ANTIMICROBIAL PHENYLPROPANOID ESTERS AND SESQUITERPENE LACTONES FROM THE LEAVES OF *TARCHONANTHUS CAMPHORATUS* (ASTERACEAE)

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A thesis submitted to Graduate School in partial fulfillment of the requirements for the Master of Science Degree in Chemistry of Egerton University

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DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and that it has not been previously presented in this or any other university for any degree.

Signature…….. Date: ……

WETUNGU MARTIN WAMALWA

RECOMMENDATION

This thesis has been presented for examination with our approval as university supervisors:

Signature…… Date……

DR. J. C. MATASYOH

Signature…… Date……

DR. T. KINYANJUI

DEDICATION

To my family

COPY RIGHT

ACKNOWLEGEMENT

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ABSTRACT

The plant *Tarchonanthus camphoratus* belongs to Asteraceae family. Its common name is wild sage or camphor bush. This study was aimed at isolating bioactive phenypropanoid esters and sesquiterpene lactones from the leaves of this plant. A powdered sample of the leaves weighing 500 g was sequentially extracted with of hexane (1.5 L), dichloromethane (1.5 L) and methanol (3 x 1.5 L). The methanolic extract was partitioned between equal volumes (250 ml each) of distilled water. The n-butanol fraction was concentrated under reduced pressure followed by addition of diethyl ether, precipitating 20 g of a dry crude extract. The extract was successively fractionated using hexane, dichloromethane, chloroform, ethyl acetate, acetone, ethanol and methanol yielding 1.6 g, 1.4 g, 1.6 g, 7.0 g, and 1.4 g, 2 g and 4 g respectively of samples soluble in each solvent. Phytochemical and chemical tests were employed in identifying the fractions that contained phenolic compounds and terpenoids. Antimicrobial activity tests of the extracts soluble in various solvents were then performed on *Escharichia coli, Staphylococcus aureus, Pseudomonas aeruginos, Klebsiella pneumoniae, Proteus mirabilis, Salmonella typhi, Candida albicans* and *Bacillus spp*. Ethyl acetate and methanol soluble fractions were found to contain phenolics, while hexane, dichloromethane, chloroform and ethyl acetate fractions contained terpenoids. Ethyl acetate fraction was active against *E. coli, P. mirabilis, K. pneumoniae, S. typhi*, *and Bacillus spp* while methanol fraction was active against *E. coli, K. pneumoniae, S. typhi*, *and Bacillus spp*. The two fractions were combined and subjected to TLC analysis, Column chromatography, HPLC analysis and sephadex LH-20 reversed phase column chromatography, affording three isomers of dicaffeoyl quinic acids: 4, 5- dicaffeoyl quinic acid, **1** (135 mg), 3, 5-dicaffeoyl quinic acid, **2** (125 mg), and 3, 4-dicaffeoylquinic acid, **3** (90 mg**)** which are known compounds, and a sesquiterpene lactone, **4** (150 mg)**.** Structural elucidation of the compounds was done using IR, MS, ^{13}C- NMR, ^{1}H- NMR, DEPT, ^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY and HMBC techniques.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACN	Acetonitrile
ATCC	American Test Culture Collection
CC	Column Chromatography
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethylsulfoxide
ESIMS	Electron Spray Ionization Mass Spectrometry
FAO	Food and Agricultural Organization
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High performance Liquid Chromatography
IR	Infra Red Spectroscopy
IUPAC	International Union of Pure and Applied Chemistry
KEMRI	Kenya Medical Research Institute
MIC	Minimum Inhibitory Concentration
NMR	Nuclear Magnetic Resonance
RPM	Revolutions Per Minute
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UNDP	United Nations Development Programme
UNESCO	United Nations Educational and Scientific Organization
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background information

Nature has been a source of medicinal agents for thousands of years. Today, despite advances in pharmacology and synthetic organic chemistry, this reliance on natural products, particularly on plants, remains largely unchanged (Trevor, 2001). Approximately 80% of the world's population currently depends on traditional system of health care that incorporates natural products, many of which are plants (Foster, 1999). Plants are commonly used in treating and preventing specific ailments and diseases. The adverse side effects of some of the modern medicines, the emergence of diseases which are incurable with these medicines and the adverse economic conditions particularly in the developing countries, have re-activated interest in traditional medicines for use in health care systems all over the world (Boukef, 1990). Presently in the developing countries, synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often have adulterations and side effects (Shariff, 2001). There is therefore need to search for plants of medicinal value.

Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity. It also facilitates pharmacological studies of more potent drug with reduced toxicity (Ebana *et al.*, 1991). The plant kingdom can indeed be regarded as perhaps the largest potential source for the development of new drugs (Mensah, 1991). Furthermore, the active components of herbal remedies have the advantage of being combined with many other substances that appear to be active. These complementary components give the plant as a whole a safety and efficacy much superior to that of its isolated and pure active components (Shariff, 2001). The information obtained from ethno medicine is therefore being put on a scientific basis and it is very important to investigate the pharmacological and phytochemical aspects of different preparations from plant sources (Hostettman and Marsten, 1996).

Currently in modern medicine, approximately 50% of the drugs in chemical use are derived from natural products (Baker *et al*., 1995), and of these, half are plant based (Posey, 1990). Consumers' preference is shifting from synthetic to natural products and this is dictating

the pace of resurgence and expansion of use of medicinal plants in therapy in industrialized countries (Akerele, 1996).

Medicinal plants play a central role not only as traditional medicines but also as commercial commodities, meeting the demand of distant markets. To cope with the growing market, there is need to expeditiously utilize and scientifically validate more medicinally useful plants (Krishna, 2004).

The use of medicinal plants thus finds its natural expression and further development in primary health care where in many cases they bridge the gap between the availability of and the demand for essential drugs (Akerele, 1996). Some of the currently known herbal medicinal plants could substitute imported drugs, which currently require foreign exchange for their purchase.

Apart from microorganisms or fungi, higher plants have also been a source of antibiotics (Trease and Evans, 1972). Plant based antimicrobials represent a vast untapped source of medicine with enormous therapeutic potential. They are effective in treatment of infectious diseases while simultaneously mitigating many of the side effects that are associated with synthetic antimicrobials (Iwu *et al.,* 1999).

The therapeutic or medicinal properties of plants are normally dependent upon presence of certain active principles (Kokwaro, 1993). However, only a handful of these secondary metabolites have been defined chemically (FAO, 1986). Proper identification of the active ingredients is invaluable in the assessment of the pharmaceutical value of the traditional medicine.

Phenolic compounds exist widely in plants. They are plant secondary metabolites, which have an important role as defense compounds. Although the exact contribution of these secondary metabolites is still unclear, phenolic compounds are known to be important in the survival of a plant in its environment (Puupponen-Pimiä *et al*., 2005). In addition to plants, phenolics exhibit several properties beneficial to humans. Several plant-derived medicines, which can prevent or cure diseases, are rich in phenolic compounds (Scalbert, 1993). The antioxidant properties of phenolic compounds have been implicated in suppressing various health related disorders, including melanogenesis (Shimozono *et al*., 1996), hepatoma invasion (Yagasaki *et al*., 2000), and human immunodeficiency virus (HIV) replication (Mahmood *et al.,* 1993; Zhu *et a*l., 1999).

Phenylpropanoids are natural products derived from the amino acid L-phenylalanine via deamination by L- phenylalanine ammmonialyase (PAL). All classes of phenylpropanoids compounds are not present in all plant species. Although the hydroxycinnamic acids and flavonoid classes are ubiquitous in higher plants, members of these classes with specific substitution patterns may be peculiar to certain genera or species (Dixon *et al.,* 2002).

The functions of phenypropanoid compounds in plants defense range from preformed or induced physical and chemical barriers against infection to signal molecules involved in local and systematic signaling for defense gene induction. Defensive functions are not restricted to a particular class of phenylpropanoid compounds, but are found in the simple hydroxycinnamic acids and monolignols through to the more complex flavonoids, isoflavonoids and stilbenes (Dixon *et al.,* 2002).

1.2 Statement of the problem

Infectious diseases and parasitic diseases, like diarrhoea, respiratory infections, typhoid and measles account for approximately one-half of all deaths in developing countries. The high cost of synthetic drugs makes them unaffordable to many people in these countries who live on less than one dollar a day. This, coupled with insufficient number of health facilities, the increasing rate of resistance of disease-causing microorganisms to conventional anti-biotics, and the Aids epidemic calls for continuous search for affordable, safe and effective herbal medicine.

1.3 Objectives

Main objective

To investigate the antimicrobial properties of the phenypropanoid esters and sesquiterpene lactones from the leaves of *Tarchonanthus camphorates.*

Specific objectives

(i) To isolate the phenylpropanoid esters and sesquiterpene lactones from the leaves of the plant.

(ii) To perform antimicrobial activity – guided fractionation of the compounds

(iii) To characterize the isolated compounds.

1.4 Justification

Most of the world's population continues to depend on herbal medicine from plants in the provision of primary health care (Trevor, 2001). There is, however, general lack of scientific evidence of the efficacy of most medicinal plants currently in use since the information about them is based on hearsay. There is therefore need to scientifically rationalize the use of these plants in order to avoid the risk of administering wrong herbs for certain diseases. The plant *Tarchonanthus camphoratus* (Asteraceae) is traditionally used to treat bronchitis, asthma, headache and stomach troubles (Kokwaro, 1993). The scientific proof of the medicinal application of the plant will create confidence in the users, therefore giving it more value. In addition, the extraction of bioactive compounds from the plant will not only permit the demonstration of their physiological activity, but may also facilitate pharmacological studies of more potent drug with reduced toxicity.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Tarchonanthus camphoratus*

Tarchonanthus camphoratus (Asteraceae) (Hilliard, 1977) is a multi–stemmed shrub species naturally occurring in Africa and Arabia. Its common name is wild sage or camphor bush and local name Leleshwa (Kalenjin). It grows in poor soils typified by water deficient sites with annual rainfall amounts of between 1500 – 2000 mm in altitudinal ecological range. It is widely spread in all the provinces of South Africa, Lesotho, Swaziland, and Namibia. In Kenya, it is found in almost all the provinces. These tree bushes form excellent tree crop that provides a sustained source of slow burning high-grade charcoal in Kenya (UNDP, 2005).

Macroscopically, the plant is a Dioecious shrub or a small tree growing to 6 m, with a moderate to strong odour of camphor. The leaves are alternate, borne on white-felted twigs, variable, 2 by 0.5 to 12 by 5 cm, obovate to lanceolate, upper surface smooth and finely reticulate with minute golden glands over the veinlets; lower surface white felted, margin entire to denticulate (Figure 2.1). Flowers are cream, borne on discoid heads in terminal or auxiliary panicles, female 1-3 flowered, male with numerous flowers.

Figure 2.1: Photograph of *Tarchonanthus camphoratus* leaves

Despite its wide geographical distribution, the medicinal use to which this species is put is remarkably similar throughout its range. It is taken orally or applied externally to relieve bronchitis, asthma, headache, inflammation, chilblains or abdominal pains (Amabeoku *et al.,* 2000).

Phytochemical tests indicate the presence of tannins, saponins and reducing sugars, but not alkaloids, cardiac or anthraquinone glycosides (Bishay, 2002). A more recent investigation of antimicrobial activity of aqueous, ethanolic and hexane extracts of dried leaf did not demonstrate *in vitro* inhibitory effects against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli or Klebsiella pneumoniae* (McGraw *et al.,* 2000).

2.2 Plant derived antimicrobial agents

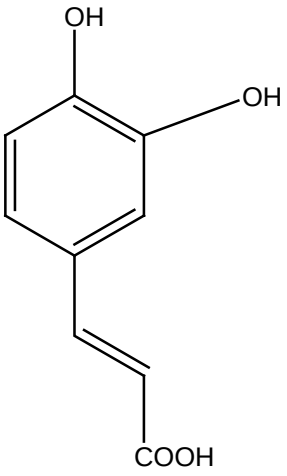
Mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics (products of microorganisms or their synthesized derivatives) become ineffective and as new, particularly viral, diseases remain intractable to this type of drug. Another driving factor for the renewed interest in plant antimicrobials has been the rapid rate of plant species extinction (Lewis and Elvin-Lewis, 1995). There is a feeling among natural-products chemists and microbiologists alike that the multitude of potentially useful phytochemical structures, which could be synthesized chemically, is at risk of being lost irretrievably.

2.3 Some groups of antimicrobial compounds from plants

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). Most are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total (Schultes, 1988). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odours; others (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavour (e.g., the terpenoid capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds

2.3.1 Phenolics and Polyphenols

Phenolics are compounds having an aromatic ring with one or more hydroxyl groups and functional derivatives (Shahidi and Naczk, 2003). They are phytochemicals consisting of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds that are in the highest oxidation state. The common herbs tarragon and thyme both contain caffeic acid **(5)**, which is effective against viruses (Wild, 1994), bacteria (Branter *et al.,* 1996; Weinmann, 1997), and fungi (Duke, 1985). Catechol **(6)** and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two -OH groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on the phenyl group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Geissman, 1963). In addition, some authors have found that more highly oxidized phenols are more inhibitory (Scalbert, 1991).



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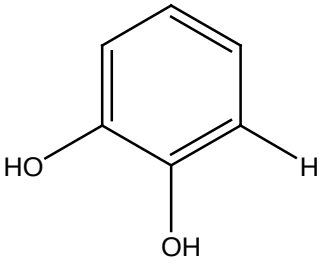

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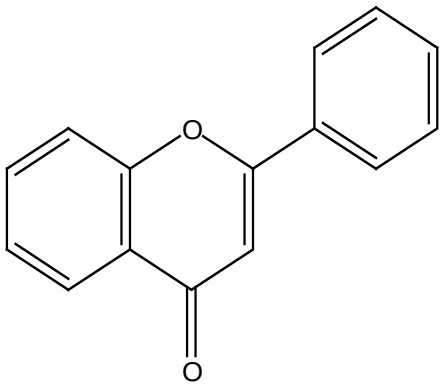
2.3.1.1 Mode of action of phenolics

The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Lewis and Elvin-Lewis, 1995).

2.3.2 Flavones, flavonoids, and flavonols.

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones) (**7**). The addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection (Dixon *et al.,* 1983) it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1996). Catechin (**8**), the most reduced form of the C3 unit in flavonoid compounds, deserve special mention. These flavonoids have been extensively researched due to their occurrence in green teas. It was noticed some time ago that teas exerted antimicrobial activity (Toda *el at.*, 1989) and that they contain a mixture of catechin compounds. These compounds inhibited in vitro *Vibrio cholerae* (Borris, 1996), *Streptococcus mutans* (Sakanaka *et al*., 1989), *Shigella* (Vijaya *et al.,* 1995).



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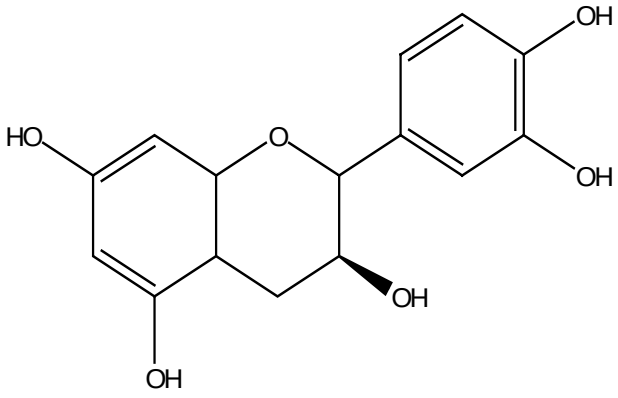


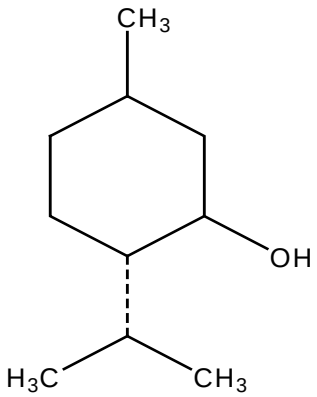
8

2.3.3 Tannins.

"Tannin" is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Their molecular weights range from 500 to 3,000 (Haslam, 1996), and they are found in almost every plant part: bark, wood, leaves, fruits, and roots (Scalbert, 1991). They are divided into two groups, hydrolysable and condensed tannins. Hydrolysable tannins are based on gallic acid, usually as multiple esters with D-glucose; while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Tannins may be formed by condensations of flavan derivatives that have been transported to woody tissues of plants. Alternatively, tannins may be formed by polymerization of quinone units. This group of compounds has received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines, can cure or prevent a variety of ills (Serafini *et al*., 1994). Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam, 1996).

2.3.4 Terpenoids and Essential Oils

The fragrance of plants is carried in the so-called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene (C_5H_8) structure. They are called terpenes. They occur as monoterpenes, diterpenes, triterpenes, and tetraterpenes (C10, C20, C30, and C40), as well as hemiterpenes (C5) and sesquiterpenes (C15). When the compounds contain additional elements, usually oxygen, they are termed terpenoids. Terpenoids are synthesized from isoprene units. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are menthol (**9**) (monoterpenoid) and artemisinin (**10**) (sesquiterpenoid).



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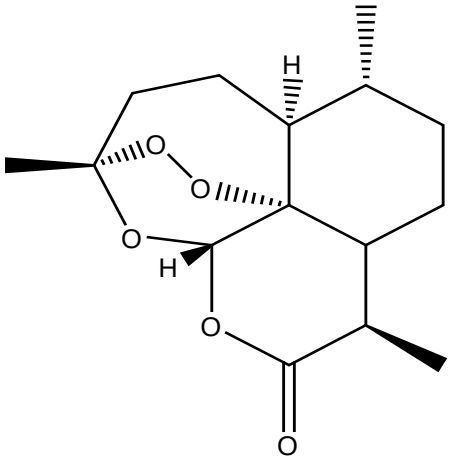

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Artemisinin and its derivative arteether, also known by the name qinghaosu, are currently used as antimalarials (Vishwakarma, 1990). Terpenes or terpenoids are active against bacteria, fungi, viruses and protozoa (Ayafor *et al.*, 1994; Mendoza *et al.*, 1997).

2.4 Biosynthetic origin of plant phenolics

The biosynthetic origin of plant phenolics is the aromatic amino acid L-phenylalanine (Robbins, 2003). The biosynthetic pathway for synthesis of the most abundant phenolic acids in sweet potato (chlorogenic acid and isochlorogenic acid) is shown in Figure 2.2.

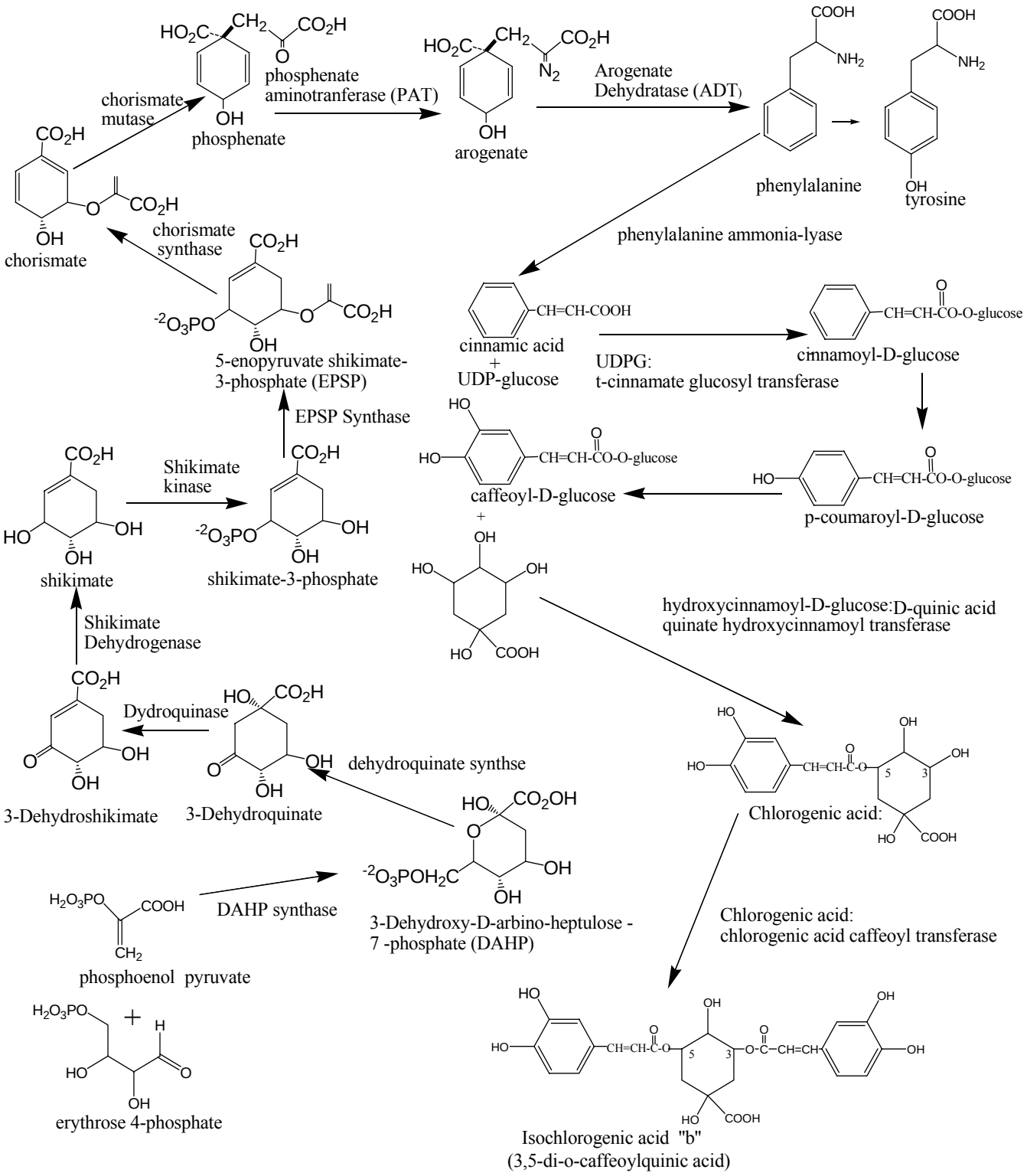
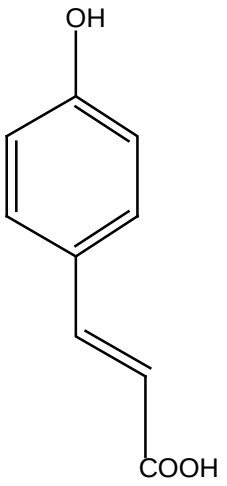


Figure 2.2: Biosynthetic pathway for production of chlorogenic acid and isochlorogenic acid (Friedman, 1997)

2.5 Cinnamic Acids

A major class of phenolic compounds is the hydroxycinnamic acids, which are found in almost every plant. They are a series of *trans* phenyl-3-propenoid acids differing in their ring substitution. The major representative of this phenolic class is caffeic (**12**) acid, which normally occurs in foods mainly as an ester with quinic acid, known as the chlorogenic acid (5-caffeoylquinic acid) **(16)** (Tapiero *et al.*, 2002). Other cinnamic acids include: *p*-coumaric acid



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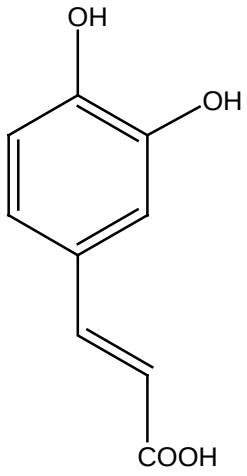

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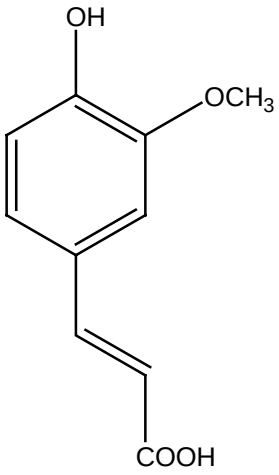

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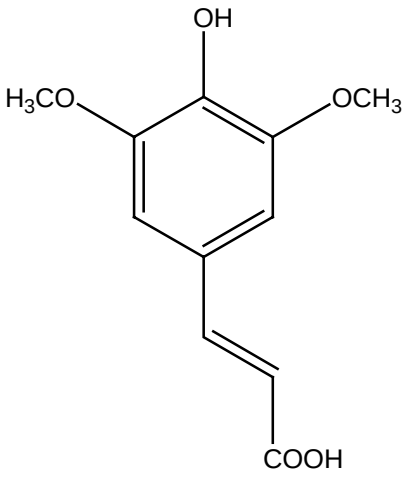


14

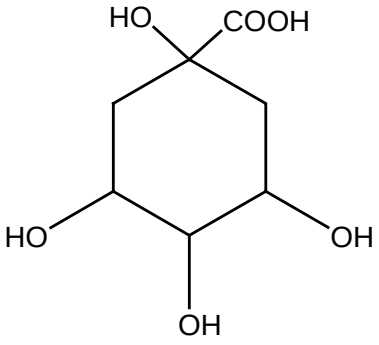
(11), ferulic acid (3-methoxy-4-hydroxycinnamic acid) **(13)** and sinapic acid (3, 5-dimethoxy-4-hydroxycinnamic acid) **(14)**.

2.6 The Cinnamate Conjugates / Phenylpropanoid esters

The cinnamate conjugates (phenylpropanoid esters) may be divided into two major categories; chlorogenic acids and other cinnamate conjugates.

2.6.1 The chlorogenic acids

Chlorogenic acids, is a family of esters formed between certain *trans* cinnamic acids and (-) quinic acid **(15)** (Clifford *et al.*, 2003). They are a major group of phenolic compounds in coffee, found widespread in plants and can be isolated from the leaves and fruit (Clifford, 2003). The best known conjugate is 5-caffeoylquinic acid (**16**). This compound, long known as an antioxidant, also slows the release of glucose into the bloodstream after a meal (Johnston *et al*., 2003).



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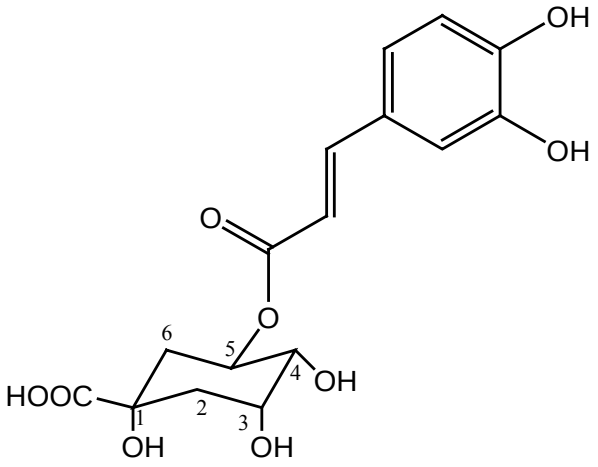

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Structurally, chlorogenic acid (CGA) is the ester formed between certain *trans* cinnamic acid and (L)-quinic acid (L-1-(OH)-3,4,5-tetrahydroxycyclohexanecarboxylic acid). Isomerisation of chlorogenic acid has been reported with 3 isomerisations of the quinic acid in postion 3, (3-CQA), 4 (4-CGA) and 5 (5-CQA). Isomerisation at postions 1 and 6 has not yet been reported. (Clifford *et al*., 2003). The identity, number and position of acyl residues may subdivide these classic CGA:

- Di-esters (diCQA), tri-esters (triCQA), and the single tetra-ester of caffeic acid (tetraCQA), the latter two groups being characteristic of Asteraceae (Agata *et al.*, 1997)
- Di-esters of ferulic acid (di FQA) in *Brachlaria* spp (Poaceae) (Wenzl *et al*., 2000)
- Mixed di-esters of caffeic and ferulic acid [caffeoylferuloylquinic acids (CFQA)] which are a characteristic of robusta coffee (*Caffea canephora*, Rubiaceae) (Clifford *et al.*, 2003), caffeic and sinapic acid [caffeoylsinapoylquinic acids (CsiQA)] as found in *Gardenia jasminoides* (Rubiaceae) (Nishizawa *et al.*, 1988) or ferulic and *p*-coumaric acid [p-coumaroylferuloylquinic acids (pCoFQA)] in *Brachiaria* spp (Poaceae) (Wenzl *et al.*, 2000)
- Mixed esters involving various permutations of between one and three residues of caffeic acid with one or two residues of a dibasic aliphatic acid (e.g glutaric, oxalic, succinic) which are a characteristic of Asteraceae (Chuda *et al.*, 1996) or various permutations of caffeic, sinapic and 3-hydroxy-3-methylglutaric acid in *Gardenia jasminoides* (Rubiaceae) (Nishizawa *et al.,* 1988)
- A mixed ester containing one residue of isobutyric acid and two residues of caffeic acid has been reported in *Buphthalmum salicifolium* (Asteraceae) (Heilmann *et al.*, 1999).

2.6.2 Other Cinnamate Conjugates

Cinnamic acid may be conjugated to many molecules other than quinic acid and its close relatives, although sometimes as very minor components of limited distribution. This second category includes:

- Esters of other hydroxy acids, particularly α-hydroxyhydrocaffeic (rosmaric), malic and tartaric. Others include galactaric, glucaric, gluconic, hydroxycitric. methoxyaldric, phenylpyruvic and tartronic.
- Amides of amino compounds including aromatic amino acids, choline, anthranilic acids and diamines;
- Esters of polysaccharides, simple sugars, sugar alcohols including glycerol and *myo*-inositol and glucosides including those of anthocyanins, flavonols and diterpenes.
- Esters of lipids including alkanols, alkanediols, ω-hydroxy-fatty acids and sterols.
- Glycosides

2.7. Medicinal uses of Phenylpropanoid esters

Chlorogenic acid has been found to posses antiviral (Jassim and Naji, 2003), antibacterial (Rodriguez and Hadley, 1998) and antifungal (Bowels and Miller, 1994) effects with relatively low toxicity and side effects, alongside properties that do not lead to antimicrobial resistance.

Dicaffeoylquinic acids (DCQAs) have been established as an important class of compounds with their potential effects of inhibiting HIV-1 integrase selectively and preventing HIV-1 replication in tissue culture at nontoxic concentrations (McDougall *et al.*, 1998; Robinson *et al*., 1996; King *et al.*, 1999). HIV-1 integrase is an essential enzyme that mediates integration of the HIV genome into the host chromosomes. (Zhu *et al*., 1999) reported that such inhibition of DCQAs on the HIV-1 integrase is irreversible toward its conserved amino acid residues in the central core domain during catalysis. The combinations of HIV integrase inhibitors with already existing inhibitors for HIV reverse transcriptase and protease have been suggested to be strongly synergetic (Robinson, 1998). Therefore, DCQAs have drawn more and more attention in the development of the therapy of HIV infection.

2.8 Sesquiterpene lactones

Sesquiterpene lactones are a major class of secondary metabolites, which occur mainly in Asteraceae (Pillay, 2006). They contain an α, β- unsaturated-γ-lactone as a major structural feature. The typical lactones are primarily classified on the basis of their carbocyclic skeletons as germacranolides (**17**, **18**, **19** or **20**), guaianolides (**21** or **22**) and eudesmanolides (**23** or **24**). However sesqueterpene lactones exhibit a variety of other skeletal arrangements (Pillay, 2006). Although over 4000 different sesquiterpene lactones are known, germacranolides represent the most primitive class and all others evolve from them (Picman, 1986).

Generally the α, β- unsaturated lactone is either *cis* or *trans*-fused to the C(5)-C(6), C(6)-C(7) or C(7)-C(8) positions of the carbocyclic skeleton containing, in many cases, an α-methylene group. Structural modification of the basic sesquiterpene skeleton involves incorporation of an epoxide ring, hydroxy groups (generally esterified), and / or a C-5 acid such as tiglic or angelic acid (Picman, 1986).

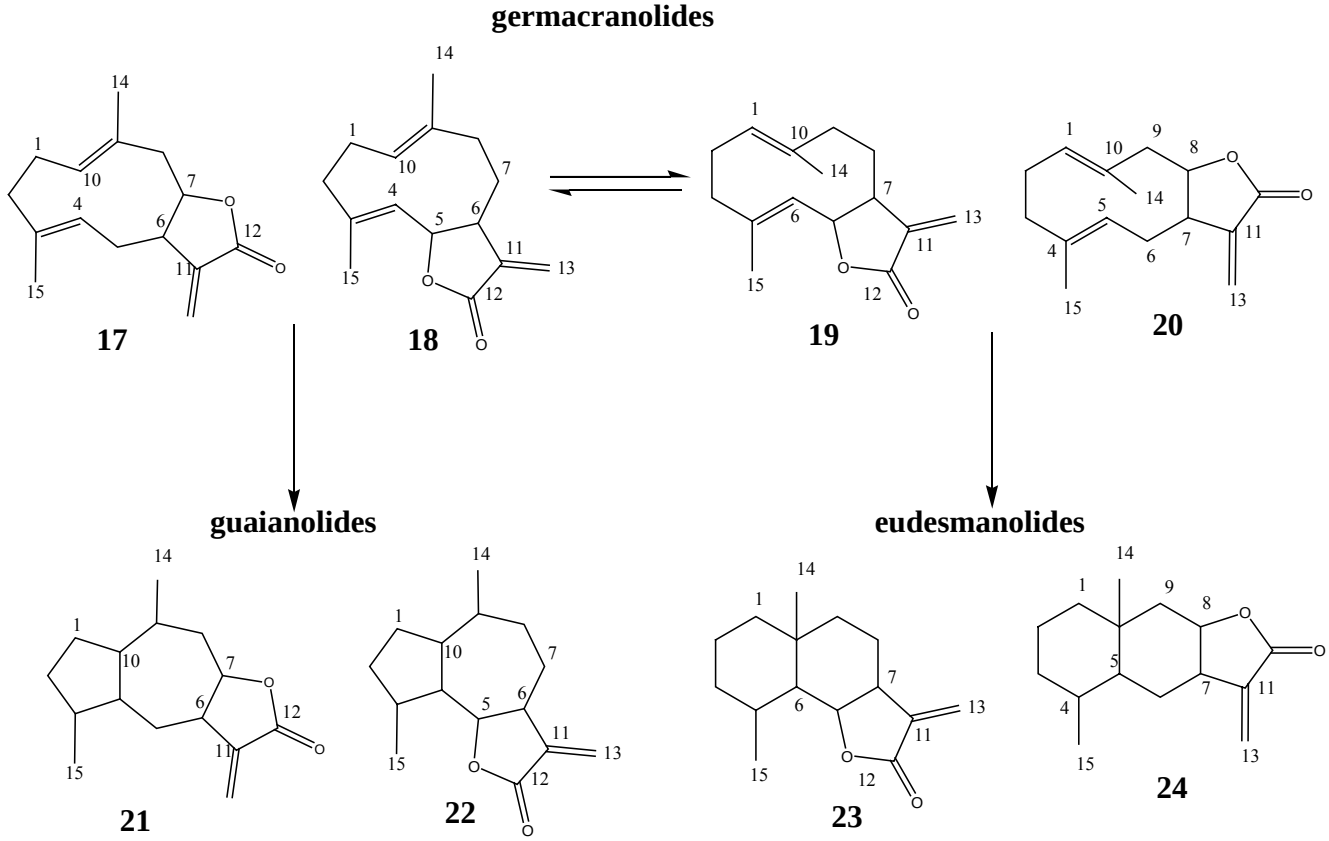
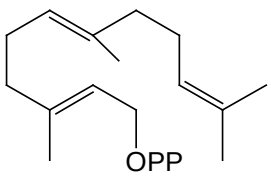


Figure 2.3: Germacranolides, guaianolides and eudesmanolides skeletal types (Pillay, 2006)

2.8.1 Sesquiterpene lactone Biosynthesis

(+)-costunolide (**19**) is generally accepted as the common intermediate of all germacrananolide-derived lactones. The committed step in the biosynthesis of (+)-costunolide (**19**) is the cyclization of fernesyl pyrophosphate (FPP) (**25**).



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The proposed biosynthetic route for germacrene-derived sesquiterpene lactones as established in chicory (*Cichorium intybus*) roots (De Kraker *et al.*, 2001) is shown in Figure 2.4.

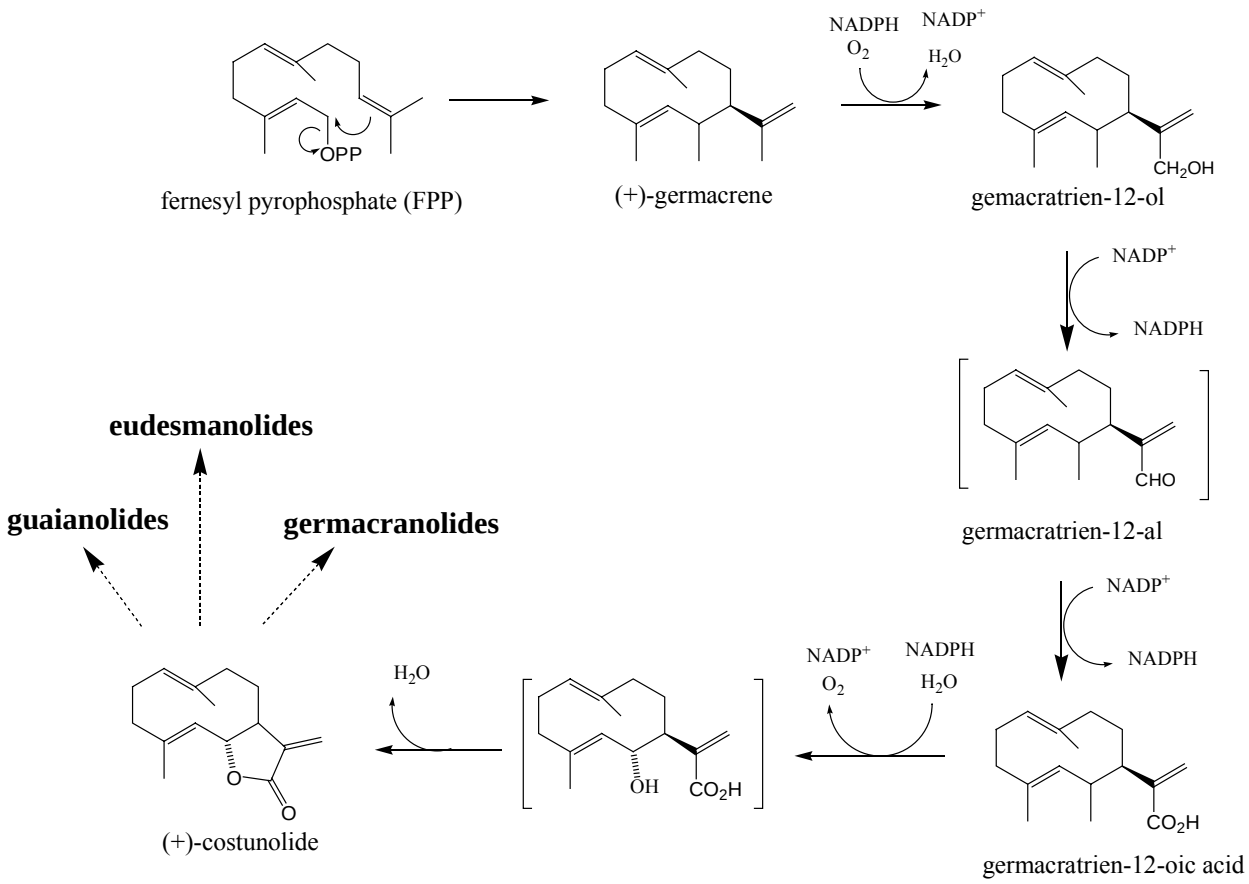
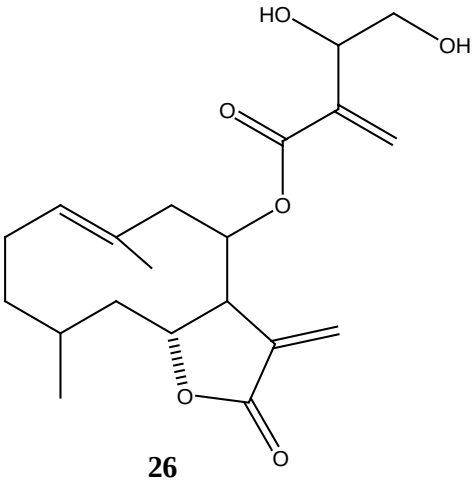


Figure 2.4: Proposed biosynthetic route for the germacranolide-derived sesquiterpene lactones (De kraker *et al.*, 2001**)**

2.8.2 Biological activities of sesquiterpene lactones

The sesquiterpene lactones from plants comprise a group of substances with a variety of biological effects. These include antibacterial, antifungal, antitumour, antiplasmodial, antihelmintic, schistosomicidal, cystotoxic, phytotoxic and analgesic activities (Burim *et al*., 1999). Artemisinin (**10**) is an antiplasmodial (Vishwakama, 1990). Cnicin (**26**) and Salonitenolide (**27**) are both germacranolides isolated from *Centaurea deusta.* Cnicin (**26)** possessed antifungal activities against *Aspergillus niger* (ATCC 6275), *Aspergillus ochraceus*

(ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus flavus* (ATCC 9643), *Penicillium ochrochloron* (ATCC 9112), *Penicillium funiculosum* (ATCC 36839) and *Cladosporium cladosporioides* (ATCC). Salonitenolide (**27**) on the other hand was found to be active against *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis, Escherichia coli* (ATCC 35218) and *Pseudomonas aeruginosa* (ATCC 27853) bacteria (Karioti *et al.,* 2001).



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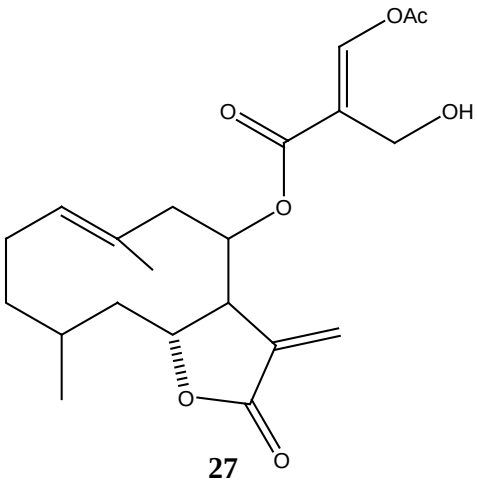

27

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemical and Working Techniques

All reagents were obtained from commercial suppliers, and were used without further purification unless otherwise stated. All solvents were distilled and/or dried prior to use by standard methodology except for those that were reagent grades. The reactions were carried out under a nitrogen atmosphere and the reaction flasks were pre-dried by heat. Chemicals that were air or water sensitive were stored under inert atmosphere.

3.2 Collection and identification of the plants

The leaves of *Tarchonathus camphoratus* were collected from Egerton University Botanical Garden and identified by a plant taxonomist. The green leaves were dried under shade to constant weight and ground to a coarse powder using a domestic miller.

3.3 Extraction and fractionation of the plant material

A powder weighing 500 g of *Tarchonanthus camphoratus* leaves was extracted sequentially with hexane (1.5 L), dichloromethane (1.5 L) and methanol (3 x 1.5 L) after soaking the sample in each solvent for 24 hours. The methanol extract was filtered through a Buchner funnel fitted with a vacuum pump with a thin layer of activated charcoal, and then concentrated using a rotary evaporator and the solvent recovered. The crude methanol extract was partitioned between equal volumes (250 ml each) of distilled water and n-butanol. The n-butanol fraction was concentrated under reduced pressure followed by addition of diethyl ether, precipitating a crude sample, which was dried in desiccators after centrifugation at 3000 RPM for 15 minutes to obtain 20 g of amorphous powder.

The dry sample was then fractionated using hexane (4 x 200 ml), dichloromethane (4 x 200 ml), chloroform (4 x 200 ml), ethyl acetate (4 x 200 ml), acetone (4 x 200 ml), ethanol (4 x 200 ml) and methanol (4 x 200 ml). The solvents were recovered using rotary evaporator to obtain 1.6 g, 1.4 g, 1.6 g, 7g, 1.4 g, 2 g and 4 g of dry hexane, dichloromethane, chloroform, ethyl acetate, acetone, ethanol and methanol soluble fractions respectively.

3. 4 Phytochemical screening of the fractions

The samples were tested for the presence of various phytochemicals, especially for phenolics, which normally include phenylpropanoid esters.

3.4.1 Test for tannins /phenolic compounds

Dried samples weighing 0.2 g were boiled in 10 ml of distilled water in a test tube and then filtered. 2 ml of 0.1% $FeCl_3$ solution was then added to the filtrate. A positive test was confirmed by formation of a characteristic blue, blue-black, green or blue-green color (tannins) and precipitate (phenolic compounds) (Faraz *et al.*, 2003).

3.4.2 Test for flavonoids

This was done by dissolving 0.2 g of each extract in 5 ml of distilled water and filtered. This was followed by addition of 5 drops of 1% aluminium chloride solution to the aqueous filtrate. Formation of a yellow coloration indicated the presence of flavonoids (Edeoga *et al.*, 2005).

3.4.3 Test for Steroids

The test was performed by dissolving 0.l g of each extract in 1 ml ethanol followed by addition of 1 ml of acetic anhydride and 1 ml H_2SO_4. The presence of steroids was indicated by the change of color from violet to blue or green (Edeoga *et al.*, 2005).

3.4.4 Test for terpenoids (Salkowski test)

0.1g of each fraction was dissolved in 2 ml $CHCl_3$ followed by addition of 2 ml conc. H_2SO_4. A positive test was confirmed by appearance of a red coloration at the interface (Edeoga *et al.,* 2005).

3.4.5 Test for cardiac glycosides (Keller-Killiani test)

Each fraction was mixed with 2 ml of glacial acetic acid containing one drop of 0.1% ferric chloride solution. The mixture was then underlayed with 1 ml of concentrated sulphuric acid. Formation of brown ring at the interface or a greenish ring in the acetic acid layer was considered a positive test (Edeoga *et al.*, 2005).

3.4.6 Test for saponins

0.2 g of powdered sample of the extract was boiled in 10 ml of distilled water on a water bath and filtered. A fraction of the aqueous filtrate measuring 2 ml was mixed with 2 ml of

distilled water and shaken vigorously for stable persistence froth. The frothing was mixed with a few drops of olive oil and shaken vigorously. Formation of an emulsion confirmed the presence of saponins (Edeoga *et al.*, 2005).

3.5 Chemical tests

The fractions were tested for the presence of some functional groups as follows:

3.5.1 The Lucas reagent

The Lucas test was used to test for presence and type of alcohol present in the samples. 0.1ml of the aqueous fraction was placed in a test tube and 1ml of the hydrochloric acid/ zinc chloride test reagent added. The mixture was vigorously shaken to ensure complete mixing. The alkyl chloride formation was noted by formation of an insoluble layer or emulsion. Tertiary alcohols form the second layer in less than a minute, secondary alcohol require somewhat longer time (5-10 min), whereas primary alcohol are essentially uncreative (Jerry *et al.*, 1998)

3.5.2 Tollen's test

The reagent was prepared by placing 2 ml of 5% aqueous solution of silver nitrate in a small test tube. A drop of 10% aqueous NaOH was added, the result being a silver oxide precipitate which was dissolved by adding just enough dilute aqueous (2%) ammonia to dissolve the silver oxide precipitate. The test was carried out by adding a few drops of the reagent to 2 ml of the unknown. Positive results were confirmed by appearance of a silver mirror or colloidal silver instantly or after gentle heating (Jerry *et al.*, 1998).

3.5.3 $KMnO_4$ test

The test was employed to detect the presence of unsaturation or –OH in the fractionated extracts. 0.2 g of each extract was dissolved in 2 ml of distilled water and 2% potassium permanganate solution added dropwise. The test was considered positive if no more than 3 drops of the reagent were decolourised (Furniss *et al.,* 1989).

3.5.4 Bromine test

This was also used to detect the presence of unsaturation. 50mg of each extract was dissolved in 1 ml carbon tetrachloride then 2% (by volume) of aqueous bromine in carbon

tetrachloride added dropwise with shaking. A positive test was indicated when the bromine was decolourised (Pavia, 1990).

3.5.5. Aqueous NaOH and $NaHCO_3$ tests

50 mg of each extract was separately dissolved in 5% aqueous sodium bicarbonate solution and 5% aqueous NaOH. A carboxylic acid was indicated when the extract was soluble in both aqueous NaOH and aqueous $NaHCO_3$ with effervescence being seen in the latter. Phenols were indicated when the extract was soluble in NaOH only (Pivia, 1990).

3.6 Antimicrobial screening of the fractions

The anti-bacterial activity was done using the American Test Culture Collection (ATCC) and clinical isolates from KEMRI. The gram-negative bacteria used were *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae, Pseudomonas aeruginosae* ATCC 27853, *Proteus mirabilis* and *Salmonella typhi* while gram–positive ones were *Staphylococcus aureus* ATCC 25923 and *Bacillus spp.* The anti-fungal activity of the extracts was done on the pathogenic fungus *Candida albicans*. The antimicrobial activity test of the fractions was done using disc diffusion method (Baker *et al*., 1995).

3.6.1 Media preparation

Mueller Hinton agar was prepared by dissolving 38 g of the solid media in distilled water to make a liter of solution, while Subouraud Dextrose Agar (SDA) was prepared by dissolving 65 g of solid powder in a liter of solution. The solutions were then sterilized in autoclave at 121^{o}C for 15 minutes, cooled then poured in Petri dishes. The solutions were then left to solidify.

3.6.2 Innoculation and incubation

Antibacterial activity was done on Mueller Hinton agar while antifungal activity on Subouraud Dextrose Agar (SDA). 1 ml of fungi / bacteria suspension was uniformly spread on the sterile Sabouraud Dextrose Agar/ Mueller Hinton Agar Petri dish. 0.4 g of each sample was dissolved in 1ml of the respective fractionation solvents. Sterile filter paper disc (Whatman no.1, diameter 6mm) was soaked with 0.01 ml of the extract and the solvent allowed to dry. The disc was placed on the Sabouraud Dextrose agar/Mueller Hinton agar Petri dish inoculated with bacteria/fungi suspension and kept at 4^{o}C for 48 hours to allow the extracts to diffuse into the media.

The Petri dish was then placed in an incubator for 24 hours at 37^{o}C. At the end of incubation period, the inhibition diameter was measured using calipers and expressed in millimeters. Chloramphenical and nystatin were used as standards. Positive antibacterial and antifungal activities were established by the presence of measurable zones of inhibition.

3.6.3 Minimum Inhibitory Concentration (MIC)

The MIC was performed on hexane, ethyl acetate and methanol fractions, which on screening, had been found to be moderately active against most of the microbes tested. A sample weighing 0.40 g of each fraction was dissolved in 1ml of the fractionating solvent to form a solution having a concentration of 400 mg/ml. Other concentrations were obtained by taking 0.1 ml of the solution containing 400 mg/ml and adding 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 ml of the solvent separately to obtain new solutions with concentrations 200 mg/ml, 133 mg/ml, 100 mg/ml, 80 mg/ml, 67 mg/ml, 57 mg/ml, 50 mg/ml and 44 mg/ml respectively. The concentration necessary to cause minimum inhibition was then determined (Tables 4.11 and 4.12).

3.7 Chromatographic techniques

The combined ethyl acetate and methanol fraction was subjected to various chromatographic techniques such as Thin Layer Chromatography (TLC), Silica gel Column Chromatography, High Performance Liquid Chromatography (HPLC), all aimed at isolating specific compounds responsible for the observed activity. Other techniques such as preparative TLC and sephadex LH-20 reversed phase chromatography were used to purify the compounds.

3.7.1 Thin Layer Chromatography

The TLC analysis with various solvent systems showed that MeOH and ethyl acetate in a ratio of 3:2 gave the most pronounced separation with distinct spots at R_f values 0.16, 0.34, 0.53 and 0.74. A repeat TLC of the fractions from the chromatographic column gave single spots at the respective R_f values.

3.7.2 Column chromatography

This was done by subjecting 1.2 g of the combined MeOH-EtAOc fraction to silica gel column chromatography using MeOH and ethyl acetate in a ratio of 3:2. Four fractions were obtained: fraction 1 (200 mg), fraction 2 (150 mg) fraction 3 (120 mg) and fraction 4 (400 mg).

All except fraction 1 tested positive for phenolics. However only fractions 1 and 4 were active, with Fraction 1 showing weak activity against *E. Coli* while fraction 4 was moderately active against *E. Coli, P. mirabilis, Bacillus spp*.

3.7.3 HPLC analysis

Fraction 4 of the column chromatography was subjected to reversed phase Semi - preparative HPLC (Figure 4.1) using Grom-Sil 120 ODS-4 HE column; 5 μm (250 mm x 10 mm ID) with H_2O: CAN solvent system with gradient elution and flow rate of 1 ml/minute for 25 minutes. A single poorly resolved peak appeared at 3 minutes, suggesting that the fraction could have contained more than one compound. On analysis using analytical HPLC on Nucleosil-100 C-18 (5 μm) 125: 125 + 20 mm x 4.6 mm I.D column with 0.1% phosphoric acid and acetonitrile solvent system (Figure 4.2), 3 peaks appeared at Retention times 5.50, 5.80 and 5.96 minutes.

3.7.4 Sephadex LH-20

This was used to purify the compounds. In the first case, 400mg of fraction 4 from the column chromatography was subjected to sephadex LH-20 column (1.5 × 60 cm) eluted with methanol affording three compounds, **1** (135), **2** (125 mg) and **3** (90 mg). Although TLC analysis of fraction 1 with dichloromethane: methanol in the ratio 4:3 (v/v) revealed a distinct spot at R_f 0.25, the fraction was purified by subjecting 200 mg of the sample to sephadex LH-20 column and eluted with methanol where compound **4** (150 mg) was obtained.

3.8 Acid hydrolysis of compound 1

Hydrolysis of compound **1** was done after NMR indicated that it was likely to be a dicaffeoyl quinic acid. The acid hydrolysis was therefore aimed at confirming whether or not compound **1** was an ester of caffeic acid. In this process, 50 mg of compound **1** was dissolved in 1M HCl and heated to 80^{o}C while stirring for 4 hrs. The mixture was then extracted with ethyl acetate. The ethyl acetate soluble fraction was purified using preparative TLC and dried with anhydrous Na_2SO_4. This product of hydrolysis was then analyzed using ^{1}H-NMR spectroscopy (Figure 4.6(b)).

3.9 Spectroscopic analysis of the compounds

The structural elucidation of the pure compounds was achieved by use various spectroscopic techniques such as MS, IR spectroscopy, one-dimensional NMR and two-dimensional NMR.

3.9.1 Mass Spectrometry

Mass spectra were recorded on Finnigan Triple- Stage-Quadrupol Spectrometer (TSQ-70) with electrospray ionisation (ESI) Method.

3.9.2 Infrared Spectroscopy

The spectra were recorded on a Fourier Transformed Infrared Spectrometer model FT/IR-430. The samples were pulverised with potassium bromide and percent reflection (R%) was measured. The percent transmittance (T%) of liquid substances was measured in film between potassium bromide plates. Absorption band frequencies were reported in cm^{-1}.

3.9.3 NMR- spectroscopy

All the spectra were measured on a Bruker Advance 400 spectrometer, which operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei. ^{1}H and ^{13}C NMR spectra were performed in deuterated solvent and chemical shifts were assigned by comparison with the residue proton and carbon resonance of the solvent and tetramethylsilane (TMS) as internal reference ($\delta = 0$). Data are reported as follows: s = singlet; d = doublet; t = triplet; dd = doublet of doublet; ddd = doublet of doublet of doublet; dt = doublet of triplet; td = triplet of doublet; m = multiplet; Br = broadened; *J* = coupling constant (Hz); integration, peak assignment in italic form.

3.9.4 Two-dimensional NMR Spectroscopy

In the ^{1}H-^{1}H COSY (Correlation Spectroscopy) the off-diagonal elements in ‘‘box-fashion’’ were used to identify spin-spin coupling interactions. The proton-carbon connectivity (three bonds) was identified using ^{1}H-^{13}C COSY and HMBC (Heteronuclear Multiple Bond Correlation) spectrum in which there was one- dimensional ^{13}C NMR spectrum along the left and the ^{1}H NMR spectrum along the top. The two-dimensional array of spots forming a ‘‘square box’’ identified the proton-carbon connectivity.

3.10 Flow chart

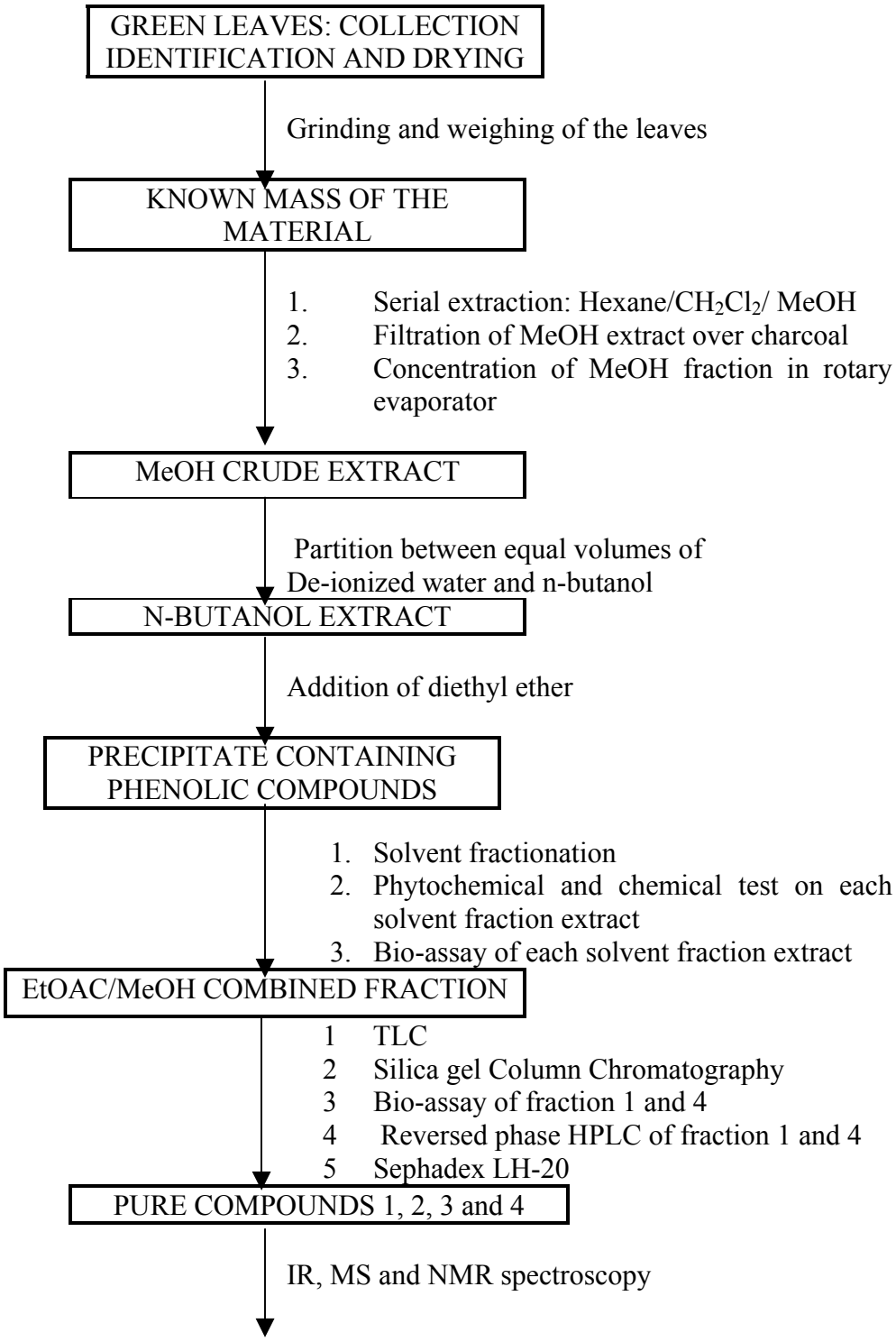

Figure 3.1: Summary of methodology

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

Antimicrobial-guided fractionation led to isolation of compounds **1**, **2**, **3** and **4**. Phytochemical tests, chemical tests and antimicrobial screening of the fractions achieved the identification of the active compounds which were purified and their structures determined using both chemical and spectroscopic (IR, MS and 1D and 2D NMR) methods.

4.1.1 Phytochemical and chemical tests

4.1.1.1 Hexane fraction

Table 4.1: Phytochemical and Chemical tests for Hexane fraction

Test	Observations	Conclusion
Tannins	No brownish green colouration formed in the yellow solution	Absent
Phenolic compounds	No blue or green precipitate formed	Absent
Flavonoids	No yellow colouration formed	Absent
Terpenoids	Reddish brown ring formed at the interface of chloroform and sulphuric acid layers	Present
Steroids	No blue or green colouration formed	Absent
Cardiac glycosides	No brown or green ring formed	Absent
Saponins	No persistence foam/froth formed	Absent
$KMnO_4$ test	$KMnO_4$ decolourised	-OH or unsaturated compound present
Bromine test	Bromine water decolourised	Unsaturated compound present
Lucas test	Immediate formation of an emulsion	Tertiary alcohol present
Tollen's test	No silver mirror deposit	aldehyde absent
NaOH test	Insoluble. Two layers formed	Phenol absent
$NaHCO_3$ test	Insoluble two layers formed. No effervescence	Acid absent

The phytochemical screening of the hexane fraction (Table 4.1) revealed the presence terpenoids and no phenolics, flavonoids steroids, cardiac glycosides and saponins. The fraction contained a tertiary alcohol but no aldehyde or acid

4.1.1.2 Dichloromethane fraction

Table 4.2: Phytochemical and Chemical tests for Dichloromethane fraction

Test	Observations	Conclusion
Tannins	No pale brownish green colouration formed in the yellow solution	Absent
Phenolic compounds	No blue or green precipitate formed	Absent
Flavonoids	No yellow colouration formed	Absent
Terpenoids	Reddish brown ring formed at the interface of chloroform and sulphuric acid layers	Present
Steroids	No blue or green colouration formed	Absent
Cardiac glycosides	No brown or green ring formed	Absent
Saponins	No persistence foam/froth formed	Absent
$KMnO_4$ test	$KMnO_4$ decolourised	-OH or unsaturated compound present
Bromine test	Bromine water decolourised	Unsaturated compound present
Lucas test	Immediate formation of an emulsion	Tertiary alcohol present
Tollen's test	Silver mirror deposit	Aldehyde present
NaOH test	Insoluble. Two layers formed	Phenol absent
$NaHCO_3$ test	Two layers formed. No effervescence	Acid absent

The dichloromethane fraction contained terpenoids but no phenolics, flavonoids, steroids, cardiac glycosides and saponins. A tertiary alcohol, an aldehyde and an unsaturated compound were present. There was neither phenol nor acid in this fraction.

4.1.1.3 Chloroform fraction

Table 4.3: Phytochemical and Chemical tests for Chloroform fraction

Test	Observations	Conclusion
Tannins	No pale brownish green colouration formed in the yellow solution	Absent
Phenolic compounds	No blue or green precipitate formed	Absent
Flavonoids	No yellow colouration formed	Absent
Terpenoids	Reddish brown ring formed at the interface of chloroform and sulphuric acid layers	Present
Steroids	No blue or green colouration formed	Absent
Cardiac glycosides	No brown or green ring formed	Absent
Saponins	No persistence foam/froth formed	Absent
$KMnO_4$ test	$KMnO_4$ decolourised	-OH or unsaturated compound present
Bromine test	Bromine water decolourised.	Unsaturated compound present.
Lucas test	Immediate formation of an emulsion	Tertiary alcohol present
Tollen's test	No silver mirror deposit	Aldehyde absent
NaOH test	Insoluble. Two layers formed	Phenol absent
$NaHCO_3$ test	Insoluble. Two layers formed No effervescence	Acid absent

Terpenoids were the only phytochemicals present in this fraction. Phenolics, flavonoids, steroids, aldehyde, cardiac glycosides and saponins were all absent. A tertiary alcohol and unsaturated compound were present, but no phenol or acid.

4.1.1.4 Ethyl acetate fraction

Table 4.4: Phytochemical and Chemical tests for Ethyl acetate fraction

Test	Observations	Conclusion
Tannins	No brownish green colouration formed in the yellow solution	Absent
Phenolic compounds	A brownish green precipitate formed	Present
Flavonoids	No yellow colouration formed	Absent
Terpenoids	A reddish brown ring formed at the interface of chloroform and sulphuric acid layers	Present
Steroids	No blue or green colouration formed	Absent
Cardiac glycosides	No brown or green ring formed	Absent
Saponins	No persistence foam/froth formed	Absent
$KMnO_4$ test	$KMnO_4$ decolourised	-OH or unsaturated compound present
Bromine test	Bromine water decolourised.	Unsaturated compound present
Lucas test	Immediate formation of a brown top layer and yellow bottom layer	Tertiary alcohol present
Tollen's test	Silver mirror deposited	Aldehyde present
NaOH test	Dissolves.	Phenol present
$NaHCO_3$ test	Effervescence seen	Acid present

The ethyl acetate fraction tested positive for phenolic compounds and terpenoids. All other phytochemicals were absent in this fraction. Chemical tests indicated the presence of a tertiary alcohol, an aldehyde, unsaturated compound and an acid.

4.1.1.5 Acetone fraction

Table 4.5: Phytochemical and Chemical tests for Acetone fraction

Test	Observations	Conclusion
Tannins	Brownish green colouration formed in the yellow solution	Present
Phenolic compounds	A brownish green precipitate formed	Present
Flavonoids	Yellow colouration formed	Present
Terpenoids	No reddish brown ring formed at the interface of chloroform and sulphuric acid layers	Absent
Steroids	No blue or green colouration formed	Absent
Cardiac glycosides	No brown ring formed	Absent
Saponins	No persistence foam/froth formed	Absent
$KMnO_4$ test	$KMnO_4$ decolourised	-OH or unsaturated compound present
Bromine test	Bromine water decolourised.	Unsaturated compound present
Lucas test	Immediate formation of a yellow top layer and brown bottom layer	Tertiary alcohol present
Tollen's test	Silver mirror deposited	Aldehyde present
NaOH test	A yellow suspension seen	Phenol absent
$NaHCO_3$ test	No effervescence	Acid absent

Flavonoids, tannins and phenolics were present in the acetone fraction. Terpenoids, steroids, cardiac glycosides and saponins were absent. A tertiary alcohol, an aldehyde, an acid and unsaturated compound were present.

4.1.1.6 Ethanol fraction

Table 4.6: Phytochemical and Chemical tests for ethanol fraction

Test	Observations	Conclusion
Tannins	Brownish green colouration formed in the yellow solution	Present
Phenolic compounds	A brownish green precipitate formed	Present
Flavonoids	Yellow colouration formed	Present
Terpenoids	No reddish brown ring formed at the interface of chloroform and sulphuric acid layers	Absent
Steroids	No blue or green colouration formed	Absent
Cardiac glycosides	No brown ring formed	Absent
Saponins	Persistence foam/froth which formed an emulsion on addition of olive oil	Present
$KMnO_4$ test	$KMnO_4$ decolourised	-OH or unsaturated compound present
Bromine test	Bromine water decolourised.	Unsaturated compound present
Lucas test	Immediate formation of a yellow top layer and brown bottom layer	Tertiary alcohol present
Tollen's test	Silver mirror deposited	Aldehyde present
NaOH test	Dissolved to form orange solution	Phenol present
$NaHCO_3$ test	Effervescence seen	Acid present

Phenolic compounds, tannins, flavonoids and saponins were present in the ethanol fraction. Terpenoids, steroids and cardiac glycosides were absent. The chemical tests revealed the presence of a tertiary alcohol, an aldehyde, an acid, phenol and unsaturated compound.

4.1.1.7 Methanol fraction

Table 4.7: Phytochemical and Chemical tests for Methanol fraction

Test	Observations	Conclusion
Tannins	Brownish green colouration formed in the yellow solution	Present
Phenolic compounds	A brownish green precipitate formed	Present
flavonoids	No yellow colouration formed	Absent
Terpenoids	No reddish brown ring formed at the interface of chloroform and sulphuric acid layers	Absent
Steroids	No blue or green colouration formed	Absent
Cardiac glycosides	No brown ring formed	Absent
Saponins	Persistence foam/froth which formed an emulsion on addition of olive oil	Present
$KMnO_4$ test	$KmnO_4$ decolourised	-OH or unsaturated compound present
Bromine test	Bromine water decolourised.	Unsaturated compound present
Lucas test	Immediate formation of a yellow top layer and brown bottom layer	Tertiary alcohol present
Tollen's test	Silver mirror deposited	Aldehyde present
NaOH test	Dissolved to form orange solution	Phenol present
$NaHCO_3$ test	Effervescence seen	Acid present

Tannins, phenolics and saponins were present in this fraction. Chemical tests indicated the presence of an aldehyde, a tertiary alcohol and an acid.

Table 4.8: Summary of phytochemical tests results

Fraction	Tannins	Phenolic compounds	flavonoids	terpenoids	Steroids	Cardiac glycosides	saponins
Hexane	–	–	–	+	–	–	–
CH_2Cl_2	–	–	–	+	–	–	–
$CHCl_3$	–	–	–	+	–	–	–
EtOAc	–	+	–	+	–	–	–
Acetone	+	+	+	–	–	–	–
EtOH	+	+	+	–	–	–	+
MeOH	+	+	–	–	–	–	+

Key: + present - Absent

Hexane, dichloromethane, and chloroform fractions contained terpenoids. Ethyl acetate fraction contained terpenoids and phenolics. Tannins, phenolics and flavonoids were present in the acetone and ethanol fractions while tannins, phenolics and saponins were present in the methanol fraction. The phenolics, which were targeted for isolation, were soluble in ethyl acetate, acetone, ethanol and methanol fractions.

4.1.2 Antimicrobial Activity

Antimicrobial activity was performed with the aim of identifying the fraction(s) containing the active compound(s). The Minimal Inhibitory Concentration (MIC) was then performed on the fractions that exhibited reasonable activity.

4.1.2.1 Fractions

The fractions were obtained after solvent fractionation of the extract using hexane, dichloromethane, chloroform, ethyl acetate, acetone, ethanol and methanol. Each fraction was screened for activity.

Table 4.9: Antimicrobial activity screening of the fractions

		Inhibition Zones (mm)							
Microorganism		**Sample (400 mg/ml)**							
Gram-negative bacteria	**Culture collection and Ref. No.**	**Hexane**	**CH_2Cl_2**	**$CHCl_3$**	**EtOAc**	**Acetone**	**EtOH**	**MeOH**	**Standard**
E. coli	ATCC 25922	14	0	9	15	0	0	14	30
S. typhi	KEMRI	10	0	0	9	0	0	12	30
K. pneumoniae	KEMRI	8	0	0	7	0	0	10	20
P. mirabilis	KEMRI	0	0	8	16	0	8	0	25
P. aeruginosae	ATCC 27853	0	0	0	0	0	0	0	0
Gram-positive bacteria									
S. aureus	ATCC 25923	11	0	0	0	0	0	0	30
Bacillus spp	KEMRI	14	0	0	18	8	0	7	18
Fungus									
C. albicans	KEMRI	10	0	0	0	0	0	10	18

Hexane, ethyl acetate and methanol fractions showed higher activity against most microbes than other fractions.

4.1.2.2 Minimum Inhibitory Concentration (MIC)

The MIC was performed on hexane, ethyl acetate and methanol fractions, which on screening, had been found to be moderately active against most of the microbes.

4.1.2.2.1 Hexane fraction

Table 4.10: Hexane fraction MIC

	Inhibition Zones (mm)									
	Concentration (mg/ml X 10^2)									
Microorganism	**4.00**	**2.00**	**1.33**	**1.00**	**0.8**	**0.67**	**0.57**	**0.50**	**0.44**	**STD (mg/ml)**
Gram-negative bacteria										
E. coli	14	9	8		0	0	0	0	0	25.0
S. typhi	10	8		0	0	0	0		0	25.0
Gram-positive bacteria									0	
S. aureus	11	9		0	0	0	0	0	0	31.3
Bacillus spp	14	11	8		0	0	0	0	0	26.3
Fungus										
C. albicans	10	9		0	0	0	0	0	0	_

-Minimum inhibitory concentration

The MIC of the hexane fraction was 100 mg/ml against *E. coli* and *Bacillus spp*, whereas against the *S. aureus*, *S. typhi* and *C. albicans* the MIC was 133 mg/ml. The hexane fraction was therefore most active against *E. coli* and *Bacillus spp*.

4.1.2.2.2 Ethyl acetate fraction

Table 4.11: Ethyl acetate fraction MIC

	Inhibition Zones (mm)									
	Concentration (mg/ml X 10^2)									
Microorganism	**4.00**	**2.00**	**1.33**	**1.00**	**0.8**	**0.67**	**0.57**	**0.50**	**0.44**	**STD (mg/ml)**
Gram-ve bacteria										
E. coli	15	13	11	9		0	0	0	0	25.0
P. mirabillis	16	15	13	12	8		0	0	0	_
S. typhi	9		0	0	0	0	0	0	0	25.0
Gram-positive bacteria										
Bacillus spp	18	15	14	12	10	9		0	0	26.3

-Minimum inhibitory concentration

The MIC of this fraction was 80 mg/ml, 67 mg/ml, 57 mg/ml and 200 mg/ml for *E. coli*, *P. mirabilis*, *Bacillus spp* and *S. typhi* respectively. The results showed that ethyl acetate fraction was most active against *Bacillus spp*.

4.1.2.2.3 Methanol fraction

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Table 4.12: Methanol fraction MIC

	Inhibition Zones (mm)									
	Concentration (mg/ml X 10^2)									
Microorganism	**4.00**	**2.00**	**1.33**	**1.00**	**0.8**	**0.67**	**0.57**	**0.50**	**0.44**	**STD (mg/ml)**
Gram-ve bacteria										
E. coli	14	10	11	9	▨	0	0	0	0	25.0
K. pnemoniae	10	9	▨	0	0	0	0	0	0	22.5
S. typhi	12	10	8	▨	0	0	0	0	0	25.0
Fungus										
C. albican	10	▨	0	0	0	0	0	0	0	_

▨ -Minimum inhibitory concentration

The Minimum Inhibitory Concentrations for *E. coli, K. pneumoniae, S. typhi* and *C. albican* were 80 mg/ml, 133 mg/ml, 100 mg/ml and 200 mg/ml respectively (Table 4.12).

4.1.3 Isolation of compounds 1, 2, 3 and 4

The active ethyl acetate and methanol fractions, both of which contained phenolics, were combined and subjected to activity- guided isolation, affording phenylpropanoid esters (**1**, **2**, and **3**) and a sesquiterpene lactone (**4**).

4.1.3.1. Thin Layer Chromatography

The TLC of the bioactive combined fraction on silica gel plates (Merck, $60F_{254}$) using solvent system MeOH- EtOAc in the ratio 3: 2 (v/v) gave four distinct spots at retention factor (R_f) 0.16, 0.34, 0.53 and 0.74. Although fraction 4 gave a single spot, it was later found out that it contained three isomers, which could not separate out during the TLC analysis (see section 4.2.3.1).

4.1.3.2 Column Chromatography

This was done using silica gel with MeOH-EtOAc solvent system gradient elution where four fractions were obtained. Antimicrobial screening of these fractions revealed fraction 1 was weakly active against *E. coli* while fraction 4 was active against *E. coli*, *P. mirabilis* and *Bacillus spp*. Fraction 4 also tested positive for phenolics. TLC of fraction 1 and 4 with MeOH and EtOAc in the ratio 3: 2 (v/v) showed distinct spots at R_f 0.74 and 0.16 respectively. The fact that three compounds were later isolated from this fraction (see section 4.2.3.1) implies that there was poor resolution with the solvent system used.

4.1.3.3 HPLC analysis

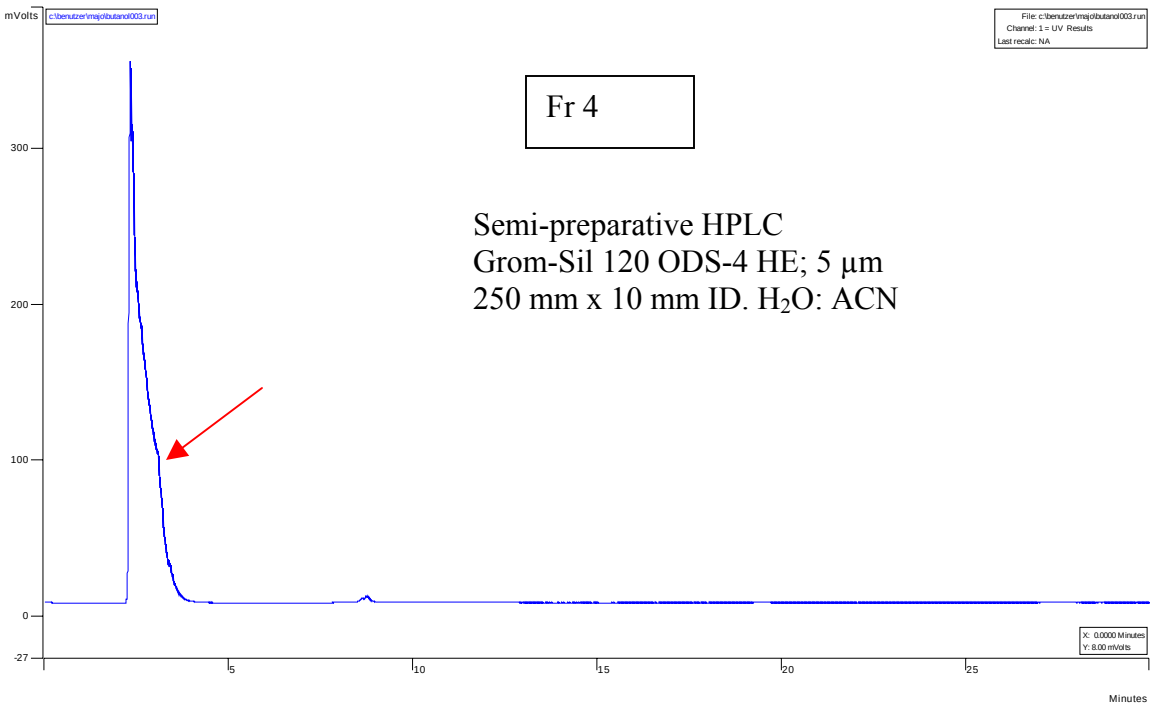


Figure 4.1: Semi-Preparative HPLC of fraction 4

Semi preparative HPLC of fraction 4 from the column chromatography (Figure 4.1) showed a single peak at 3 minutes with a broad base (see the arrow on the chromatogram) that indicated incomplete resolution.

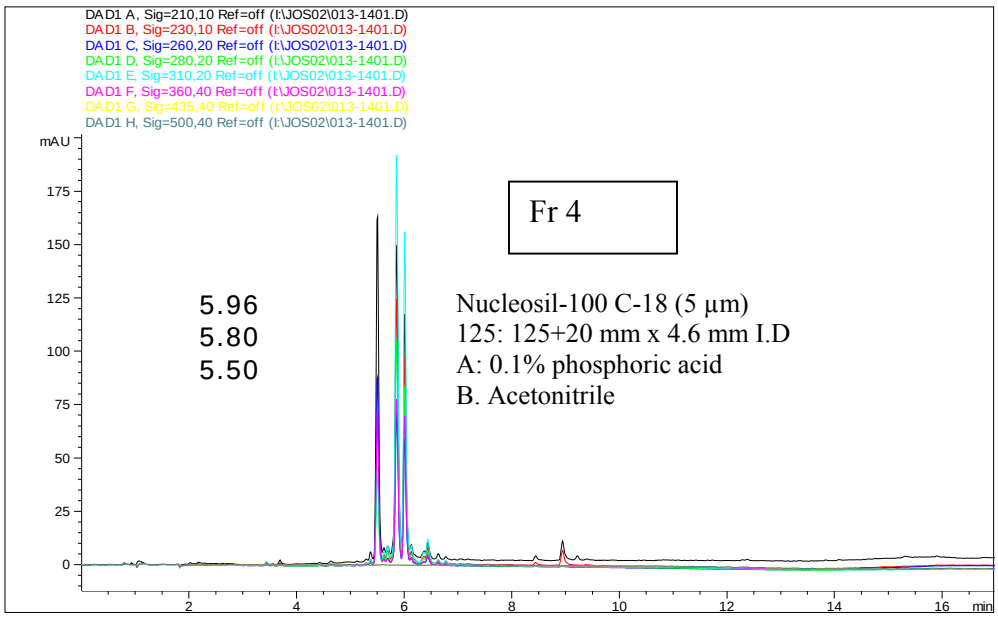


Figure 4.2: Analytical HPLC of fraction 4

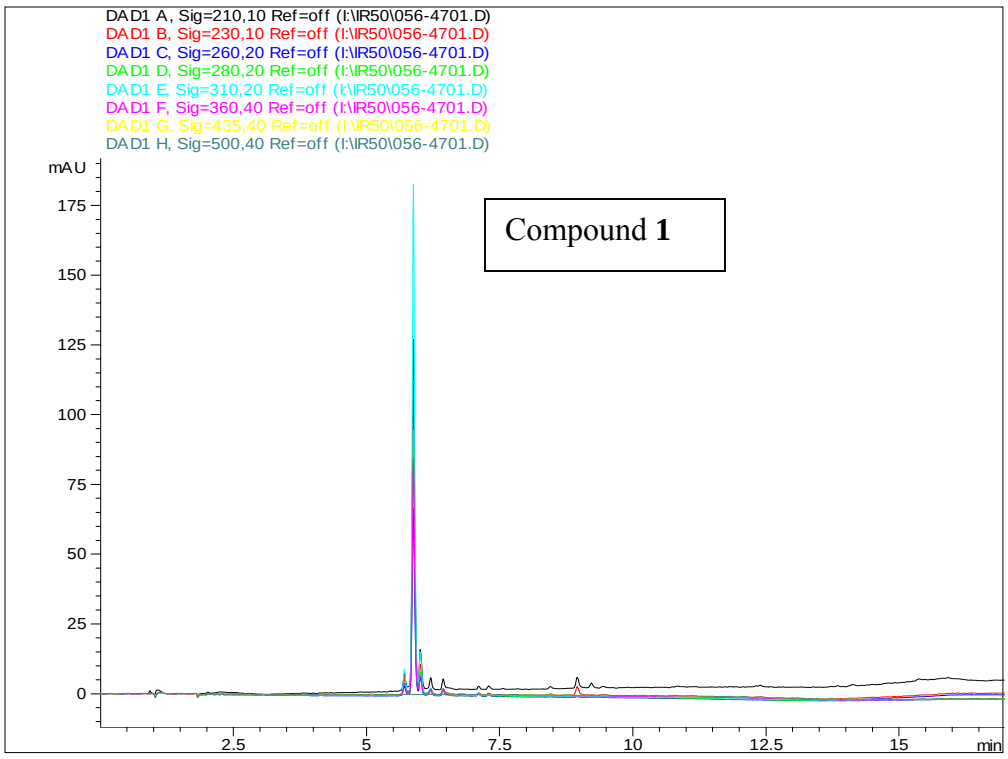


Figure 4.3: HPLC chromatogram of compound **1**

Analytical HPLC of fraction 4 from the column chromatography (Figure 4.2) showed three peaks at Retention times 5.50, 5.80 and 5.96 minutes**.** HPLC of compound **1** (Figure 4.3) after fraction 4 had been purified on sephadex LH –20, showed a single peak at 5.50 minutes.

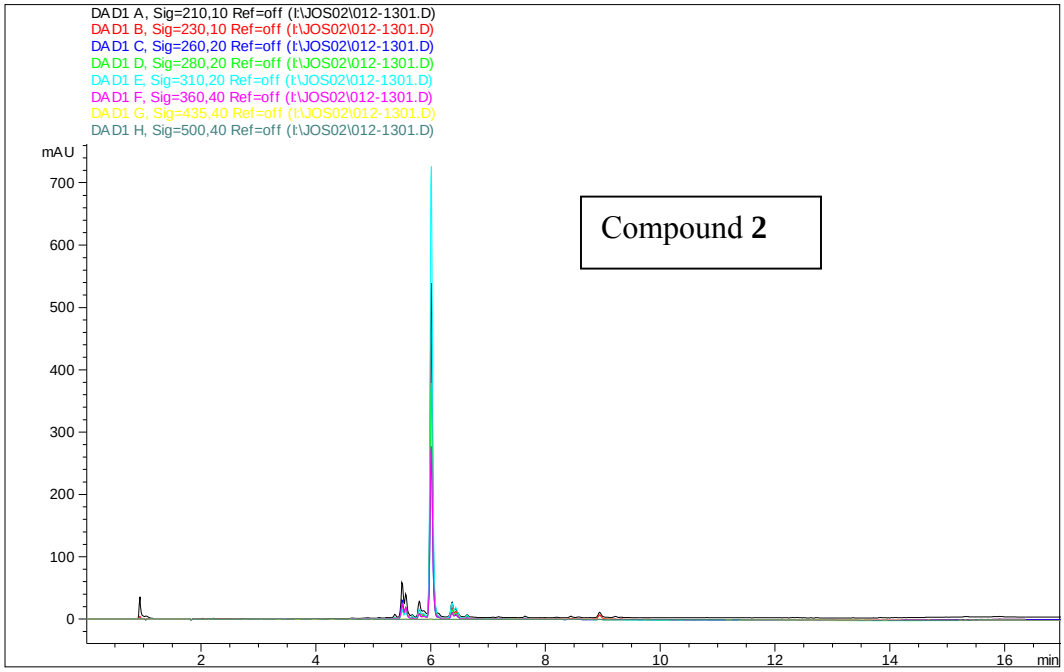


Figure 4.4: The HPLC chromatogram of compound **2**

HPLC of compound 2 (Figure 4.4) showed a single peak at retention time 5.80 minutes. The last of the three compounds was labelled Compound **3**.

4.1.3.4 NMR Structural elucidation of the compounds

^{13}C NMR, ^{1}H NMR, DEPT ^{1}H-^{1}H COSY and HMBC techniques were applied in determining the structures of the pure compounds.

4.1.3.4.1 Compound 1

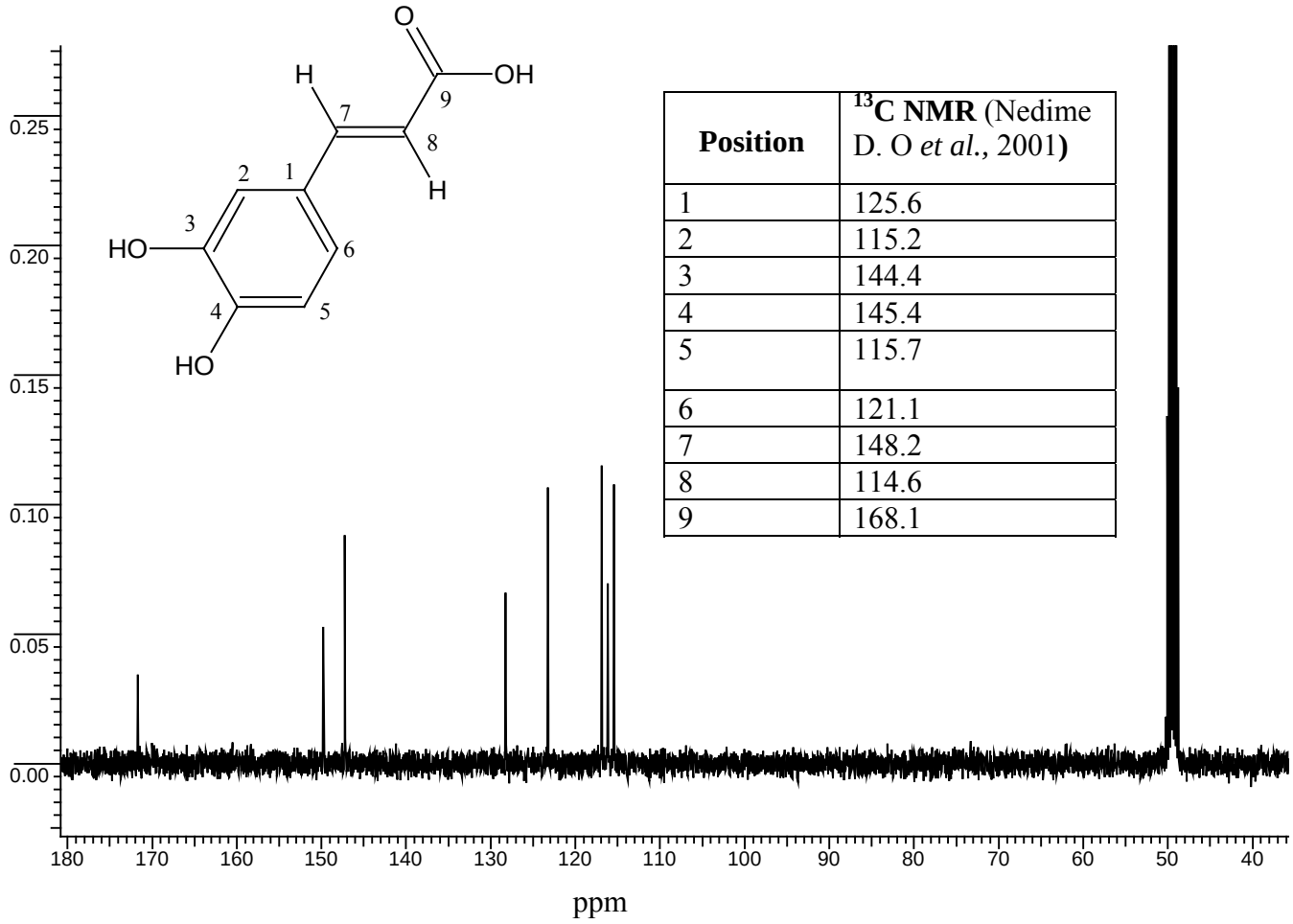
Table 4.13: NMR spectral data of compound **1**

Position	**^{1}H NMR 1** (See appendix 3)	**^{13}C NMR 1** (See Appendix 4)	**DEPT 1** (See Appendix 5)	**^{1}H-^{1}H COSY 1** (See Appendix 6)	**HMBC 1** (See Appendix 7)	**LITERATURE VALUES** (Yue *et al.,* 2000)	
						^{1}H NMR	**^{13}C NMR**
1	-	73.93	C-q			-	75.50
2	1.9-2.44 (2.13), m 2H	36.45	CH_2			2.22 2H, m	36.31
3	3.96, ddd 12Hz, 8Hz	70.48	C-H			3.97	71.16
4	5.27, d, 8Hz 1H	72.07	C-H			5.43	72.91
5	5.23, ddd, 8.1, 8.0, 4.0 Hz	72.59	C-H			5.50	72.06
6	1.8-2.42 (2.13), m	37.97	CH_2			2.17-2.32	38.31
7	-	177.09	C-q			-	178.30
1′	-	128.34	C-q			-	127.97
2′	6.87, s	115.60	C-H		H - 2′→C-4′, C-6′, C-7	7.05	115.26
3′	-	147.55	C-q			-	146.69
4′	-	149.98	C-q			-	149.47
5′	6.60, d, 8Hz	116.88	C-H	H-5′→H- 6′	H -5′ → C-1′, C-3′		116.52
6′	6.77, d, 8Hz	123.41	C-H	H-6′→ H-5′	H- 6′ → C-2′, C-7′		123.00
7′	7.40, 2d, 16Hz	147.55	C-H	H-7′ →H- 8′	H 7′→ C-2′, C- 6′, C-9′	7.60	147.22
8′	6.20, 2d, 16Hz	115.68	C-H	H- 8′′→ H-7′	H- 8′ → C-1′	6.35	115.66
9′	-	169.63	C-q			-	168.87
1′′	-	128.21	C-q			-	127.86
2′′	6.87, s	115.60	C-H		H-2′′→C-4′′, C-6′′ C-7′′	7.05	115.26
3′′	-	147.19	C-q			-	146.69
4′′	-	149.90	C-q			-	149.41
5′′	6.60, d, 8Hz	116.88	C-H	H-5′′→ H-6′′	H- 5′′→ C-1′′, C-3′′	6.77	116.52
6′′	6.77, d, 8Hz	123.41	C-H	H-6′′→ H-5′′	H- 6′′→ C-2′′, C-7′′	6.29	123.00
7′′	7.40, 2d, 16Hz	147.19	C-H	H-7′′ → H-8′′	H -7′′→ C-2′′, C-6′′, C-9′′	7.56	147.06
8′′	6.20, 2d, 16Hz	115.68	C-H	H-8′′→ H-7′′	H- 8′′ → C-1′′	6.35	115.66
9′′	-	168.26	C-q			-	168.52
OH	4.08, s						

The ^{1}H NMR and ^{13}C NMR spectral data of compound **1** (Table 4.13) matched that of dicaffeoyl quinic acid shown in columns 7 and 8 of the table (Yue J. M *et al.,* 2000). The ^{1}H NMR multiplicities of compound **1** were also similar to those in literature.

4.1.3.4.2 Hydrolysis product of compound 1

Hydrolysis of compound **1** was done in order to confirm the type of the cinnamoyl group in compound **1.**



Position	**^{13}C NMR** (Nedime D. O *et al.*, 2001**)**
1	125.6
2	115.2
3	144.4
4	145.4
5	115.7
6	121.1
7	148.2
8	114.6
9	168.1

Figure 4.5: ^{13}C NMR of Hydrolysis product of compound **1**

The ^{13}C NMR spectrum of Hydrolysis product of **1** (Figure 4.5) was in agreement with the ^{13}C NMR spectral data of caffeic acid shown on the spectrum (Nedime D.O *et al.,* 2001). This confirmed that the compound **1** was caffeoylquinic acid.

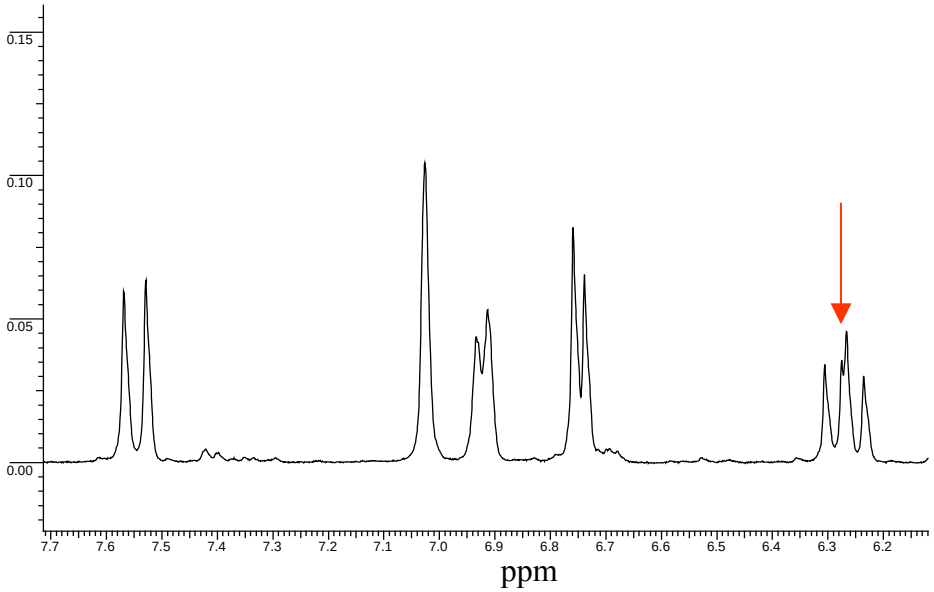


Figure 4.6(a): ^{1}H NMR spectrum of compound **1**

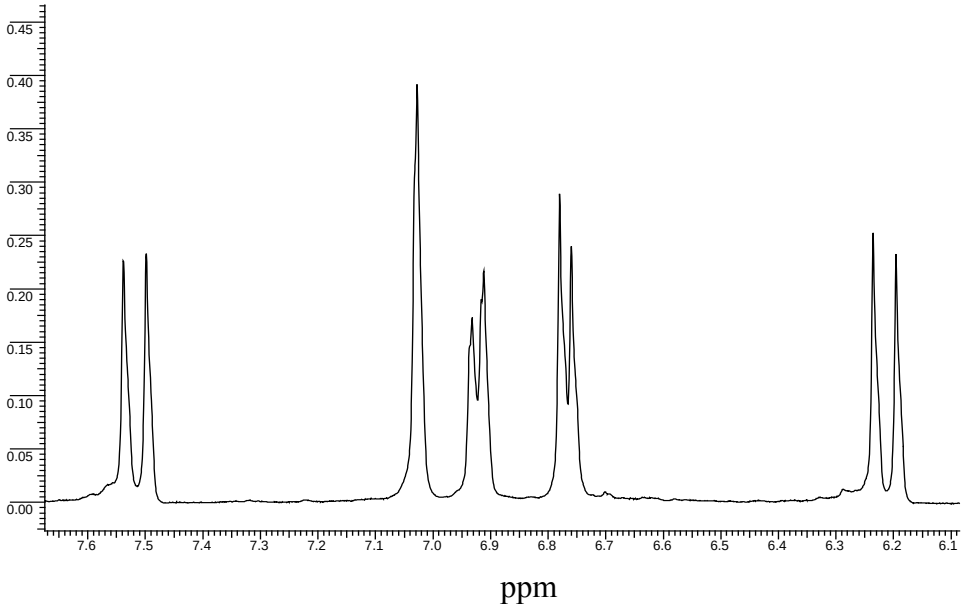


Figure 4.6 (b): ^{1}H NMR spectrum of the hydrolysis product of **1**

Comparison of the ^{1}H NMR of compound **1** (Figure 4.6 a) and its product of hydrolysis (Figure 4.6 b) showed overlapping doublets in the former at δ 6.20 (as shown by the arrow) and a single doublet in the latter. The presence of two doublets at δ 6.20 pointed to the fact that two caffeoyl groups were present. This, together with the downfield shift of H-4 and H-5 (Table 4.13) led to the conclusion that compound **1** was 4,5-O-dicaffeoylquinic acid.

4.1.3.4.3 Compound 2

Table 4.14: NMR spectral data of compound **2**

Position	**^{1}H NMR 2** (See appendix 9)	**^{13}C NMR 2** (See appendix 10)	**DEPT 2** (See appendix 11)
1	-	73.34	C-q
2	1.84-197 (1.91), m	38.15	CH_2
3	5.10, dd, 12Hz, 8Hz	71.22	C-H
4	3.52 d, 8Hz	70.98	C-H
5	5.09, dd, 12Hz, 8Hz,	71.91	C-H
6	1.84-197 (1.91), m	38.70	CH_2
7	-	176.99	C-q
1′	-	127.72	C-q
2′	6.83, s	115.11	C-H
3′	-	147.02	C-q
4′	-	149.51	C-q
5′	6.56, d, 8Hz	116.40	C-H
6′	6.73, d, 8Hz	122.93	C-H
7′	7.36, 2d, 16Hz	146.73	C-H
8′	6.05, 2d, 16Hz	115.18	C-H
9′	-	168.60	C-q
1′′	-	127.72	C-q
2′′	6.83, s	115.11	C-H
3′′	-	147.02	C-q
4′′	-	149.51	C-q
5′′	6.56, d, 8Hz	116.40	C-H
6′′	6.73, d, 8Hz	122.93	C-H
7′′	7.36, 2d, 16Hz	146.73	C-H
8′′	6.05, 2d, 16Hz	115.18	C-H
9′′	-	168.60	C-q
OH	4.08, s		

The ^{1}H NMR and ^{13}C- NMR spectral data of compound **2** (Table 4.14) were very similar to that of compound **1** (Table 4.13). The main differences were in the splitting pattern of H-3, H-4 and H-5 where the resonance frequencies of protons bonded to the same carbon as the cinnamoyl group were higher than the one with a carbon bearing an –OH group. In this respect the resonance frequencies of H-3 (δ 5.10) and H-5 (δ 5.09) in compound **2** were higher than that of H-4 (δ 3.52).

4.1.3.4.4 Compound 3

Table 4.15: NMR spectral data of compound **3**

Position	**^{1}H NMR 3** (See appendix 13)	**^{13}C NMR 3** (See appendix 14)	**DEPT 3** (See appendix 15)
1	-	73.44	C-q
2	1.85-2.50, m	36.75	CH_2
3	5.47, dd, 12Hz, 8Hz	71.91	C-H
4	5.24, dd, 8Hz	71.22	C-H
5	3.17, dd, 12Hz, 8Hz, 4.0Hz	70.98	C-H
6	1.85-2.50 m	37.70	CH_2
7	-	177.19	C-q
1′	-	128.72	C-q
2′	6.95, s	115.51	C-H
3′	-	147.35	C-q
4′	-	149.90	C-q
5′	6.67, d, 8Hz	116.40	C-H
6′	6.86, d, 8Hz	123.93	C-H
7′	7.48, 2d, 16Hz	147.73	C-H
8′	6.19, 2 d, 16Hz	115.20	C-H
9′	-	169.60	C-q
1″	-	128.72	C-q
2″	6.95, s	115.11	C-H
3″	-	147.22	C-q
4″	-	149.51	C-q
5″	6.67, d, 8Hz	116.40	C-H
6″	6.86, d, 8Hz	122.93	C-H
7″	7.48, 2d, 16Hz	146.73	C-H
8″	6.19, 2 d, 16Hz	115.18	C-H
9″	-	169.60	C-q
OH	4.16, s		

The ^{1}H NMR and ^{13}C- NMR spectral data of compound **3** (Table 4.15) were very similar to that of compound **1** (Table 4.13) and compound **2** (Table 4.14). The resonance frequencies of H-3 (δ 5.47) and H-4 (δ 5.24) in compound **3** were higher than that of H-5 (δ 3.17).

4.1.3.4.5 Compound 4

One and two –dimensional NMR spectral data for compound **4** shown in Table 4.16.

Table 4.16: NMR Spectral data of compound **4**

Position	**^{1}H NMR 4** (See Appendix 16)	**^{13}C NMR 4** (See Appendix 17)	**DEPT 4** (See Appendix 18)	**^{1}H-^{1}H COSY 4** (See Appendix 19)	**^{13}C NMR LITERATURE VALUES** (Motoo et *a.l*, 2002; Pentes *et al*, 2003)
1	6.93 (dd 14.1,13.0 Hz)	128.31	CH	H-1→H-2	127.3
2	2.36 (m)	31.47	CH_2	H-2→H-1, H-3	31.6
3	3.63 (d, 3.6Hz)	50.74	CH	H-3→ H-2	52.7
4	-	158.07	C-q		
5	6.72 (d, 3.3 Hz)	112.61	CH		
6	3.65 (m)	54.14	CH		54.2
7	-	129.21	C-q		130.8
8	7.11(dd, 6.3, 1.1 Hz)	143.97	CH	H-8 → H -9	141.8
9	4.09 (d, 10.2 Hz)	60.90	CH_2	H-9 → H-8	61.7
10	-	131.09	C-q		132.7
11	-	169.19	C-q		170.0
12	4.31 (2d, 12.1 Hz)	72.53	CH_2		73.4
13	3.65 (d, 3.6 Hz)	62.19	CH_2		62.0
14	1.46 (s)	12.31	CH_3		12.8
15	1.47 (s)	18.36	CH_3		18.4

^{13}C NMR spectrum of **4** (Appendix 17) showed 15 carbon signals, comprising one lactone carbonyl (δ 169.19), three olefinic methine carbons (δ 143.97, 128.31 and 112.61,), two oxygenated methylenes (δ 62.19 72.53), one methylene, C-2 (δ 31.47), two methyl groups (δ 12.31, 18.36) and three quaternary carbons (δ 129.21, 131.09, 158.07).

4.2 Discussion

4.2.1 Phytochemical and chemical tests

Phytochemical and chemical tests (Tables 4.1 to 4.8) revealed that the phenolic compounds, which were targeted for isolation in this work, were soluble in the polar solvents. This is because most phenolics contain –OH groups, which make them more soluble in polar than non-polar solvents. Ethyl acetate and methanol extracts also tested positive for $NaHCO_3$, bromine and Lucas tests. These findings supported the structures of the pure isolated compounds **1**, **2**, **3** and **4**, as discussed in sections 4.2.3.4.1 and 4.2.3.4.3, 4.2.3.4.4, 4.2.3.4.6 respectively.

4.2.2 Antimicrobial activity

The extracts soluble in some solvents demonstrated moderate activity when tested against some Gram-positive and Gram-negative bacteria and fungus, as detailed below.

4.2.2.1 Hexane fraction

The hexane fraction demonstrated reasonable activity (Table 4.9) against *E. coli, S. typhi, K. pneumoniae, S. aureus, Bacillus spp* and *C. albicans* while *P. aeruginosae* and *P. mirabillis*, were resistant. *P. aeruginosae* was resistant even to the standard chloramphenicol drug. *P. aeruginosae* and *P. mirabillis* are both gram-negative bacteria which are less susceptible to antimicrobial agents than gram–positive ones because they possess outer membrane surrounding the cell wall (Ratledge and Wilkinson, 1988). A previous study on the aqueous, ethanol and hexane extracts of the same plant species did not demonstrate in vitro inhibitory effects against *S. aureus, Bacillus subtilis, E. coli and K. pneumoniae* (McGraw *et al*., 2000). The differences in the antimicrobial properties of the same plant species may be due to the fact that geographical areas of the plants play a role in the availability of the bioactive secondary metabolites in plants (Kitagawa *et al.*, 1999). The active metabolites may also be generated only during specific developmental period of the plant, and also these compounds partition exclusively in particular solvents (Marjorie, 1999).

The MIC of the hexane fraction was 100 mg/ml for *E. coli* and *Bacillus spp*., while for *S. typhi, S. aureus*, and *C. albicans* it was 133 mg/ml (Table 4.10). The MIC of the standard chloramphenicol for the same microorganisms was in the range of 25 mg/ml to 31.3 mg/ml. The standard was therefore approximately four times more active than the hexane soluble fraction.

4.2.2.2 Dichloromethane, chloroform, acetone and ethanol fractions

Almost all the microorganisms were resistant to these fractions (Table 4.9). The medicinal properties of plant extracts normally depend upon the presence of active compounds (Kokwaro, 1993) possessing specific functional groups that are soluble only in solvents of particular polarity. The active compounds in the extract of the leaves of *Tarconanthus camphoratus* were therefore not soluble in these solvents.

4.2.2.3 Ethyl acetate fraction

Ethyl acetate fraction was one of the two fractions from which the pure compounds **1**, **2**, **3** and **4** were isolated. It exhibited the highest activity against *E. coli, P. mirabillis* and *Bacillus spp* compared to all other fractions. The activity of 4 X 10^{3} μg of the extract against *P. mirabillis* and *Bacillus spp* compared favourably with that of 30 μg of the standard chloramphenical. The big difference with the reference antibiotic could be due to the fact that the active compound was only a small percentage of the extract since no purification had been done at this stage. The fraction was moderately active against *E.* coli, *P. mirabilis* and *Bacillus spp.* and weakly active against *S. typhi* as compared to the standard (see table 4.11). The gram-positive bacteria, *S. aureus*, the gram-negative bacteria, *P. aeruginosae* and the fungus, *C. albicans* were resistant to the ethyl acetate fraction (see Table 4.9).

The MIC of the fraction (Table 4.11) ranged from 57 mg/ml to 200 mgl/ml as compared to the standard values of between 25 mg/ml and 26.3 mg/ml for the same microorganisms. The MIC of the fraction for *E. coli* was 80 mg/ml compared to 25.0 mg/ml of the standard. *Bacillus spp* was most susceptible to the fraction with MIC of 57 mg/ml compared to 25.0 mg/ml for the standard. The standard was therefore only twice more active than the fraction against *Bacillus spp.* The fraction was least active against *S. typhi* with MIC of 200 mg/ml compared to 25.0 mg/ml of the standard.

4.2.2.4 Methanol fraction

The fraction representing 4 X 10^{3}μg showed lower activity than 30 μg of the standards (Table 4.9) with the highest activity being recorded against *E. coli* and the lowest activity against *Bacillus spp.* On the other hand, *S. aureus*, *P. aeruginosae* and *P. mirabillis* were all resistant to the fraction.

The MIC of the methanol fraction was between 80 mg/ml and 200 mg/ml compared to 22.5 mg/ml and 25.0 mg/ml for the standard (Table 4.12). The fraction was most active against *E. coli* with MIC of 80mg/ml and least active against *C. albicans* with MIC of 200mg/ml.

4.2.2.5 Activity of compounds I, 2, 3 and 4

Since compounds **1**, **2**, **3** and **4** were obtained as a result of activity-guided fraction of the extract, the activity reported in this work is therefore attributed to the isolated compounds either independently or working synergistically with others. However the activity of each individual compound was not tested because of the insufficient amount of the compounds left after spectroscopic analysis.

4.2.3 Isolation and structure elucidation of the compounds

Antimicrobial activity tests were carried out after every separation step so as to keep track of the active compounds in the fractions. TLC, HPLC and Column Chromatographic techniques were applied in separating the compounds. The structures of the pure compounds were elucidated by means of spectroscopic methods (IR, MS, 1D and 2D NMR).

4.2.3.1 HPLC analysis

Although the TLC of the fractions from column chromatography indicated that separation of the compounds had been achieved, it was necessary to subject them to HPLC to confirm the results. Fraction 4, which showed only one spot on TLC analysis, also gave a single peak at 3 minutes with a broad base at 3 minutes in semi preparative HPLC (Figure 4.1), suggesting that it contained more than one compound. Furthermore, initial ^{13}C NMR and ^{1}H NMR analysis of the same fraction resulted in many overlapping peaks, an indication that more than one compound was present. The fraction was then subjected to analytical HPLC leading to identification of three peaks at retention times 5.50, 5.80 and 5.96 minutes (Figure 4.2). These findings pointed to the fact that the fraction actually contained three closely related compounds. The three compounds, **1**, **2** and **3** were isolated and purified on sephadex LH-20. A repeat of HPLC of the separated compounds showed single peaks at retention times 5.50 minutes (Figure 4.3), 5.80 minutes (Figure 4.4) for compounds **1** and **2** respectively. The last of the three compounds was labelled compound **3.**

4.2.3.2 Mass Spectroscopy of compound 1

Compound **1** gave a base peak at 516 $(M+H)^+$ in the positive ion ESIMS (Appendix 1) suggesting a molecular formulae of $C_{25}H_{24}O_{12}$.

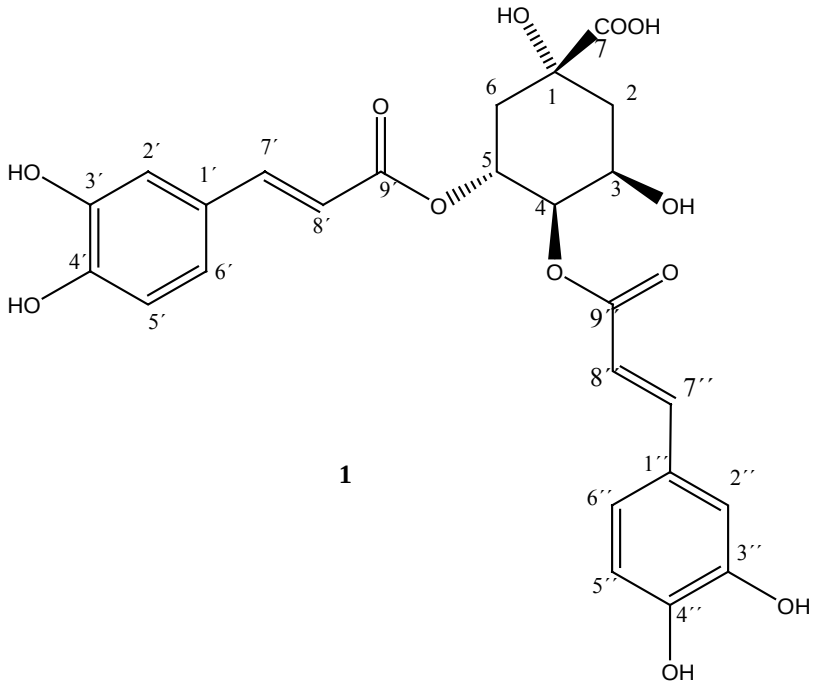
4.2.3.3 IR Spectroscopy of 1, 2 and 3

The IR spectroscopy of compound **1** (Appendix 2) showed absorptions typical of hydroxyl groups (3432.67 cm^{-1}) and a carbonyl (1658.48 cm^{-1}). The peak at 1000 cm^{-1} was due to C-O stretch while the two peaks at 829.24 and 767.53 cm^{-1} were due to the presence of a disubstituted aromatic system. This was in agreement with the chemical tests that had indicated the presence of a tertiary alcohol and phenolics in the compounds (see Tables 4.4 and 4.7). The IR spectra of compound **2** (see appendix 8) and compound **3** (see appendix 12) were very similar to that of compound **1**. The O-H stretch and C=O stretch in compound **2** appeared at 3432.67 cm^{-1} and 1656.56 cm^{-1} respectively while in compound **3** they appeared at 3438.46 cm^{-1} and 1656.55 cm^{-1} respectively.

4.2.3.4 NMR spectroscopy of compound 1, 2, 3 and 4

Both one and two-dimensional NMR were used to determine the structure of the pure compounds **1**, **2**, **3** and **4**

4.2.3.4.1 4, 5-O-dicaffeoyl quinic acid, 1



1

Com

pound **1** (135mg) was isolated as yellow amorphous powder. The ^{1}H-NMR spectrum (Appendix 3) showed signals by two *trans*-caffeoyl groups 7.40/7.42 (1H each, d, J = 16.0 Hz), 6.87/6.87 (1H each, s), 6.77/6.77 (1H each, d, J = 8.0 Hz), 6.60/6.60 (1H each, d, J = 8.0 Hz) and 6.20/6.20 (1H each, d, J= 16.0 Hz) and -OH (4.08 (1H, s). The ^{13}C-NMR spectrum (Appendix 2) showed two methylene carbons at δ 36.45 and 37.97, two oxygenated carbons at δ 72.07, and 73.93, and three carbonyl carbon signals at δ 177.09, 169.63 and 168.26. The above ^{1}H- and ^{13}C NMR spectral data were typical of dicaffeoyl quinic acid derivatives (Yue J. M *et al.*, 2000; Clifford *et al.*, 2003). The position of two caffeoyl groups was established by the downfield shift of the H-4 (δ 5.37) and H-5 (δ 5.23) as compared to H-3 (δ 3.96) in the ^{1}H-NMR spectrum. There was also a downfield shift of C-4 (δ 72.07) and C-5 (δ 72.59) in the ^{13}C-NMR spectrum as compared to C-3 (70.48). Thus, the structure of compound **1** was determined as 4, 5-O-dicaffeoyl quinic acid since its NMR spectral (see Table 4.13) and physical data were in good agreement with those reported previously (Annie, 1988, Lin *et al*., 1999; Sang Zin Choi *et al*, 2004).

DEPT, ^{1}H-^{1}H COSY, ^{1}H-^{13}C NMR (HMBC) techniques achieved the structural elucidation and complete proton and carbon assignments. Comparisons of DEPT spectrum with a broadband decoupled carbon spectrum, the carbon peaks were firstly classified into methyl, methylene, methine and quaternary carbons. The DEPT experiment (Appendix 3) revealed that compound **1** had two methylene carbons, which were assigned to C-2 and C-6 of the quinic acid ring of the compound. The quaternary carbons were assigned to carbons 1, 3, 4, and 9 of the two caffeoyl groups and C-1 of the quinic acid. The methine carbons were assigned to C-3, 4 and 5 of the quinic acid part of the molecule and carbons 2, 5, 6, 7 and 8 of the two caffeoyl groups.

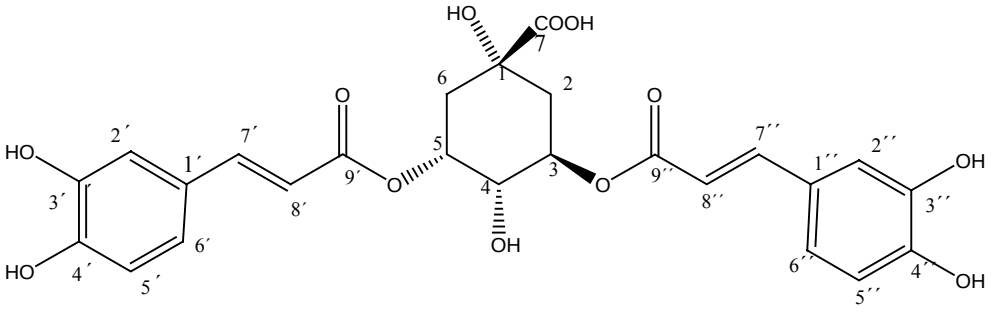
In the ^{1}H-^{1}H COSY, the coupling correlation of protons was used to determine their carbon bonding. Spectrum (Appendix 4) showed that the olefinic protons H-7 and H-8 were coupled with a magnitude of 16 Hz indicating that the two protons were *trans*-oriented. The aromatic proton H-5 was coupled with H-6. There were no correlations involving the four quaternary carbons, C-1′, C-3′ C-4′ and C-9′. The ^{1}H-^{13}C NMR correlations was performed to reveal the direct attachments between protons and carbons, and thus afforded an initial assignment of the protons in the quinic acid unit and the caffeoyls. The proposed structure of compound **1** and the assignment of NMR signals were both confirmed by HMBC (Appendix 5). Important correlations from the HMBC spectrum of the caffeoyl group established the point of attachment of the protons as well as the C-C connectivity therein. There were long range

Correlations (3 bonds) between H-2′ and C-4′, C-6′ and C-7′; H-5′ to C-1′ and C-3′; H-6′ to C-2′ and C-7′; H-7′ was correlated to C-2′, C-6′ and C-9′ while H-8′ to C-1′. According to the chemical shifts of carbons and protons, the oxygen-bearing and non-oxygen-bearing carbons could be distinguished.

4.2.3.4.2 Hydrolysis product of 1

The hydrolysis of compound **1** was done in order to confirm the identity of the cinnamoyl group in the compound. The ^{13}C NMR data of ethyl acetate soluble product of hydrolysis (Figure 4.6 (b)) was in good agreement with that of caffeic acid (Nedime *et al.*, 2001). Comparison of the ^{1}H NMR spectra of the hydrolysis product and compound **1** revealed the absence of one of the two overlapping doublets at δ 6.20 in the hydrolysis product. The arrow in the ^{1}H NMR spectrum of **1** (Figure 4.6a) points at the two doublets while there is only one doublet in the product of hydrolysis (Figure 4.6b), indicating the presence of a single caffeoyl. The overlapping doublets were attributed to the two caffeoyl groups with little or no difference in NMR data.

4.2.3.4.3 3, 5-O-dicaffeoyl quinic acid, 2

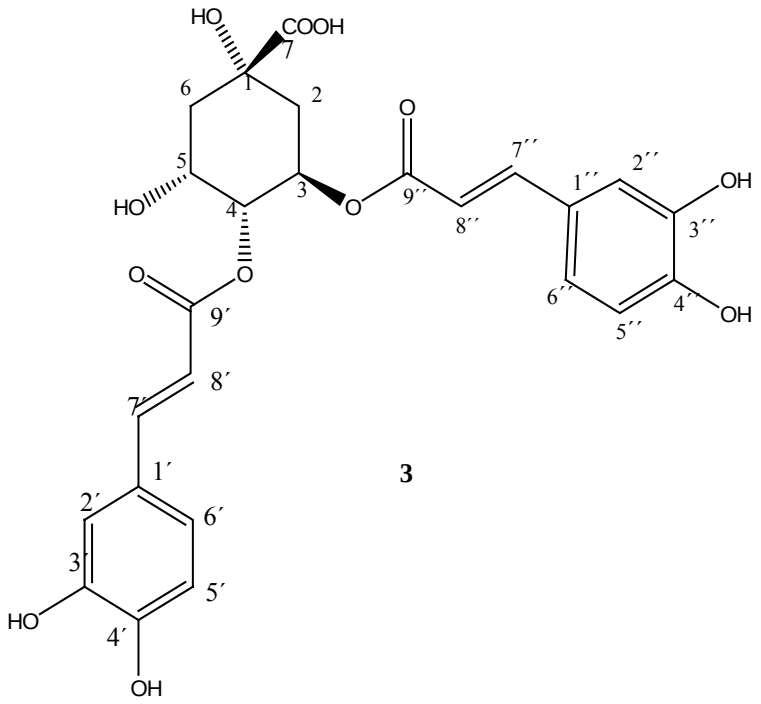


2

Compound **2** (125mg) was isolated as a yellow amorphous powder. NMR spectra of compound **2** (Table 4.14) were very similar to those of compound **1**. The main differences among the spectra of **1** and **2** were the ^{1}H-NMR absorptions of H-3, H-4, and H-5, which were easily recognized in the spectra by their very different splitting patterns. The chemical shifts of these protons then served to distinguish the three compounds from one another. The upfield

proton in **1** is H-3 at δ 3.96 as compared to δ 5.37 and δ 5.23 in H-4 and H-5 respectively. In ^{1}H NMR spectrum of compound **2** (see appendix 9), H-4 is the upfield proton at δ 3.52 as opposed to H-3 (δ 5.10) and H-5 (δ 5.09). The caffeoyl groups on C-3 and C-5 have a greater deshielding effect on the proton of these carbons than the –OH group on C-4. H-3 and H-5 therefore come to resonance at higher frequencies than H-4.

4.2.3.4.4 3, 4-O-dicaffeoylquinic acid, 3



3

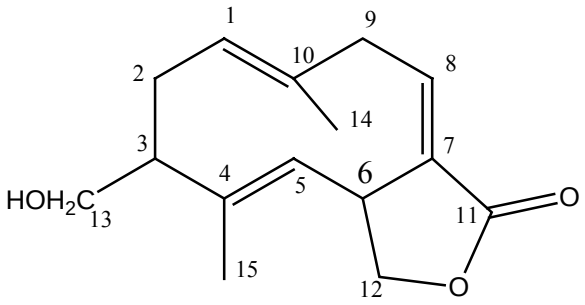
Compoud **3** (90mg) was isolated as a yellow amorphous powder. The NMR spectra of compound **3** (see Table 4.15) were very similar to those of compound **1** and **2**. There were differences in the chemical shifts of H-3, H-4 and H-5. The upfield proton was H-5 (δ 3.17) in compound **3**. H-3 (δ.5.24) and H-4 (δ 5.47) were the downfield protons in this compound. The differences in the chemical shifts of these protons were attributed to the greater deshielding effect of the caffeoyl groups on H-3 and H-4, which increased the resonance frequenies of these

particular protons. All the three compounds **1**, **2** and **3** have previously been isolated together from *Erigeron breviscapus* (Asteraceae), (Yue Jian-Min *et al.*, 2000).

4.2.3.4.5 Stereochemistry

The coupling constant of 16 Hz between the two olefinic protons H-7 and H-8 of **3** indicated a trans-configuration. The two isomers of **1**, both of which have been previously isolated with **1** from *Erigeron breviscapus* (Asteraceae), (Yue Jian-Min *et al*, 2000), differ from **1** in having the two caffeoyl groups at positions 3, 4 and 4, 5 of the quinic acid respectively. Furthermore, the order of elution and the nature of the chromatogram in HPLC were in agreement with previous study (Makiko *et al.*, 2006).

4.2.3.4.6 Sesquiterpene lactone, 4



4

Compound **4** (150mg) was obtained as a white solid. The ^{13}C NMR including the DEPT measurements (Appendix 17 and 18 respectively) showed 15 carbon signals, comprising two methyls (δ12.31, 18.36), two methylene (δ 31.47, 60.90), two methines (δ50.74, 54.14) two oxygenated methylenes (δ, 62.19, 72.53), three olefinic methine carbons (δ 112.61, 128.31, 143.97), three quaternary carbons (δ 129.21, 131.09, 158.07) (see Table 4.16) and a carbonyl carbon (δ 169.19) (Table 4.14). Structural elucidation of **4** was achieved by interpretation of the obtained 1D NMR and 2D NMR spectral data and comparing with that given in literature. The ^{13}C NMR resonance of the two methyl groups at δ 12.31 and δ18.36 on C-14 and C-15 respectively are similar to those of sonchuside A isolated from *Cicerbita alpina* (Asteraceae) (Zidorn *et al.*, 2005), Eupaglehnin A, B, C, D, E and F isolated from *Eupatorium glehni*

(Asteraceae) (Motoo *et al.*, 2002), a sesqueterpene lactone isolated from *Tithonia diversifolia* (Asteraceae) (Obafemi *et al*, 2006) and the prepared dihydrosesquiterpene lactones (Pentes *et al.*, 2003). Furthermore the ^{13}C NMR spectral data for C-1 (δ 128.31) and C-8 (δ 143.97) also corresponded to that of the said compounds. The similarities of these selected spectra of **4** with the named compounds led to the suggestion that **4** is a garmacrene derivative, as all the mentioned compounds have a germacrene parent unit.

The absorption around δ 169.19 (C-11) in the ^{13}C NMR is characteristic of α, β-unsaturated-γ-lactones typical in sesquiterpene lactones such as germacranolides (Motoo, *et al.*, 2002; Pentes *et al.*, 2003; Zidorn *et al.*, 2005), guaianolides and eudesmanolides (Seto *et al.,* 1988; Van Beek *et al.*, 1990). This led to the conclusion that **4** could be containing a lactone moiety. Most of the sesquiterpene lactones contain an α-methylene group outside the 10-membered ring as opposed to **4** which has an extra double bond inside the ring. The DEPT spectrum of **4** (Appendix 7) revealed a highly deshielded methylene carbon at δ 72.53 (C-12), an indication that it was bonded to oxygen in an epoxide framework. The distinguishing features of compound **4** from the conventional germacranolides are the methylene carbon in the lactone ring and the additional unsaturation in the 10-membered ring in **4**, which are missing in germacranolides.

^{1}H NMR (Appendix 8) showed signals of the two methyl protons H-14 and H-15 appearing as a singlet at δ 1.46). The resonance of the oxymethylene (C-12) and hydroxymethylene (C-13) appeared at δ 4.31 and δ 3.65 respectively. The three olefinic protons, H-1, H-5 and H-8 resonated at δ 6. 93, 6.72 and 7.11 respectively while the two methine protons signals on C-3 and C-6 appeared at δ3.63 and 4.31 respectively.

In the ^{1}H-^{1}H COSY spectrum (Apendix 9) the methylene proton, H-2, at δ 2.36 was vicinally coupled to the olefinic proton H-1 at δ 6.93 and the methine proton H-3 at δ 3.63. There was also correlation involving H-8 at δ 7.11 and H-9 at δ 4.09. The NMR spectra and literature survey led to the conclusion that compound **4** was a sesquiterpene lactone.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

- Phytochemical screening of the extracts showed that *Tarconanthus camphoratus* leaves are rich in phenolic compounds, among them, the phenypropanoid esters.
- The bioactivity guided fractionation led to the isolation of the phenypropanoid esters (**1**), (**2**), and (**3**), which are known compounds, and a sesquiterpene lactone (**4**) for the first time from *Tarconanthus camphoratus*.
- The EtOAc fraction containing phenypropanoid ester exhibited relatively high activity against *E. coli*, *Bacillus spp* and *P. mirabillis*.
- All active fractions exhibited highest activity against *E. coli*.
- Ethyl acetate fraction was less active against *S. typhi* (200 mg/ml) compared to the methanol fraction (100 mg/ml).
- Ethyl acetate fraction has same activity (80 mg/ml) against *E. coli* as that of methanol fraction.
- The antimicrobial activity tests results contradicted the findings of a previous study, which showed that the extracts of the leaves of this plant are not active against most Gram-positive and Gram- negative bacteria.
- The findings of this work support, at least in part, the validity of the use of *Tarconanthus camphoratus* in traditional medicine.

5.2 Recommendations

- Further work should be done to isolate more compounds from the plant and assess their antimicrobial activity, including MIC.
- Since dicaffeoylquinic acid derivatives have been shown to inhibit the replication of HIV, a study should be carried out to determine the effect of the extract from the leaves of *Tarconanthus camphoratus* on the progression of HIV *in vitro.*

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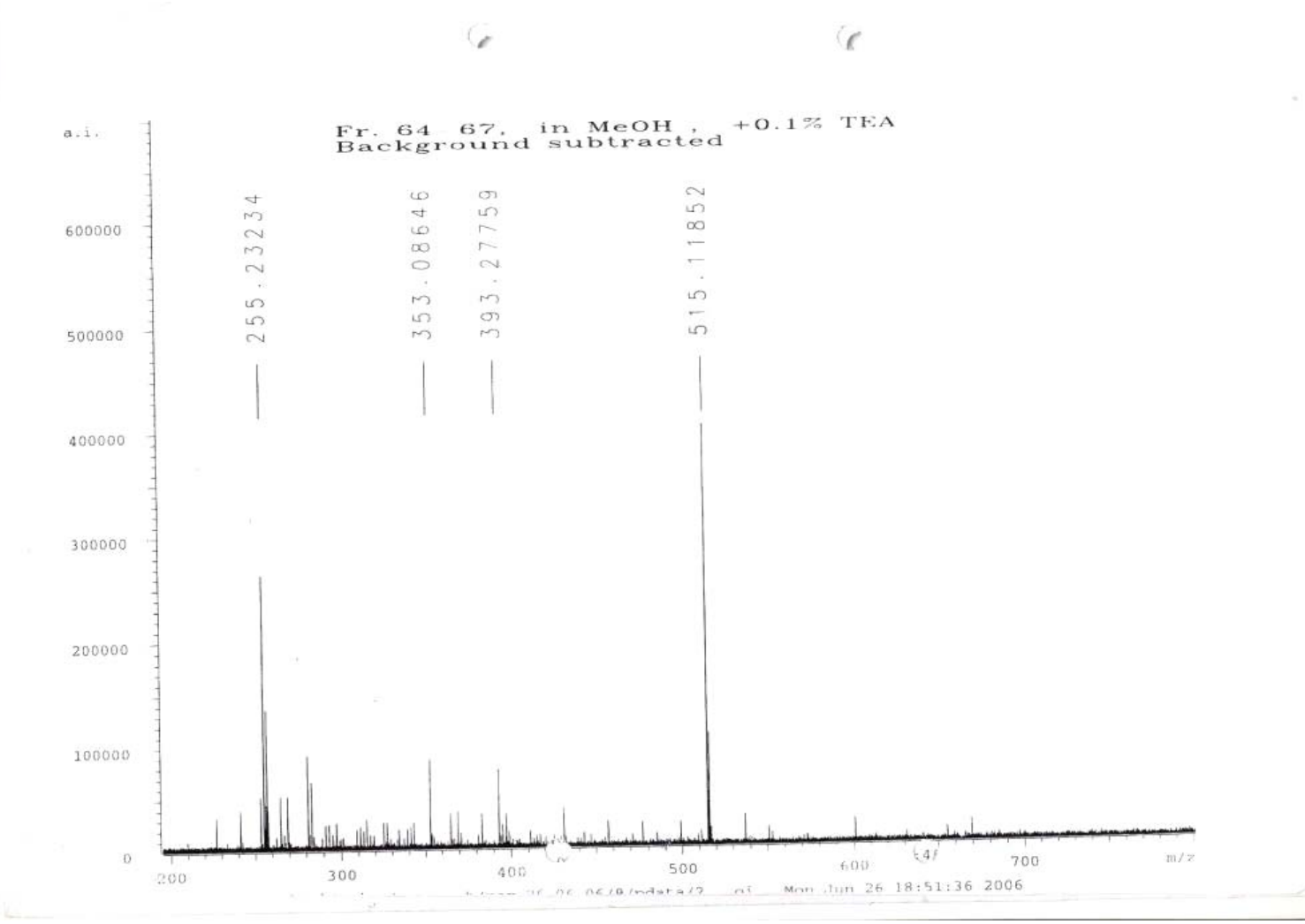
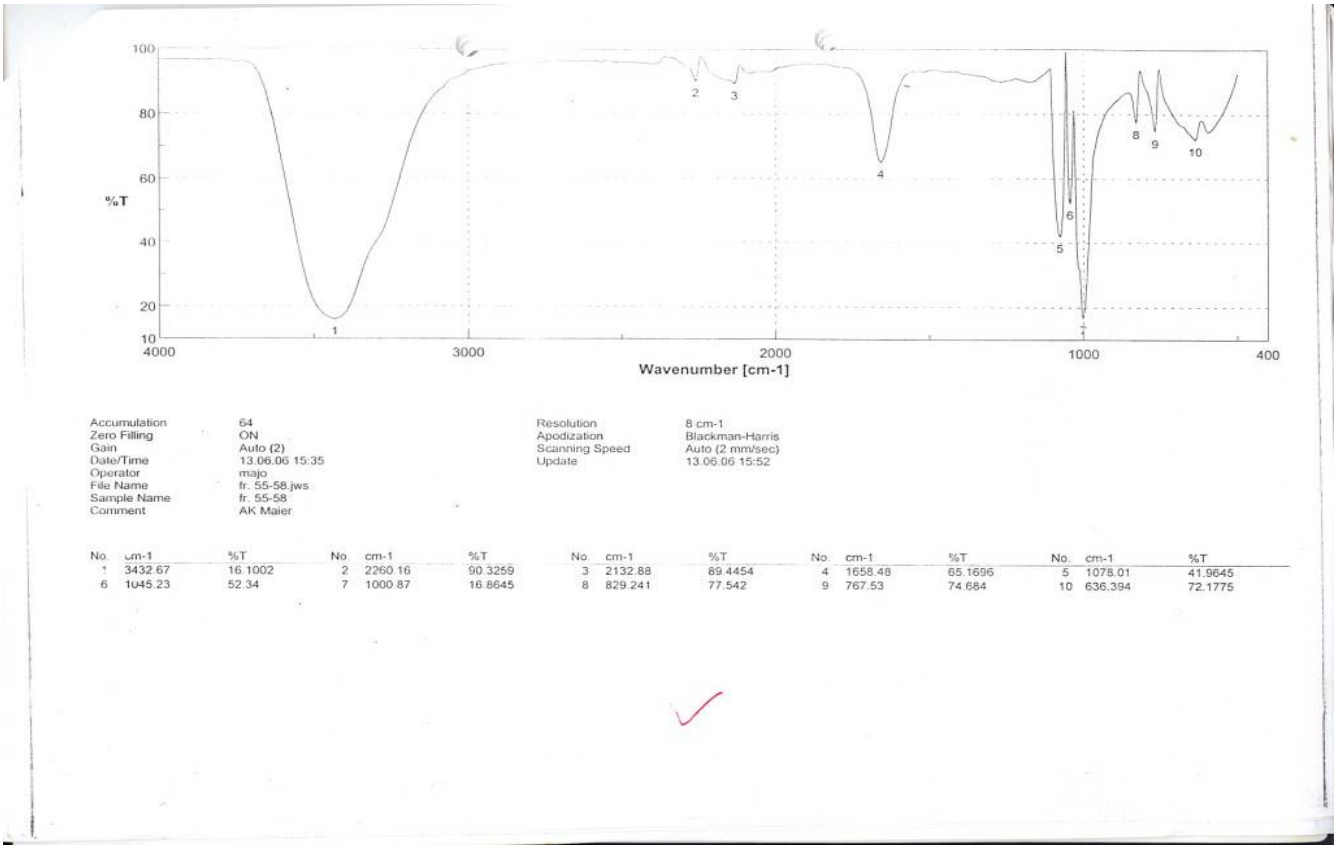
APPENDICES



Appendix 1: Mass spectrum of compound **1**



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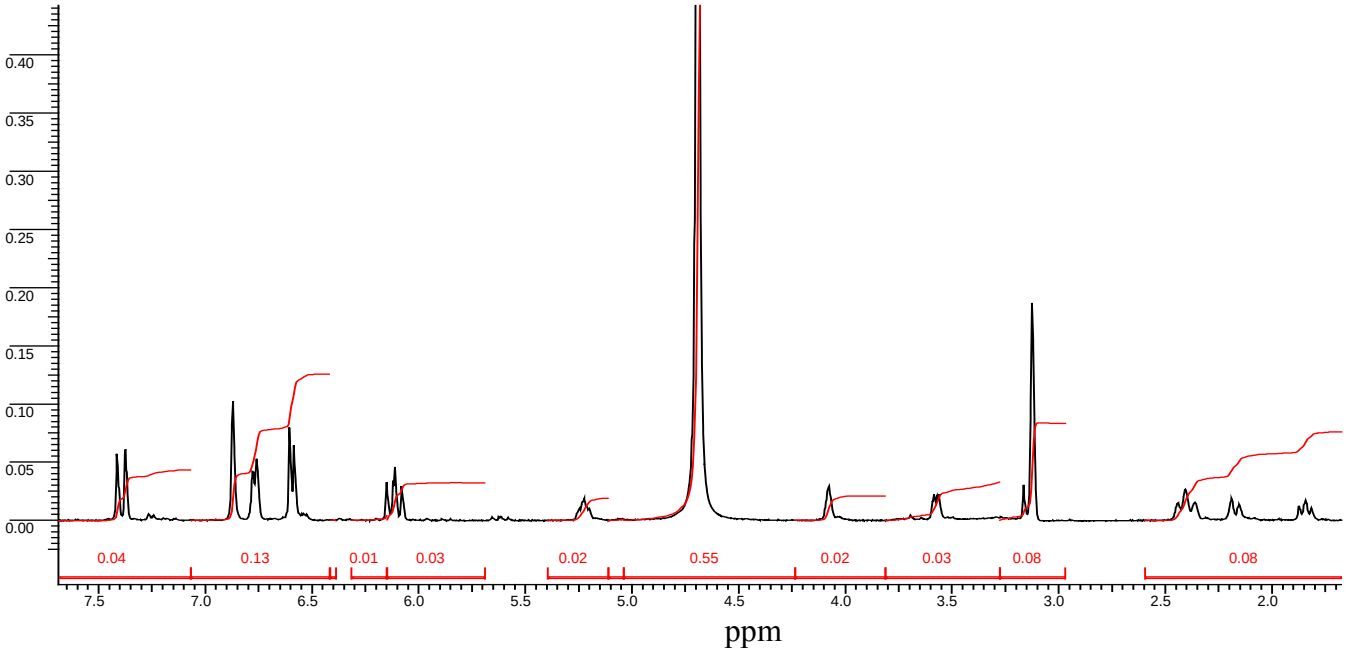
Date/Time	13.06.06 15:35	Update	13.06.06 15:52
Operator	majo		
File Name	fr. 55-58.jws		
Sample Name	fr. 55-58		
Comment	AK Maier		

No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T
1	3432.67	16.1002	2	2260.16	90.3259	3	2132.88	89.4454	4	1658.48	65.1696	5	1078.01	41.9645
6	1045.23	52.34	7	1000.87	16.8645	8	829.241	77.542	9	767.53	74.684	10	636.394	72.1775

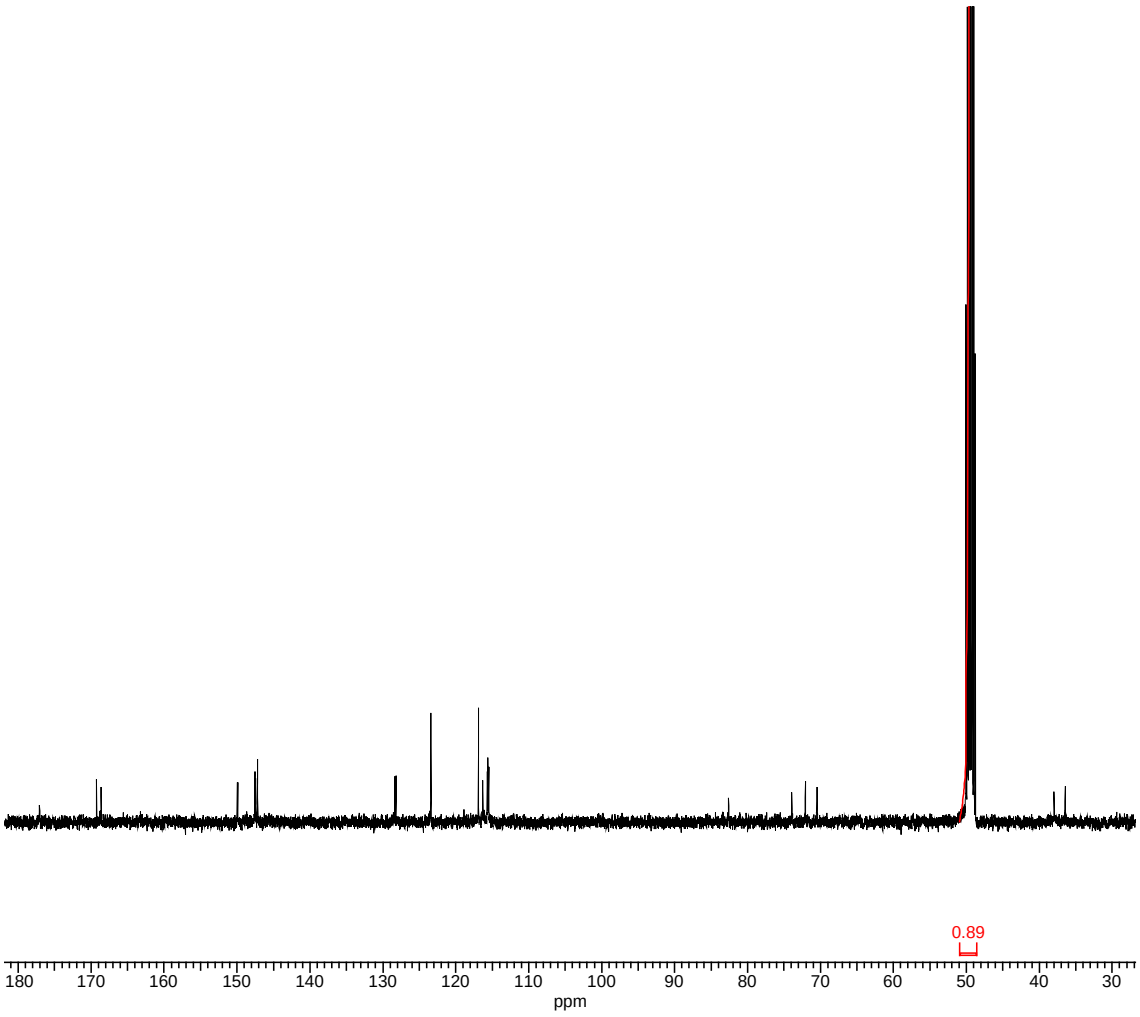
Appendix 2: IR spectrum of compound **1**



Appendix 3: ^{1}H NMR spectrum of **1**

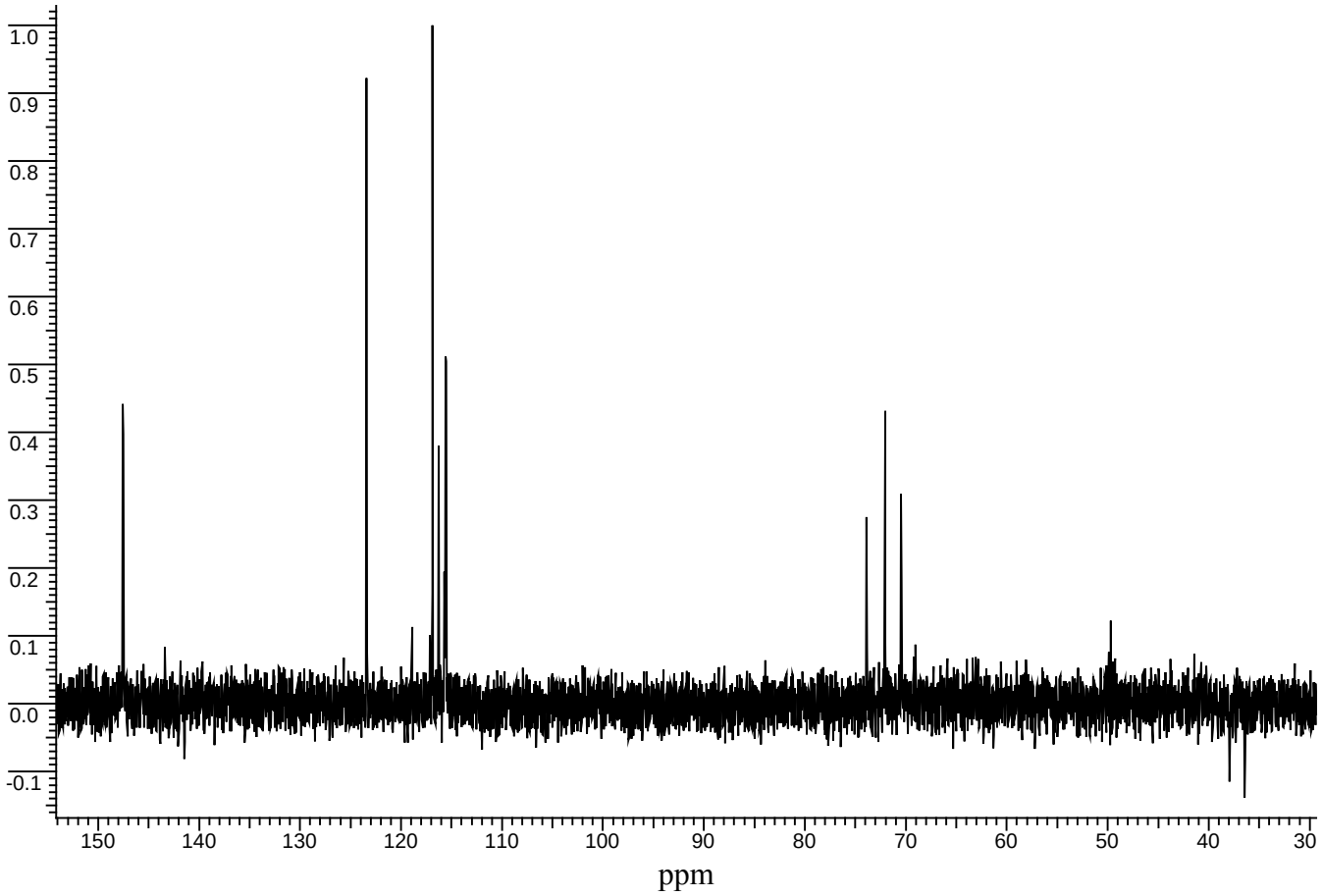
24 Aug 2006

Acquisition Time (sec)	1.3664	***Comment***	fr. 80-95	***Date***	27 Jun 2006 14:43:14
File Name	C:\Documents and Settings\WETUNGU\Desktop\nmr\20060623-28-majo_001001r				
Frequency (MHz)	100.62	***Nucleus***	13C	***Number of Transients***	2048
Original Points Count	32768	***Points Count***	32768	***Pulse Sequence***	***zgpg 30***
Sweep Width (Hz)	23980.81	***Temperature (degree C)***	27.000		

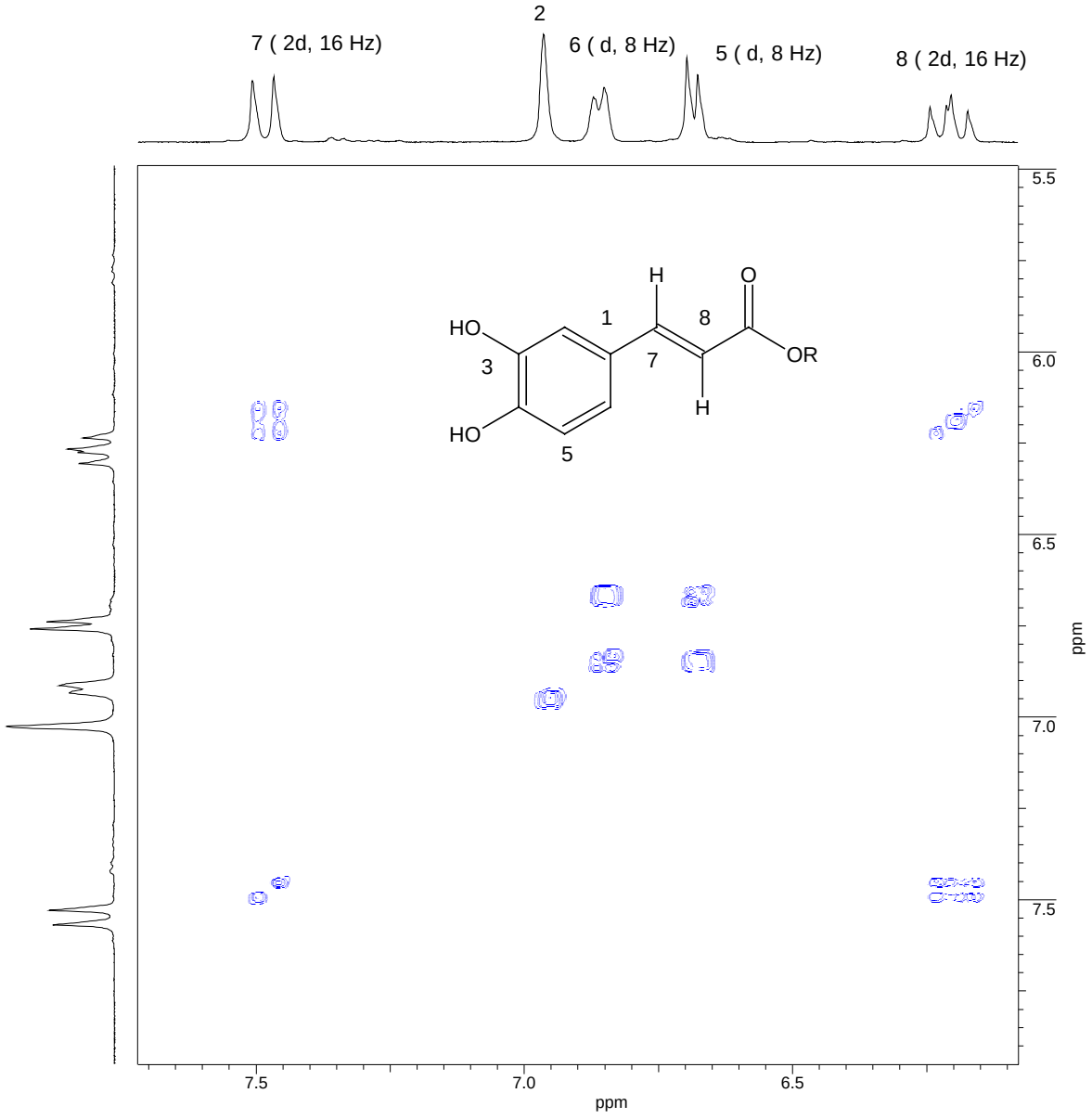


No.	(ppm)	Value	Absolute Value
1	[48.59 .. 50.96]	0.893	6.95355e+9

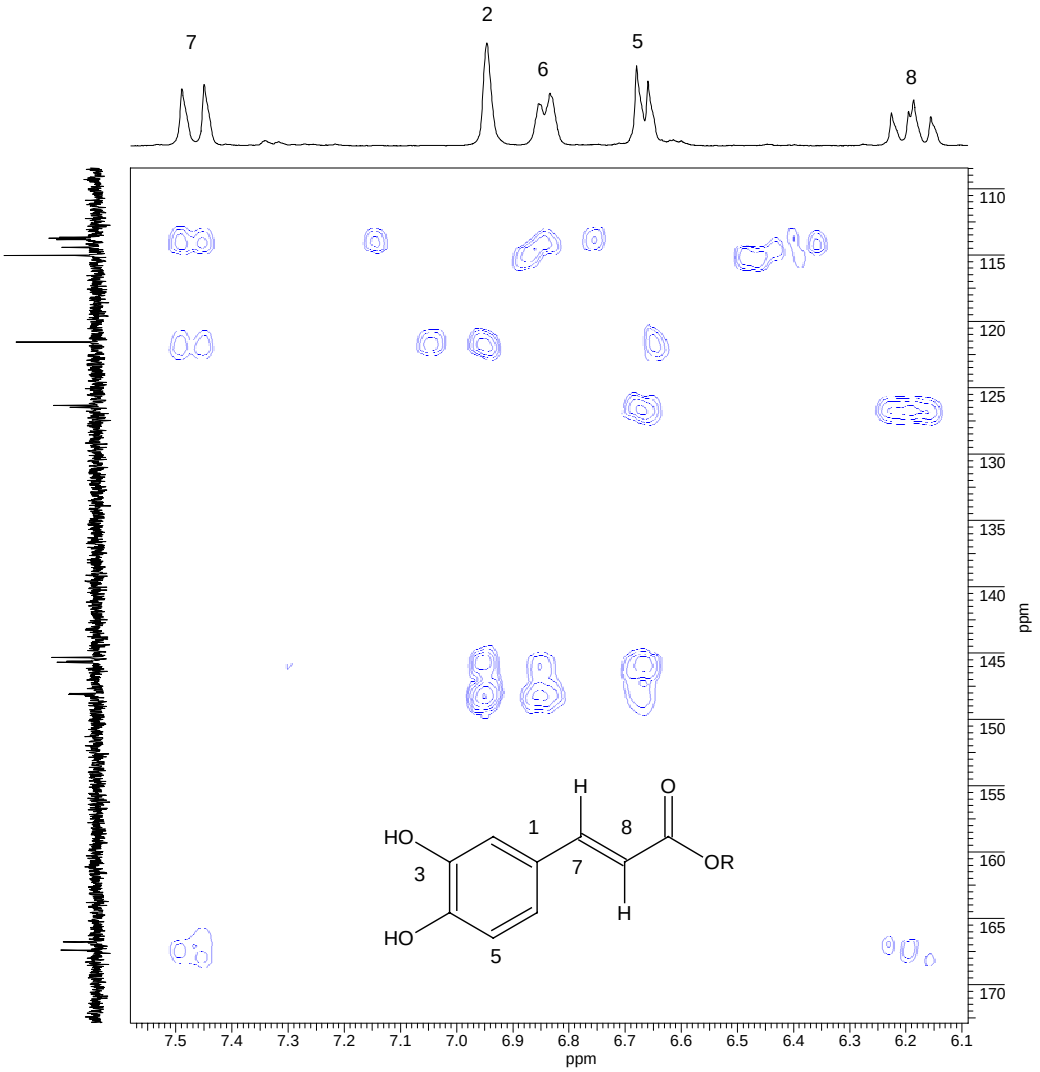
Appendix 4: ^{13}C NMR spectrum of compound **1**



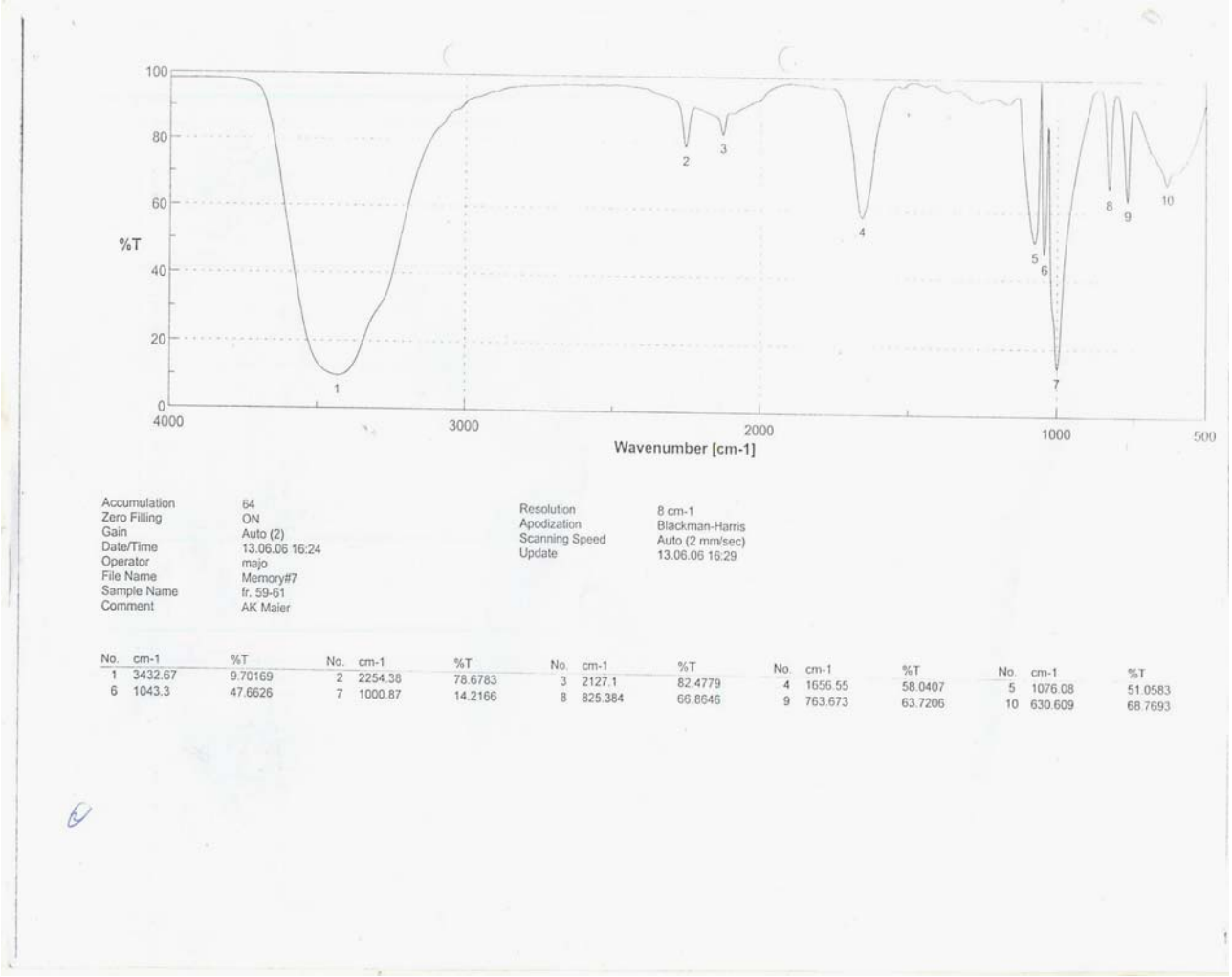
Appendix 5: ^{13}C NMR DEPT of compound **1**



Appendix 6: ^{1}H-^{1}H COSY of hydrolysis product of **1**



Appendix 7: HMBC of the hydrolysis product of **1**



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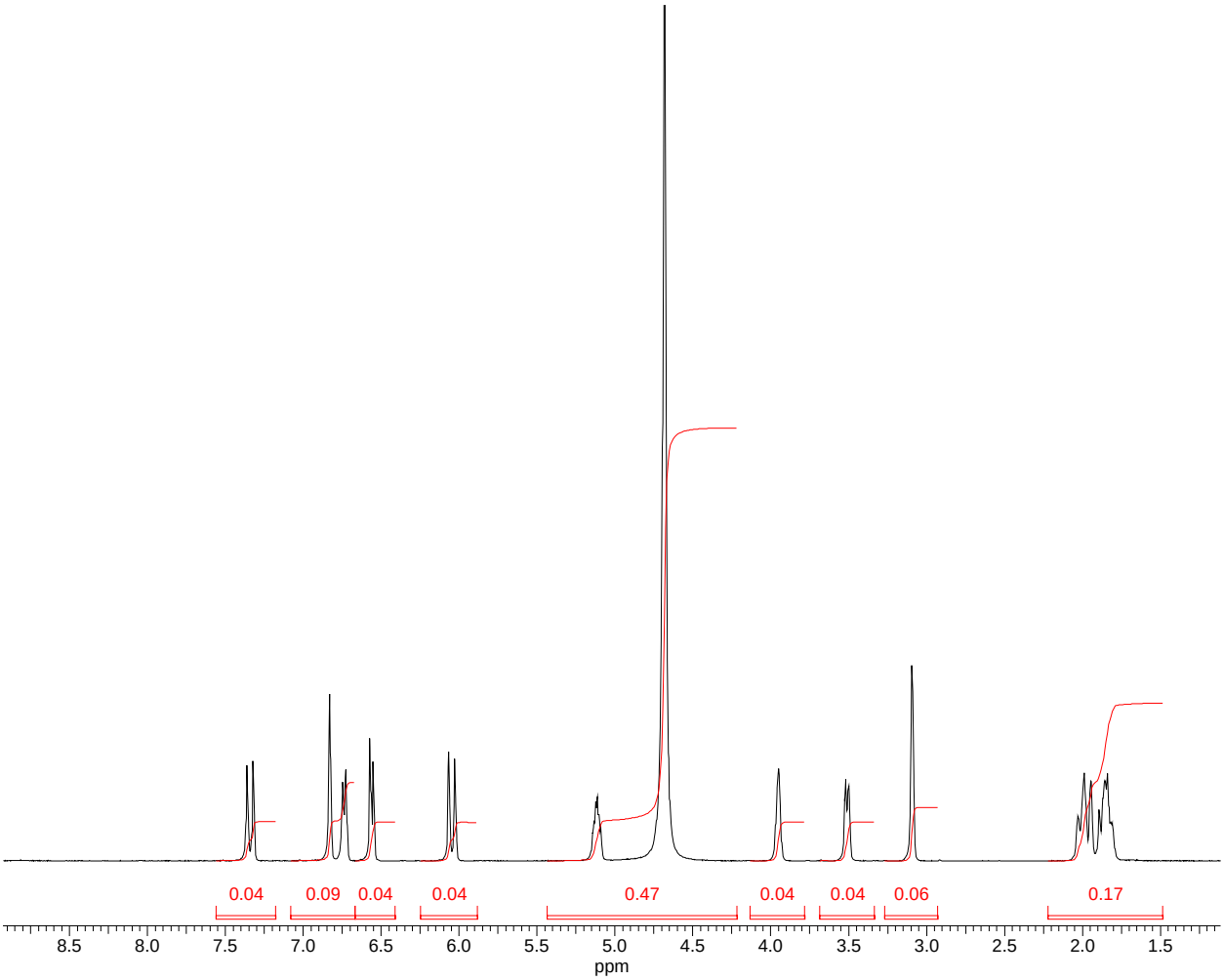
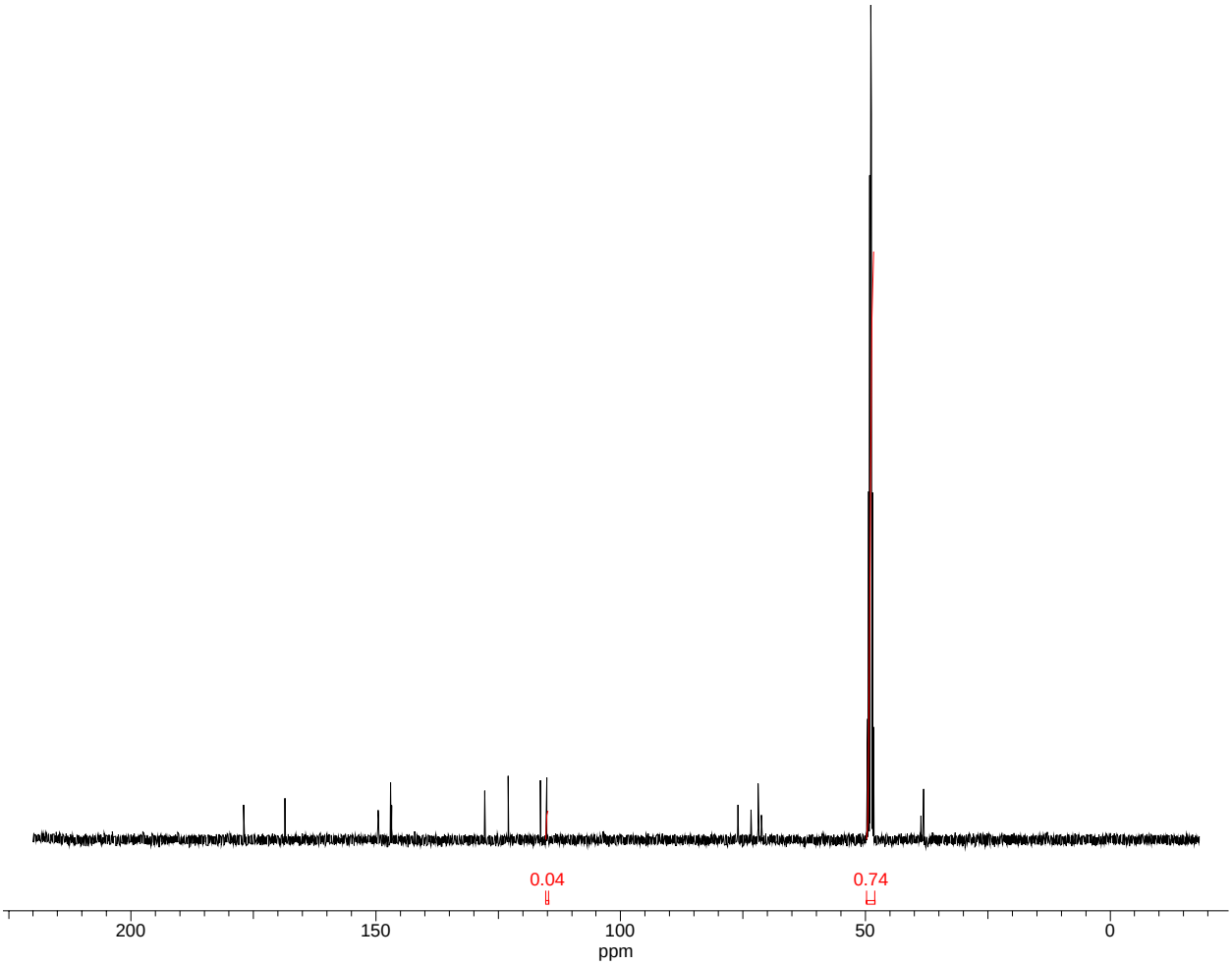
Operator	majo		
File Name	Memory#7		
Sample Name	fr. 59-61		
Comment	AK Maier		

No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T
1	3432.67	9.70169	2	2254.38	78.6783	3	2127.1	82.4779	4	1656.55	58.0407	5	1076.08	51.0583
6	1043.3	47.6626	7	1000.87	14.2166	8	825.384	66.8646	9	763.673	63.7206	10	630.609	68.7693

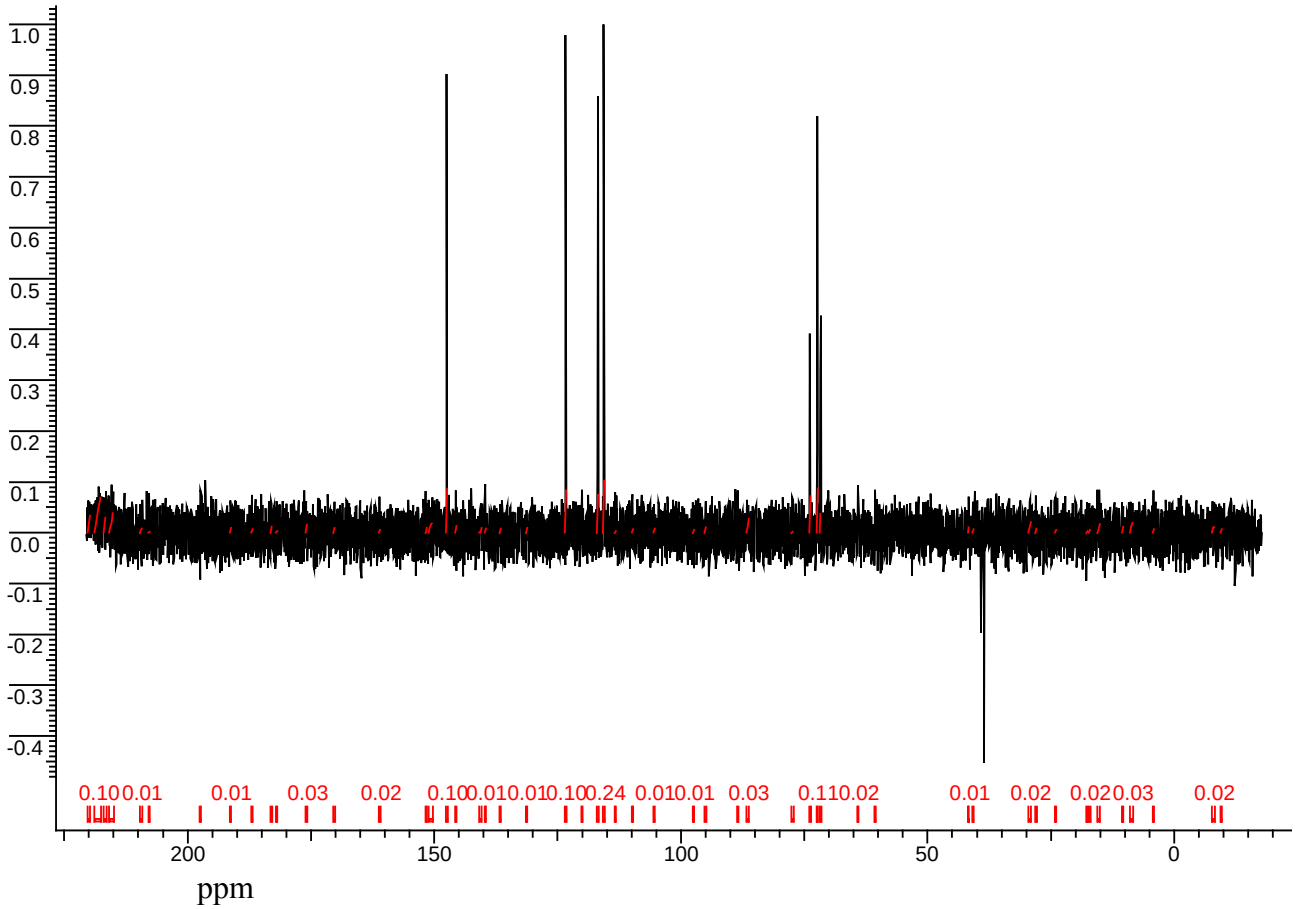
Appendix 8: IR Spectrum of compound **2**



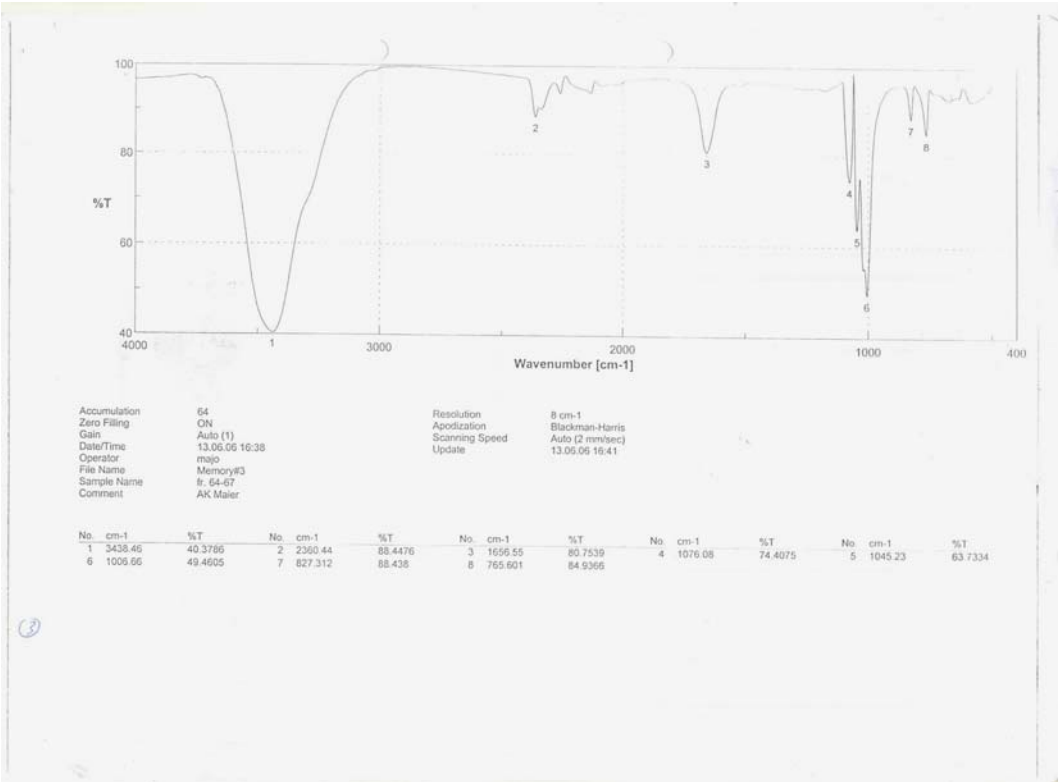
Appendix 9: ^{1}H NMR spectrum of compound **2**



Appendix 10: ^{13}C NMR spectrum of compound **2**



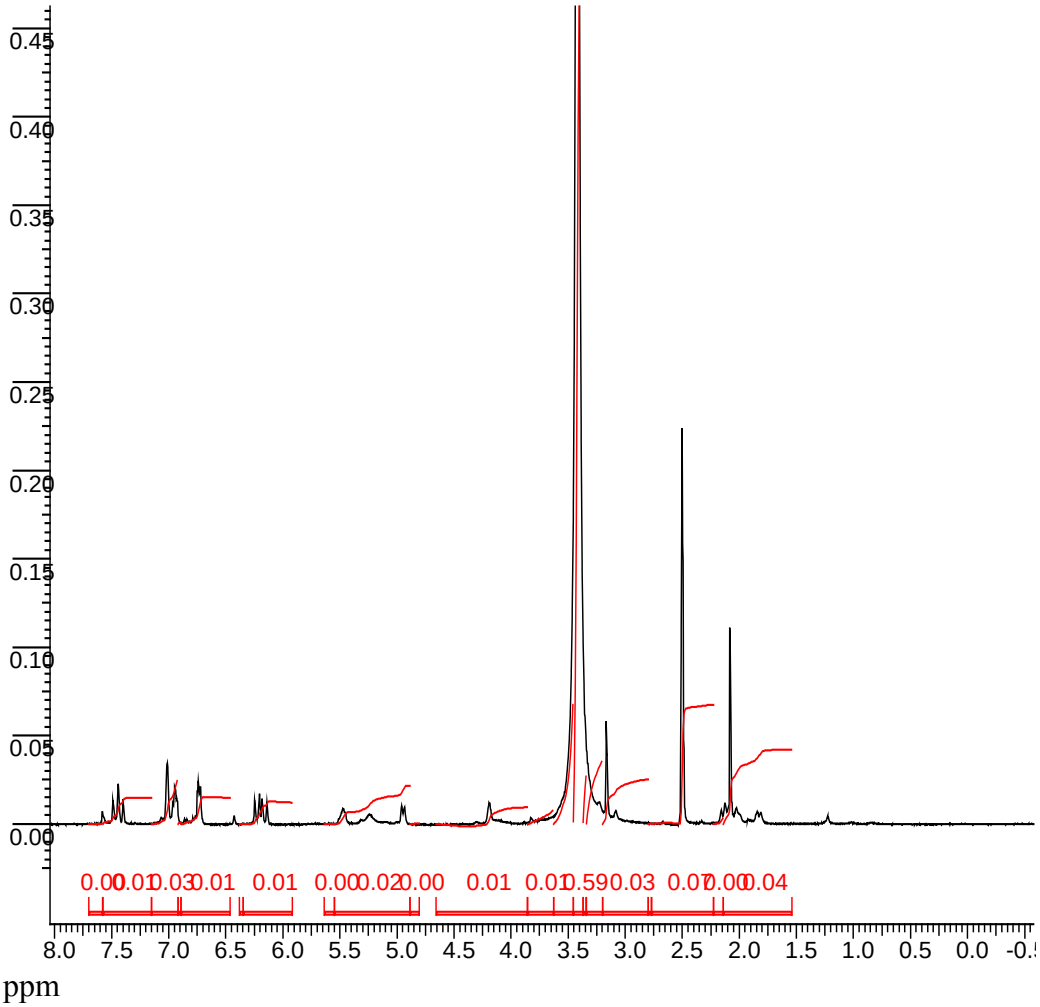
Appendix **11**: DEPT Spectrum of compound **2**



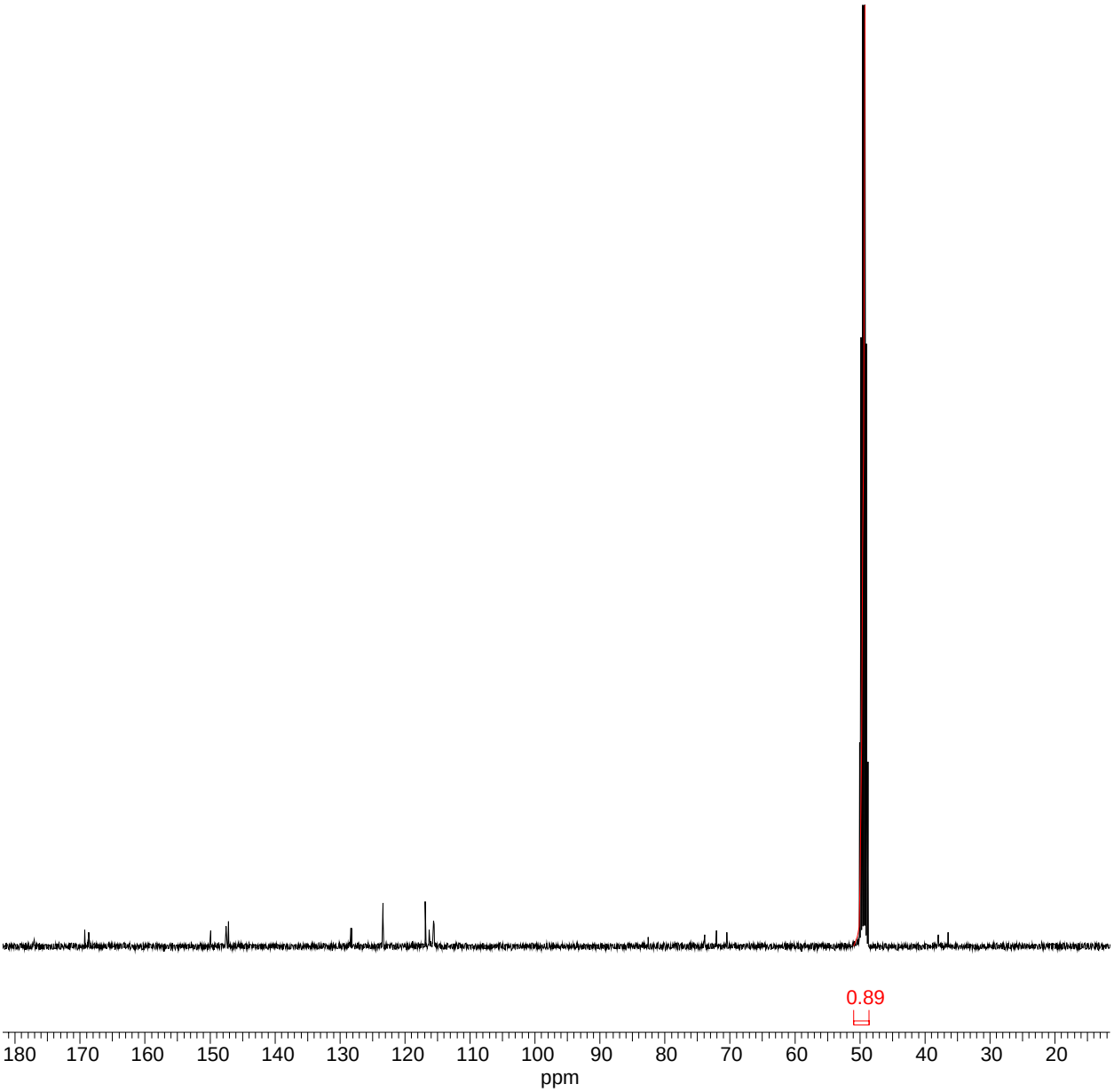
Accumulation	64	Resolution	8 cm-1
Zero Filling	ON	Apodization	Blackman-Harris
Gain	Auto (1)	Scanning Speed	Auto (2 mm/sec)
Date/Time	13.06.06 16:38	Update	13.06.06 16:41
Operator	majo		
File Name	Memory#3		
Sample Name	fr. 64-67		
Comment	AK Maier		

No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T
1	3438.46	40.3786	2	2360.44	88.4476	3	1656.55	80.7539	4	1076.08	74.4075	5	1045.23	63.7334
6	1006.66	49.4605	7	827.312	88.438	8	765.601	84.9366						

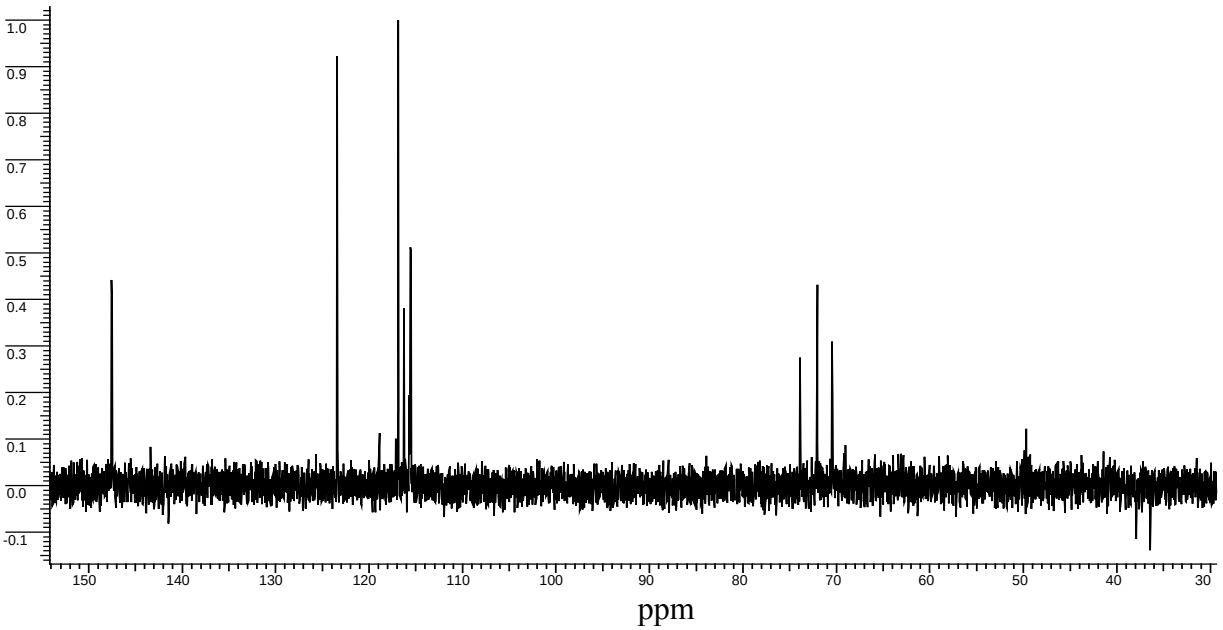
Appendix 12: IR Spectrum of compound **3**



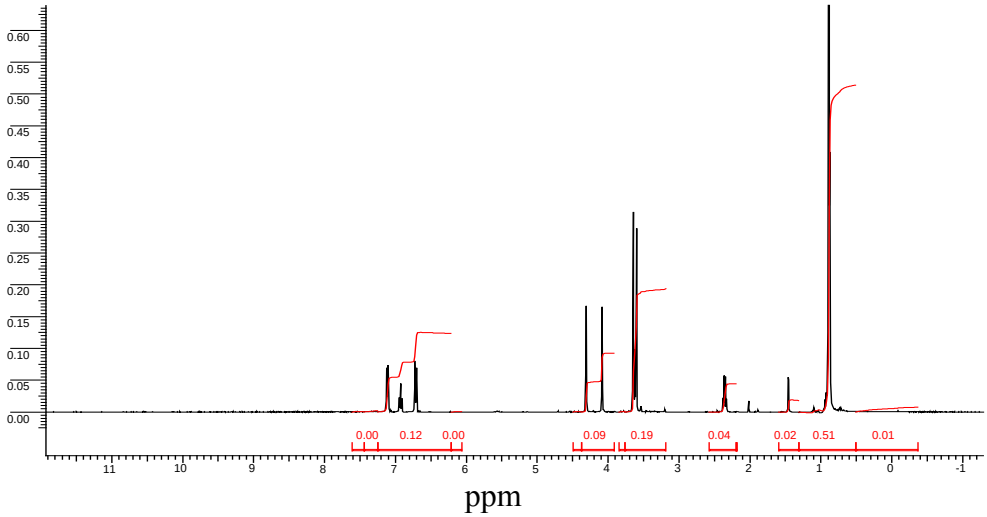
Appendix 13: ^{1}H NMR spectrum of **3**



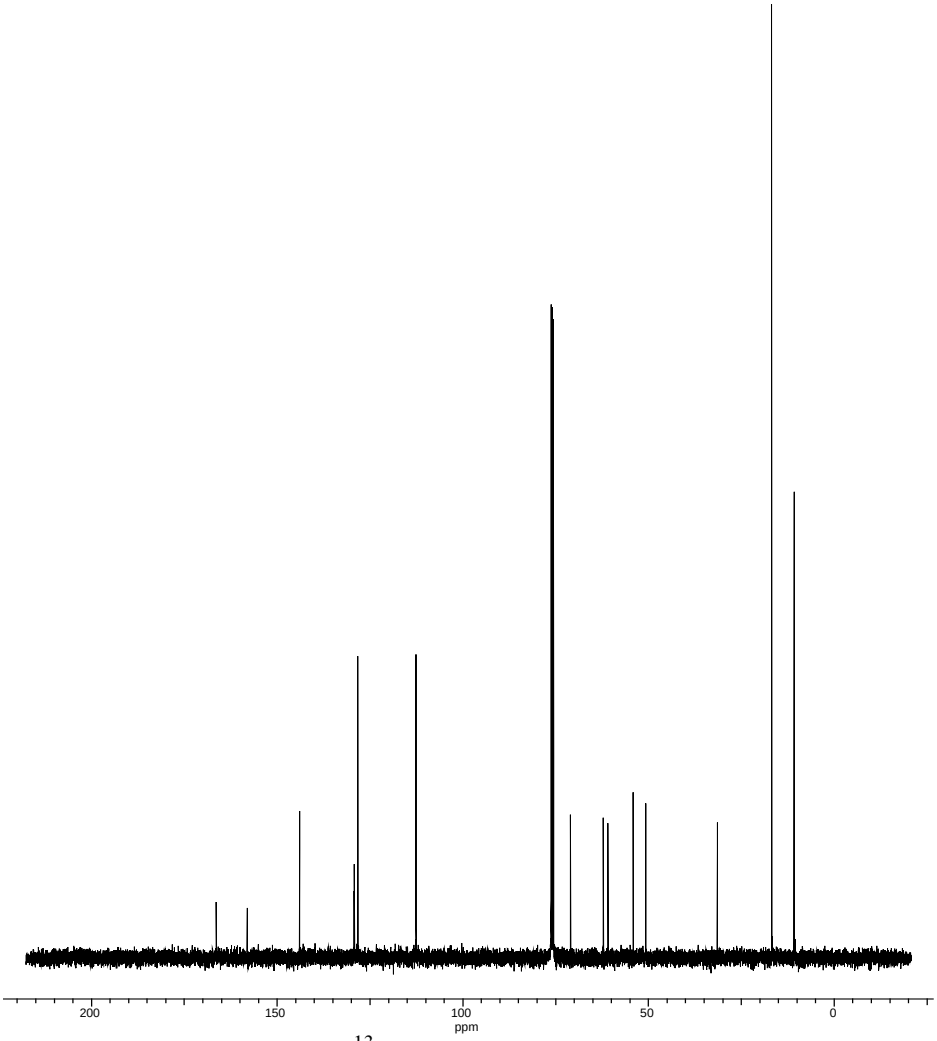
Appendix 14: ^{13}C NMR spectrum of **3**



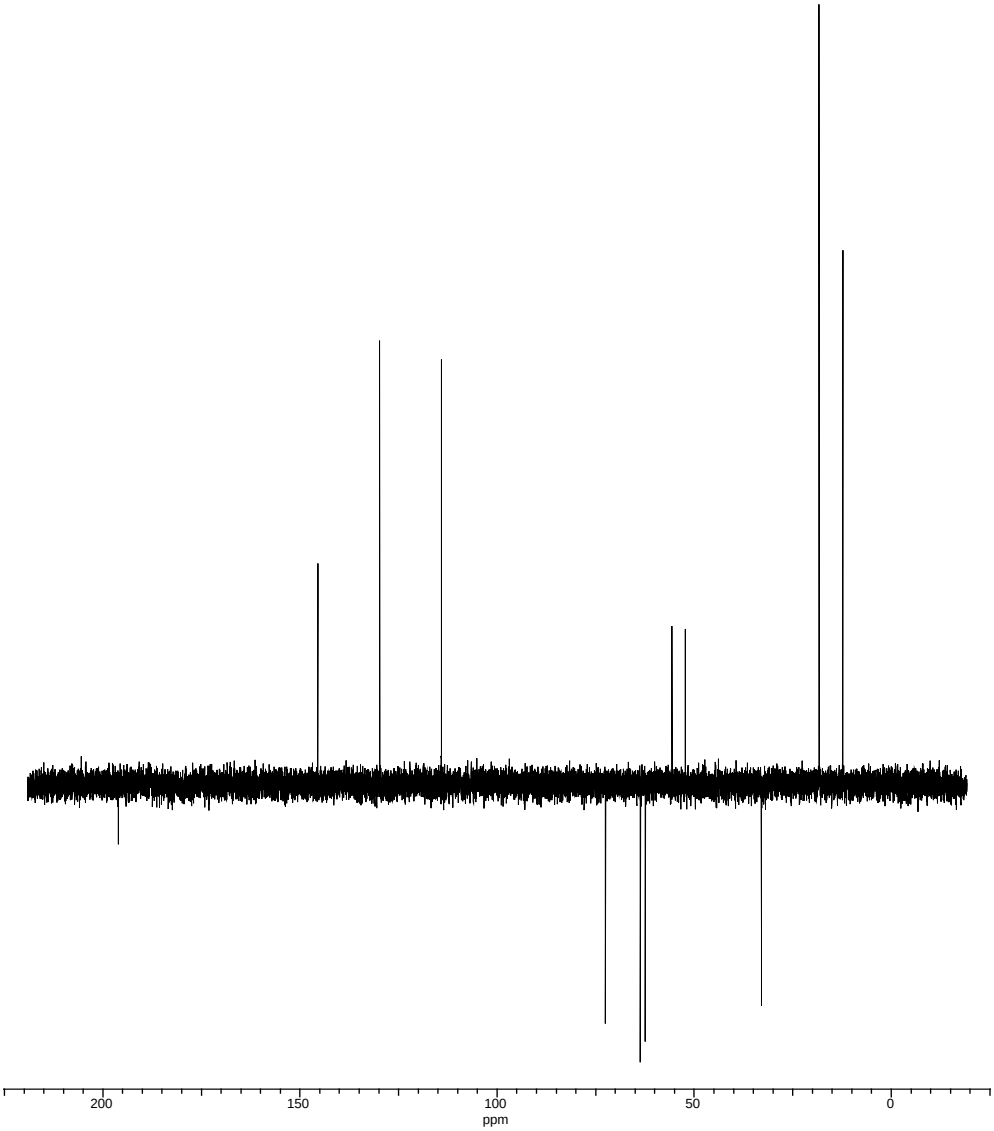
Appendix 15: ^{13}C NMR DEPT of compound **3**



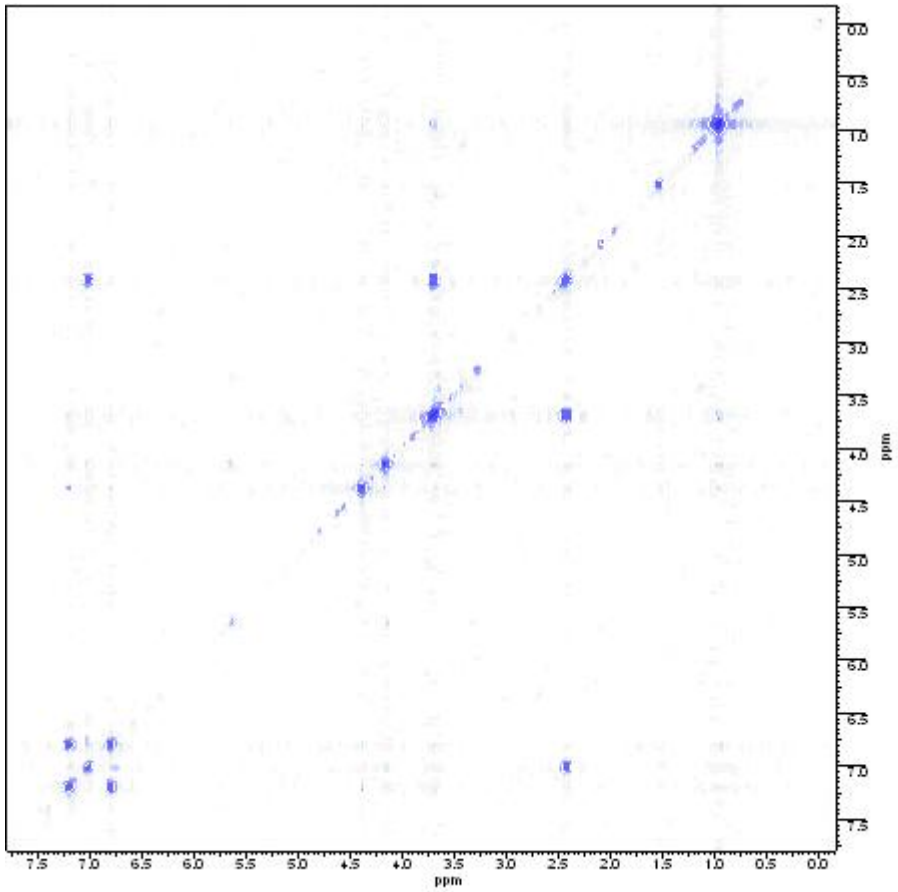
Appendix 16: ^{1}H NMR Spectrum of compound **4**



Appendix 17: ^{13}C NMR Spectrum of compound **4**



Appendix 18: DEPT Spectrum of compound **4**



Appendix 19: ^{1}H-^{1}H COSY Spectrum of compound **4**