to C-1 (136.8) of the other aromatic ring. H-1 showed NOESY correlation with 2 aromatic protons at δ6.99 (H-6') and δ7.18 (H-2'). H-5 showed similar NOESY correlations to H-6" (δ7.10) and H-2" (7.27). H-6' showed a ^{3}J correlation to C-4' (δ147.4) which in turn showed a ^{3}J interaction to H-1 of the glucose, thus showing that the glucose is attached to the aromatic ring at C-4'. This was further supported by COSYLR correlation of H-5' with the anomeric proton of the glucose. H-1 and H-5 appeared as multiplets due to coupling with each other, oxymethylenes (CH_2-8/CH_2-4) and methines (H-2 and H-6). The latter were assigned to the doublets at δ4.93 (J=5.0 Hz) and δ4.97 (J=5.0Hz) respectively. These protons showed NOESY correlations to H-4 and H-8 (δ4.02). The chemicals shifts for the benzylic protons H2/H6 are in accordance with that reported in which the two aryl substituents are in the equatorial position (Pelter, 1976, Lee *et al.*, 1999).

The aromatic protons for the benzene ring bearing the glucose were assigned the signals at δ7.56, doublet, (J=8.4Hz) for H-5' (*ortho* coupled to H-6') which was the most deshielded of the aromatic protons. H-2' appeared as a *meta* coupled doublet at δ7.18 (J=1.7Hz). One methoxy δ3.93 showed ^{3}J to the C-3'(δ151.1). This pattern was also observed for the aromatic protons of the other benzene ring (Table 3.14). The protons of the methoxy at δ3.83 showed ^{3}J interaction to C-3'' (δ149.4).

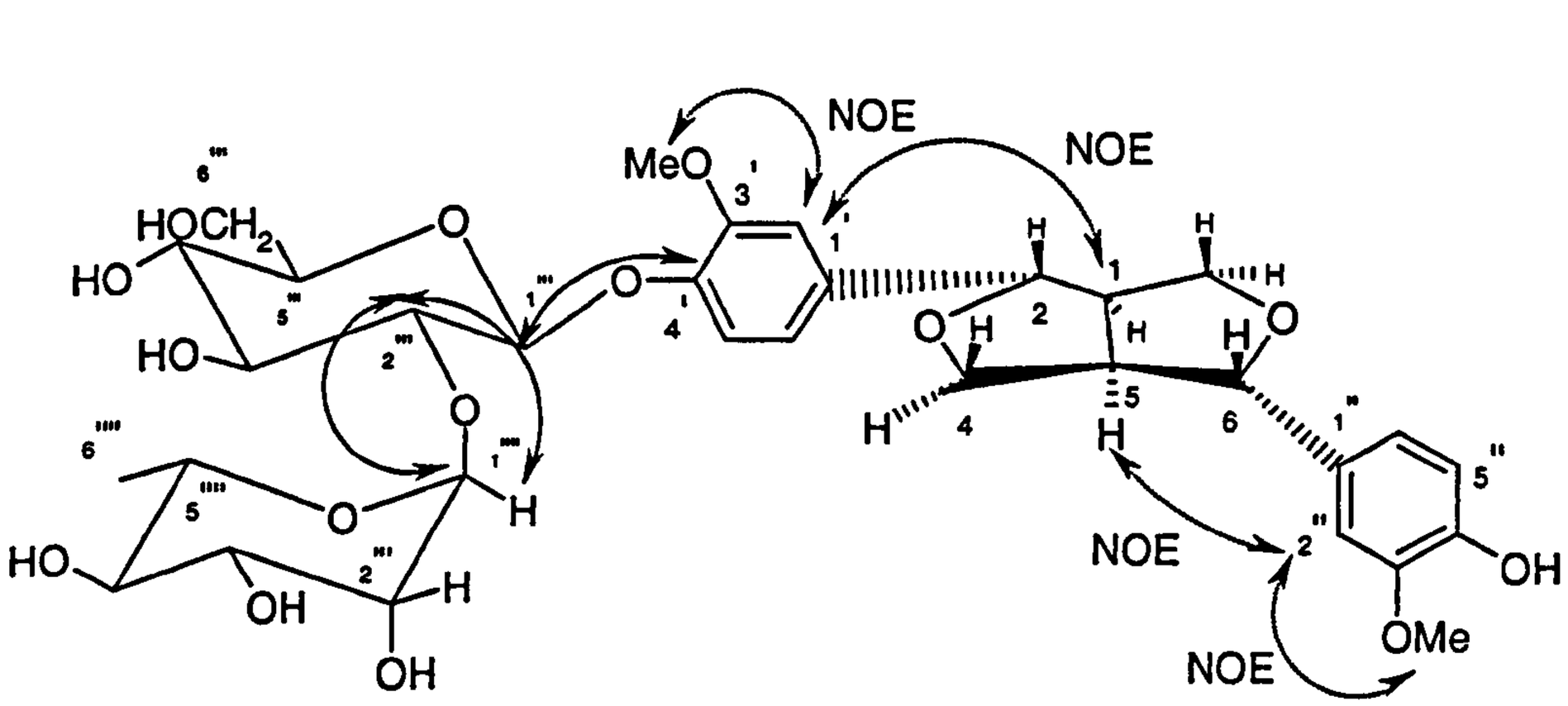
The J-modulated ^{13}C spectrum exhibited 32 carbon signals, those for the aglycone showing pairs of carbons with close chemical shifts. There were 3 methylene signals; 2 very close ones for the lignan oxymethylenes and 1 for the glucose methylene. The signals for the methoxys were observed at δ56.4 and δ56.5, almost as one signal.

In the ^{1}H-^{1}H COSY the glucose anomeric proton showed correlation toH-2 at δ4.65. This latter proton showed correlation in the HMBC to the glucose anomeric carbon (^{2}J) at δ100.7 and also to another carbon (^{3}J) at δ102.3, the rhamnose anomeric carbon, C-1'''' (see Figure 3.42). This data thus indicated that the rhamnose was attached to the glucose at C-2 of the glucose. This was further revealed by the COSY, NOESY and HMBC spectra. H-1 of the rhamnose showed ^{3}J correlation to C-2 of the glucose and H-2 (δ4.65) of the glucose also to C-2 of the rhamnose. Further, H-1 of the rhamnose showed NOESY and COSYLR correlations to H-2 of the glucose.

^{1}H-^{1}H COSY data further provided coupling information that was useful in assigning the rest of the protons for the sugars (Table 3.14). The information provided by the TOCSY (Figure 3.43) spectrum further simplified the assignment of these signals as athe individual spin systems for each part of the molecule's sugar moieties were separated from each other. The anomeric proton of the glucose showed correlation to a triplet at δ4.65 assigned to H-2 of the glucose. This in turn showed coupling to another triplet at δ4.39 assigned to H-3 of the glucose. H-3 showed coupling to a triplet at δ4.25 attributed to H-4 and this in turn showed coupling to a multiplet at δ4.04 assigned to H-5 of the glucose molecule. H-5 showed coupling to another multiplet at δ4.36 assigned to H-6 of

the glucose. HC-COBI, NOESY and HMBC data were also used to confirm these assignments. As expected there was no correlation between H-5/H-6 to H-1 in the TOCSY.

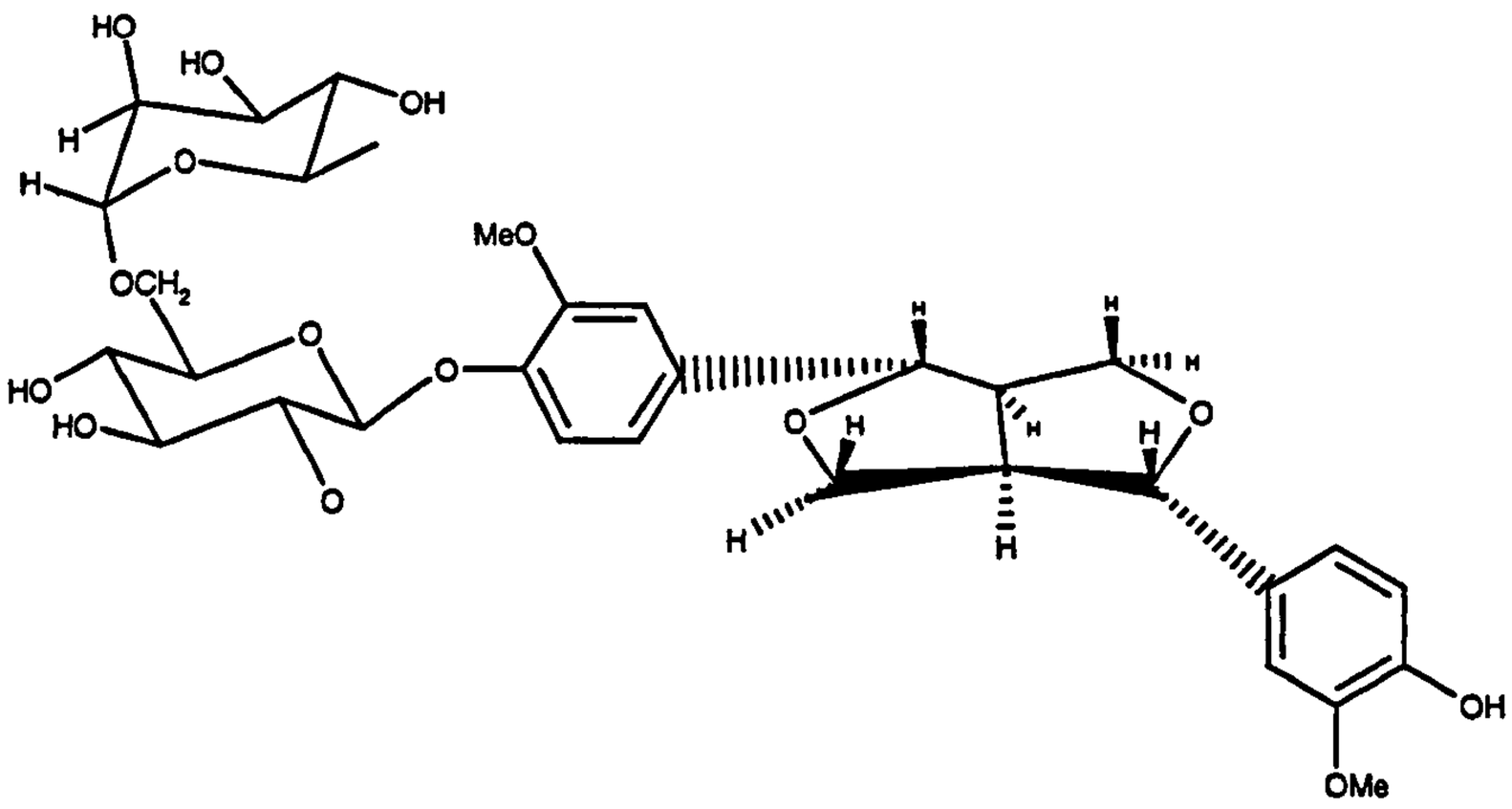
Simillar couplings were observed for the rhamnose; H-1 coupled with a doublet of doublets at δ4.81 assigned to H-2, which in turn coupled with another doublet of doublets at δ4.62, assigned to H-3. H-3 showed coupling to a doublet of doublets at δ4.39 attributed to H-4. The doublet of quartets at δ5.07, assigned to H-5 of the rhamnose, showed coupling to the methyl doublet at δ1.78. Based on these data, the compound was thus identified as 6-(4-hydroxy-3-methoxyphenyl)2-[3-methoxy-4-(α-L-rhamnopyranosyl{1→2}-β-D-glucopyranosyloxy)phenyl]3,7-dioxabicyclo[3:3:0]-octane or (ξ)-pinoresinol 4-*O*-[α-L-rhamnosyl(1→2)-β-D-glucopyranoside], the structure of which is shown below including some of the important correlations. Arrows on the structure indicate ^{3}J HMBC and NOESY correlations.



ξ= not assigned

This appears to be a new natural product. Having searched the literature databases such as Beilstein, the Dictionary of Natural Products etc. two similar compounds were discovered that are both pinoresinol derivatives. These were: rhamnosyl (1→6) glucoside

from *Haplophyllum versicolor* (versicoside, **174,** Batirov, *et al.*, 1986) and glucosyl (1→2) rhamnoside (hibiscuside, **175,** from *Hibiscus syriacus*, Lee *et al.*, 1999). This latter reference, Lee, *et al.* (1999), was consulted and it appears that hibiscuside was incorrectly named in the paper because the structure they present in the paper is actually (ξ)-pinoresinol 4-*O*-[α-L-rhamnosyl(1→2)-β-D-glucopyranoside], the same as the compound reported here. These authors state that the HMBC gave correlation between H-2 of glucose and C-1 of rhamnose, which were also observed with the present compound. It therefore appears that the present discovery of (ξ)-pinoresinol 4-*O*-[α-L-rhamnosyl(1→2)-β-D-glucopyranoside], is in fact hibiscuside. The biological activity of this compound as an anti-oxidant has been reported (Lee, *et al.*, 1999, see section 3.4.1).



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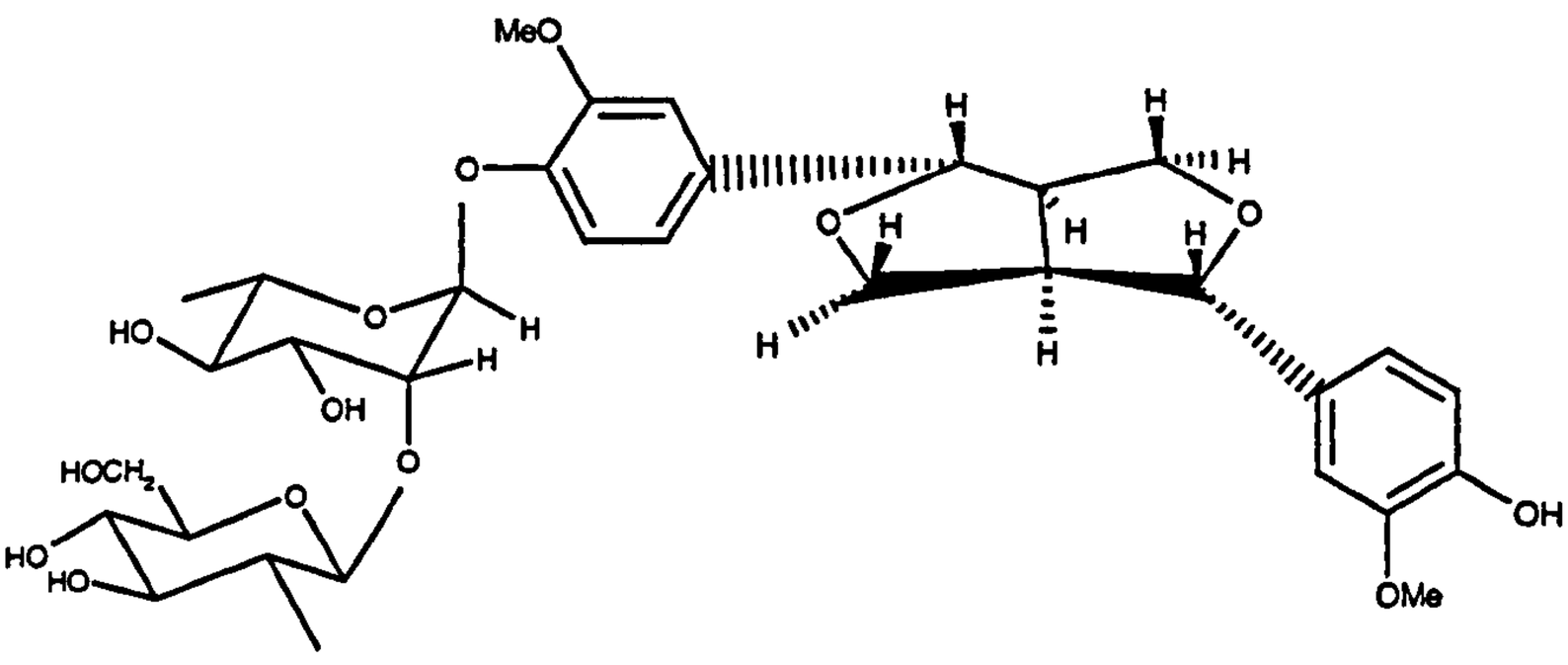


175

Table 3.14: ^{1}H and ^{13}C NMR 400 MHz data for AB Fn 41-50 in C_5D_5N

Posisition		^{1}H δ(ppm)	^{13}C δ(ppm)
C-1	CH	3.17, m	55.2
C-2	CH	4.93, d, (J=5.0)	86.7
C-4	CH_2 β	4.02, dd (J=9.0, 4.2)	72.5
	α	4.30, dd (J=9.0, 7.3)	
C-5	CH	3.24, m	55.3
C-6	CH	4.97, d, (J=5.0)	86.9
C-8	CH_2 β	4.02, dd, (J=9.0, 4.2)	72.5
	α	4.40, dd (J=9.0, 7.3)	
C-1'	C	-	136.8
C-2'	CH	7.18, d (J=1.6)	111.3
C-3'	C	-	151.1
C-4'	C	-	147.4
C-5'	CH	7.56, d (J=8.4)	117.1
C-6'	CH	6.99, dd, (J=8.4, 1.6)	119.1
OCH_3	CH_3	3.93	56.4
C-1"	C	-	133.7
C-2"	CH	7.27, d, (1.6)	111.5
C-3"	C	-	149.4
C-4"	C	-	148.4
C-5"	CH	7.28, d, (J=8.3)	117.0
C-6"	CH	7.10, dd, (J=8.3,1.6)	120.3
OCH_3	CH_3	3.83	56.5
Glucose moiety			
C-1'''	CH	5.65, d (J=7.8)	100.7
C-2'''	CH	4.65, t (J=8.8, 3.0)	77.4
C-3'''	CH	4.39, t (J=8.8, 3.2)	80.3
C-4'''	CH	4.25, t (J=9.1)	72.0
C-5'''	CH	4.04, m	79.2
C-6'''	CH_2 a	4.36, m	62.76
	b	4.51, dd (J=11.9,2.0)	
Rhamnose moiety			
C-1''''	CH	6.58, br singlet	102.3
C-2''''	CH	4.81, dd, (J=3.2, 1.3)	74.7
C-3''''	CH	4.62, dd (J=8.5, 3.2)	73.1
C-4''''	CH	4.39, dd, (J=8.5,9.5)	74.7
C-5''''	CH	5.07, dq, (J=9.5, 6.0)	70.4
C-6''''	CH_3	1.78, d (J=6.0)	19.5

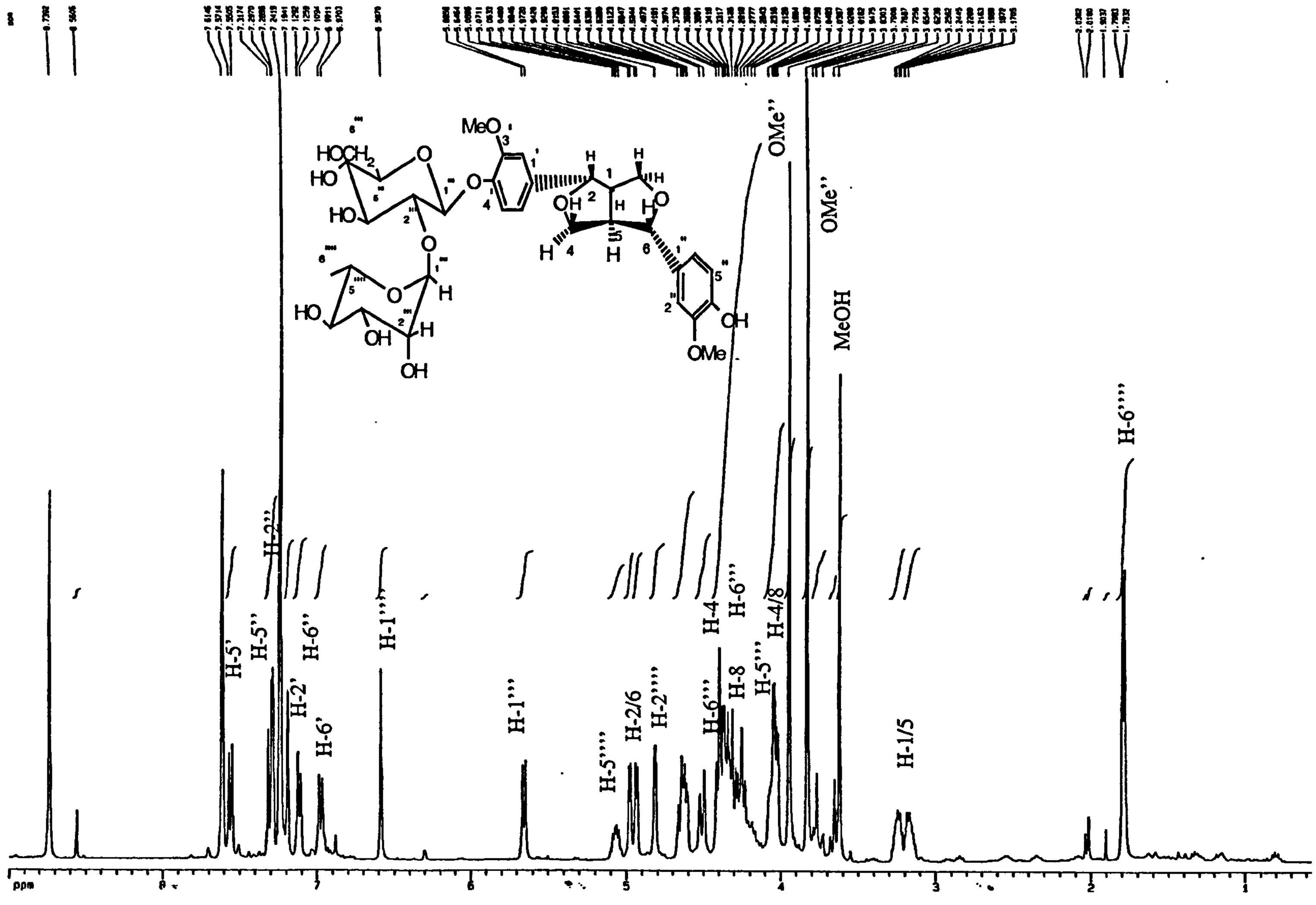


Figure 3.38: ^{1}H NMR spectrum (400 MHz, C_5D_5N) of ABFn41-50

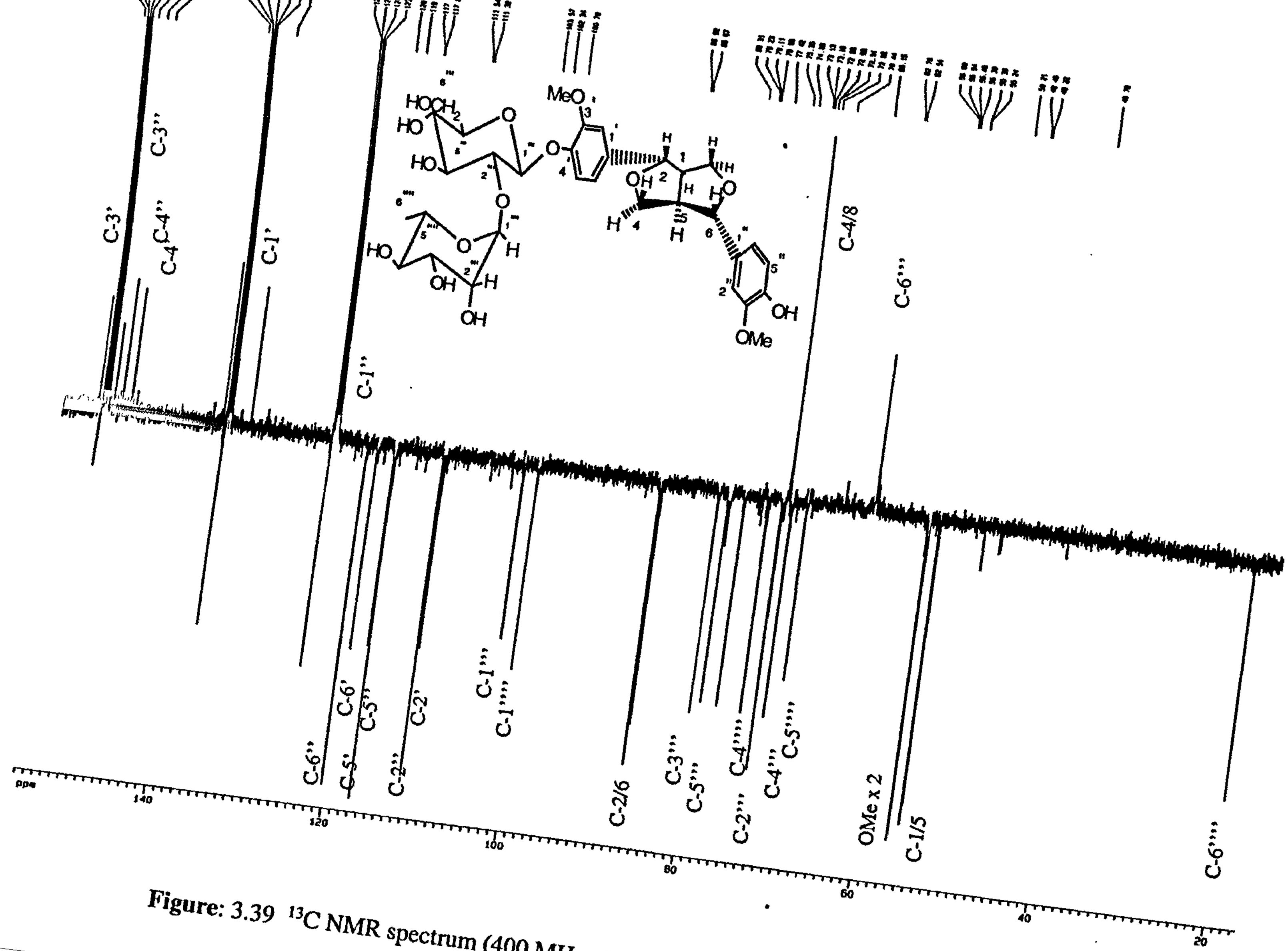


Figure: 3.39 ^{13}C NMR spectrum (400 MHz, C_5D_5N) of ABFn41-50

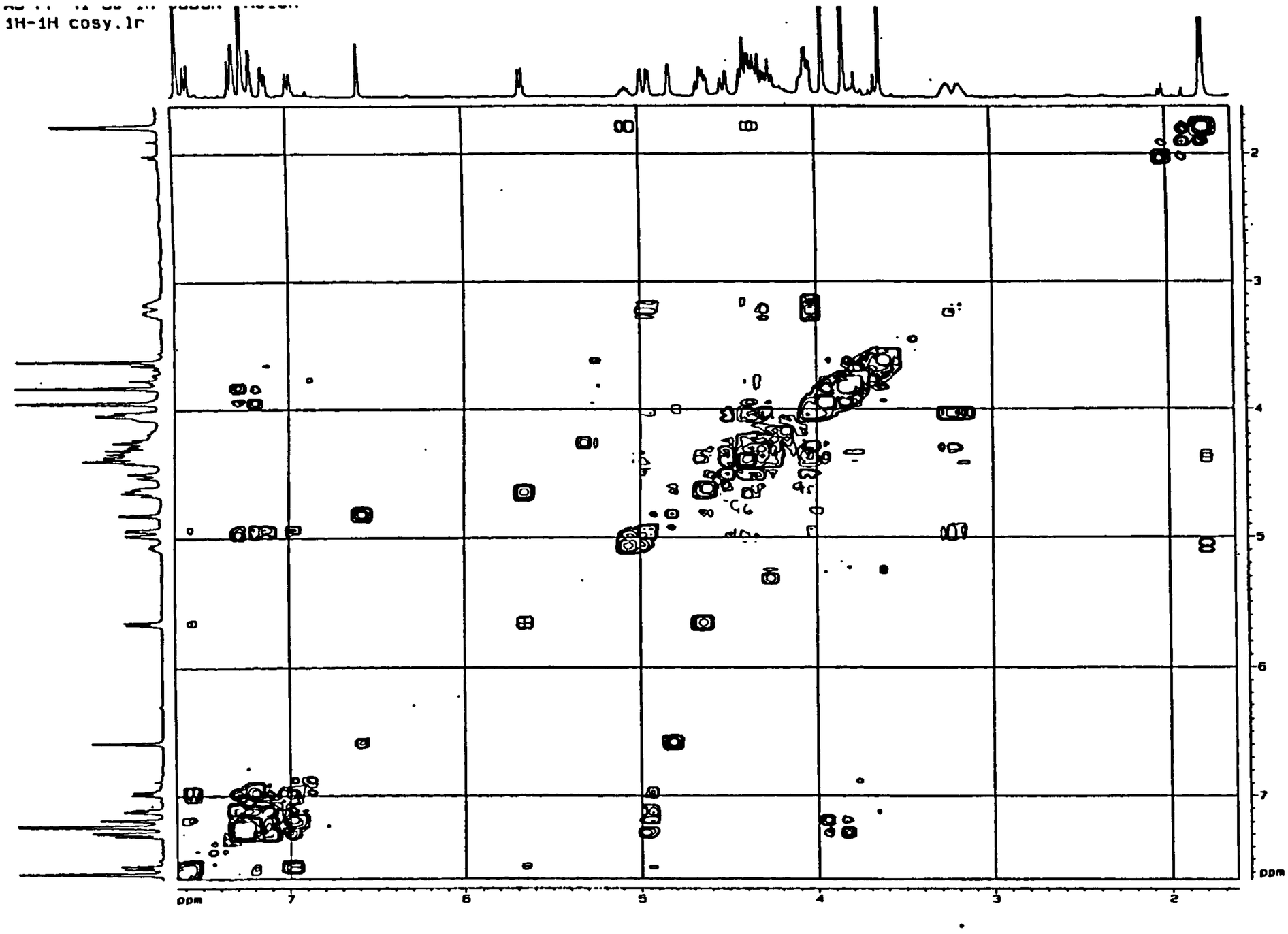


Figure: 3.40 ^{1}H-^{1}H COSYLR NMR spectrum (400 MHz, C_5D_5N) of ABFn41-50

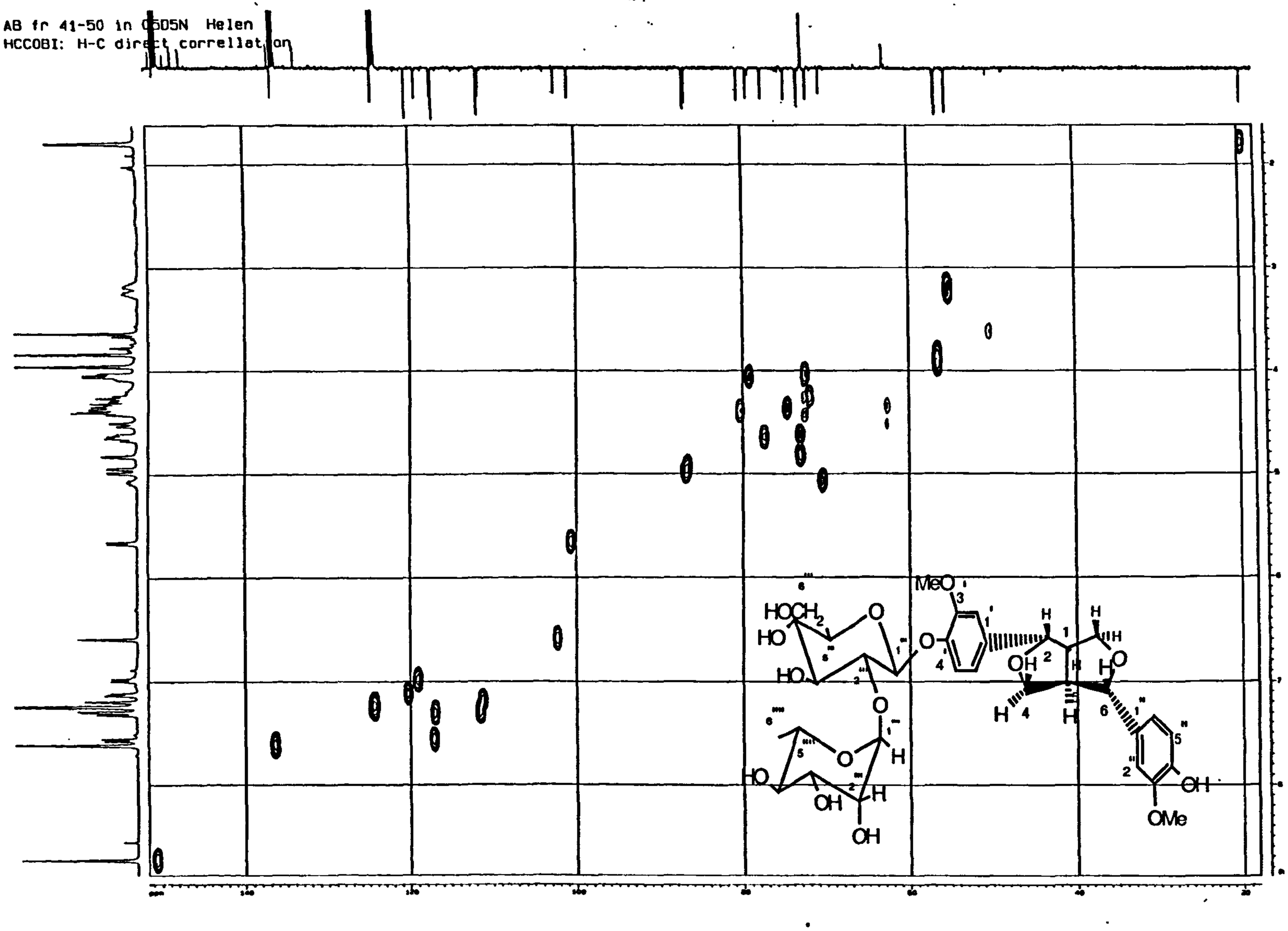


Figure 3.41: HC-COBI NMR spectrum (400 MHz, C_5D_5N) of ABFn41-50

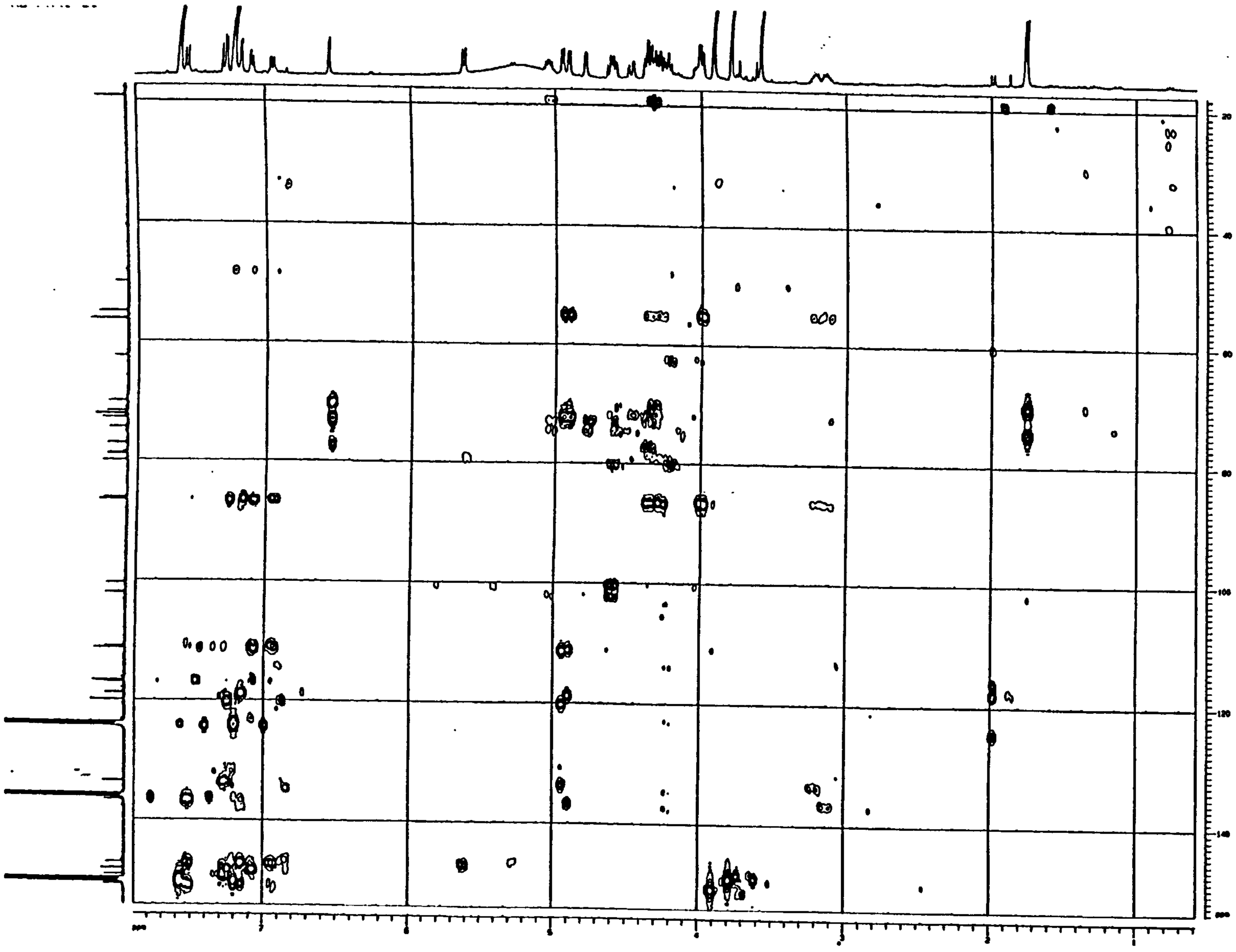


Figure 3.42: HMBC NMR spectrum (400 MHz, C_5D_5N) of ABFn41-50

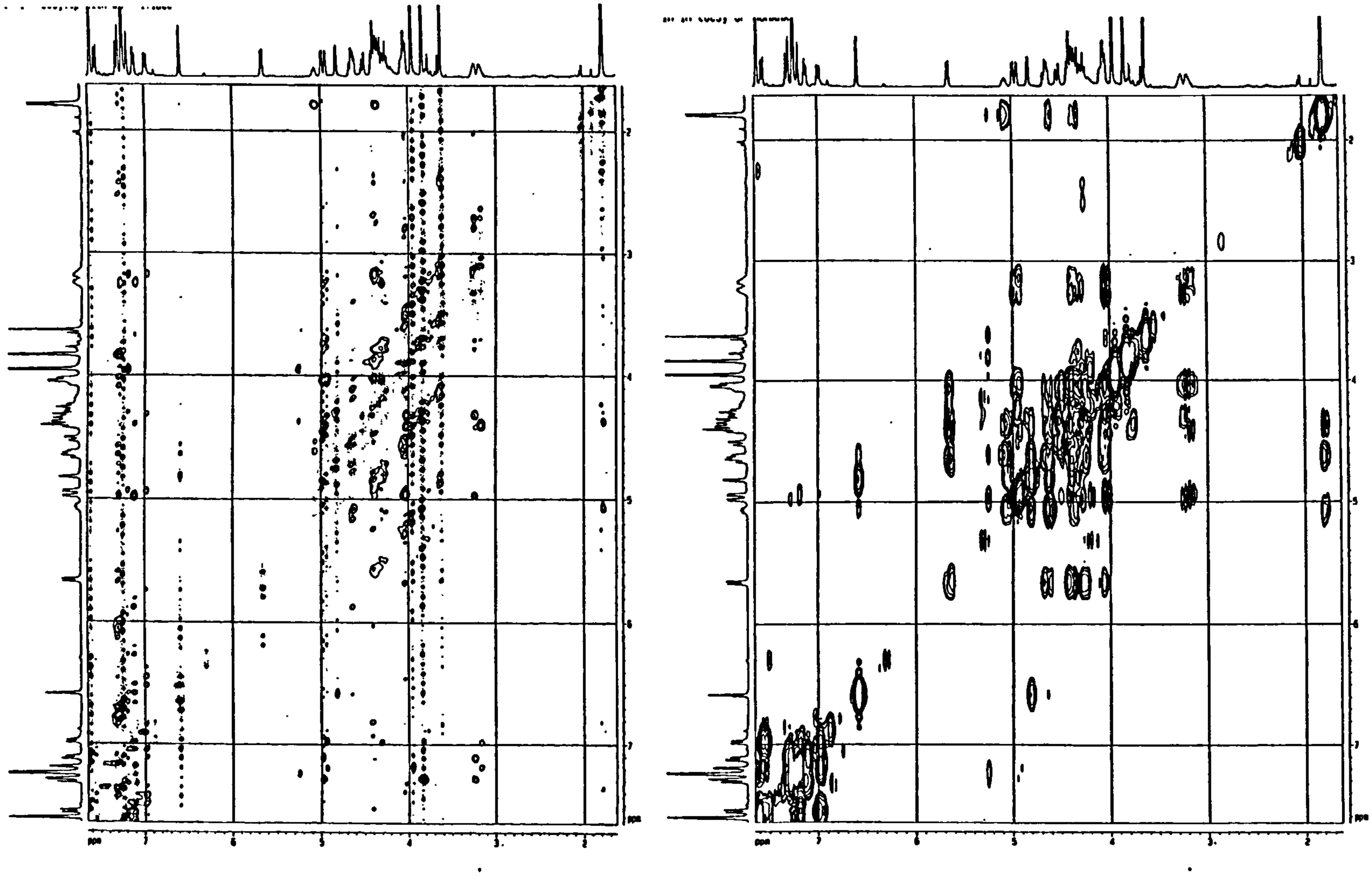


Figure 3.43: ^{1}H-^{1}H NOESY and TOCSY NMR spectra (400 MHz, C_5D_5N) of ABFn41-50

3.3.1.3.2 Characterisation of ABFn64xs as a mixture of sucrose and another sugar(s)

The ^{1}H NMR and ^{13}C NMR spectra (Figures 3.44 and 3.45) indicated the presence of sucrose evidenced by the presence of the doublet for the anomeric proton of glucose at δ5.90 in the proton spectrum and the signal at δ99.9 for the anomeric carbon in the J-modulated spectrum. The quaternary carbon for the fructose moiety appeared at δ103.9 in the J-modulated spectrum. The ^{13}C NMR spectrum however showed the presence of 24 carbon atoms and thus indicating that there were two other hexose sugars in addition to sucrose.

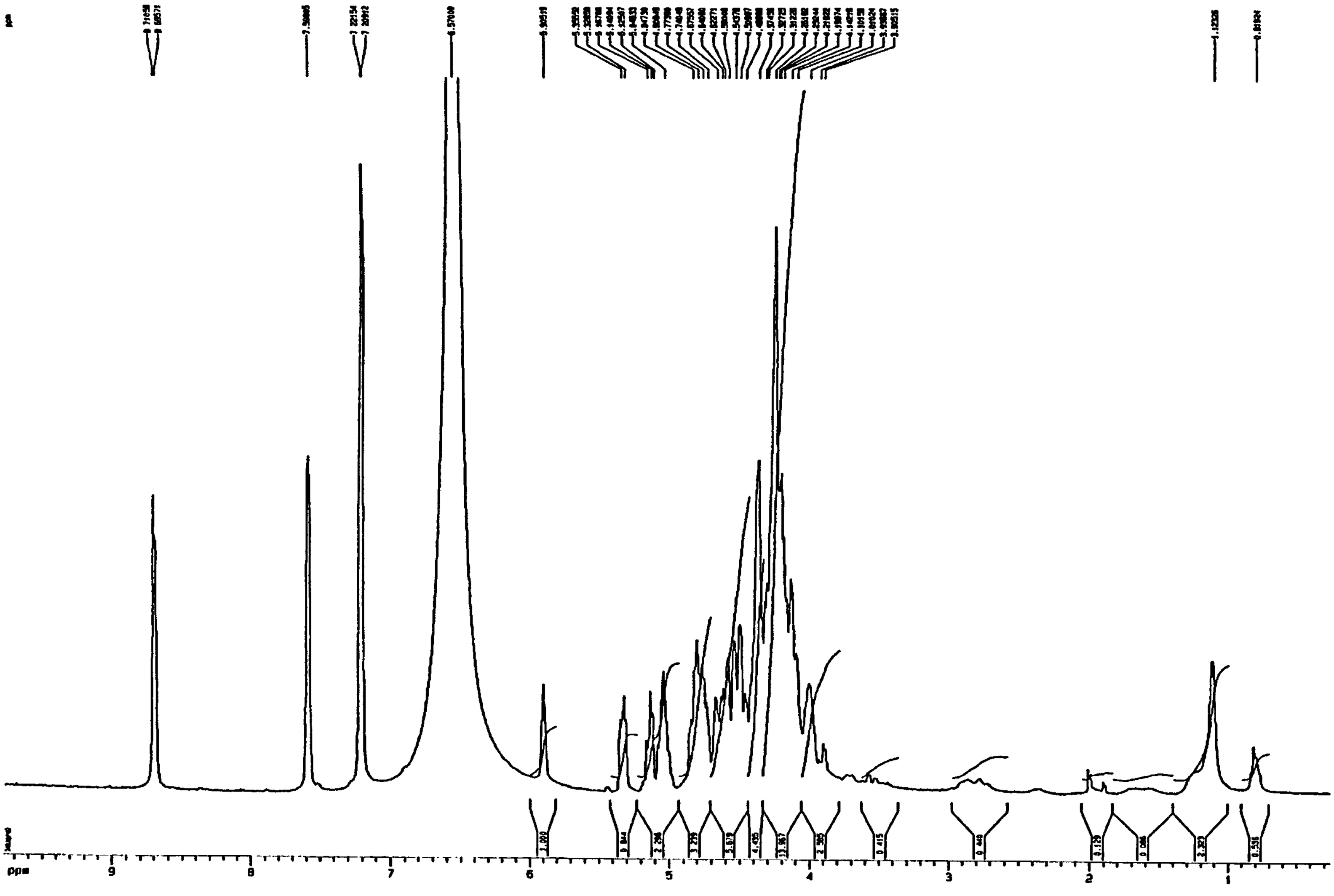


Figure 3.44: ^{1}H NMR spectrum (400 MHz, C_5D_5N) of AB fn64xs

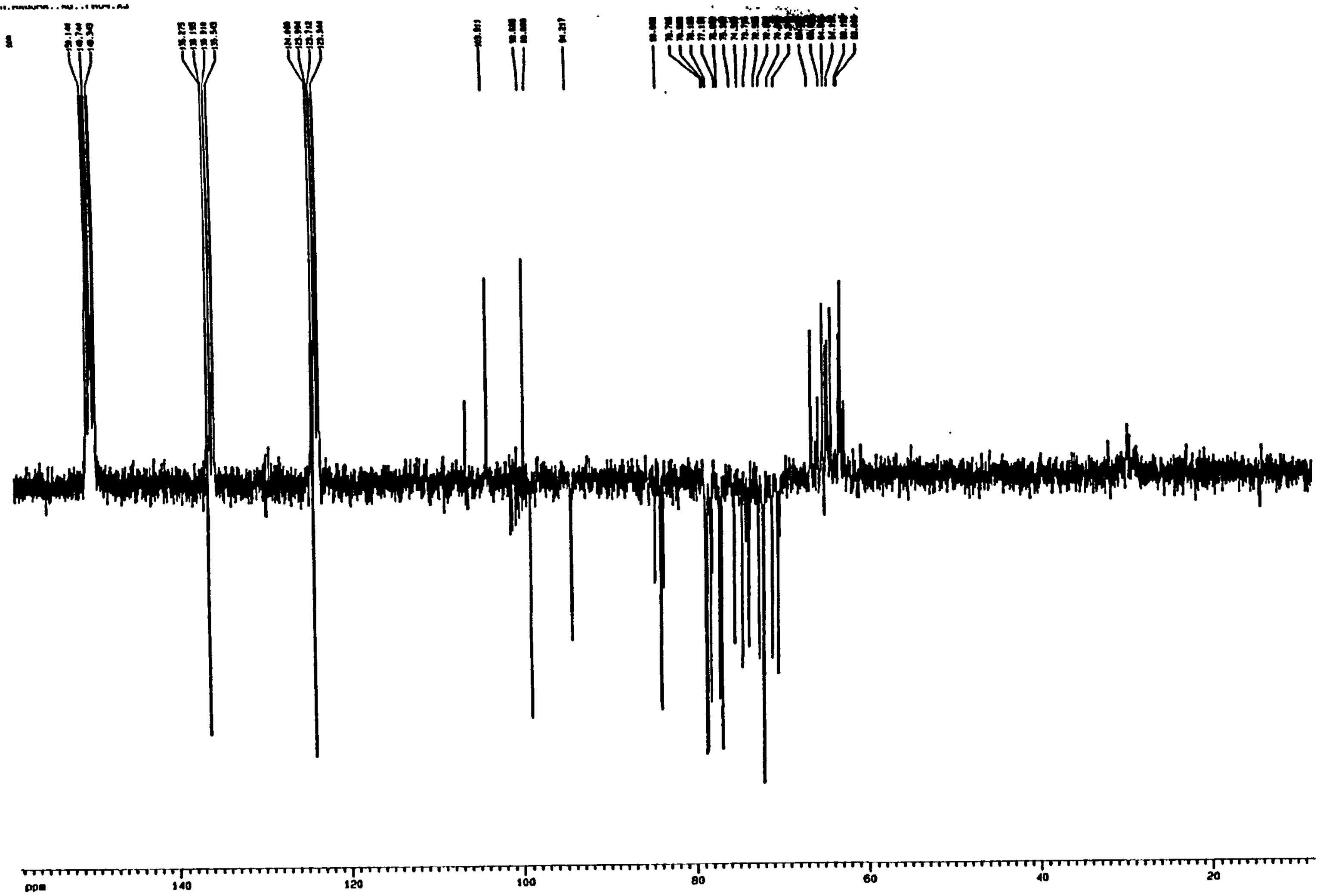


Figure 3.45: ^{13}C NMR spectrum (400 MHz, C_5D_5N) of AB64xs

3.3.2 *Enicostemma axillare*

The plant is being investigated chemically for the first time but the genus has been shown to produce a wide range of substances (see section 1.6.6.1). Whole plant material was powdered and extracted for 48 hours with dichloromethane and to exhaustion with methanol. The extraction, fractionation and isolation of compounds is summarized in the following diagram.

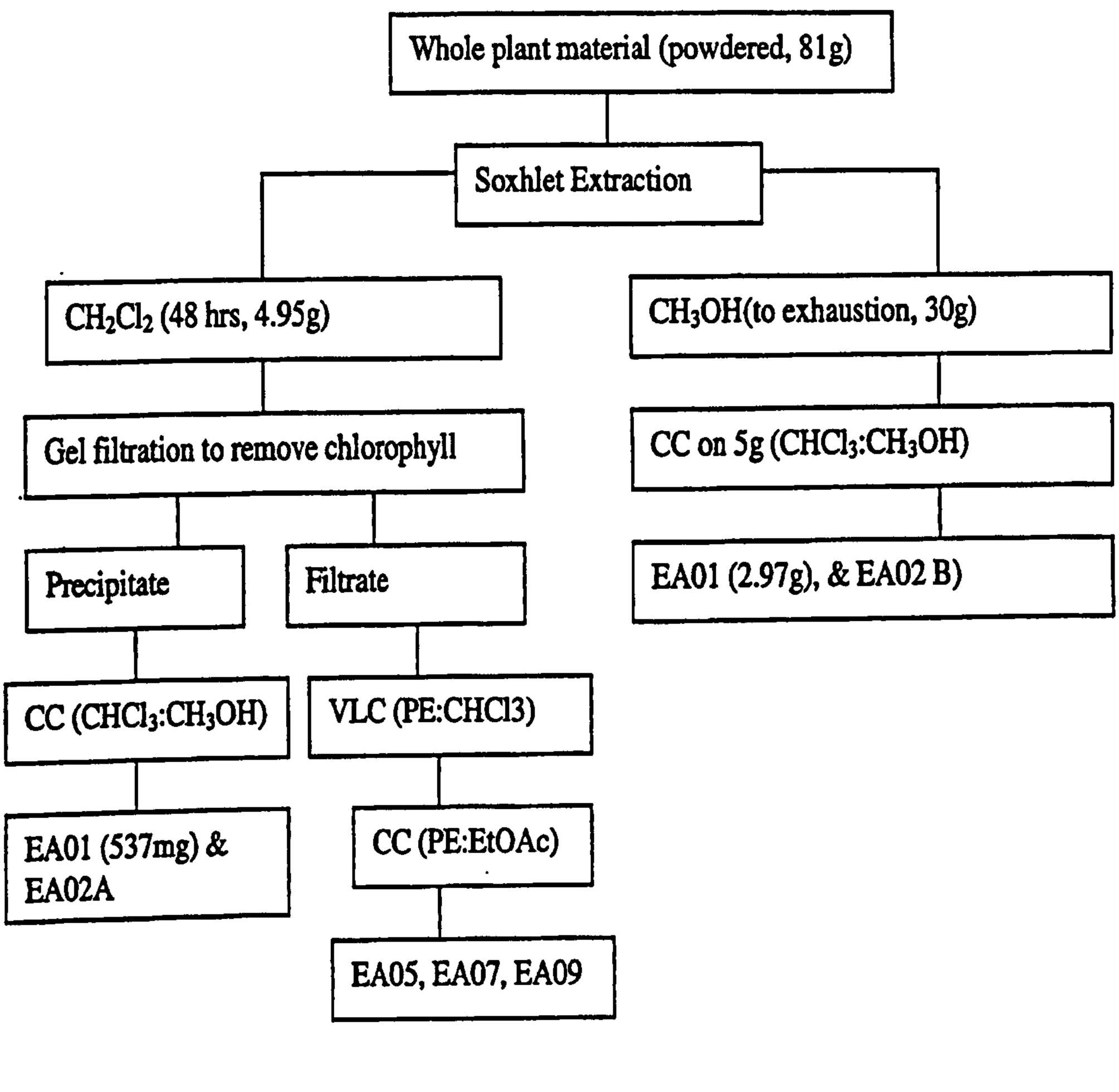


Figure 3.46: Extraction and fractionation scheme for *E. axillare* plant material

3.3.2.1 Investigation of the Dichloromethane extract

Chlorophyll was removed by CC on Sephadex LH-20 (50cm x 2.5 cm i.d.) using 30% DCM in cyclohexane as the eluting solvent. Compound EA01 precipitated from the solvent during rotary evaporation and was subjected to further purification by CC on silica gel 60 (0.063-0.200 mm particle size, 48.5 cm x 2.2 cm i.d) with 500 ml each of 10, 15 and 20% MeOH in $CHCl_3$ in 50 ml fractions. Fractions containing EA01 were pooled and the solvent removed. EA01 (537.9mg) was collected, as an amorphous white solid. Compound EA02A was also isolated during this separation as colourless crystals.

The filtrate was concentrated under reduced pressure and subjected to VLC eluting with 2.5% increments of chloroform in petroleum ether. Fractions that showed major compounds were pooled accordingly and subjected to CC employing gradient elution with petroleum ether in ethyl acetate. Compounds EA04, EA05, EA06, EA08, EA09 and EA10 were isolated.

3.3.2.1.1 Characterisation of EA01 as swertiamarin

The compound melted at 124-126°C and had an $[\alpha]_D$ of –127 measured in methanol. On TLC, this compound turned purple and then to dark brown after spraying with vanillin-sulfuric acid reagent and heating. It was UV active and the FTIR spectrum showed a very broad absorption band at 3397 cm^{-1} indicating the presence of hydroxyl groups. The spectrum also showed a peak at 1701 cm^{-1} indicating the presence of carbonyl functionality. The HRMS spectrum showed molecular ion at m/z 375.1 and another at m/z 194 due to the loss of the glucose component.

The structure was determined on the basis of ^1H NMR, ^{13}C J-modulated, ^{13}C DEPT 135, ^1H-^1H COSY90, HCCOBI and HMBC NMR. The ^1H NMR spectrum (Figure 3.47 and Table 3.14) displayed resonances characteristic of a sugar molecule, thus indicating that the compound was a glycoside. The spectrum showed the presence of two methylene signals and six other protons besides the sugar signals. The ^{13}C J-modulated spectrum

(Figure 3.48 and Table 3.15) displayed the presence of ten carbons beside those of the sugar. HCCOBI spectrum (Figure 3.49) provided information as to which protons were attached to which carbons (Table 3.16). In the ^{1}H NMR spectrum, the singlet at δ7.61 was attributed to H-3, which does not couple to any other proton. The doublet at δ5.96 was attributed to H-1 which shows axial-equatorial coupling to H-9 (J=1.4 Hz). H-8 appeared at δ5.36 as a doublet of doublets of doublets, this being due to *trans* coupling to one of the protons at C-10 (J=17.1 Hz), *cis* vicinal coupling to the other C-10 proton (J=8.1 Hz), and coupling to H-9. The exomethylene protons at C-10 appeared at δ5.29 as a multiplet, which is a result of *trans* coupling of one of them with H-8 and vicinal coupling of the other with H-8. Other important signals were those at δ4.52 and δ4.40 attributed to the protons at C-7, one of which was being deshielded by the oxygen and therefore appearing downfield to the other. The signal at δ3.03 was assigned to the proton at C-9, which appeared as a doublet of doublets being split by H-8 (J=7.9 Hz) and H-1 (J=1.4 Hz). The methylene resonance around δ1.80 was assigned to the protons at C-6, one of which appeared at δ1.74 and the other at δ1.82. The two also showing as non-equivalent because of the deshielding effect exerted by the oxygen atom of the –OH group at C-5.

HMBC (Figure 3.50) correlations are summerised at Table 3.15. These were useful in the assignment of the quaternary carbons and in supporting other carbon and proton assignments. H-3 showed ^{3}J correlation to C-5 and C-11. Other protons which showed ^{3}J to the carbonyl carbon were those at C-7. C-5 showed ^{3}J correlations to H-1, H-3 and the two protons at C-7. C-4 showed ^{3}J correlation to H-9 and to the protons at C-6. C-10 showed ^{3}J correlation only to H-9, this supported its position as an exomethylene carbon and the fact that it was much downfield for a CH_2 carbon.

COSY correlations (Figure 3.51) were also used to assist in assigning the protons. These were shown by H-1 to H-9, H-8 to H-9 and C-10 protons and by the protons at C-6 to those at C-7.

The DEPT135 experiment was carried out to help distinguish quaternary carbons from methylene carbons. In the spectrum (Figure 3.52) the quaternary carbons C-4, C-5 and C-11 are visibly missing while the CH_2 carbons are present.

^{13}C J-modulated spectrum showed 6 signals for the sugar moiety, which was identified as β-D-glucose. The β-linkage was evidenced by the fact that the anomeric proton, the doublet at δ4.64 showed a large coupling constant of J=7.9 Hz. The anomeric proton showed ^{3}J to C-1 of the aglycone and thus indicating the sugar attachment. The HCCOBI spectrum was used to assist in assigning the carbons and protons of the sugar molecule.

The compound was identified as swertiamarin, the structure of which is shown below. The assignments were in agreement with published values for the compound (Ma *et. al.*, 1994).

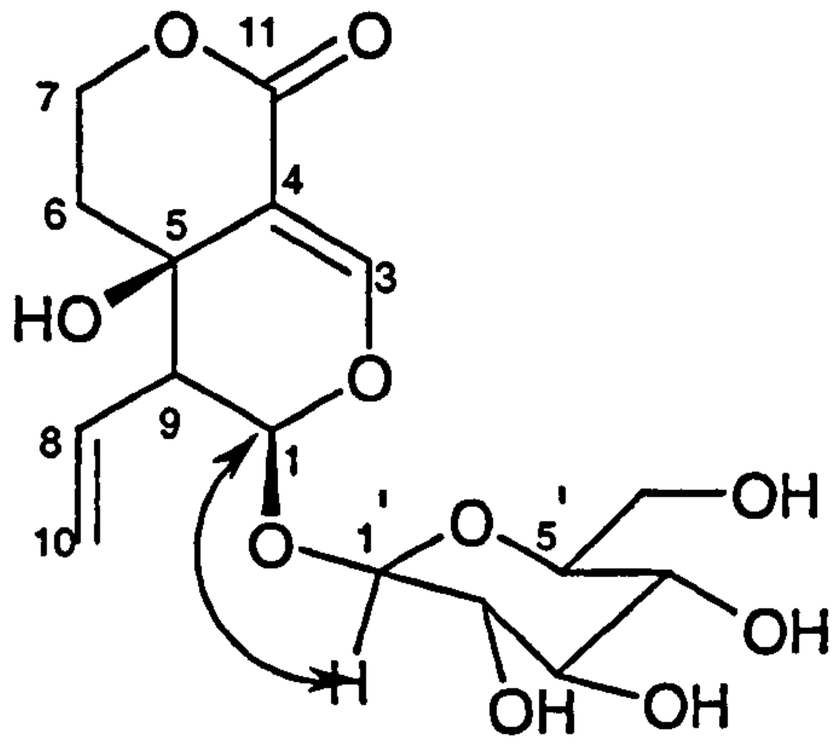


Table 3.15: ^{1}H and ^{13}C NMR (400 MHz) data for swertiamarin in CD_3OD

Posisition	^{1}H δ(ppm)	^{13}C δ(ppm)
C-1	5.72, d (J=1.4)	99.6
C-3	7.63, s	154.9
C-4	-	109.0
C-5	-	64.5
C-6 (a)	1.90, m	33.9
C-6 (b)	1.74, m	
C-7 (a)	4.32, dd (J=5.0, 1.5)	66.8
C-7 (b)	4.35, dd (J=5.0, 1.5)	
C-8	5.45, ddd (J=17.1, 8.1, 8.1)	133.9
C-9	2.91, dd (J=7.9, 1.4)	52.1
C-10 (a)	5.30, m	121.0
C-10 (b)	5.40, m	
C-11	-	168.2
Sugar moiety		
C-1'	4.64, d (J=7.9)	100.4
C-2'	3.37	71.6
C-3'	3.35	73.1
C-4'	3.31	77.9
C-5'	3.28	78.7
C-6'	3.89, dd	62.7

Table 3.16: HCCOBI (O, Direct) and HMBC (X, long-range carbon-proton) correlations for swertiamarin aglycone

H/C	1	2	3	4	5	6	7	8	9	10	11
H-3			O	X	X				X		X
H-1		O		X		X					
H-8	X					X			O	X	
H-10(a)									X	X	O
H-10(b)									X	X	O
H-7(b)			X	X	O						X
H-7(a)			X	X	O						X
H-9	X		X	X					X	O	X
H-6(a)			X		O	X					
H-6(b)			X		O	X					

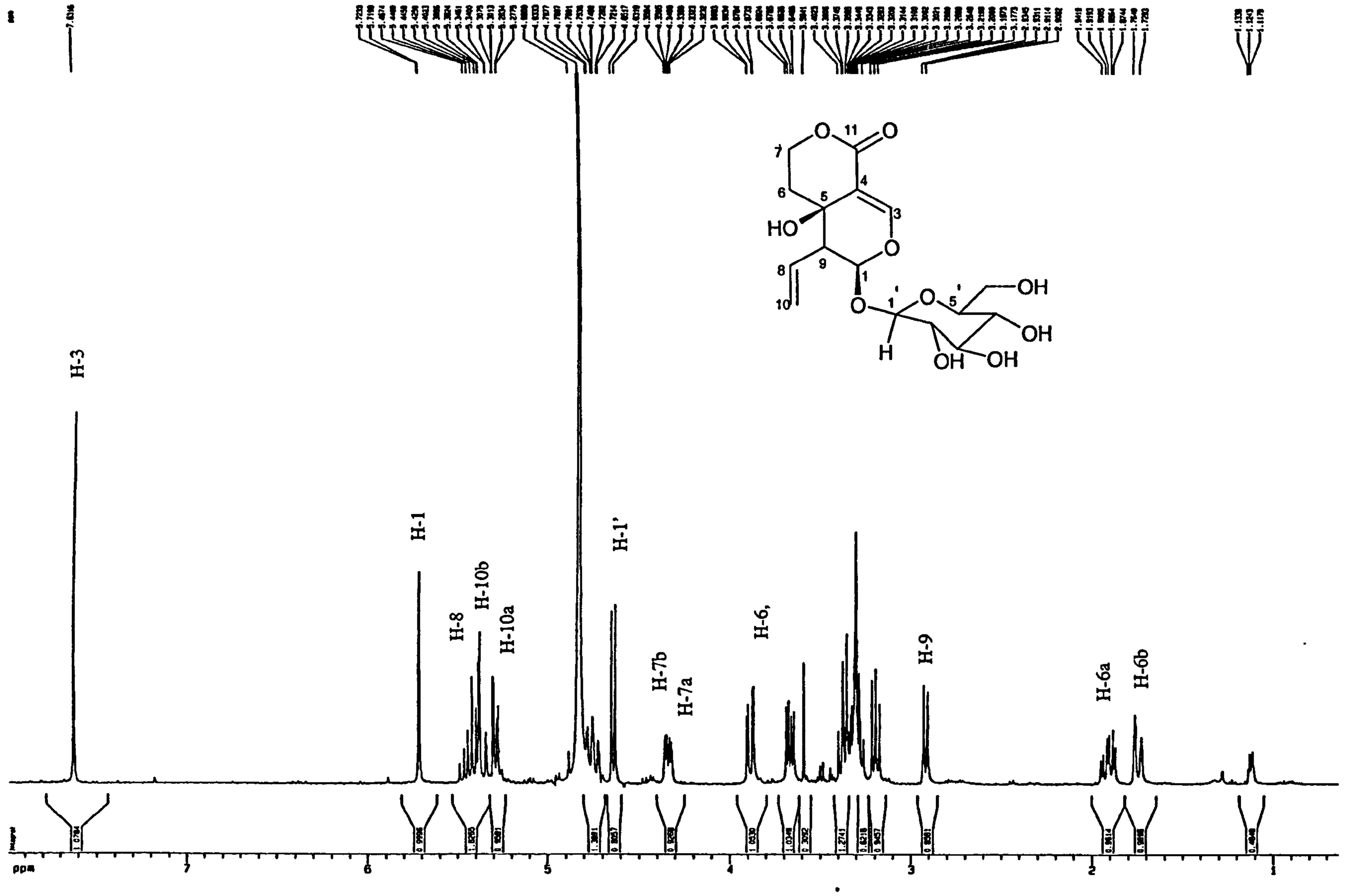


Figure 3.47: ^{1}H NMR spectrum (400 MHz, CD_3OD) of EA01

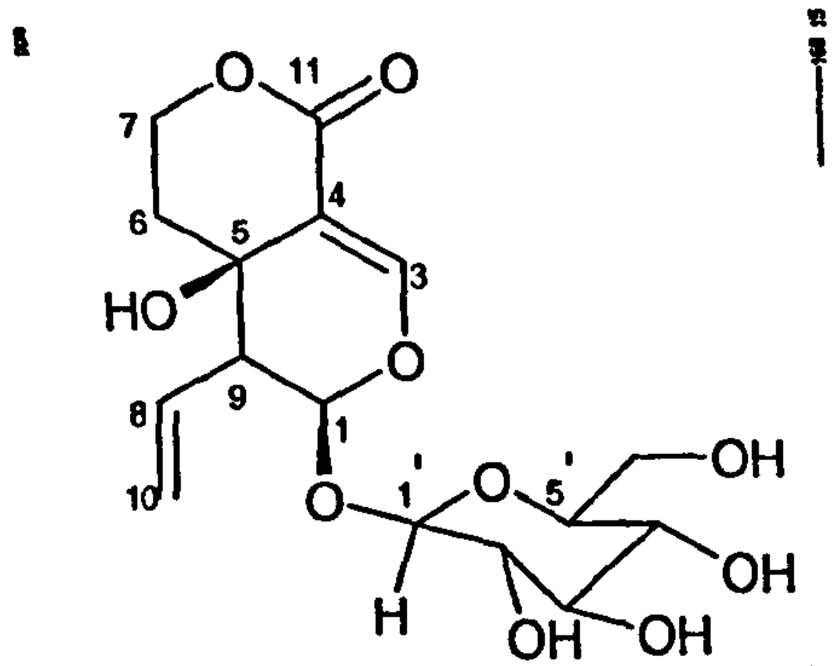


Figure 3.48: ^{13}C NMR spectrum (400 MHz, CD_3OD) of EA01

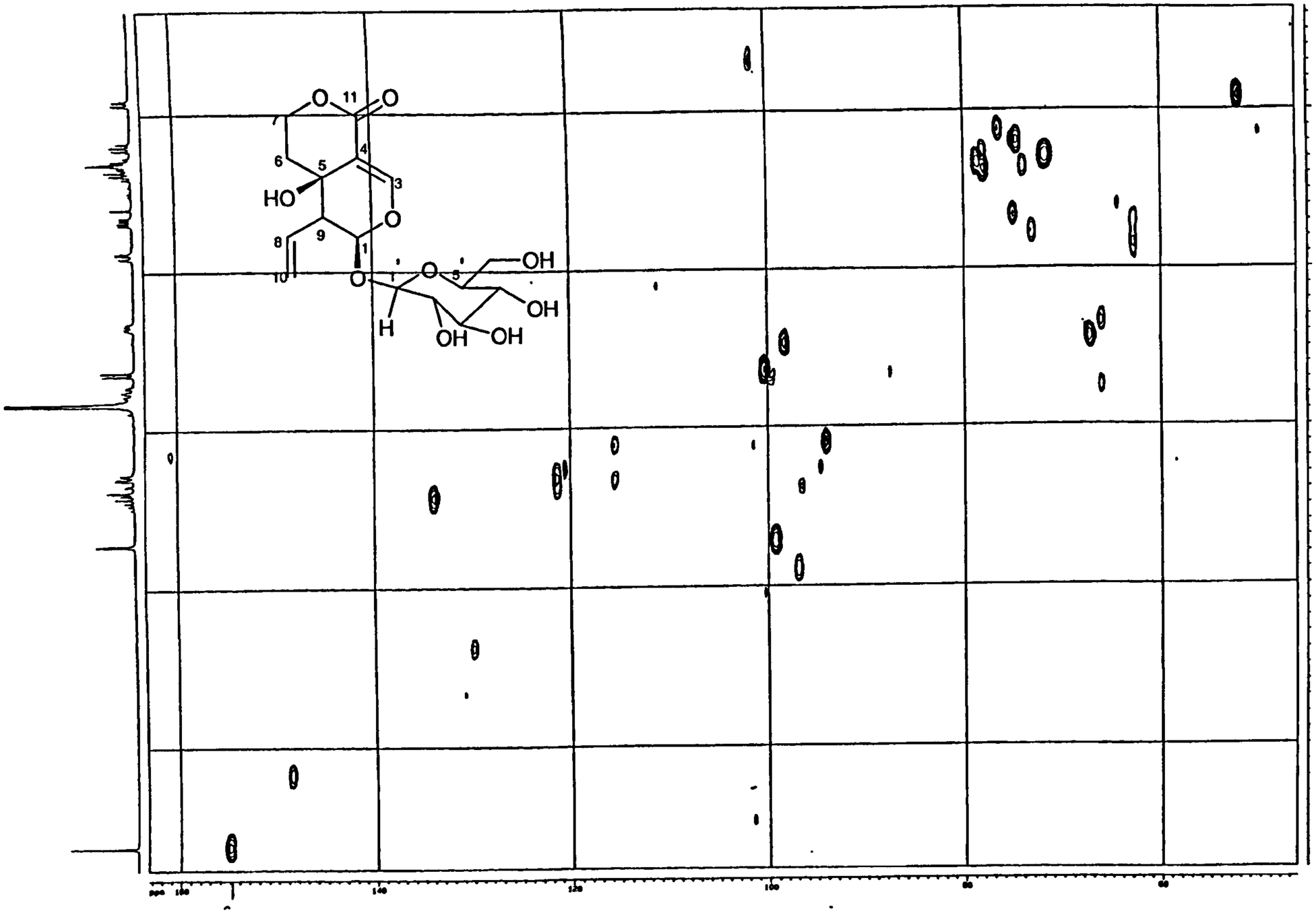


Figure 3.49: HCCOBI NMR spectrum of EA01

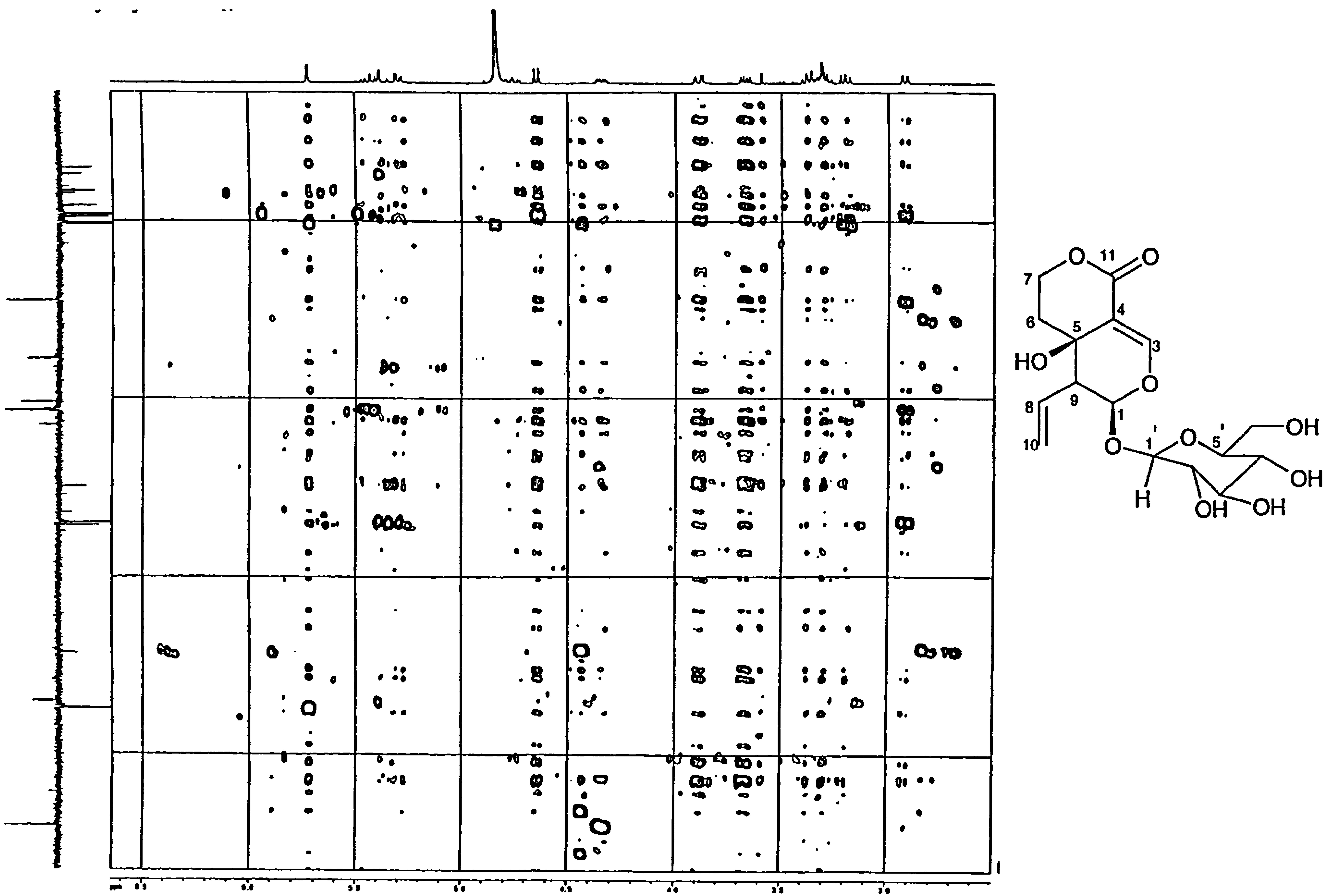


Figure 3.50: HMBC NMR spectrum of EA01

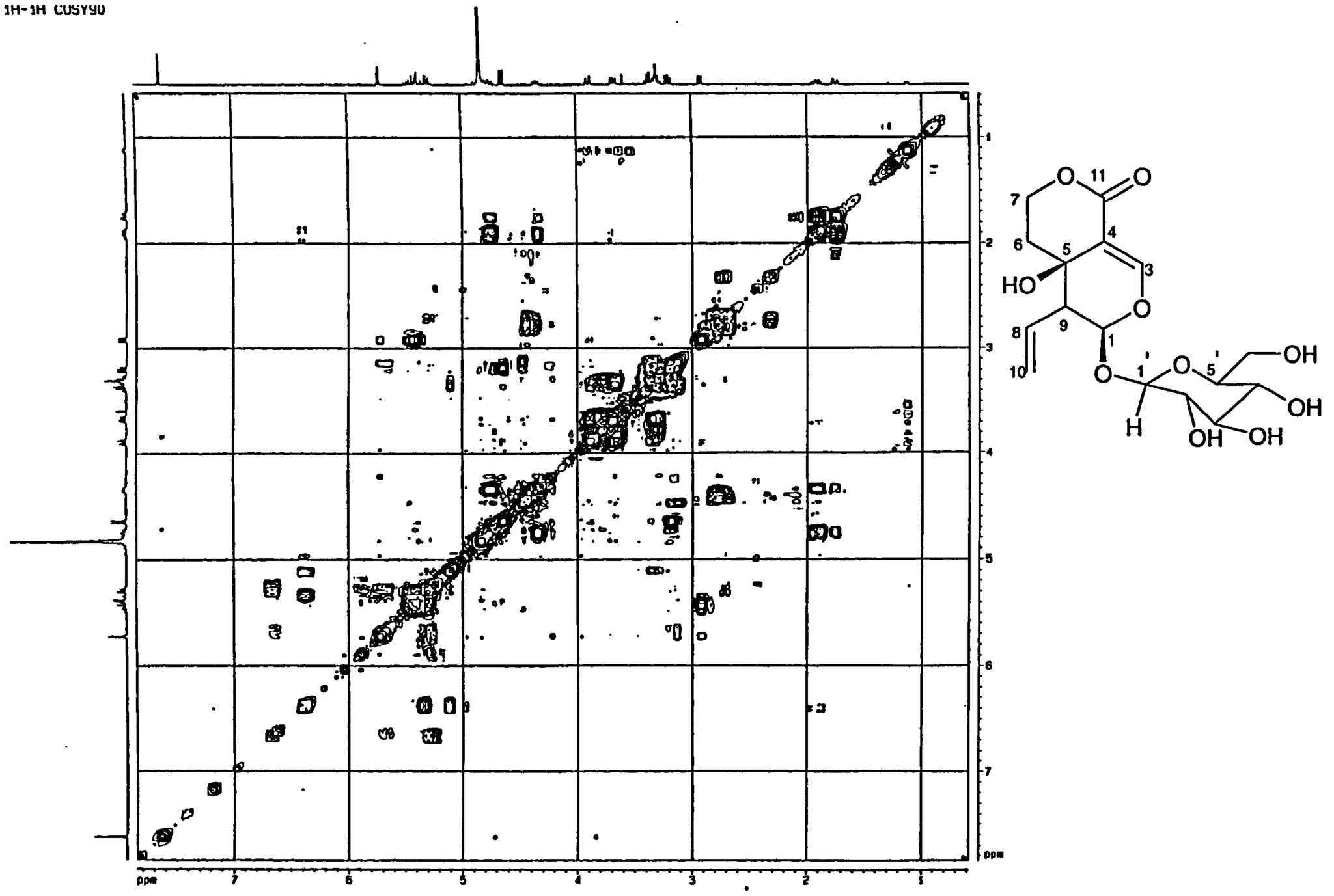


Figure 3.51: ^{1}H-^{1}H COSY NMR spectrum (400 MHz, CD_3OD) of EA01

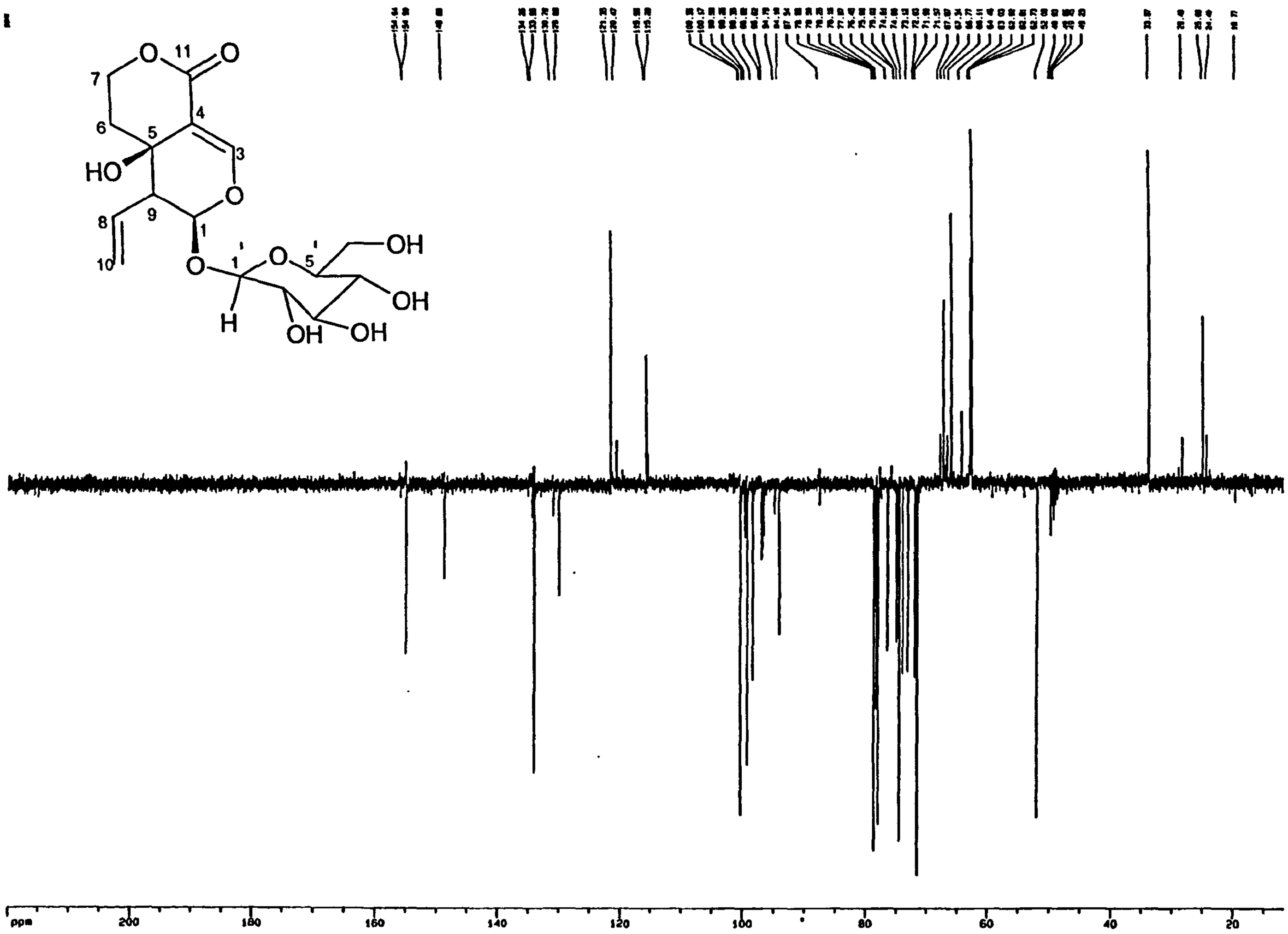


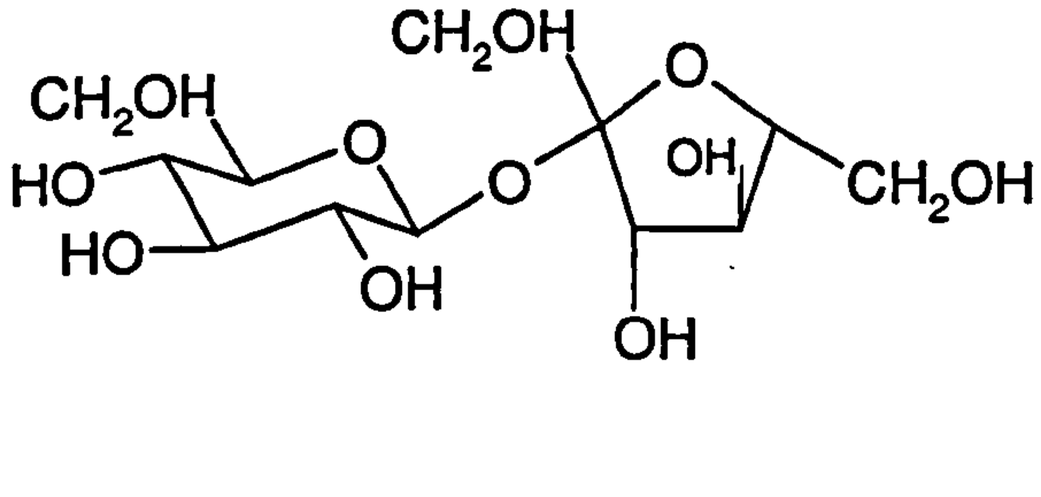
Figure 3.52: ^{13}C DEPT NMR spectrum (400 MHz, CD_3OD) of EA01

3.3.2.1.2 Characterisation of compounds EA06 and EA10 as a mixture of β-sitosterol and stigmasterol

These compounds were isolated (in a mixture) from the dichloromethane extract as colourless needles. The spectra (e.g. Figure 3.53) were very similar to that of the stigmasterol/sitosterol mixture (Section 3.3.1.1.3). The characteristic signals for the axial proton at C-3 (at δ3.55) and the doublet at δ5.36 were present with integration indicating the presence of a mixture of two compounds. The identity of the compounds was confirmed to be stigmasterol and β-sitosterol in a mixture.

3.3.2.1.2 Characterisation of EA02 (A) as sucrose

This compound was isolated from the DCM extract. It was sparingly soluble in methanol and on TLC it showed as a black spot after spraying with vanillin-sulfuric acid reagent and heating. The FTIR spectrum showed a very broad absorption band at 3350 cm^{-1} indicating the presence of hydroxyl groups. A quick ^{13}C J-modulated spectrum (Figure 3.55) revealed the presence of twelve carbon signals, 1 quaternary, 3 CH_2's and 8 CH's. The ^{1}H NMR spectrum (Figure 3.54) showed the presence of 12 protons (the –OH protons did not show) and that the doublet for the anomeric proton had a coupling constant of 3.84 Hz, hence showing that the *O*-linkage for glucose was α. Based on this data and comparison with literature values, the compound was identified as sucrose (α-D-glucopyranosyl-β-D-fructofuranoside) having a molecular formula of $C_{12}H_{22}O_{11}$).



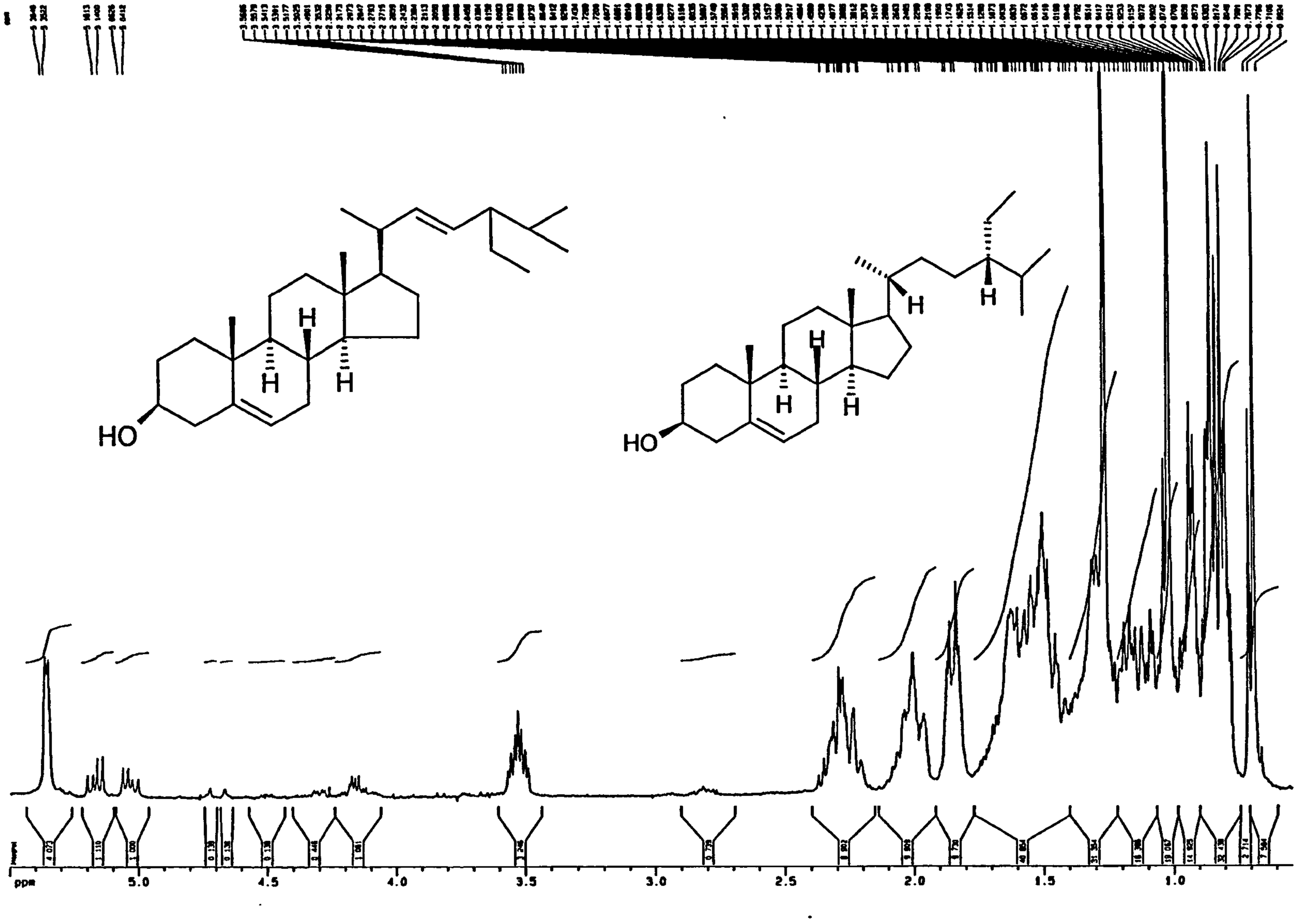


Figure 3.53: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of EA10 and EA06

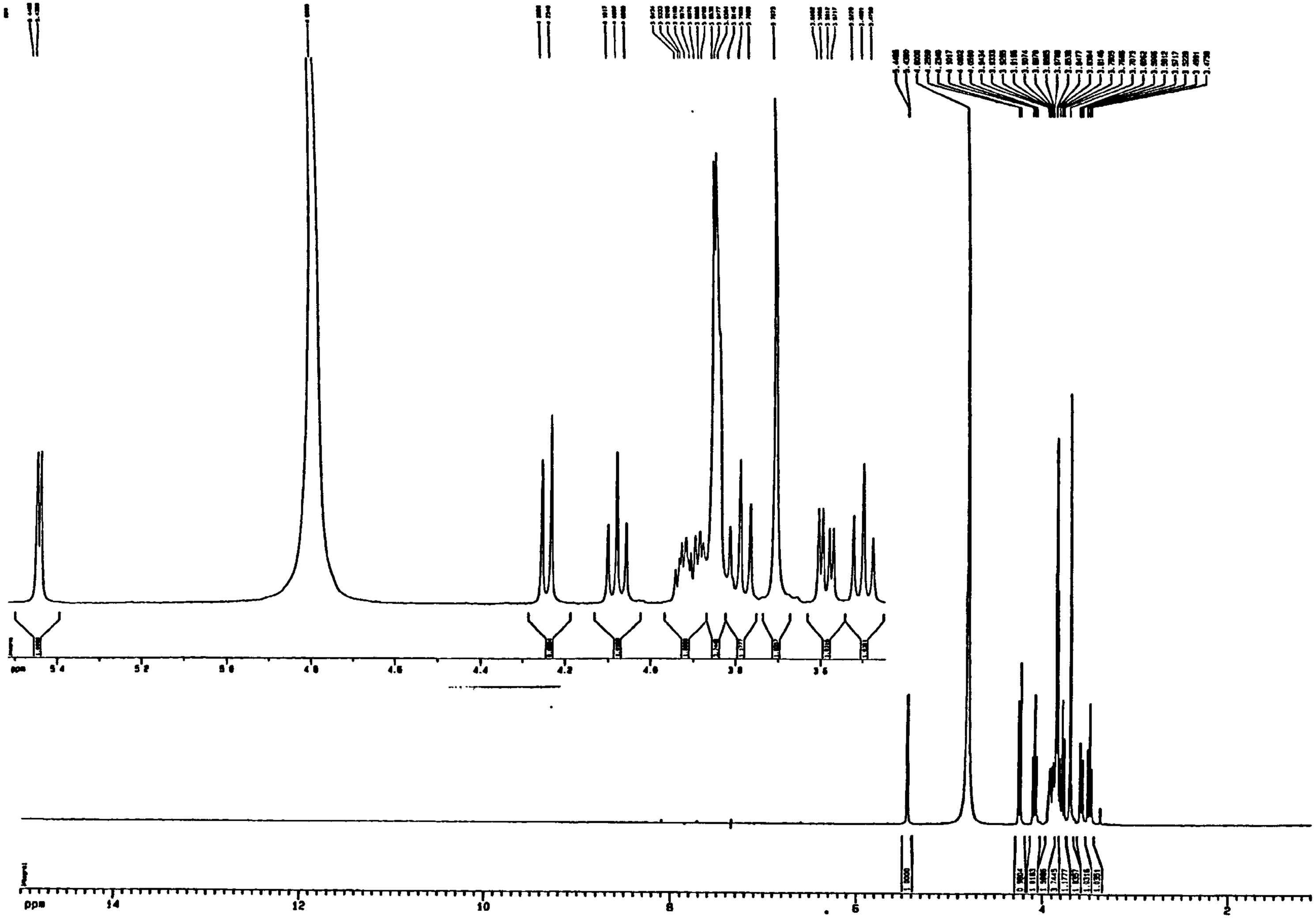


Figure 3.54: ^{1}H NMR spectrum (400 MHz, CD_3OD) of EA02A

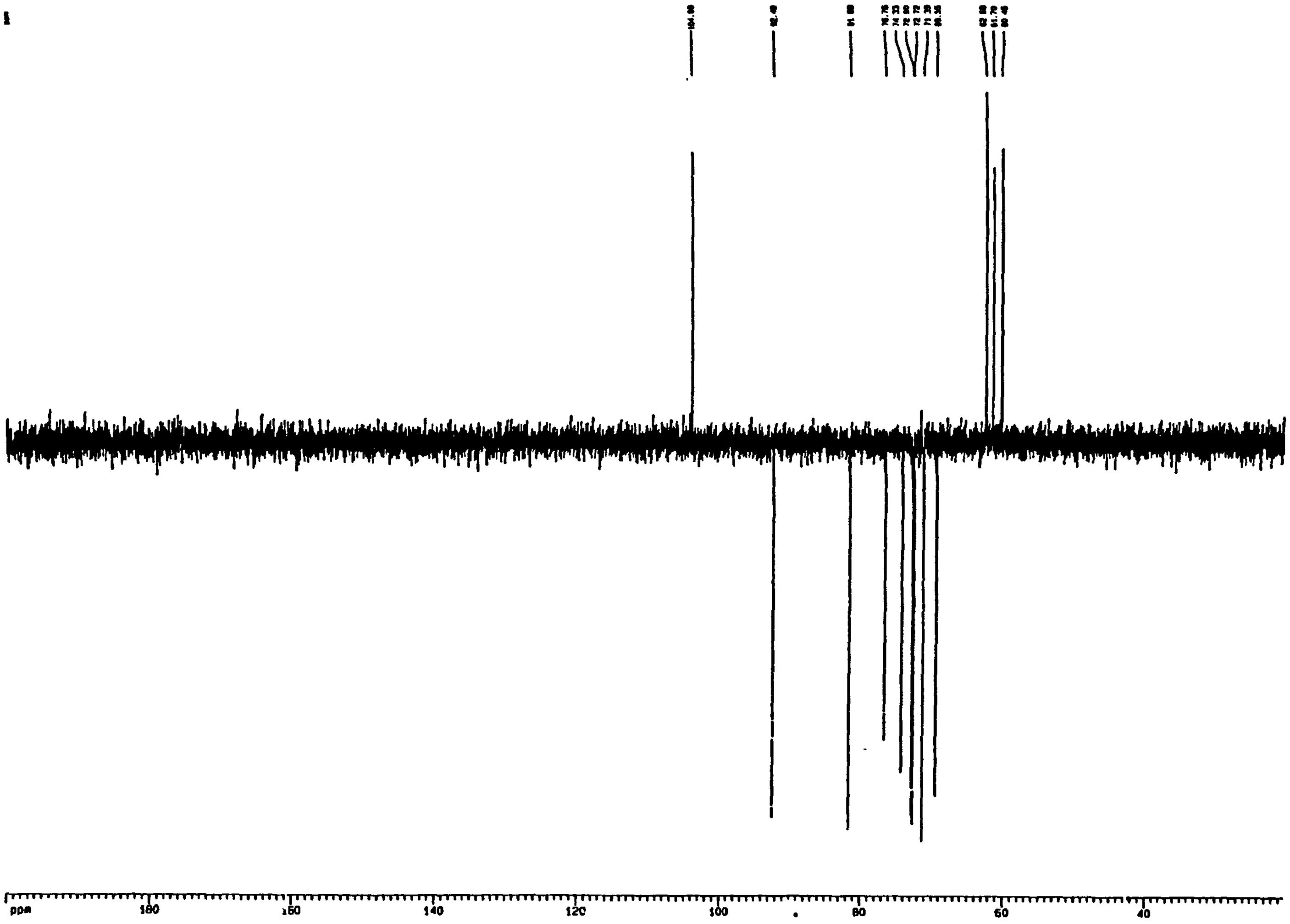


Figure 3.55: ^{13}C NMR spectrum (400 MHz, CD_3OD) of EA02A

3.3.2.2 Methanol Extract

This weighed 30g, 5 g was subjected to CC eluting with different proportions of methanol in chloroform, starting with 100% chloroform. More of compound EA01 (2.97g) was isolated and 220mg of EA02 (B).

3.3.2.2.1 Characterisation of EA02 (B) as α/β "mutarotated" glucose

This compound was isolated from the methanol extract as colourless crystals, which were insoluble in methanol. ^{1}H NMR and ^{13}C J-modulated spectrum of the compound were obtained in deuterated water. The ^{1}H NMR spectrum (Figure 3.56) displayed two doublets one centred at δ4.48 (J=7.5 Hz) and another at δ5.11 (J=2.3 Hz) indicating the presence of glucose in the α- and β- form. These signals were assigned to the anomeric protons.

The J-modulated ^{13}C NMR spectrum (Figure 3.57) revealed the presence of 12 carbon signals, which had very close chemical shifts. It showed two methylene signals and 10 methine signals, which suggested the presence of two molecules of a pyranose or a disaccharide of six carbon sugars. The signals at δ100.6 and δ96.3 were assigned to the anomeric carbons, β- and α- respectively. The methylene signals resonated at δ65.1 and δ65.2.

3.3.2.2.2 Other Isolated Compounds

Compounds EA05, EA07 and EA09 were isolated as white crystalline solids from the dichloromethane extract. Proton NMR analysis of these showed that there were terpenoids, however because they were isolated in small amounts (due to limitation of plant material), it was not possible to obtain informative carbon NMR spectra. As a result the compounds could not be identified. This therefore calls for further chemical investigation of the plant.

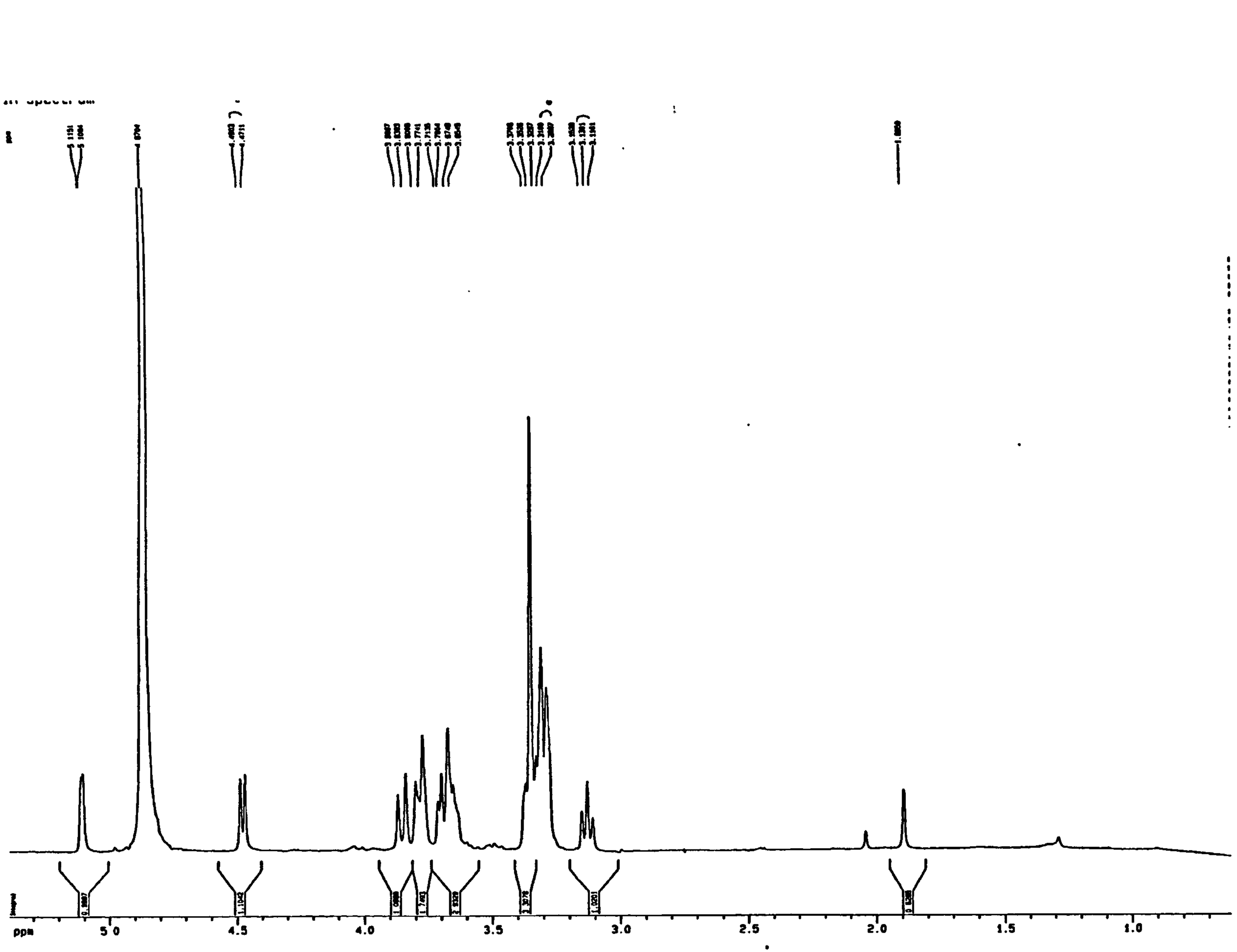


Figure 3.56: ^{1}H NMR spectrum (400 MHz, CD_3OD) of EA02B

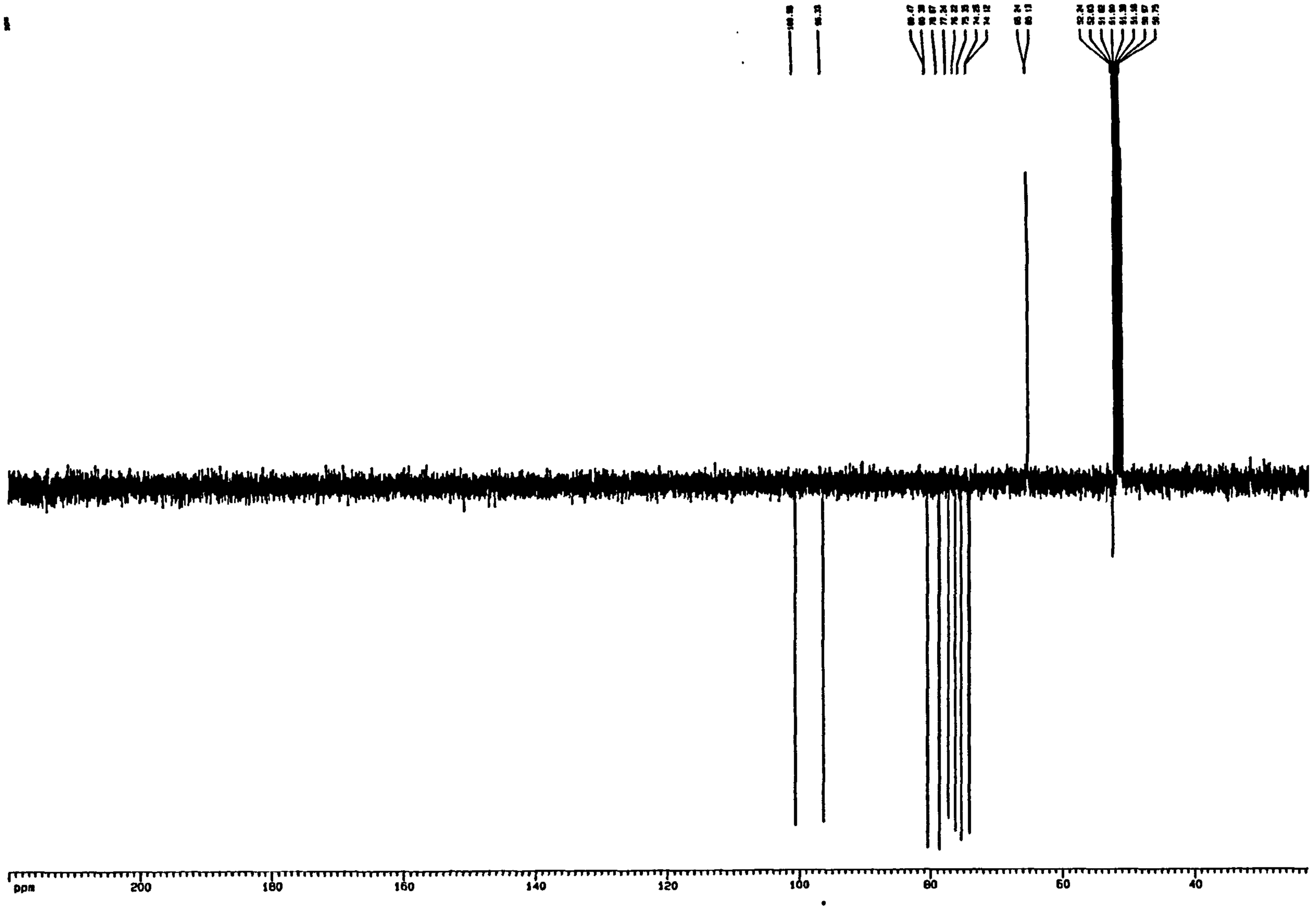


Figure 3.57: ^{13}C NMR spectrum (400 MHz, CD_3OD) of EA02B

3.3.3 *Argemone mexicana*

The leaves of this plant were investigated. A pilot scale extraction was carried out on 0.5g of the plant material to assess the efficiency of two extraction procedures: acid-base extraction and extraction with methanol for alkaloids. In the first extraction, the plant material was extracted in 1% HCl for 2 hours. This was filtered and the filtrate basified with ammonia solution. Chloroform was used to extract the compounds and the extract was concentrated. For the second extraction the plant material was macerated in methanol overnight. This was filtered, defatted with hexane and concentrated. The two extracts were compared by TLC. The acid-base extract showed a cleaner profile than the methanol. However the methanol extract showed one or two more spots than the acid-base and therefore was chosen for the large-scale extraction since more compounds would presumably be extracted. Therefore, 200g of the leave plant material was extracted in a Soxhlet apparatus to exhaustion. Chlorophyll was removed and the resulting extract weighed 11.8 g. 5g of this was fractionated using VLC. Fractions containing major compounds were combined (2.5g) and subjected to CC (column L: 30cm, i.d. 2 cm) using silica gel. The column was eluted with 400ml of 5% MeOH in $CHCl_3$, 500 ml of 7% MeOH and then increasing the polarity by increments of 2.5% to 40% MeOH in chloroform. Semi-preparative HPLC and PTLC were used to further to further separate and purify compounds that could not separate by CC. Compounds AM2S, AM07 (Fn47-55), AM12, Fn 31-35, and Fn 8 BP were isolated.

3.3.3.1 Characterisation of Componds AM 2S and Fn 8 BPk as protopine

The compound was isolated as white crystals melting at 208-210°C. On TLC it gave a positive reaction with Dragendorff reagent. The FTIR spectrum showed an absorption band at 1675 cm^{-1} suggesting the presence of carbonyl functionality. HRMS showed ions at m/z 354.13363 (M+1) corresponding to the molecular formula $C_{20}H_{19}NO_5$. The UV spectrum showed λ_{max} at 290 nm and a shoulder at 240 nm. The identity of the compound was determined on the basis of this information together with ^1H NMR and ^{13}C NMR experiments.

The ^{1}H NMR spectrum (Figure 3.58 and Table 3.17) displayed two singlets at δ5.94 and δ5.96, typical for methylenedioxy groups. These were assigned to the two methylene dioxy groups at positions 2,3 and 9,10. The spectrum also displayed signals for four aromatic protons. The singlet at δ6.89 was assigned to the proton at C-1 whereas the proton at C-4 resonated at δ6.63. The proton at C-1 resonates downfield to the one at C-4 because of the proximity of the carbonyl at C-14. Two signals for *ortho* coupled protons appeared at δ6.68, doublet, (J=7.8 Hz) and δ6.66. These were assigned to H-11 and H-12 respectively. Other important signals were observed, including the two singlets at δ3.80 and δ3.59 attributed to the CH_2's at C-13 and C-8 respectively. These signals for these methylenes originally appeared as a broad mass of undistinguishable peaks but by raising the temperature to 323K, their resolution was dramatically improved to the two singlets seen in Figure 3.58. This behaviour is due to the flexibility of the 10-membered ring of the molecule. At low frequencies (60 MHz) the protons are well defined but become broader as the frequency increases and may not be visible at high frequencies (360 MHz) (Guinaudeau and Bruneton, 1993), which was the case here. The two signals at δ2.89 and δ2.53 were assigned to the CH_2's at C-5 and that at C-6. The singlet at δ1.95 was assigned to the N-CH_3 group.

The ^{13}C J-modulated spectrum (Figure 3.59) displayed a ketonic carbon signal at δ209.4, which was assigned to the carbonyl carbon, C-14. The signals for the methylene dioxy groups appeared at δ101.2 and δ101.5, thus showing that they were attached to oxygen by being much downfield for methylenes. The signals arising from the methylenes at C-5, C-6, C-8 and C-13, on the other hand, appeared at the expected range fro methylene carbons, i.e. δ30 to δ60. Other signals were also in agreement with the proton assignments, the aromatic methines were present, the N-CH_3 carbon was displayed at δ41.9 and the nine signals assigned to the quaternary carbons (Table 3.17), the most deshielded being the four attached to the oxygen atoms at 2,3,9 and 10. Examination of the HCCOBI and HMBC (Figure 3.61) revealed information that supported these assignments. For instance, the methylenedioxy at 2,3 showed clear ^{3}J to the quaternary carbon signals and so did those at 9,10. The aromatic protons at C-11 and C-12 showed ^{3}J

to C-9 and C-10. H-4 showed ^{3}J to C-2. Significant NOESY (Figure 3.60) correlations were observed between H-5 and the methylene protons at C-13 and between these methylene protons and those at C-8. These correlations give an idea of the conformation of the molecule. Overall the data was in agreement with that published for the compound (Brossi, 1988). The structure and model of the compound are shown below.

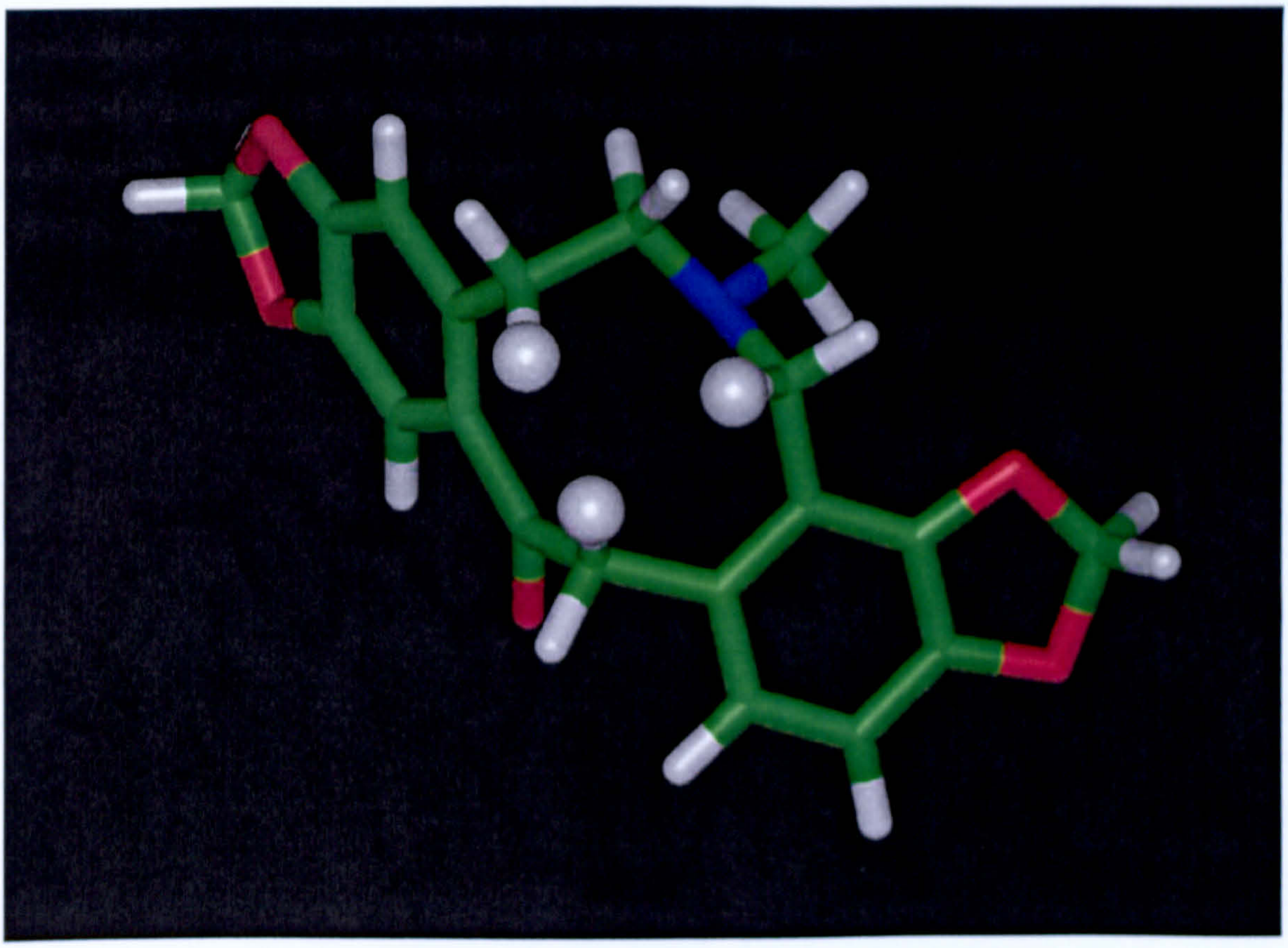


Figure 3.62: Computer model of protopine, the protons showing NOE correlations are shown as balls.

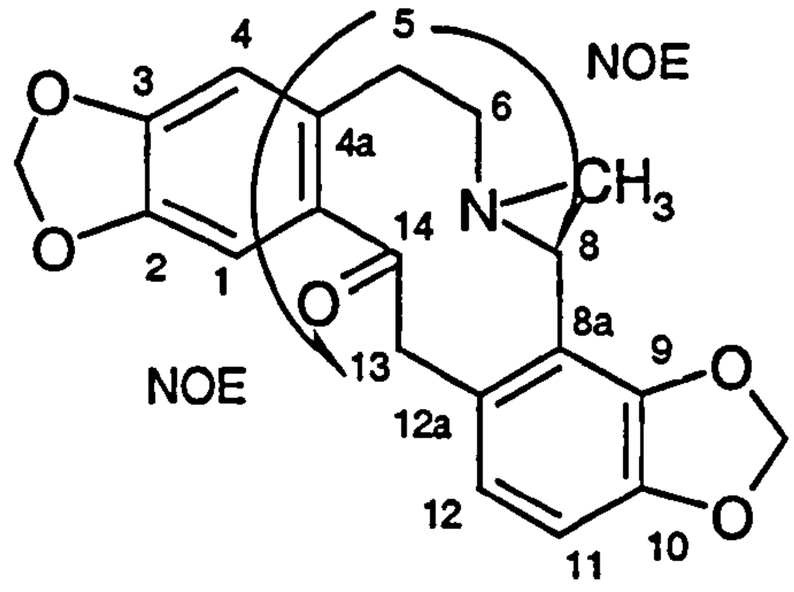


Table 3.17: ^{1}H and ^{13}C NMR (400 MHz) data for protopine in $CDCl_3$

Position		^{1}H δ(ppm)	^{13}C δ(ppm)
C-1	CH	6.89,s	108.2
C-2	C	-	146.3
C-3	C	-	148.3
C-4	CH	6.63, s	110.5
C-4a	C	-	132.6
C-5	CH_2	2.89, s	31.4
C-6	CH_2	2.53, s	57.8
C-7	N-CH_3	1.95, s	41.9
C-8	CH_2	3.59, s	51.8
C-8a	C	-	117.4
C-9	C	-	146.4
C-10	C	-	145.5
C-11	CH	6.68, d (J=7.8)	107.2
C-12	CH	6.66, d	125.1
C-12a	C	-	128.8
C-13	CH_2	3.80, s	37.6
C-14	C=O	-	209.4
C-14a	C	-	136.1
2.3	–OCH_2O-	5.94, s	101.2
9,10	–OCH_2O-	5.91, s	101.5

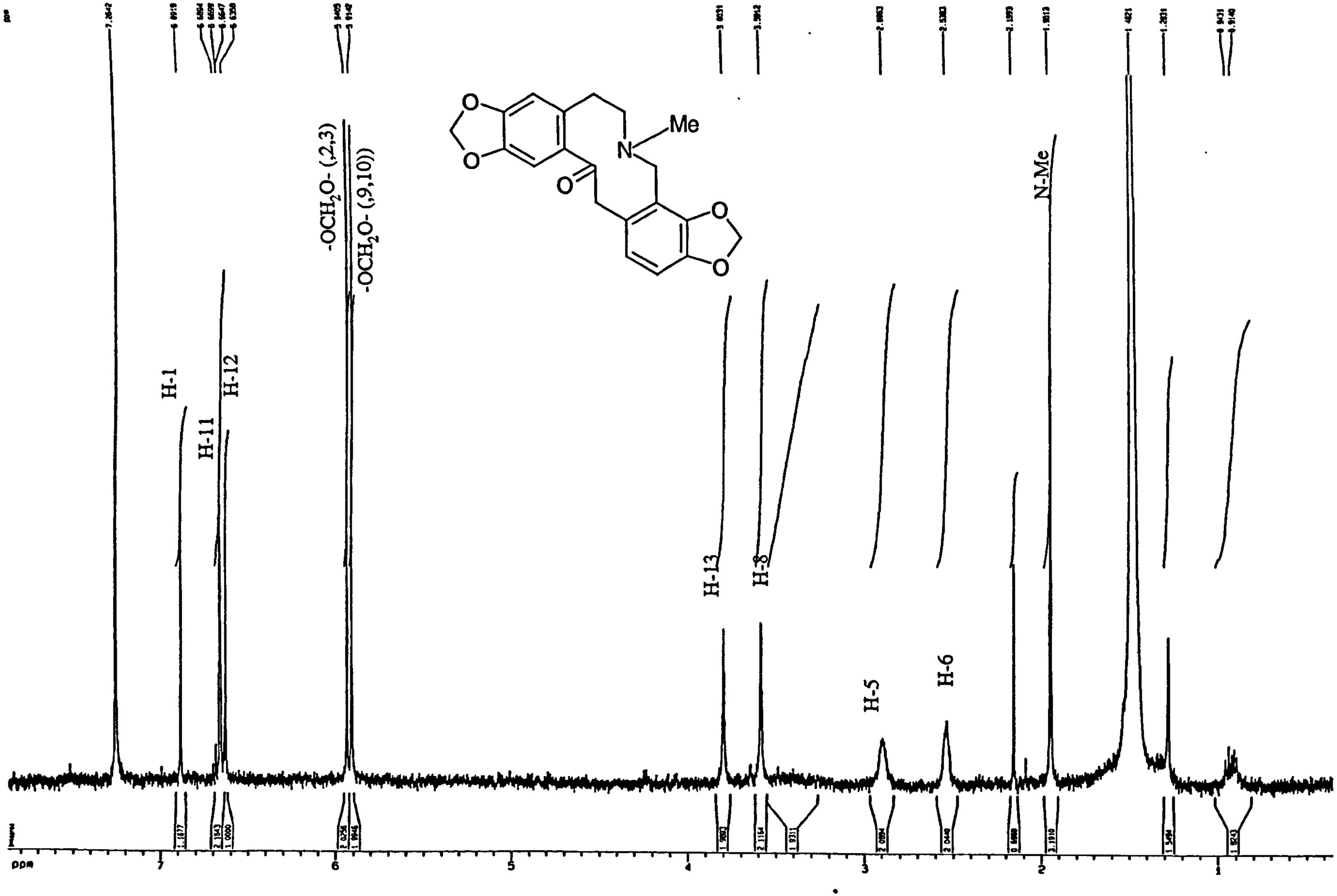


Figure 3.58: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of protopine

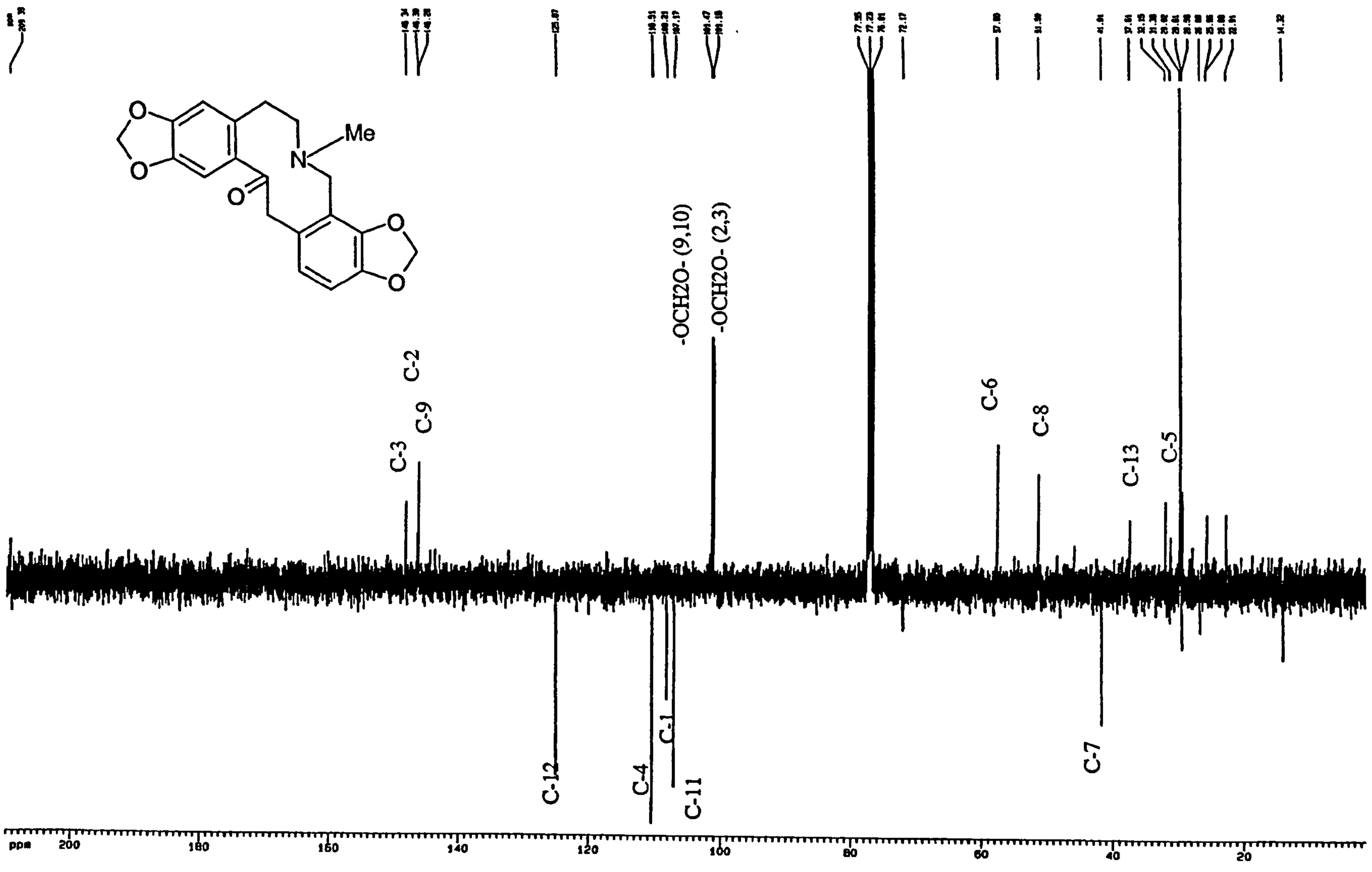
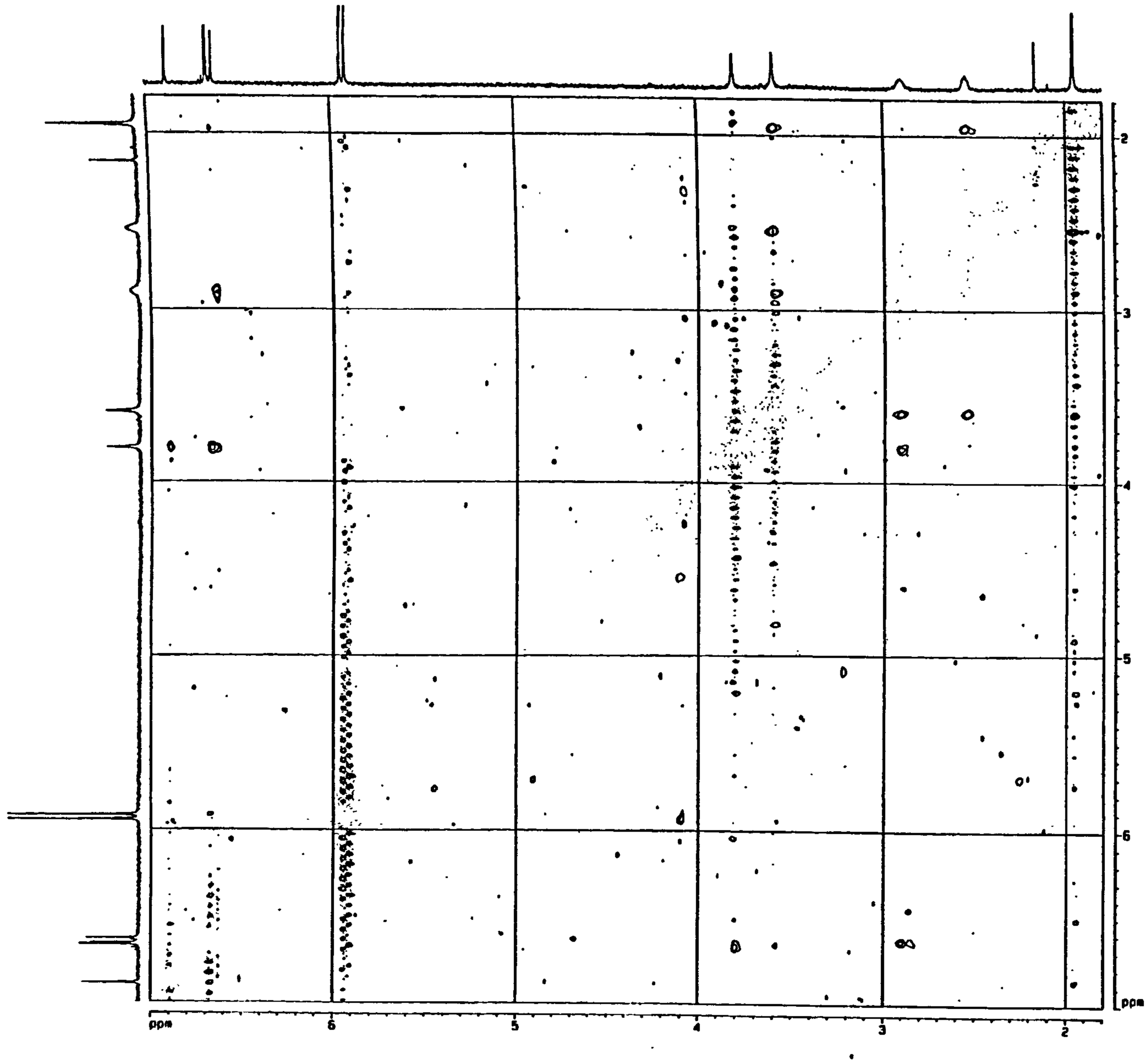


Figure 3.59: ^{13}C NMR spectrum (400 MHz, $CDCl_3$) of protopine



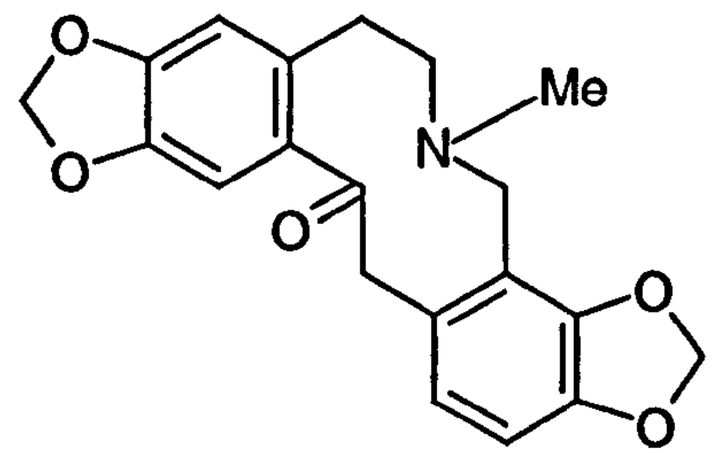


Figure 3.60: ^{1}H-^{1}H NOESY NMR spectrum (400 MHz, $CDCl_3$) of protopine

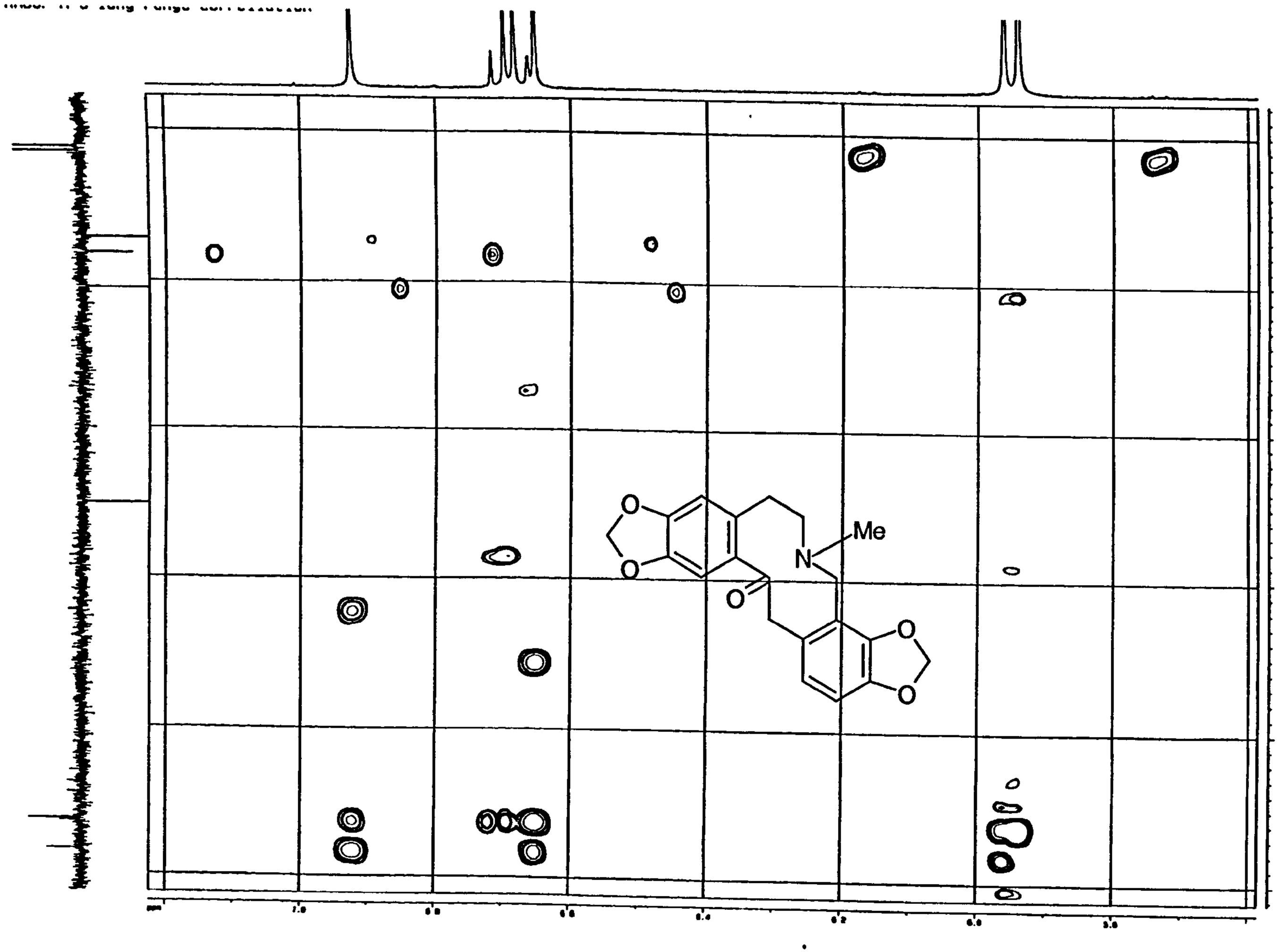
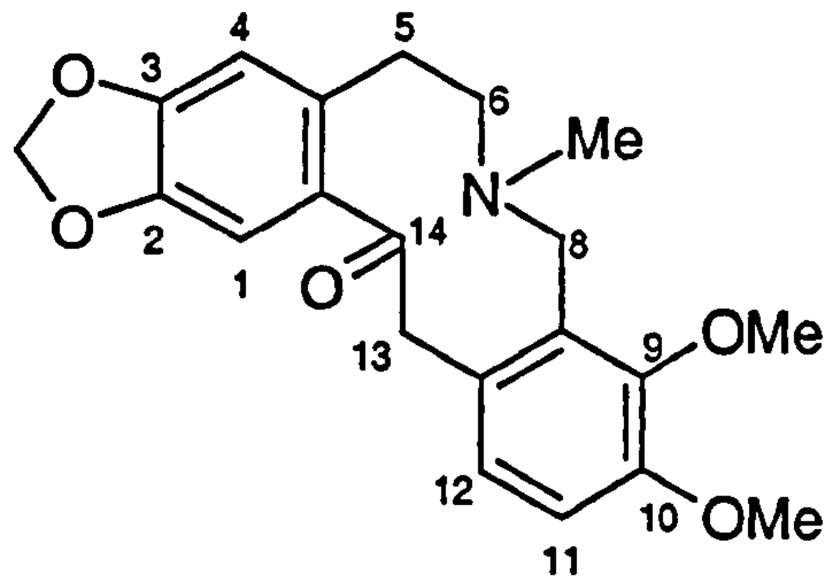


Figure 3.61: HMBC NMR spectrum (400 MHz, $CDCl_3$) of protopine

3.3.3.2 Characterisation of Compound AM Fn 31-35 as Allocryptopine

The compound was isolated as a crystalline solid melting at 174-176°C. It gave a positive reaction for an alkaloid on TLC. HRMS spectrum showed a molecular ion at 369.15 corresponding to the molecular formula $C_{21}H_{23}NO_3$. The FTIR spectrum showed the presence of a carbonyl group at 1671 cm^{-1}. The UV spectrum was similar to that of protopine.

The ^{1}HNMR spectrum (Table 3.18) was similar to that of protopine, but differed in that the two signals for the methylenedioxy groups had now been reduced to one, appearing at δ5.93. Another difference was that of the appearance of two singlets at δ3.82 and δ3.86 attributed to the two methoxy groups at C-9 and C-10. The signal for the N-CH_3 appeared at δ1.96 and those for the four aromatic protons were also present. Table 25 shows the ^{1}H NMR data for this compound and that of protopine in deuterated methanol. This data is in agreement with that published for the compound, (Brossi, 1988). ^{13}C NMR spectrum showed a δ212.01 confirming the presence of a carbonyl group in the molecule. The compound was thus identified as allocryptopine (**131**), the structure and model of which are shown below.



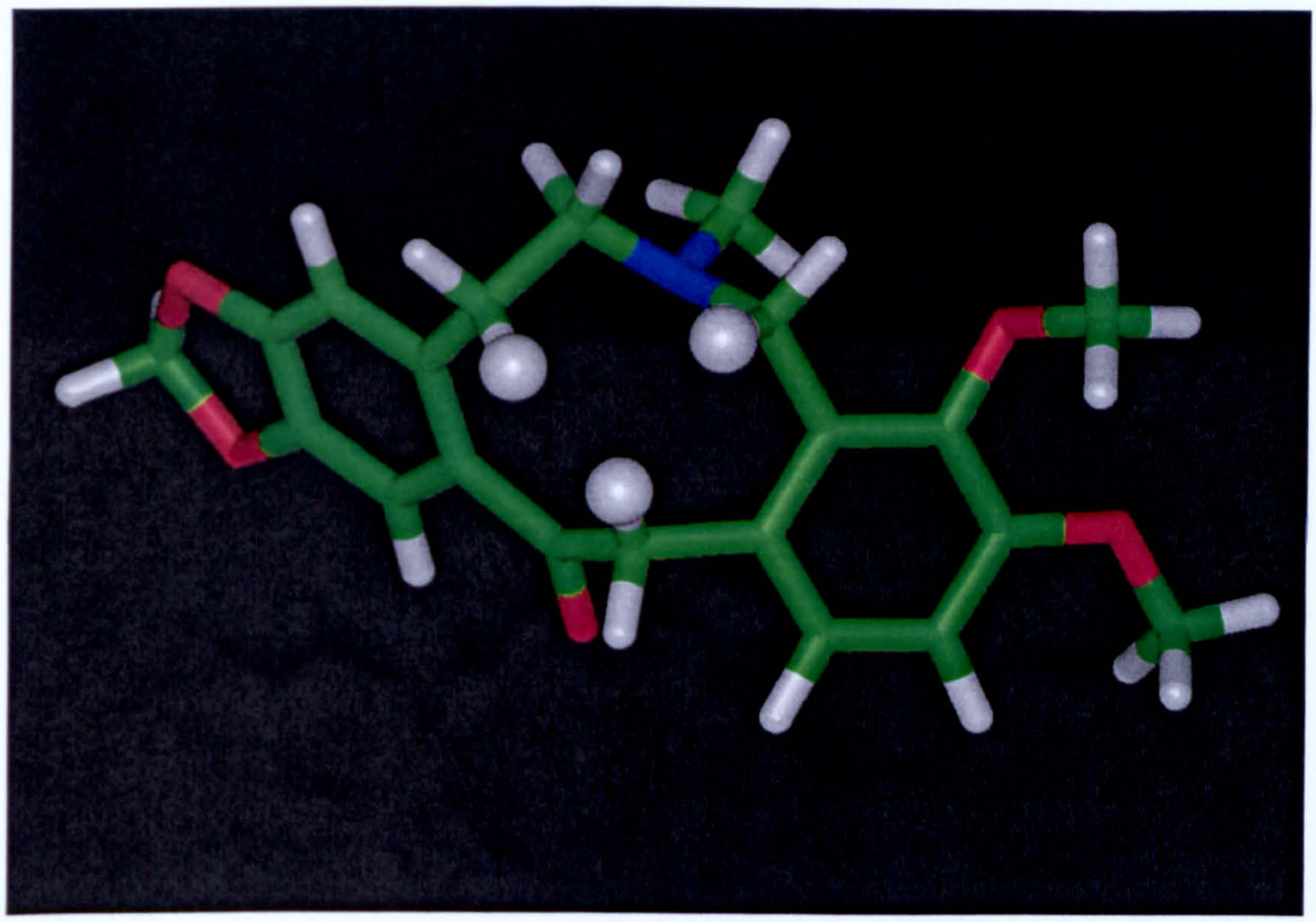


Figure 3.63: Computer model of allocryptopine, the protons showing NOE correlations are shown as balls.

Table 3.18: ^{1}H NMR (400 MHz) data for allocryptopine and protopine in CD_3OD

Position		Allocryptopine (δ)	Protopine (δ)
1	CH	7.01, s	7.01, s
4	CH	6.78, s	6.77, s
5	CH_2	3.00	2.99
6	CH_2	3.34	Not seen
7	$N\text{-}CH_3$	1.91	1.92
8	CH_2	3.66	3.86
11	CH	6.78, d	6.83, d
12	CH	6.66, d	6.80, d
13	CH_2	3.68	3.89
$-OCH_2O-$		6.01	6.00
$-OCH_2O-$		-	6.03
$-OCH_3$		3.87	-
$-OCH_3$		3.89	-

3.3.3.3 Characterisation of AM CC Fn 47-55 as berberine

The compound was isolated as yellow crystals melting at 210-212°C. It gave a positive reaction on TLC after spraying with Dragendorff reagent. The carbonyl group absorbance band was missing from the FTIR spectrum. HRMS showed an ion at 336.13 corresponding to the molecular formula $C_{20}H_{14}NO_4$ expected for berberine. The UV spectrum showed λ_{max} values at 230, 266 and 350 nm. Comparison of this data and that provided by ^{1}H NMR (Table 3.19) with literature values (Janssen *et al.*, 1989) assisted in identifying the compound.

Examination of the proton spectrum revealed the typical methelenedioxy signal at δ5.99 appearing as a singlet. The signals at δ4.20 and δ4.11 were assigned to the two methoxy

groups at C-9 and C-10 respectively. The signals at δ7.01 and δ6.75 were assigned to the *para* protons at C-1 and C-4. The *ortho* coupled protons H-11 and H-12 appeared as doublets at δ8.12 (J=9.1 Hz) and δ7.98 (J=9.2 Hz) respectively. The proton at C-8 appeared as a much deshielded singlet at δ9.76. This was because the positively charged Nitrogen atom draws electrons away from C-8 and thereby exerting a deshielding effect. H-13 also experienced similar deshielding from the double bond and the Nitrogen atom and therefore appeared downfield at δ8.70. The spectrum also displayed two triplets, one at δ3.26 (J=6.4Hz, 12.4Hz, 6.4Hz) attributed to the methylene at C-5 and the other at δ4.91. Overall the data agreed with that published for berberine (**125**) and thus the compound was identified as such.

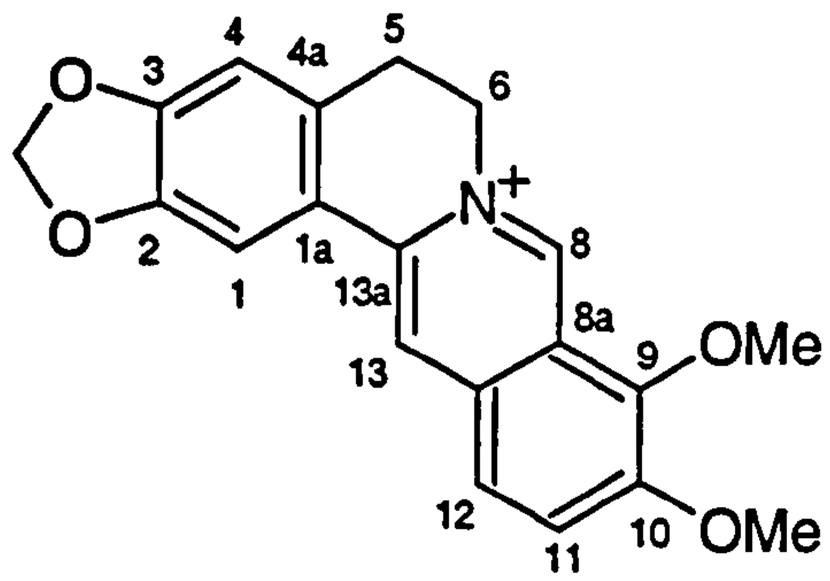


Table 3.19: ^{1}H NMR (400 MHz) data for berberine in CD_3OD

Position	^{1}H δ(ppm)
H-1	7.1, s
H-4	6.75, s
H-5	3.26, t (2H, J=6.4, 12.4, 6.4)
H-6	4.91, t (2H)
H-8	9.76,s
H-11	8.12, d, (J=9.2)
H-12	7.98, d (J=9.1)
H-13	8.70, s
$-OCH_2O-$	5.99, s
C-9, $-OCH_3-$	4.20, s
C-10, $-OCH_3-$	4.11, s

3.3.3.4 Charecterisation of AM12 as sanguinarine

The compound was isolated as an orange crystalline solid. It gave a positive reaction with Dragendorff reagent on TLC. The HRMS spectrum showed a molecular ion of 332.09 corresponding to the molecular formula $C_{20}H_{14}NO_4$. The UV spectrum showed λ_{max} values at 236, 284, and 324 nm.

The ^1H NMR spectrum (Table 3.20 and Figure 3.65) showed the presence of the methylenedioxy groups signals at δ6.28 and δ6.53 as singlets. The signal at δ3.35 was assigned to the N-CH_3 group, which was much more deshielded than in the protopines because of the positively charged nitrogen atom. The deshielded methine singlet at δ9.91 was assigned to H-6 also being deshielded by the nitrogen atom. Other important signals were those seen around the aromatic region of the spectrum. The singlet at δ7.52 was assigned to the proton at C-1. The proton at C-4 appeared at δ7.94. The two *ortho* coupled protons at C-9 and C-10 appeared as a doublet each. H-9 was seen at δ8.13 (J=8.1Hz) and H-10 at δ8.17 (J=8.9Hz). The other pair of *ortho* coupled protons H-11 and H-12 also appeared as doublets. The signal at δ8.56, (J=8.9Hz) was assigned to H-11 and that at δ8.48, (J=8.9 Hz) to H-12. The data was in agreement with that published for sanguinarine (Ishi *et al.*, 1978). The ^{13}C J-modulated spectrum (Figure 3.66) showed the signal for the N-methyl at δ52.9. The signal for the two methylenedioxy's appeared at δ104.5 and δ105.1. There were seven methine signals and 10 other signals attributed to the quaternary carbon atoms. The compound was thus identified as sanguinarine (**130**). The structure and computer model of the compound are shown below.

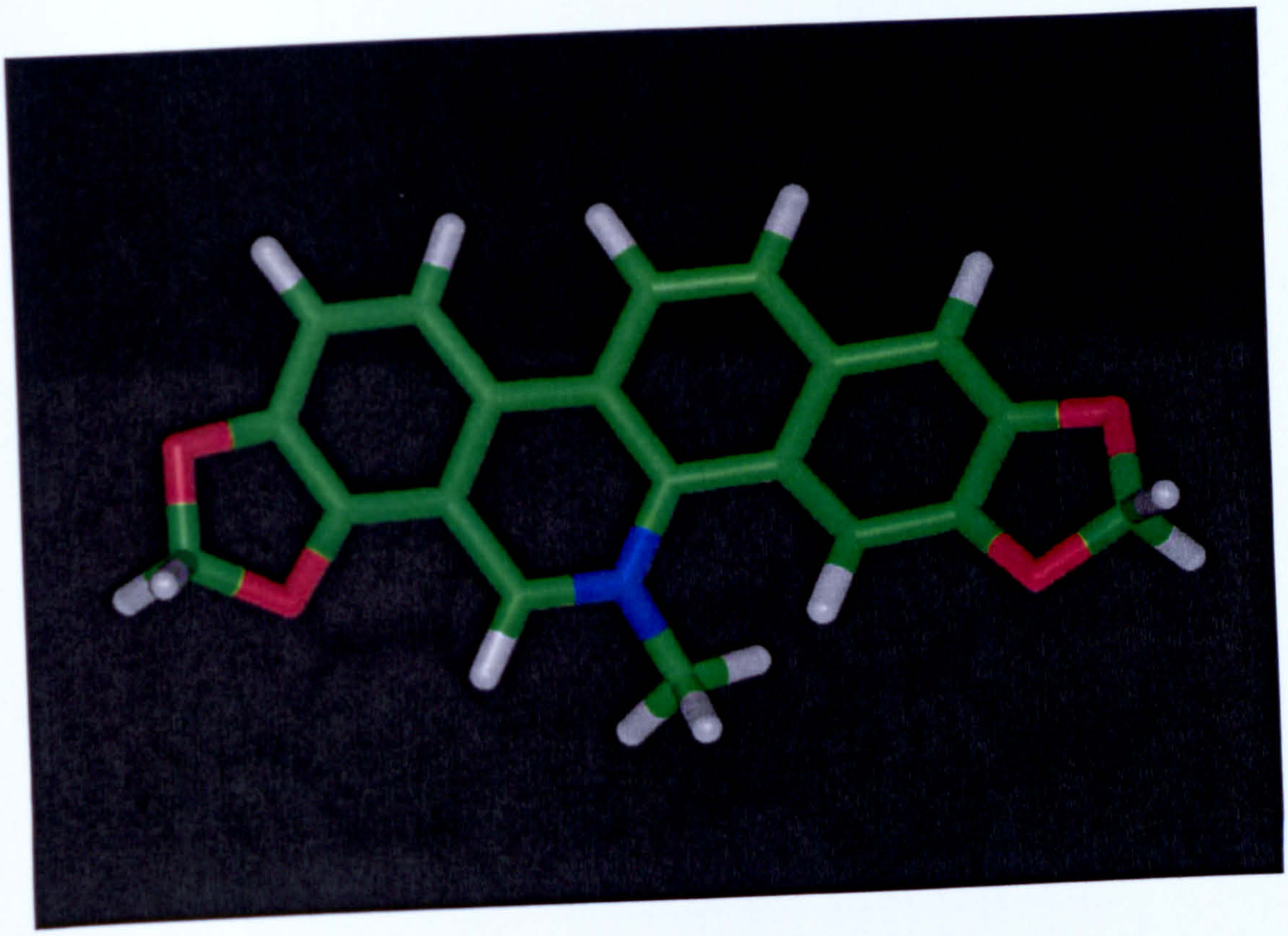


Figure 3.64: Computer model of sanguinarine

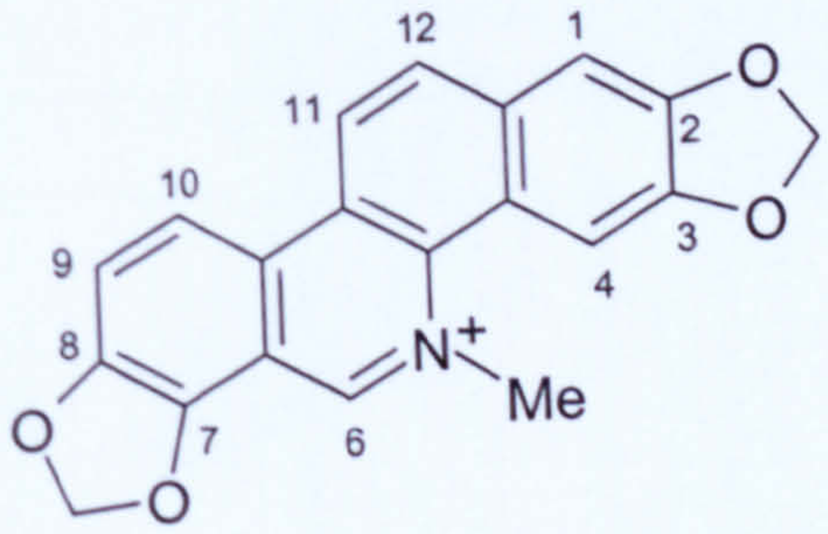


Table 3.20: ^{1}H NMR (400 MHz) data for sanguinarine in CD_3OD

Position	^{1}H δ(ppm)
H-1	7.52, s
H-4	7.94, s
N-CH_3	3.35, s
H-6	9.91, s
H-9	8.13, d (J=8.2)
H-10	8.17, d (J=8.2)
H-11	8.56, d (J=8.9)
H-12	8.48, d (J=8.9)
2,3, -OCH_2O-	6.28, s
7,8, -OCH_2O-	6.53, s

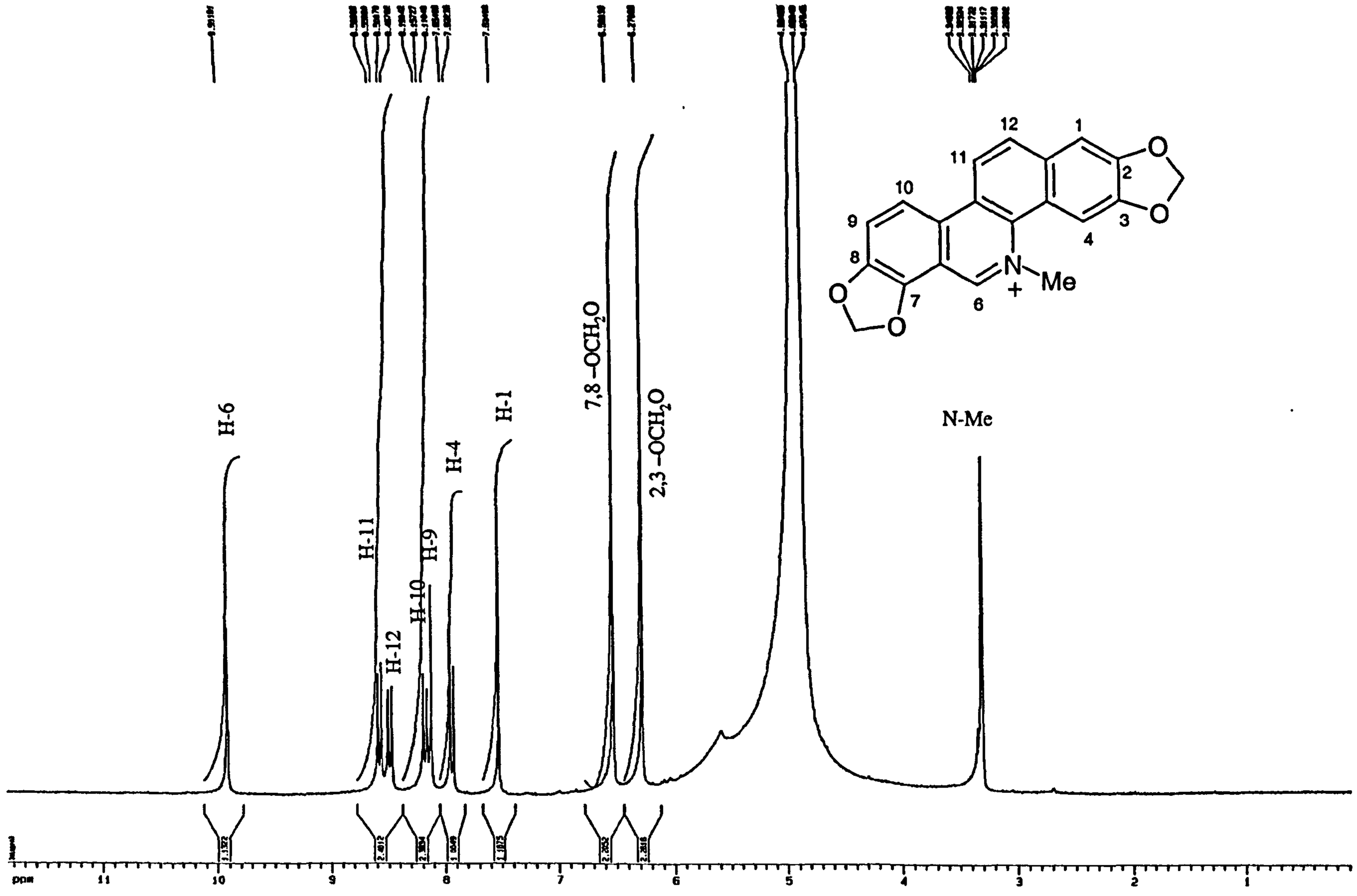


Figure 3.65: ^{1}H NMR spectrum (400 MHz CD_3OD of AM12 (Sanguinarine)

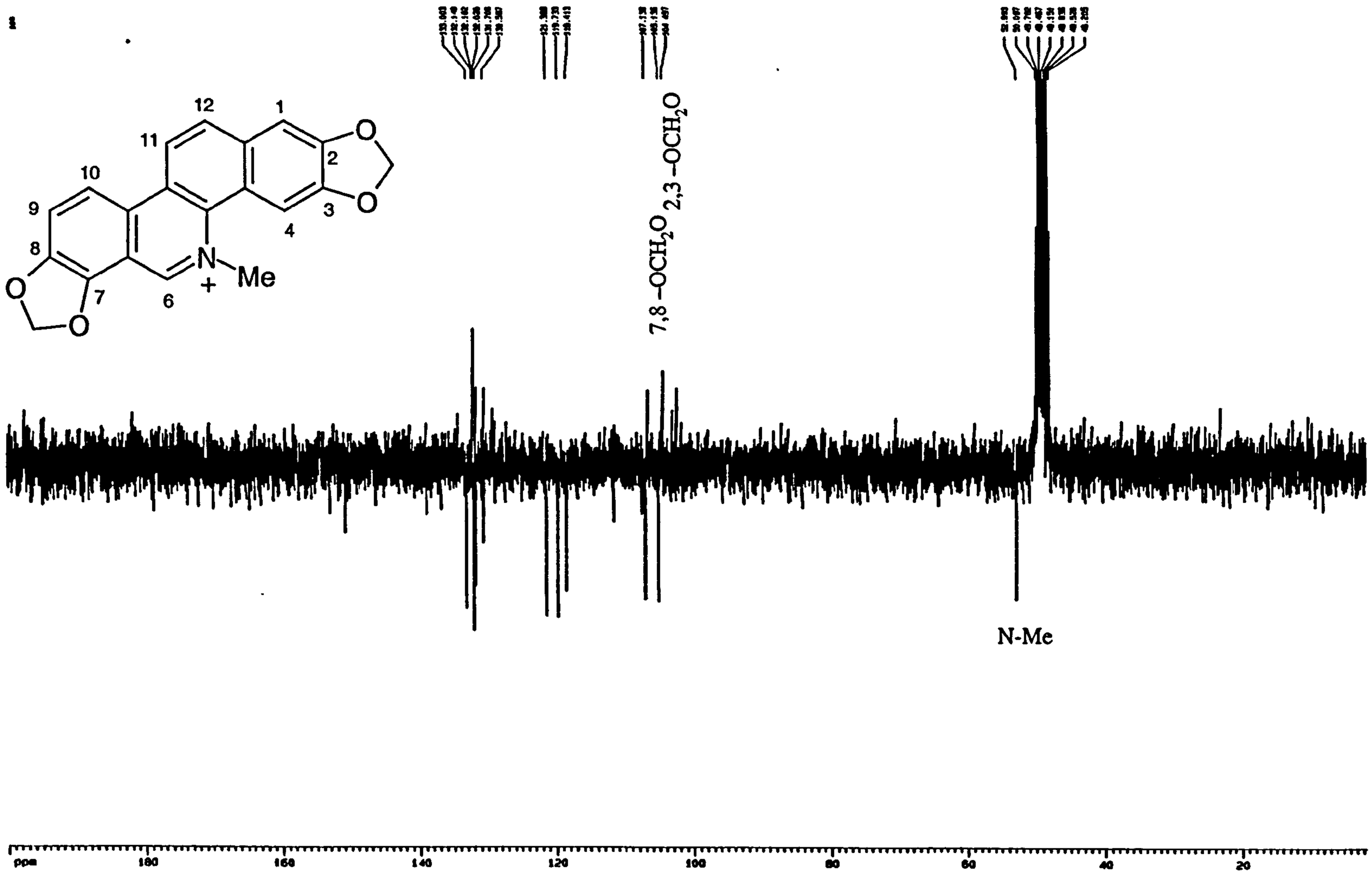


Figure 3.66: ^{1}H NMR spectrum (400 MHz CD_3OD of AM12 (Sanguinarine)

3.3.4 *Jatropha erythropoda*

TLC examination of the DCM and MeOH extracts of the tubers of this plant was carried out. Spraying the plates with Dragendorff reagent gave a negative result for alkaloids. When the plates were sprayed with vanillin-sulfuric acid a whole series of compounds was detected mainly in the DCM extract (Figure 3.58). The compounds gave reactions typical of terpenoids (mainly) and a few phenolics. This was determined by comparison with known samples of representative compounds. The MeOH (Figure 3.59) extract revealed the presence of polar compounds concentrated closer to the baseline.

Figure 3.67: TLC analysis of *J. erythropoda* and *C. italica DCM* extract, system: 100% $CHCl_3$

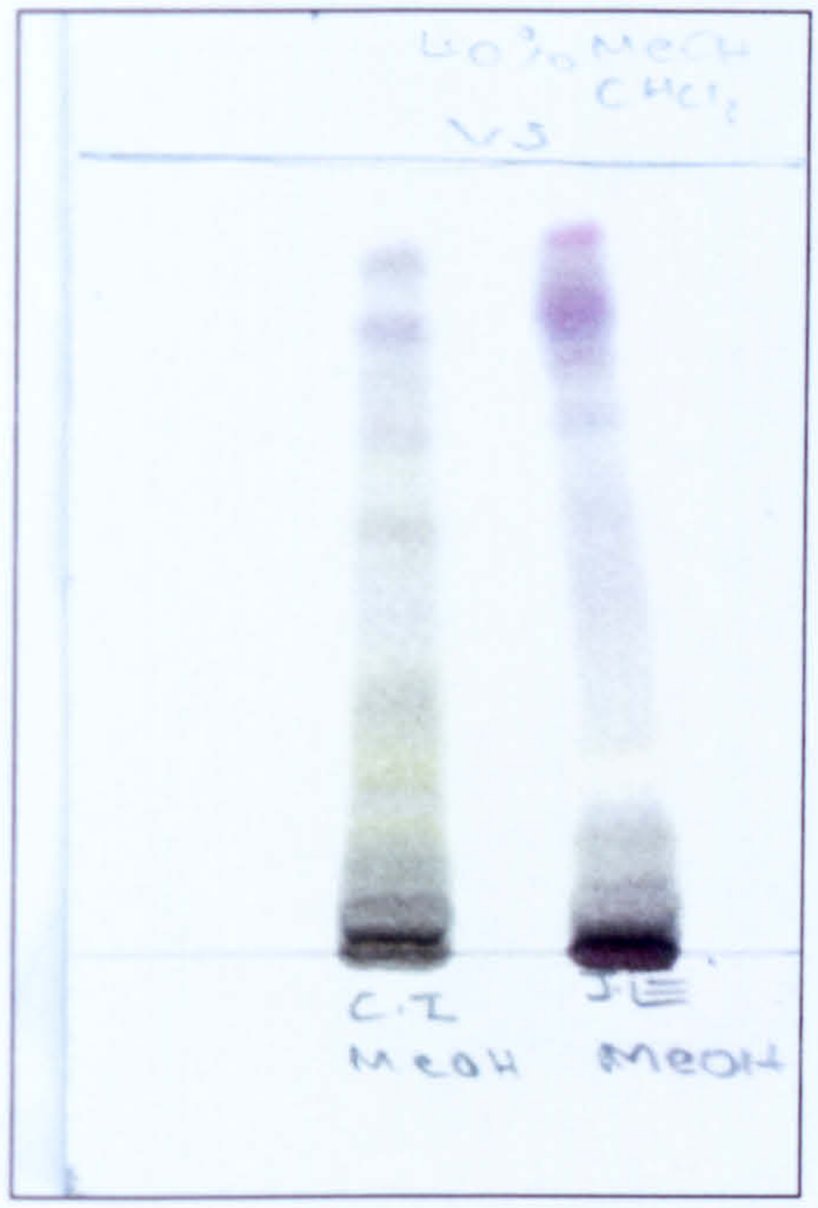


Figure 3.68: TLC analysis of *J. erythropoda* and *C. italica* MeOH extract, system: 40% MeOH in $CHCl_3$

HPLC analysis of the extracts was also carried out to obtain chemical profiles and develop a good system for LC-MS analysis. Extracts were analysed at a concentration of 1mg/ml. Chromatograms were obtained in the wavelength range 200 to 380 nm, using a mobile phase of acetonitrile and water. The separation was achieved using a gradient, starting with 20% acetonitrile and ramping it up to 100% in 25 minutes. Figure 3.69 shows the profile for DCM extract and Figure 3.70 that for the MeOH extract. The results indicate that there were more compounds in the DCM extract than in the MeOH extract. UV spectra of the major peaks were obtained. Figures 3.71 and 3.72 display the spectra for a few of the peaks. Judging by the TLC results and UV absorption properties of the constituents, the DCM extract contains mainly terpenoids, a few phenolics (absorbance maximas around 220 to 280) and a few flavonoids (absorbance maximas around 250 to

360). For both extracts, plotting the chromatograms at 360 nm showed better detection than when plotted at 220 nm.

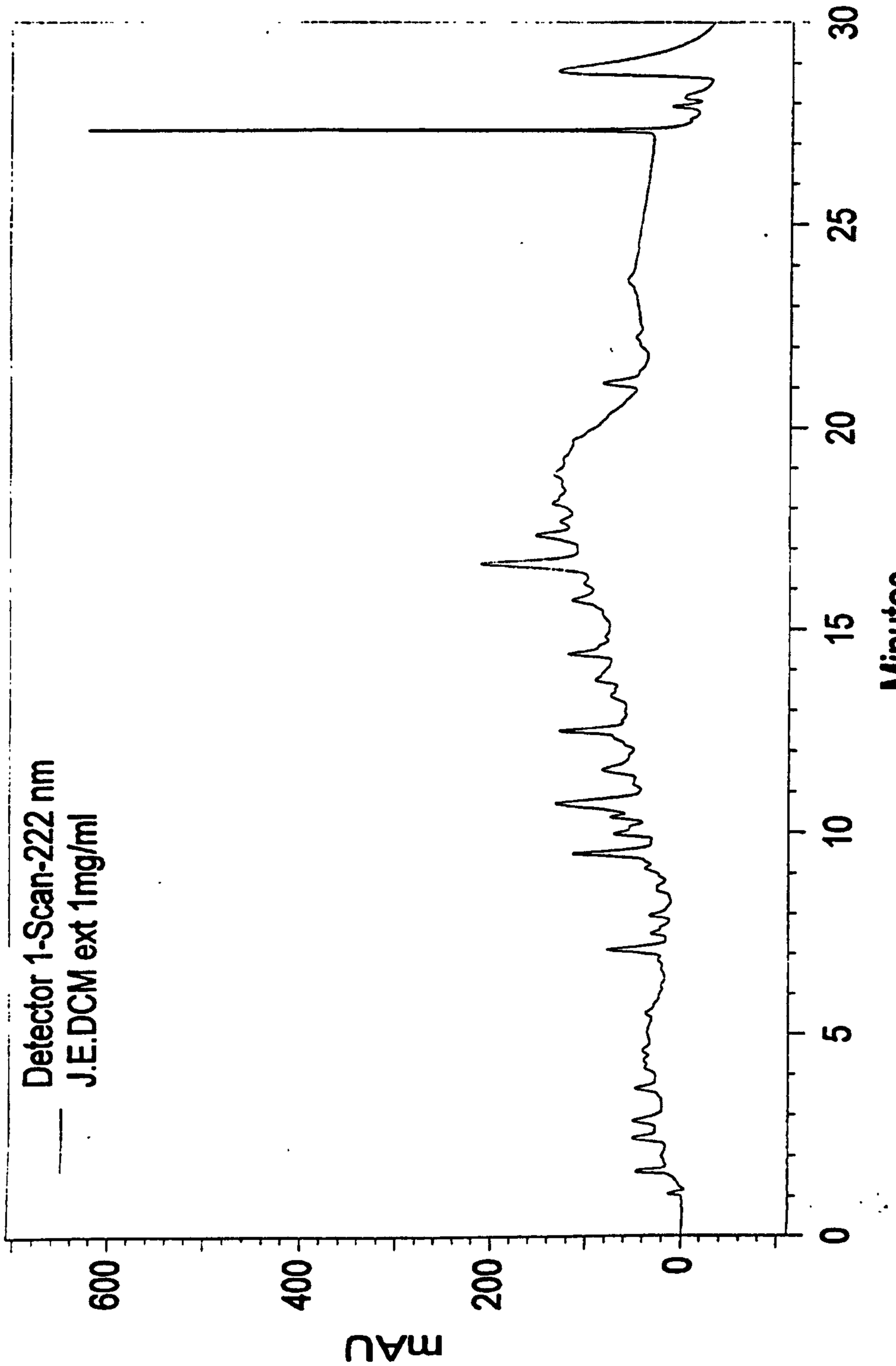


Figure 3.69: *J. Erythropoda* DCM extract HPLC chromatogram

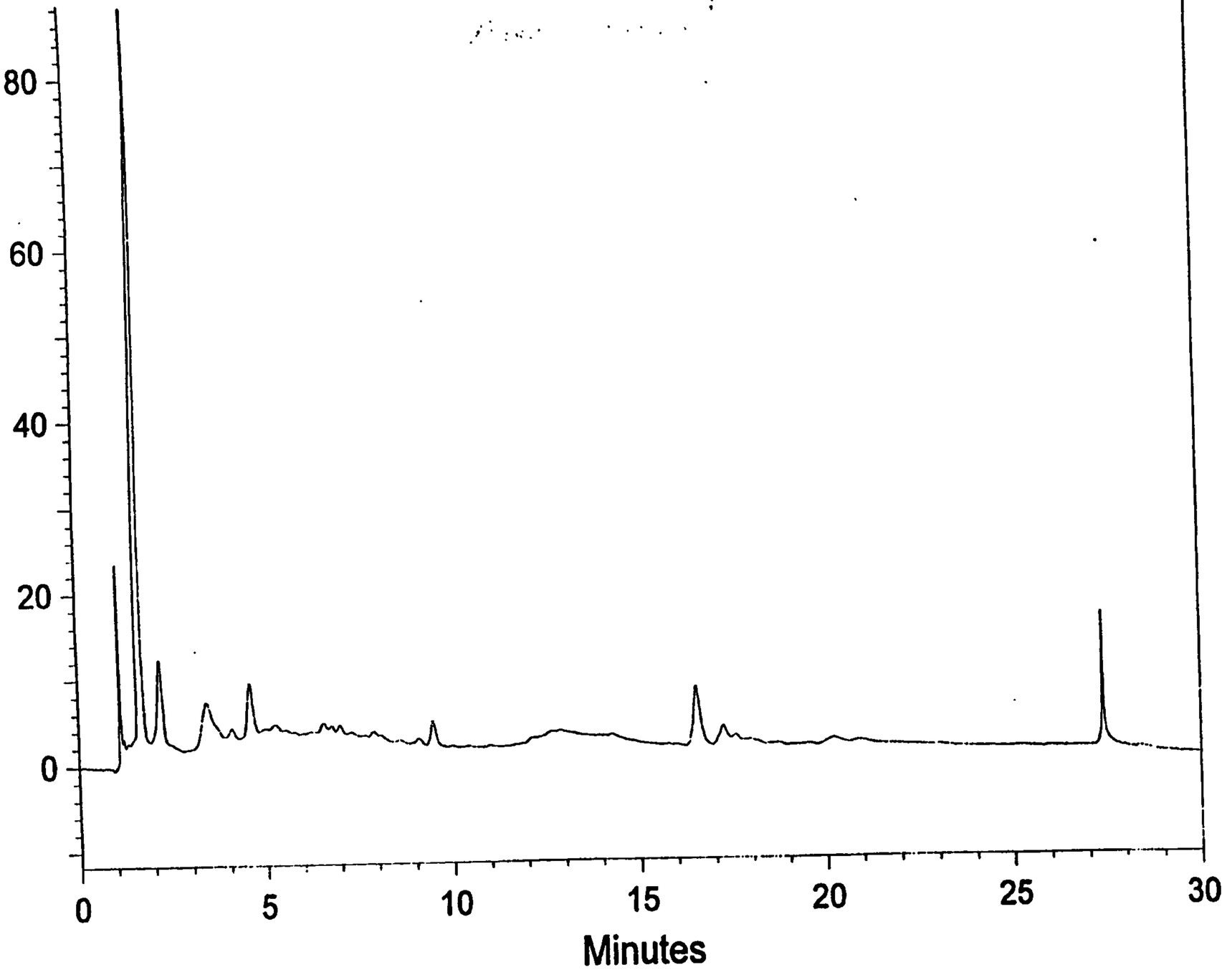


Figure 3.70: *J. Erythropoda* MeOH extract HPLC chromatogram

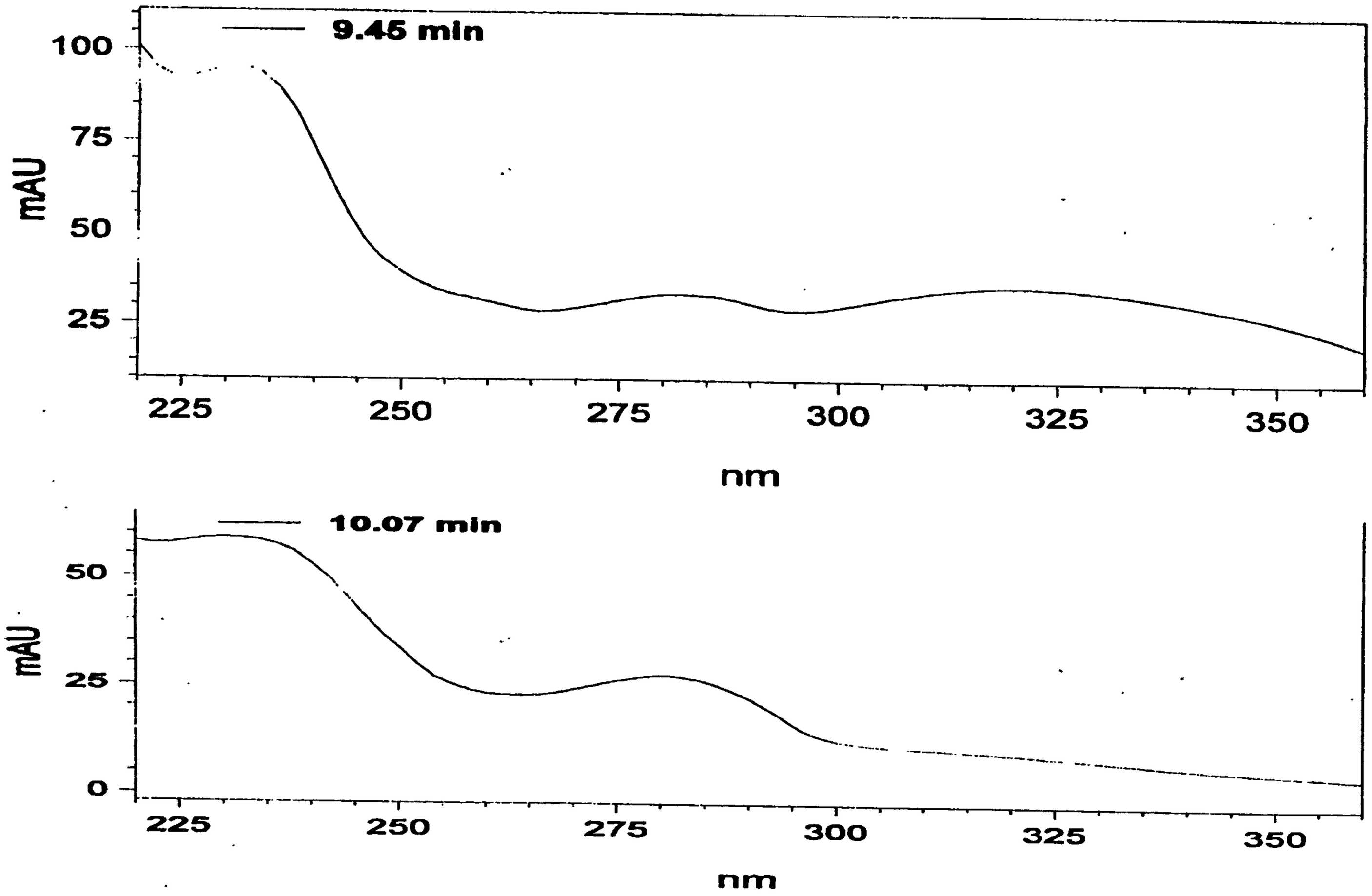


Figure 3.71: Representative UV spectra of compounds from *J.erythropoda* DCM extract

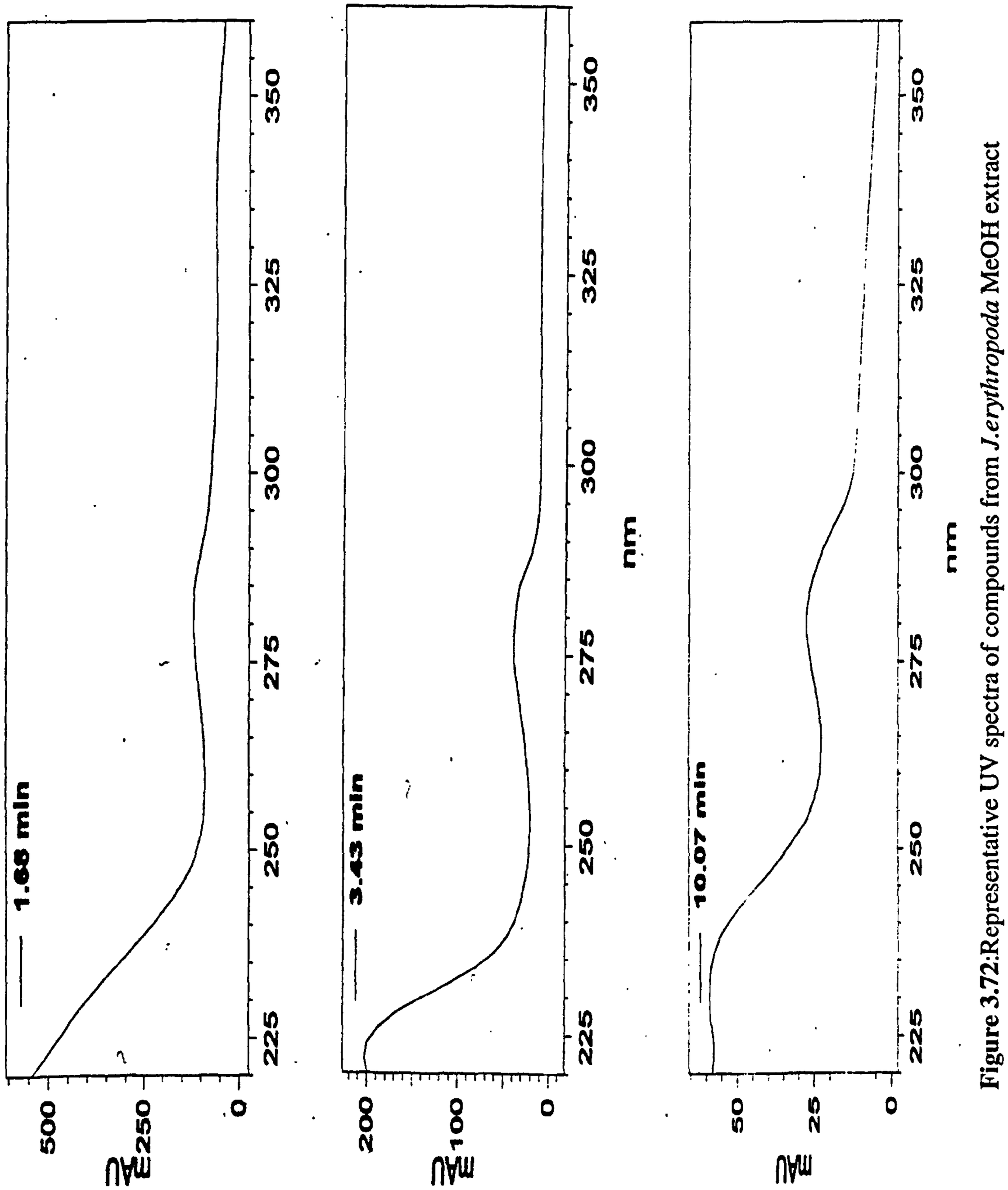


Figure 3.72: Representative UV spectra of compounds from *J.erythropoda* MeOH extract

3.3.5 *Cassia italica*

Extracts of the stems of this plant were analysed in the same way as *J. erythropoda*. Tests for alkaloids on TLC were negative. Results from spraying the plates with vanillin-sulfuric acid are shown in Fig 3.73 for the DCM extract and Figure 3.74 for the MeOH extract. Results indicate the presence of terpenoids (mainly) and flavonoids in the DCM extract and more flavonoids in the methanol extract than were detected in the *J. erythropoda* extract.

HPLC profiles of the extracts are provided at Figures 3.73 for DCM and 3.74 for MeOH. UV spectra of the major peaks in each extract are shown at Figures 3.75 and 3.76. Most of the compounds detected in this plant absorbed around 250, 275 to 360, thus indicating the presence of phenolics and flavonoids. Clearly these extracts contain more UV active compounds than *J. erythropoda.*

The methanol extract of this plant was subjected to NMR analysis and the ^{1}H NMR and ^{13}C NMR spectra are shown at Figures 3.77 and 3.78. The spectra display three sets of signals, the prominent being the sugar region. The other groups of signals are those around the aliphatic and the aromatic regions of the spectrum. In the sugar region, the doublet at δ5.40 (J=3.8 Hz) and that at δ4.40 (J=7.8 Hz) indicate the presence of the two mutarotational forms of glucose, α- and β-. In the ^{13}C NMR spectrum, the signal at δ61.8 indicates the presence of a methyl and thus the presence of a methylated sugar or sugar-like compound. Comparing this spectrum with that of inositol (Rahman, 2003) suggest that it could be the major compound could in the extract.

The presence of signals in the aliphatic region indicate the presence of terpenoid glycosides such as saponins especially since in the ^{1}H-^{1}H COSY some of the signals are coupling with the sugar region. The signals in the aromatic region indicate the presence of some phenolic compounds such as flavonoids and the presence of sugars (and their appearance in this polar medium) indicate that some of these occur as glycosides.

From the literature review it has been shown that this plant, in addition to anthracene derivatives, produces terpenoids, flavonoids and their glycosides (Table 1.2). These results support those reports.

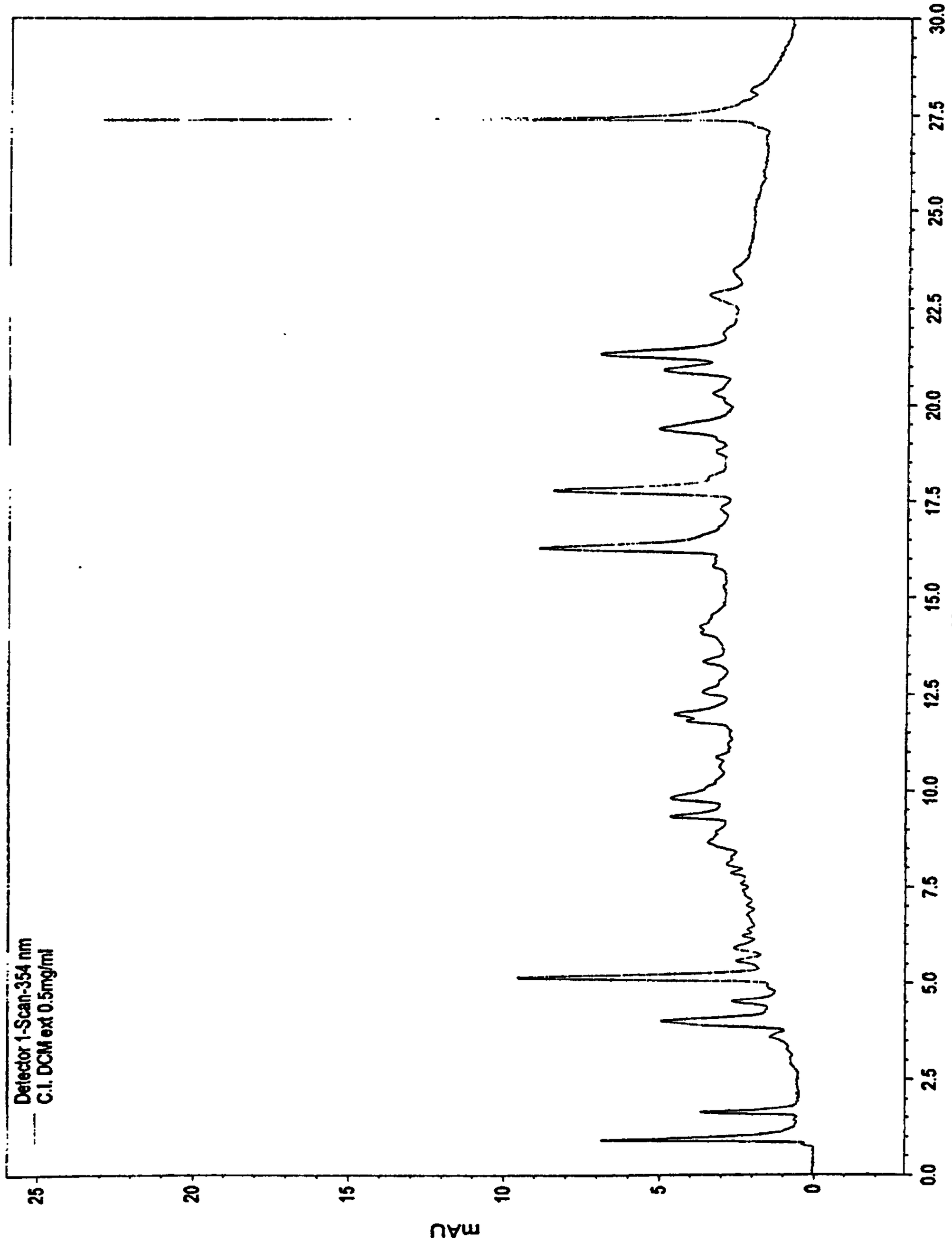


Figure 3.73: *C. Italica* DCM extract HPLC chromatogram

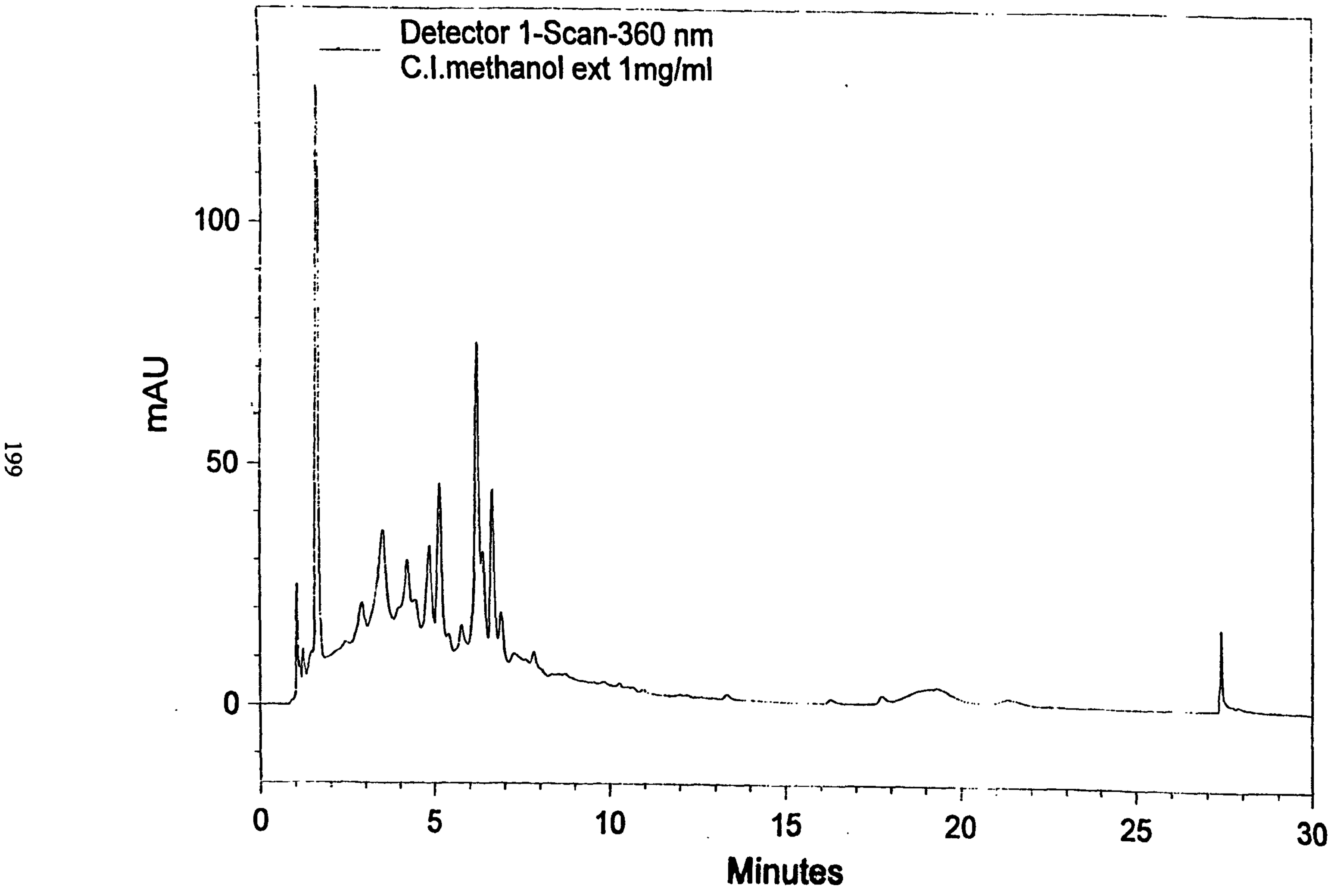


Figure 3.74: *C. Italica* MeOH extract HPLC chromatogram

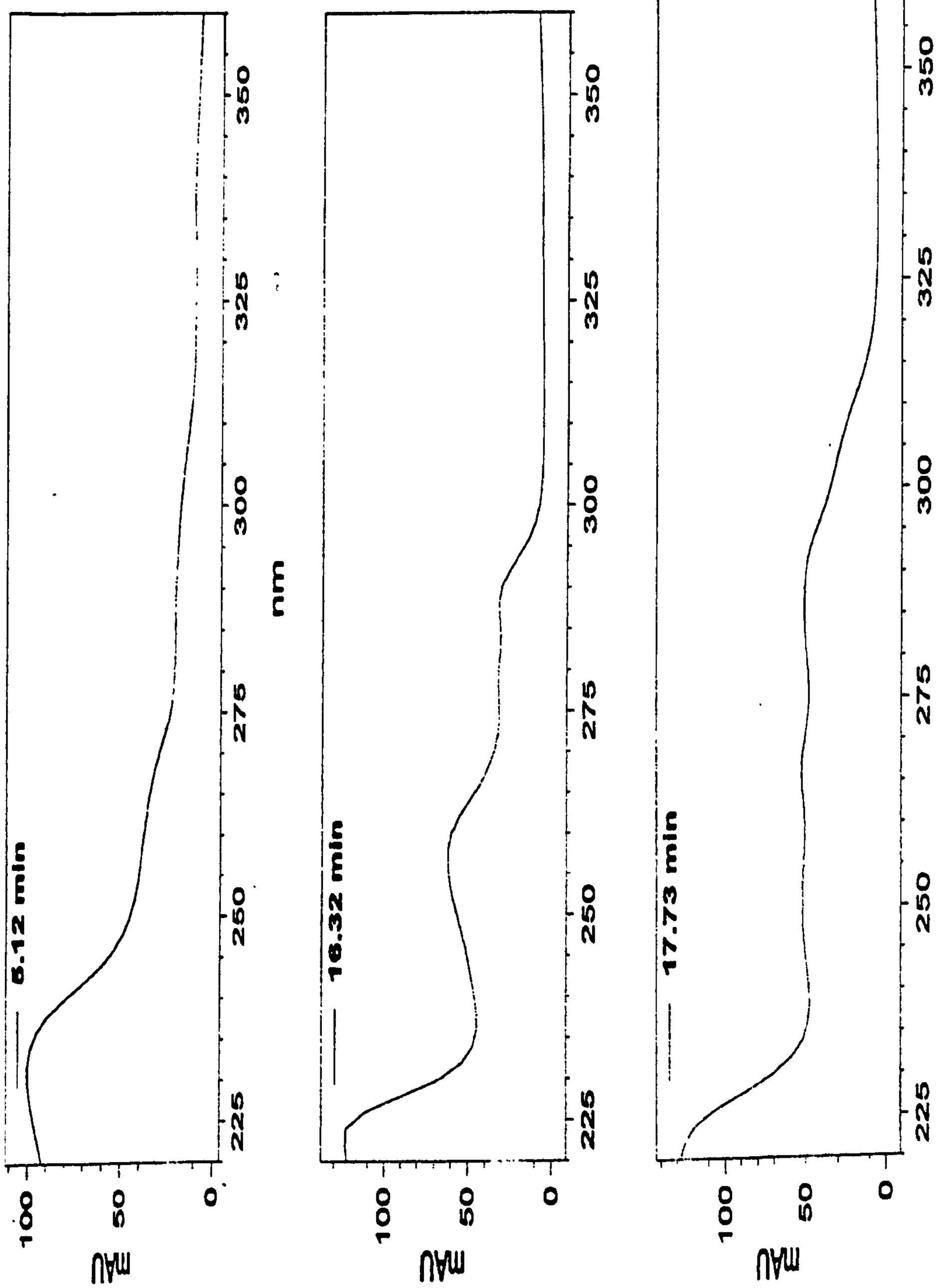


Figure 3.75: Representative UV spectra of compounds from *C. italica* DCM extract

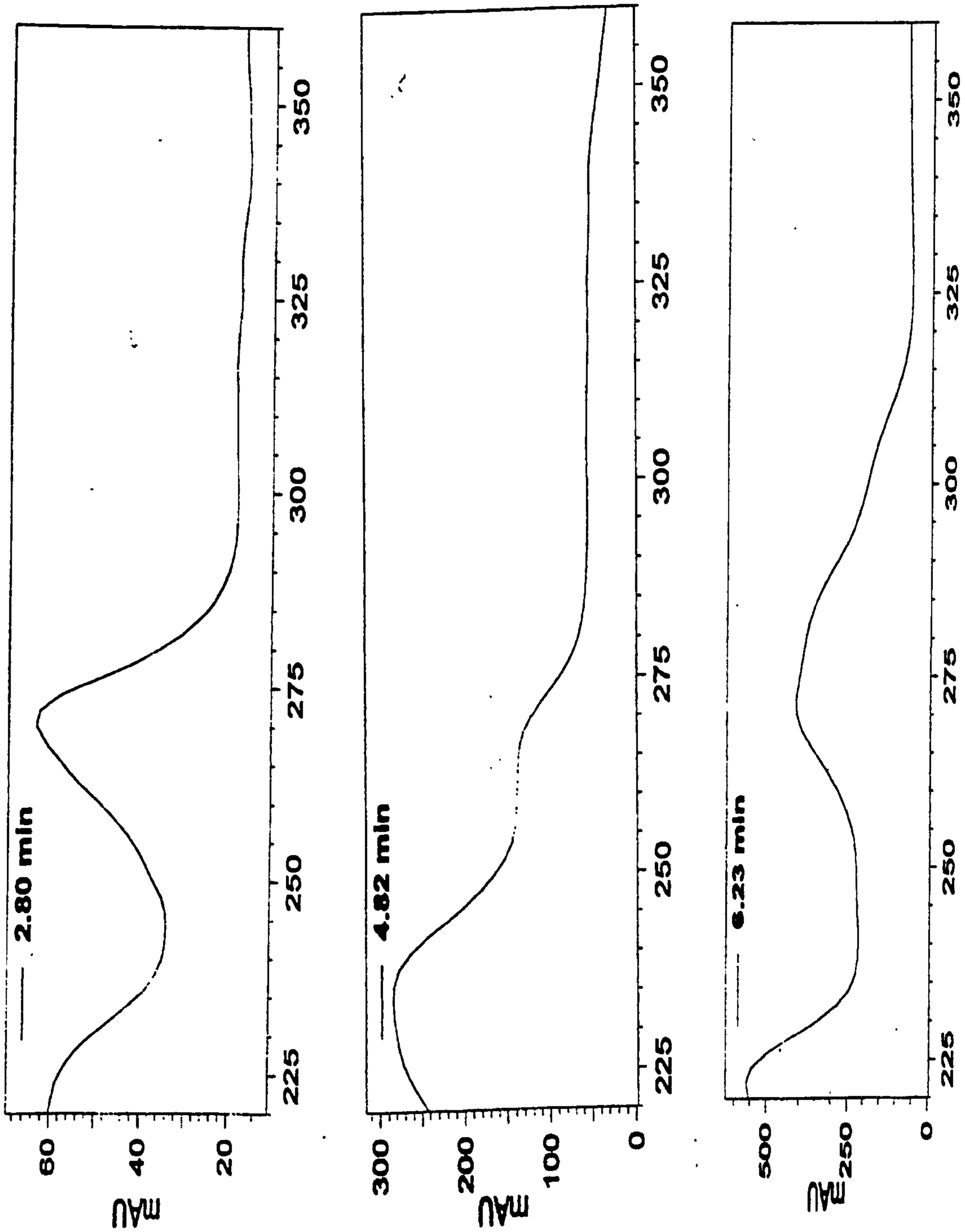


Figure 3.76: Representative UV spectra of compounds from *C. Italica* MeOH extract

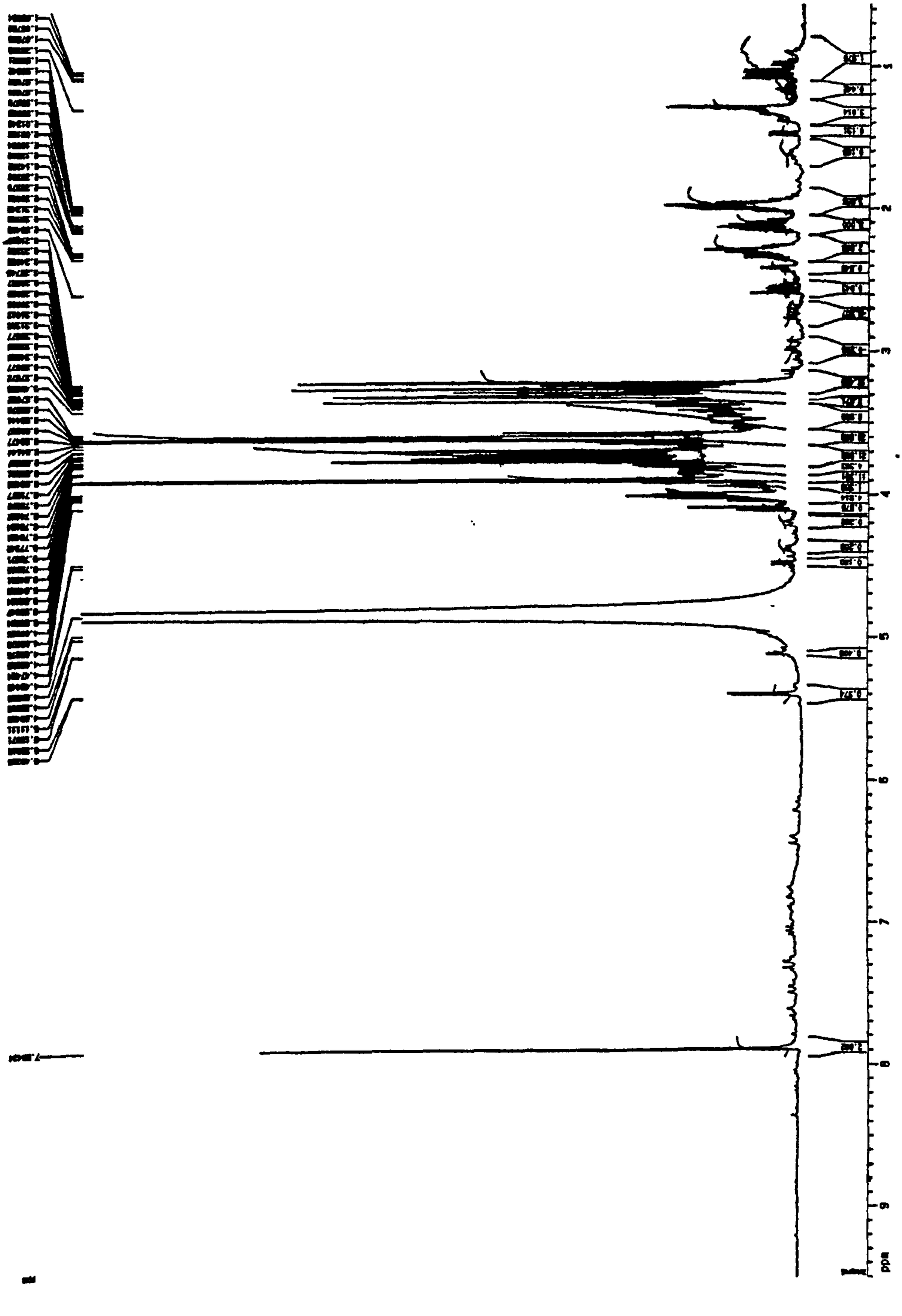


Figure 3.77: ^{1}H NMR spectrum of *C. italica* MeOH extract

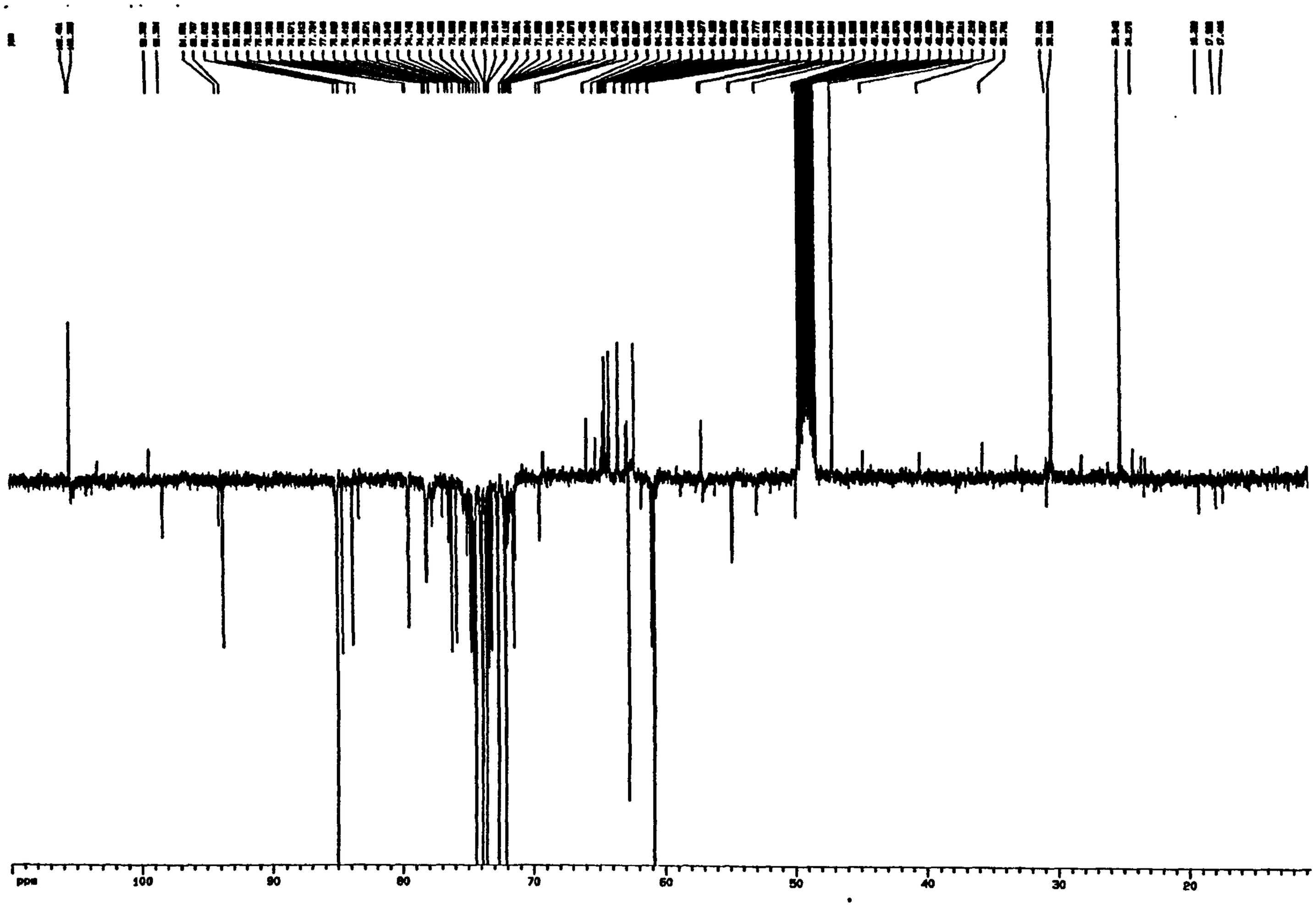


Figure 3.78: ^{13}C NMR spectrum of *C. italica* MeOH extract

3.3.6 *A. fruticosa*

The chemistry of this plant has been investigated extensively (Section 1.6.3.1). In this study 70% ethanol extract of the leaves of the plant was subjected to NMR analysis. The ^{1}H spectrum (Figure 3.79) shows signals in the aliphatic and mainly sugar regions. The ^{13}C NMR spectrum (Figure 3.80) also displays resonances in the methylene, methyl and sugar regions. There are some deshielded methine signals in the carbon spectrum. The sugars present appear to possess some methyl attachments thus supporting the presence of sugars such as oleandrose, digitoxose, cymanose etc. as part of the glycosides (Table 1.3). Overall the findings support reports that the plant produces cardiac glycosides whose aglycones are mainly aliphatic.

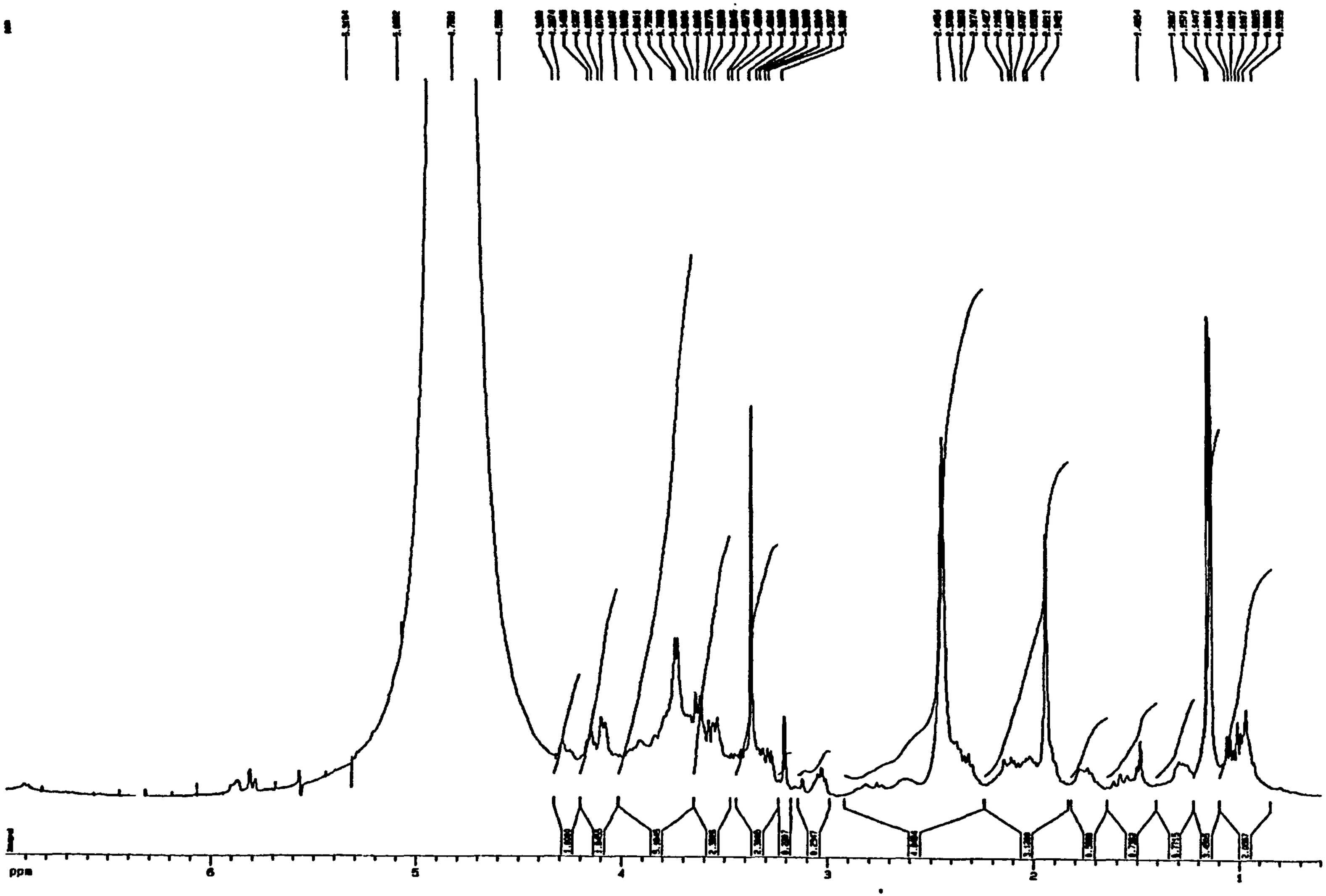


Figure 3.79: ^{1}H NMR spectrum of *A. fruticosa* extract

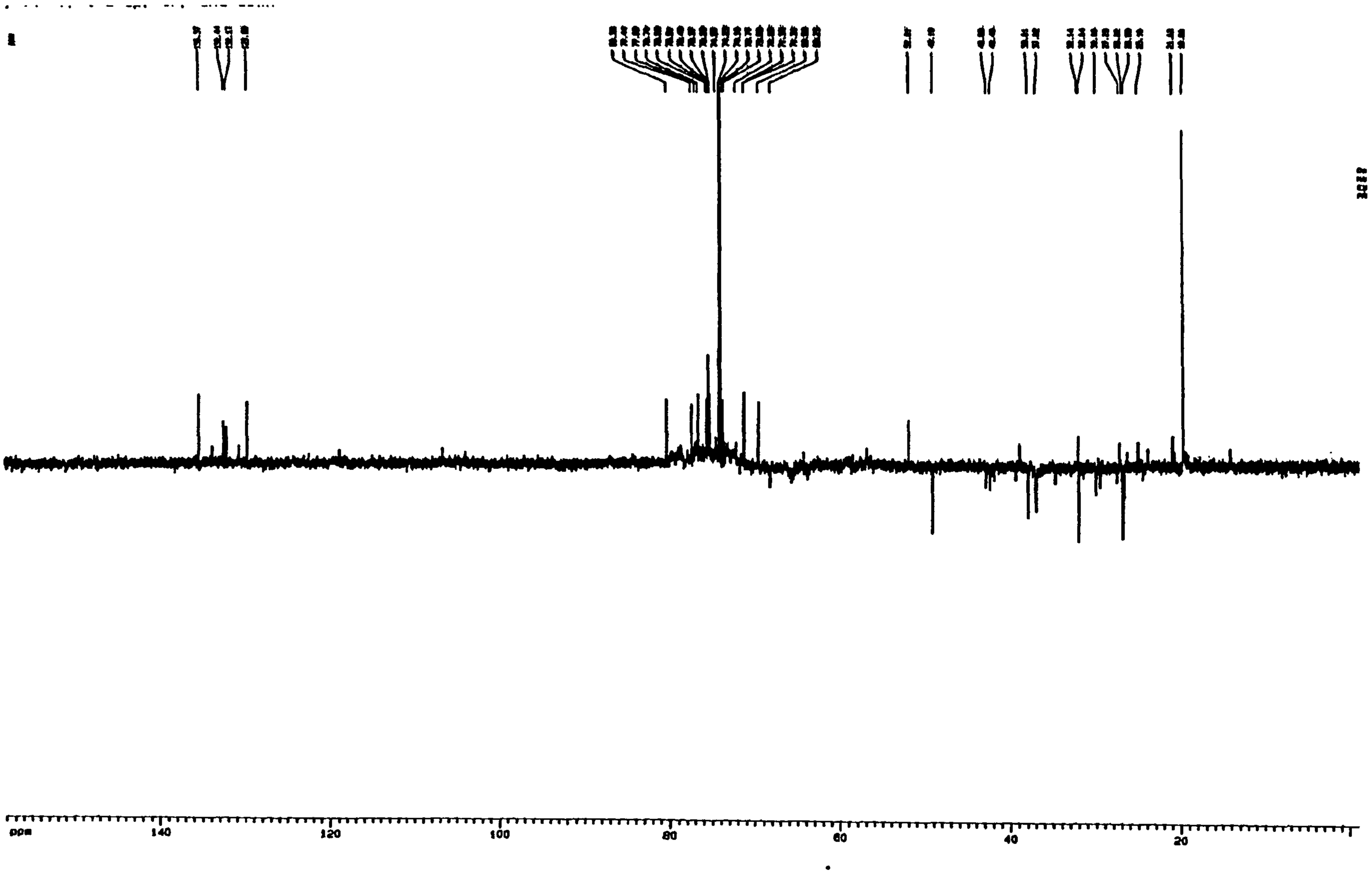


Figure 3.80: ^{13}C NMR spectrum of *A. fruticosa extract,* CH and CH_3 up, CH_2 and C down

3.4 Toxicology Results of isolated compounds

Some of the isolated compounds were subjected to cytotoxicity testing as described at Section 2.3. A stock solution of 1mg/ml of the compounds was made and then serially diluted two-fold.

3.4.1 Compounds from *Albizzia brevifolia*

Compounds isolated from this plant can be grouped into four types; triterpenoids, simple phenolics, flavan-3-ols (component of procyanidins), and a lignan glycoside. Four triterpenes were isolated, two of these, stigmasterol and β-sitosterol, are widespread in higher plants and their lack of toxicity is well known. For instance, stigmasterol is found in Soya beans (*Glycine max*), eaten by people. It is an essential component of plant cell walls. However, the glycoside of sitosterol, daucosterol (**100**) is reported to exhibit some cytotoxicity (Harrigan, 1990). The other two triterpenes are lupanes, betulinic acid and lupeol. Both are reported to have antitumour activities (Phytochemical Dictionary, 1999). In this study betulinic acid showed some cytotoxicity. The LD_{50} value for betulinic acid against the stomach cancer line and the melanoma cell line were 15.7 and 11.2 μg/ml respectively (Table 3.21). Lupeol was not tested because of sample amount limitation. Wada *et al.*, (2001) found that it expresses toxicity by acting as a catalytic inhibitor of human topoisomerase II activity (*in vitro*) with IC_{50} values in the range of 10-39μM.

Of the simple phenolics isolated, only 2-(3'-β-D-glucopyranoside,4'-hydroxyphenyl)ethanol was tested. The LD_{50} values were 51.2 and 63.5 μg/ml for the melanoma and SCL lines (Table 3.21). The results indicate that it is not really toxic at these levels. Feruladehyde and vanillin were not tested as they were isolated as a mixture and the yield could not allow further separation of the two. They are reported to have antibacterial (feruladehyde) and antifungal (vanillin) activity. There are no reports of them being toxic. Vanillin is used as a flavouring agent in confectionary, beverages,

foods and in perfumery (Phytochemical Dictionary, 1999). It is also used as a pharmaceutical aid.

The flavan-3-ol, (+)-catechin, was tested and the LD_{50} values were 40.18 and 38.03 μg/ml against the melanoma and SCL lines respectively. The results indicate that it showed very weak cell suppression at these concentrations and was even seen to promote cell growth at low concentrations (Figure 3.81). (+)-catechin is known to be biologically active. It is known to have antioxidant properties and is used as a haemostatic drug and in the treatment of various liver diseases. It shows strong liver-protective and potent antiperoxidative activities, so that it can act as a "radical scavenger" by neutralising free radicals produced by hepatotoxic substances. (Phytochemical dictionary, 1999). However it is reported that prolonged use of the compound can lead to several adverse reactions including haemolysis, acute renal failure and skin rashes. Lignans of the type isolated in this study are also known to have antioxidant properties (Lee, *et al.*, 1999; Phytochemical Dictionary, 1999).

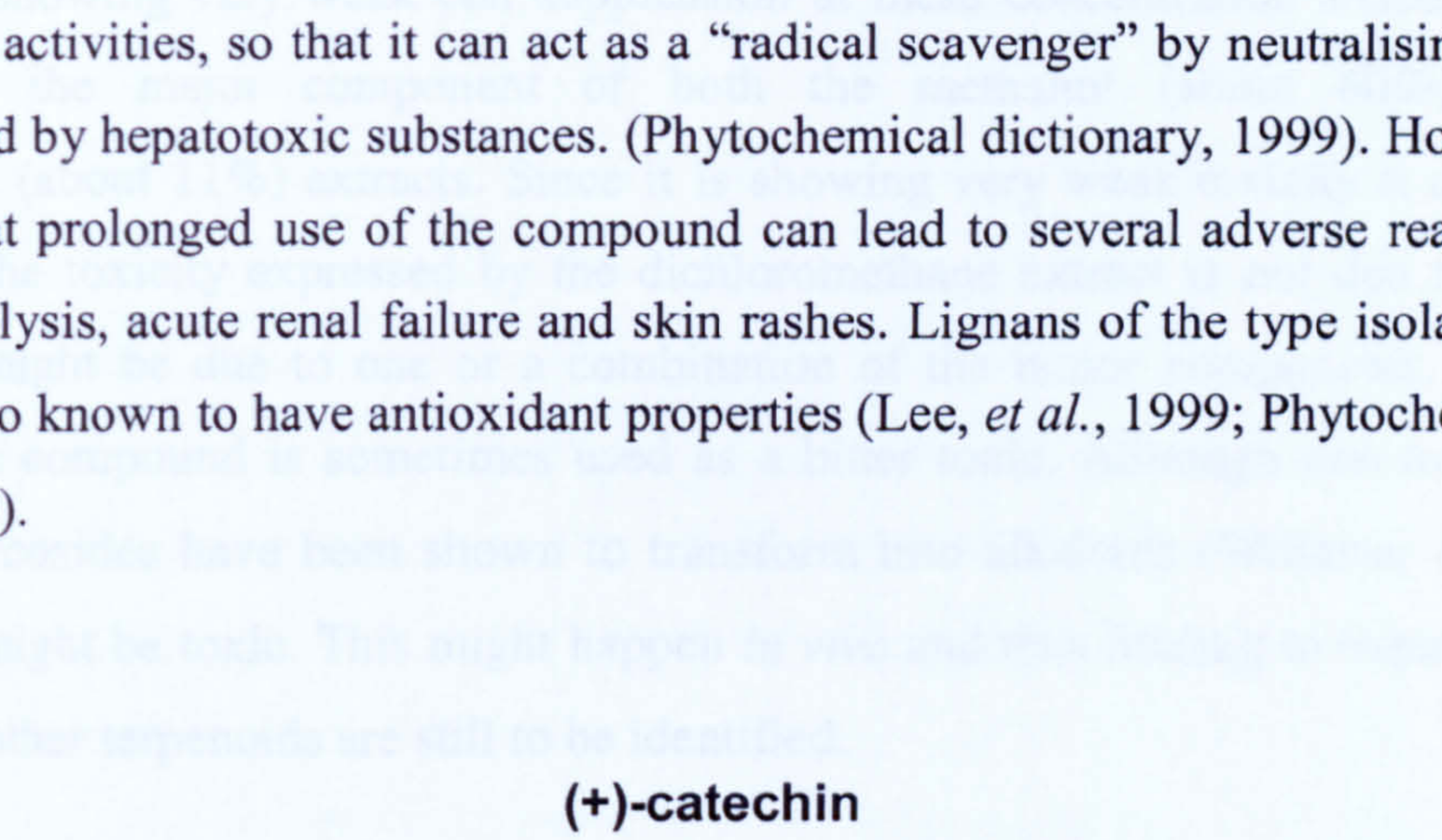


Figure 3.81: Dose-response curve for (+)-catechin

3.4.2 Compounds from *Enicostemma axillare*

Three groups of compounds were isolated from this plant: terpenoids including the triterpenes stigmasterol and β-sitosterol, a secoiridoid, swertiamarin and sugars. It has already been mentioned that stigmasterol and sitosterol are not known to be toxic and that the glycoside of β-sitosterol, is reported to be cytotoxic. The toxicity of swertiamarin was evaluated against two cell lines, melanoma and SCL. LD_{50} values were 32 µg/ml for the melanoma and 35 µg/ml for the SCL line (Table 3.21, p 213). The results show that the compound was showing very weak cell suppression at these concentration tested. This compound was the major component of both the methanol (about 60%) and dichloromethane (about 11%) extracts. Since it is showing very weak toxicity it can be concluded that the toxicity expressed by the dichloromethane extract is not due to this compound but might be due to one or a combination of the minor components. It is reported that the compound is sometimes used as a bitter tonic. Although non-toxic *in vitro*, iridoid glycosides have been shown to transform into alkaloids (Willems, 1987), some of which might be toxic. This might happen *in vivo* and thus leading to expression of toxicity. The other terpenoids are still to be identified.

3.4.3 Compounds from *Argemone mexicana*

Four alkaloids were isolated from this plant. These were evaluated against the two cell lines, melanoma and stomach cancer line. The LD_{50} values are provided at Table 3.21. Among the four alkaloids, sanguinarine was found to be the most toxic with LD_{50} values of 1.4 µg/ml (melanoma) and 0.215 µg/ml (SCL). Berberine also showed some toxicity, though not as much as sanguinarine. The LD_{50} values for berberine were 15.8 µg/ml for the melanoma and 16.8 µg/ml for the SCL. Protopine and allocryptopine showed the least toxicity with LD_{50} values around 30 µg/ml.

Both allocryptopine and protopine are reported to have antiarrythmic (Brossi, 1988) and anticholinergic (Ustunes, *et al.*,1988) properties. Protopine is reportedly used as a muscle

relaxant and sedative in animals. Reports indicate that it is active against gram-positive bacteria. Allocryptopine is reported to possess oxytocin properties.

Berberine is used as an antimalarial, antimicrobial, antipyretic and anthelmintica agent. It is reported to be cytotoxicity and moderately toxic to humans causing cardiac damage, and lowered blood pressure (Phytochemical dictionary, 1999). It is reported to inhibit acetylcholine easterase (Piancente, *et al.*, 1997; Ustunes, *et al.*, 1988).

Sanguinarine is reported to have antibacterial, antifungal, antitumour and anti-inflammatory properties (Das and Khanna, 1997). It is used a lot in dentrifices and mouth washes because of its anti-plague activity (Phytochemical Dictionary, 1999). It is known to have a positive inotropic effect to the heart. Its adverse effects include inhibition of enzymes such as Na^+/K^+ ATP-ase), diamine oxidase and some alanine and aspartate aminotranseferases ((Das and Khanna, 1997). It has also been shown to bind non-covalently to DNA by intercalating between GC-rich regions of the DNA (Maiti, *et al.*, 1982; Sen, *et al.*, 1996, and Schmeller, *et al.*, 1997). In this process, the planar aromatic rings of the compound get inserted between adjacent DNA base pairs (Figure 3.82) and this results in impairment of DNA polymerase activity. It is also known to stimulate smooth muscles and to have oxytocic properties. At high doses it reportedly causes glaucoma over a prolonged period. Its toxicity has been demonstrated in mice (LD_{50}: 19.4mg/kg) and has been linked with outbreaks of dropsy in India (Das and Khanna, 1997). These authors report that sanguinarine produces a metabolite, 3-4-benzacridine, which is also toxic and that the elimination of the two from the body is slow as they have been detected 96 hours after administration. This is thought to be probably due to the fact that sanguinarine and maybe its metabolite bind to plasma proteins, which in turn may lead to slow elimination.

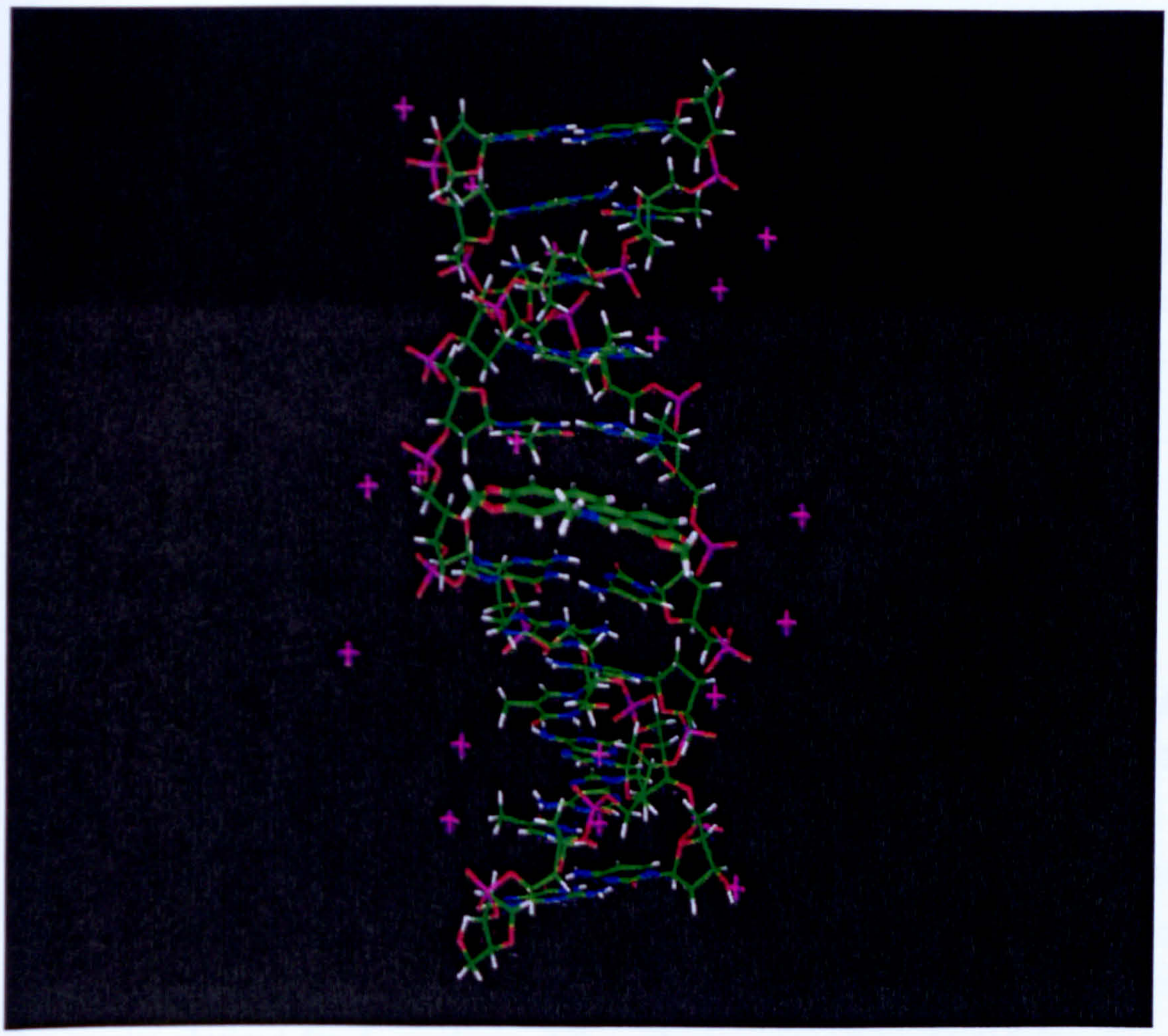


Figure 3.82: Computer model showing the binding of sanguinarine to DNA

3.4.4 Compounds from *Asclepias fruticosa*

As seen from section 3.1.6, extracts from this plant were the most toxic with LD_{50} values ranging from 1.3 to 3.4 μg/ml. The cardiac glycosides produced by this plant are responsible for the toxicity. This group of secondary metabolites is among man's oldest drugs. They have been used not only as medicines, but also as poisons (Section 1.6.3.2). Primitive people used them as arrow poisons. Cardiac glycosides have a pronounced effect on the heart. At therapeutic concentrations they increase the contractibility of cardiac muscles and are used to treat congestive heart failure. However a slight increase in the concentration results in detrimental effects to the user (Section 1.3). The toxic

doses are only slightly above the therapeutically effective doses and this applies to all of them (Bowman and Rand, 1980). At toxic levels cardiac glycosides cause cardiac dysrhythmias, which is the most life threatening of the toxicological manifestations. Other signs of toxicity include anorexia, nausea and diarrhoea, drowsiness and fatigue and visual disturbances (Rang and Dale, 1987). The major enzymatic effect of cardiac glycosides is the inhibition of membrane Na^+/K^+-ATPase and it is generally agreed that the main toxic actions of cardiac glycosides on the heart is a consequence of inhibition of this enzyme (Bowman and Rand, 1980). This results in the malfunctioning of the electrical conduction system.

3.4.5 Compounds from *Jatropha erythropoda*

The methanol extract of this plant showed most activity (Table 3.4). This suggests that the polar components of this plant are responsible for the observed effects. From the TLC results, the methanol extract showed the presence of glycoside, and some such as saponins, are known to be toxic. The toxicity observed, however, is not as strong as that exhibited by *A. fruticosa*; (LD_{50} values from 0.0013–0.0034 mg/ml as compared to those of *J. erythropoda* which range from 0.268-0.661 mg/ml). However, as demonstrated by the results obtained for *A. mexicana* (LD_{50} values ranging from 0.557-0.735 mg/ml), extracts may exhibit mild toxicity even though the plant produces a very toxic compound, but because these would be present at low concentrations in the extract, their effects are masked by other components.

3.4.6 Compounds from *Cassia italica*

The toxicity of this plant has been reported by a number of authors (Bakhiet and Adam, 1996; Galal *et al.*, 1985, Section 1.6.2.2). Compounds that are reported to have been isolated from this plant in previous studies are listed at Table 1.2 (Section 1.6.2.1). In this study TLC and HPLC analysis of extracts of this plant have shown evidence of the presence of terpenoids, phenolics and flavonoids. The ^1H NMR data has further shown that some of these compounds occur as glycosides. Toxicity was seen to be expressed

mainly by dichloromethane extract (LD_{50}: 0.250–0.481 mg/ml), which is of medium polarity. It can be concluded therefore that some of the terpenoids and flavonoids could be responsible for the observed activity. The polar extracts were less toxic (LD_{50} values for water extract ranging from 0.734-0.850 mg/ml and for methanol extract around 1mg/m).

Summary of the results

A summary of LD_{50} values is provided in the following table. Figure 3.83 provides a graphical comparison of LD_{50} values of the tested compounds. As can be seen sanguinarine is very toxic compared to the other compounds.

Table 3.21: LD_{50} values for some of the isolated compounds

LD_{50} (µg/ml)		
Compound	**Cell lines**	
	Melanoma	**SCL 4°**
Betulinic acid (BA)	11.2	15.7
(+)-Catechin	40.18	38.03
2-(3'-β-D-glucopyranoside,4'-hydroxyphenyl)ethanol (PE)	51.2	63.47
Swetiamarin	32.0	35.4
Protopine	29.7	35.0
Allocryptopine	28.6	29.1
Berberine	15.8	16.8
Sanguinarine	1.4	0.215

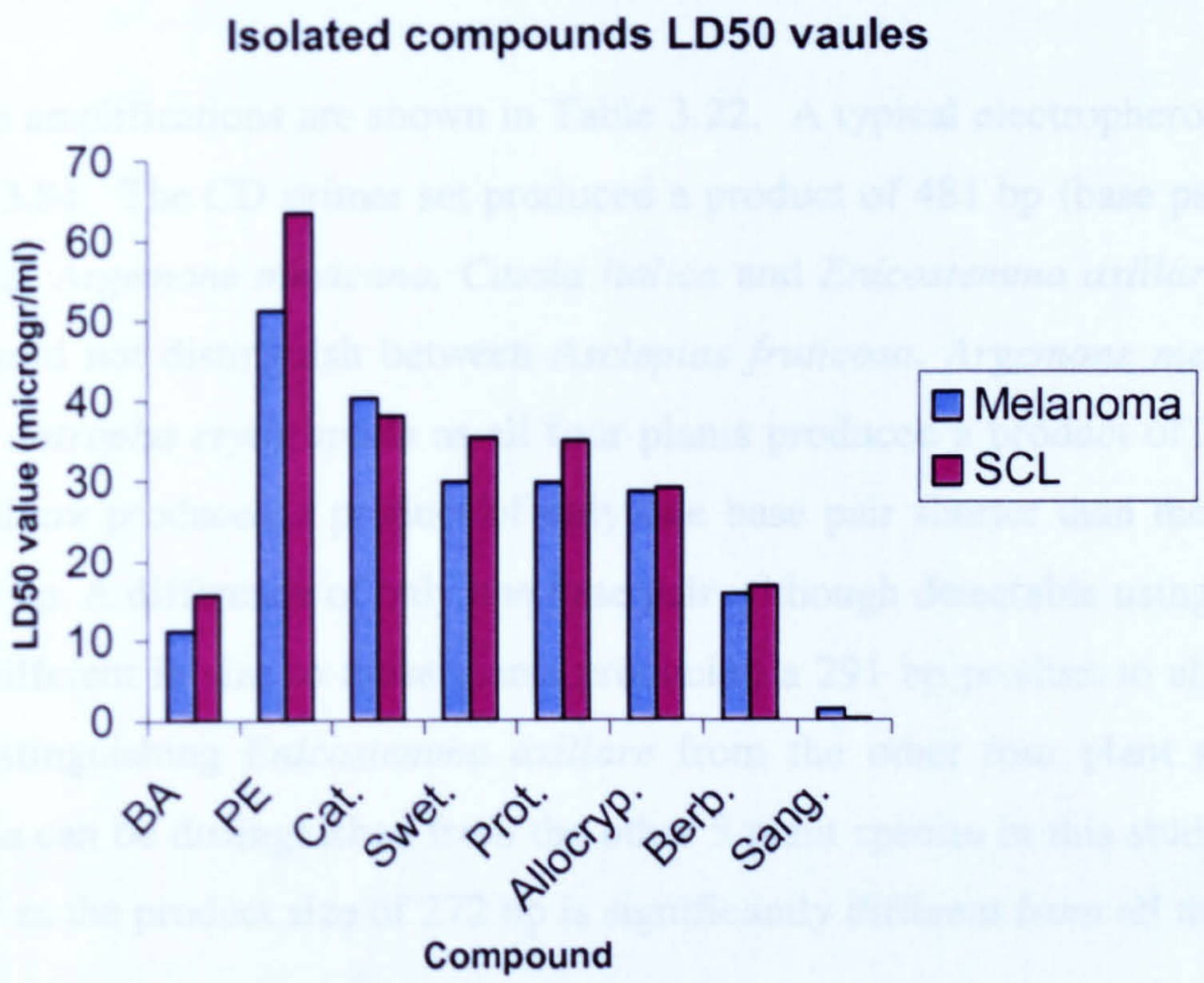
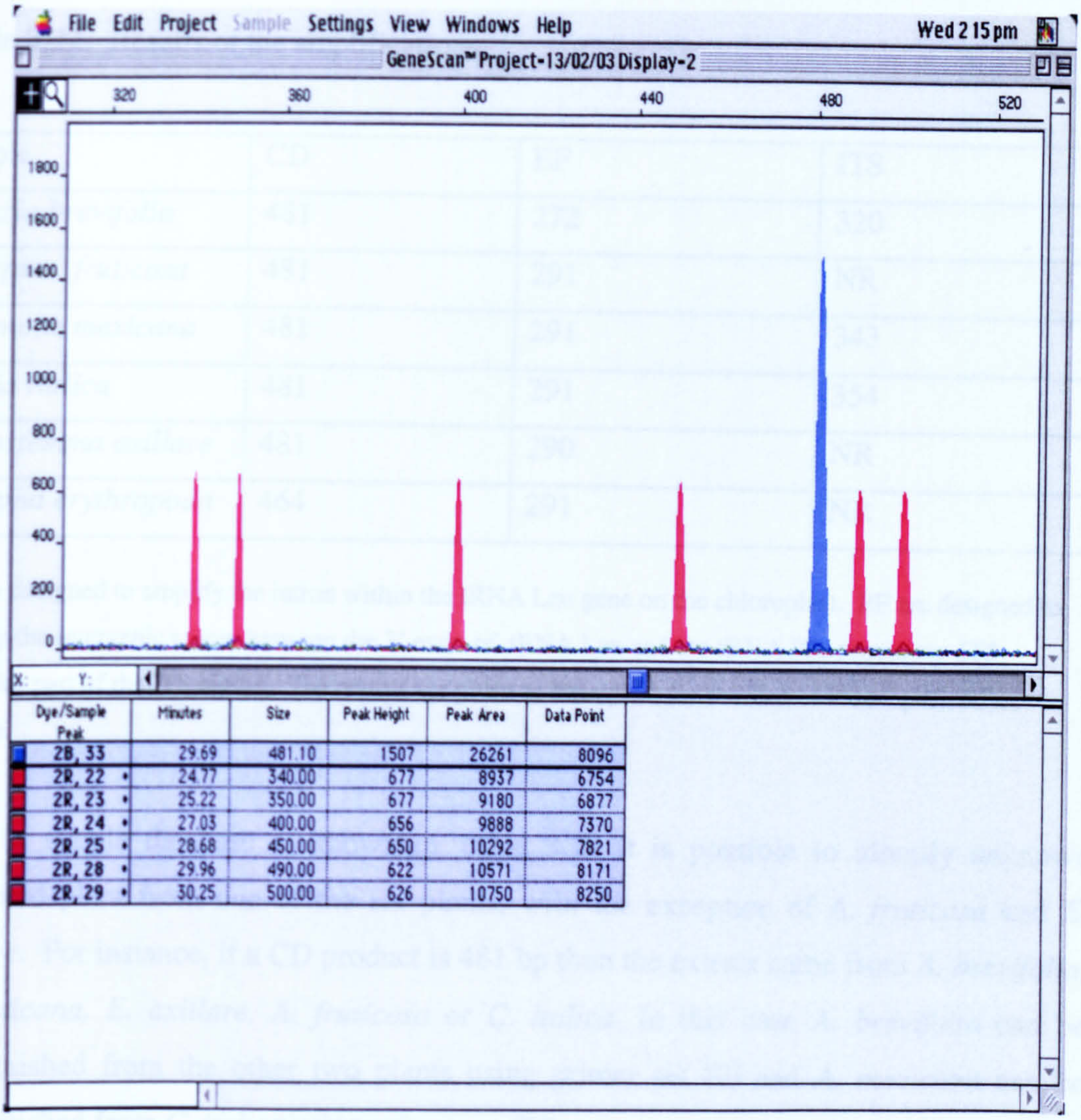


Figure 3.83: Comparison of LD_{50} values for isolated compounds

3.5 DNA Results

The results of the amplifications are shown in Table 3.22. A typical electropherogram is shown in Figure 3.84. The CD primer set produced a product of 481 bp (base pairs) for *Albizzia brevifolia, Argemone mexicana, Cassia italica* and *Enicostemma axillare.* The primer set EF could not distinguish between *Asclepias fruticosa, Argemone mexicana, Cassia italica* or *Jatropha erythropoda* as all four plants produced a product of 291 bp. *Enicostemma axillare* produced a product of only one base pair shorter than these four plants, being 290 bp. A difference of only one base pair, although detectable using CE, is not sufficiently different in size to those plants producing a 291 bp product to allow for confidence in distinguishing *Enicostemma axillare* from the other four plant species. *Albizzia brevifolia* can be distinguished from the other 5 plant species in this study using the primer set EF as the product size of 272 bp is significantly different from all the other plant species.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
2B, 33	29.69	481.10	1507	26261	8096
2R, 22 •	24.77	340.00	677	8937	6754
2R, 23 •	25.22	350.00	677	9180	6877
2R, 24 •	27.03	400.00	656	9888	7370
2R, 25 •	28.68	450.00	650	10292	7821
2R, 28 •	29.96	490.00	622	10571	8171
2R, 29 •	30.25	500.00	626	10750	8250

Figure 3.84: showing the amplification product using primer set CD on *A. fruticosa*. The blue coloured peak represents the amplification product. The size is shown as being 481.10 base pairs in mass. This is calculated by the software on the computer based upon the size standards shown in red (340, 350, 400, 450, 490 & 500).

Table 3.22: Results of the amplifications

Sample	CD	EF	ITS
Albizzia brevifolia	481	272	320
Asclepias fruticosa	481	291	NR
Argemone mexicana	481	291	343
Cassia italica	481	291	354
Enicostemma axillare	481	290	NR
Jatropha erythropoda	464	291	NR

CD are designed to amplify the intron within the tRNA Leu gene on the chloroplast. EF are designed to amplify the intergenic spacer between the 3' exon of tRNA Leu and the tRNA Phe sequence. ITS amplifies part of the ITS region. The results are given as base pairs for the size of the amplification product.

Using a simple database as shown in Table 3.22 it is possible to identify unknown powdered plant from one of the six plants, with the exception of *A. fruticosa* and *E. axillare*. For instance, if a CD product is 481 bp then the extract came from *A. brevifolia*, *A. mexicana, E. axillare, A. fruticosa* or *C. italica*. In this case *A. brevifolia* can be distinguished from the other two plants using primer set EF and *A. mexicana* can be distinguished from *C. italica* using primer set ITS.

In some instances one primer set would be sufficient for identity, but a combination of all three primer sets will yield more confidence. It is possible to amplify all three primer sets in the same reaction, called a multiplex, thus reducing the time and effort.

In cases where there is a mixture of plant species, a mix of the different sized products will be obtained. It is possible to determine the species present, although mixtures of more than two plant species may prove problematic.

Summary of the results

A simple DNA test has been developed that is capable of identifying the species of plant present from powdered fragments. The test uses allele specific amplicons that show polymorphisms in the length of DNA sequences between two conserved primers. The simple PCR test can be performed in one day from extraction of DNA through to analysis of the fragment. The database of fragment sizes will grow as more plants are tested and it will be possible to add more loci if required.

CHAPTER 4:

CONCLUSIONS

4.0 CONCLUSIONS

The primary objectives of this study were to investigate the toxicity and chemistry of some of the medicinally used plants that have been presented to the Botswana Police Forensic Science Laboratory as exhibits in cases of suspected poisoning. Six plants from different genera were studied. Crude extracts of the plants and compounds isolated from them were evaluated for toxicity using established human cancer cell lines and a murine macrophage cell line. These objectives were achieved and while, no claims can be made that any of the plants investigated was studied to exhaustion, a number of useful observations were made during the study. The secondary objective was to develop a DNA-based method that can be used for species identification where the exhibit offered insufficient material for taxonomical and chemical identification. This has also been achieved.

Traditional medicinal uses of each of the plants in Botswana and other countries are outlined at section 1.6. Extracts from all of them were shown to exert stress and/or kill cells to varying degrees depending on the solvent used for extraction and hence the nature of the groups of secondary metabolites extracted. In general activity was not limited to extracts of a particular polarity. The most toxic among all the plants was *Asclepias fruticosa L.* (Asclepiadaceae). The general toxicity exhibited by extracts of the other five plants when compared to that of *A. fruticosa* ranged from medium to low. During the course of this study a variety of secondary metabolites were isolated. A total of eighteen compounds were identified. Not all of them were tested for toxicity due, in some cases, to their low yield from the material available. However, among those selected for testing, some exhibited toxicity.

All extracts from *Albizzia brevifolia* (Leguminosae) exhibited toxicity, the most toxic being the methanol extract. Ten compounds were isolated from the stem bark of this plant. Most of these have been isolated from the genus *Albizzia* before but are isolated for the first time from this plant in this study. These compounds comprised triterpenoids, simple phenolics, a procyanidin component, a lignan glycoside and other phenolic

glycosides. Most of the compounds isolated from *A. brevifolia* did not show significant toxicity at the levels tested. The most active was found to be betulinic acid (LD_{50}: 11.2 - 15.7 µg/ml). Lupeol and the glycoside of sitosterol, daucosterol, are known to be cytotoxic (Wada *et al.*, 2000 and Harrigan, 1990, respectively). The toxicity expressed by the extracts, especially the dichloromethane extract, could therefore be attributed to compounds such as betulinic acid, lupeol (see Section 3.3.1.1) or some other components that were not isolated (see Section 1.6.4). The toxicity might also be due to synergetic activity of the constituents. The genus *Albizzia* is known to produce cytotoxic saponins (Zou *et* al, 2000 and Ideda, *et al.*, 1997) and alkaloids (Rukunga, 1996). *A. brevifolia* in this study, however tested negative for alkaloids. These results show that there is need for caution when administering the plant material; at high concentrations it can cause serious toxicity problems.

Polar extracts of *Enicostemma axillare* L. (Gentianaceae) did not show significant toxicity at the tested concentrations whereas the dichloromethane extract displayed toxicity. Five compounds were isolated from this plant. These comprised terpenoids, a secoiridoid glucoside, swertiamarin and sugars. These compounds have been isolated before from some members of the family, but are isolated for the first time from this plant. Swertiamarin, which was isolated from the polar components of *E. axillare* (see Section 3.3.2.1) showed no toxicity at the tested concentrations. This compound was the major component of the plant amounting to about 60% of the methanol extractive. The observed toxicity of the dichloromethane extract of which about 10% was swertiamarin was therefore more likely to be due to other minor components. These results also suggest that, taken in aqueous medium, extracts of this plant would probably cause little harm, but were they to come into contact with a lipophilic medium they would pose danger to users. These dangers may also be a reality where mixtures of plants are used as medicine. For example, a plant that produces saponins may facilitate the extraction of a wide range of chemical components and therefore amplify the potency of toxic extractives. Methanol extracts of the only other species of genus, *E. hyssopifolium* (synonymous with *E. litorale*), are reported by Kavimani and Mansenthikumar (2000) to have exhibited cytotoxic properties. The authors did not isolate swertiamarin from this

plant (Ghosal, *et al.*, 1974). These findings support the division of the genus *Enicostemma*, which was originally thought to be monospecific (Ghosal, *et al.*, 1980) into more than one species (Magora *et al.*, 2003).

The dichloromethane and methanol extracts of *Argemone mexicana* L. (Papaveraceae) were seen to be toxic whereas the water extract did not show significant toxicity at the tested concentrations. Four alkaloids were isolated from *A. mexicana* leaves. These have been isolated from the plant before, mostly from the seeds. The most toxic of these was sanguinarine (LD_{50}: 0.22 – 1.4 µg/ml). This compound was also the most toxic of all the isolated compounds. The other alkaloids also showed some cytotoxicity, though not as strong as sanguinarine. Outbreaks of toxicity attributed to this plant have been reported (Section 1.6.5.2). These were mainly due to contamination of seed oil with *A. mexicana* seeds, which resemble mustard seeds (Das and Khanna, 1997; Meaker, 1950, Sakar, 1926 and 1948; Singh, *et al.*, 1983; Sohrab, *et al.*, 1961). The results from this study support reports that toxicity of this plant is mainly due to sanguinarine and it has been shown that toxicity is due to its enzyme inhibitory (Na^+/K^+ ATPases) properties and its ability to intercalate with DNA bases (Das and Khanna, 1997, Maiti, *et al.*, 1982, Sen, *et al.*, 1996; Schmeller, *et al.*, 1997) and thus interfering with the replication process (See Section 3.4.3 and Figure 3.82). Even though the compound has some medicinal uses, the use of this plant as medicine is not safe, as doses are not controlled.

The methanol extract of *Jatropha erythropoda* (Euphorbiaceae) showed most toxicity. The dichloromethane and methanol extracts of this plant were shown to contain terpenoids, flavonoids and glycosides. Some glycosides, such as saponins are known to be toxic and these have been isolated from other species of the genus that have been associated with toxic properties including *J. curcas* (Oluwole and Bolarinwa, 1997; Ghandi, *et al.*, 1995; El-Badwi, *et al.*, 1995, Section 1.6.1.2). For *Cassia italica* Lam. Ex. (Leguminosae) the dichloromethane extract showed most toxicity whereas the methanol extract was the least toxic. *C. italica* also has shown the presence of terpenoids, more flavonoids than *J. erythropoda* and glycosides of these. The toxicity of *C. italica* has been reported by a number of authors (Section 1.6.2.2, e.g. Bakhiet and Adam, 1996;

Galal *et al.*, 1985). The results of this study thus support these reports that *C. italica* exhibits toxic properties. The observed activity of extracts of these two plants could therefore be attributed to any of the detected secondary metabolites, especially the terpenoids and glycosides. Flavonoids are known to have antioxidant properties and have not yet been associated with acute toxicity. It would be useful to isolate compounds from these plants in the future and establish which exactly are responsible for the observed activity. In the meantime, results from this study call for caution to be exercised in the use of these plants as medicines.

Asclepias fruticosa extracts were the most toxic (LD_{50}: 1.3 –3.4 µg/ml) encountered in this work. The plant displayed the presence of cardiac glycosides and these are responsible for the observed toxicity of its extracts. Their main toxicity effect is also by inhibition of the Na^+/K^+ ATPases, which results in the malfunctioning of the electrical conduction system, affecting mostly the cardiac muscles and leading to arrythmias (Bowman and Rand, 1980). Clearly this plant as a medicine can only be used under controlled conditions and its use by traditional healers should be discouraged as mistakes in dosage can result in fatalities. This problem could be exacerbated by seasonal variations in the production of the toxic constituents by the plant. This problem applies to other plants as well.

For those extracts and compounds that did not exhibit much acute toxicity, the possibility of chronic toxicity still exists and has to be taken into consideration. Another potential danger with herbal medicine is the possibility of cross contamination especially in developing or the so-called third world countries, where only minimal forms of hygiene and quality control are exercised. If for instance, *A. fruticosa* plant material was accidentally allowed to contaminate plant material that is ordinarily taken in large quantities, life-threatening toxicity could result. As with any other poisonous substance, malicious use of such toxic materials is a possibility, though that would be a matter for the courts to establish and deal with. The toxicologist's concern is to produce data regarding the toxicity of the plant material.

It has been shown (Section 3.2) that some of the randomly selected actual case samples were toxic. This clearly indicates that these toxic materials are dispensed out to the general public and that they do find their way into vital processes *in vivo*. This is supported by the findings by forensic pathologists (Section 1.2) that these materials do cause a significant percentage of deaths. This proves that there is a basis for the concern raised regarding the safety of herbal medicines.

The DNA investigation has revealed three markers that were able to differentiate four of the six plant species. This has provided the basis for the creation of a database to which more species will be added as more markers are investigated in the future. This is going to prove useful in the future where only small amounts of materials are available and in powder form and especially where there are no unique phytochemical markers that could be used to identify the plants. Once the species is known more of it can be collected from the source or the wild to allow detailed toxicological and phytochemical work.

This work has provided experience in the isolation and structure elucidation of natural products, especially since a diverse collection of compounds was isolated and characterised. Spectral libraries of the commonly encountered secondary metabolites will be compiled in the future and this is going to simplify and speed up casework. Experience has also been gained in the area of toxicity evaluation of plant extracts and their components, so that in the future, facilities permitting, an indication of whether the plant material in question is potentially toxic or not could be obtained in a short time and would thus provide the basis for further toxicological investigation. The methods used and developed in this study are applicable to the Botswana situation.

Plants are an important source of therapeutic agents (Harborne, 1988; Sneader, 1996) and useful drugs will continue to be isolated from them. However, this study has shown that some of the plants used in traditional medicine in Botswana are toxic and therefore the dose should be regulated as well as the supply. The key issue is that there is need for quality control and controlled administration of herbal medicines.

The work forms the platform in the endeavour to assist the criminal justice system in Botswana, and traditional healers, in issues relating to the safety of herbal medicines. Some of the questions raised by the Justice System in Botswana can now be answered and thus assisting the courts in making informed decisions. Herbalists would also be exonerated from wrongdoing where possible. A step has therefore been taken in the right direction as to how such cases should be dealt with in the future. There is, of course, need for *in vivo* work to study fully the pharmacological as well as toxicological properties of these herbal medicines.

Finally, on a positive note, toxic compounds are also useful "pharmacological probes" and their isolation during casework and research on herbal medicines may lead to the discovery of further useful drugs.

Future work:

This would include:

- The isolation and toxicity evaluation of compounds from *J. erythropoda* and *C. italica*
- Further DNA work to investigate more markers and characterise more species to add to the database
- *In vivo* work to study the pharmacological and toxicological properties of the plants.
- Phytochemical and toxicological investigation of more plants.

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Publications and Communications

1. Magora, B. H., Gray, A. I., and Cole, M.D., (2003), Swertiamarin from *Enicostemma axillare* subsp. *Axillare* (Gentianaceae), *Biochemical Systematics and Ecology,* Vol. 31, pp. 553-555.

2. Magora, B.H. and Cole, M. D., (2001), Phytochemical and Toxicological Studies of Some Botswanan Plants Used in Traditional Medicine, *Problems of Forensic Science*, Vol. XL VII, pp. 358-362.

3. Magora, B.H., Gray, A.I. and Carter, K.C., (2002), Phytochemical and Toxicological Studies of *Enicostemma axillare*: a paper presented at the Second Congress of the Arab Union of Pharmacology, Fez, Morocco September 5-8, 2002.

4. Magora, B.H., Cole, M.D. and Linacre, A., (2003), DNA Typing of Plants Used in Traditional Medicines. A paper to be presented at the Third European Academy of Forensic Sciences Meeting, Istanbul, Turkey, September 22-27, 2003.

5. Magora, B.H., Gray, A.I. and Cole, M.D., Phytochemical and Toxicological Studies of Some Botswanan Plants Used in Traditional Medicine. An oral Presentation given at the Strathclyde University, School of Pharmacy Research Seminar, Glasgow, Scotland, June 2002.