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ISOLATION AND IDENTIFICATION OF POISONOUS TRITERPENOIDS FROM *ELAEODENDRON CROCEUM*

by

THEMBELA YELANI

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Department of Plant Science
Faculty of Natural and Agricultural Sciences
University of Pretoria
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SUPERVISOR: Prof J.J.M Meyer

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I declare that the thesis, which I hereby submit for the degree 'Magister Scientiae' at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE  DATE 11 / 11 / 2009

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LIST OF ABBREVIATIONS

^{13}C NMR	: Carbon nuclear magnetic resonance
^{1}H NMR	: Proton nuclear magnetic resonance
CO_2	: Carbon dioxide
Dept	: Distortionless enhancement by polarization transfer
DMSO	: Dimethyl sulphoxide
DNA	: Deoxyribonucleic acid
IC_{50}	: Concentration of an inhibitor that is required for 50 % inhibition of its target.
LRMS	: Low resolution mass spectra
MLD	: Minimum lethal dose
MEM	: Minimal essential medium
NMR	: Nuclear magnetic resonance
SD	: Standard deviation
STZ	: Streptozotocin
TB	: Tuberculosis
TBCA	: Tubulin cofactor A
TBCB	: Tubulin cofactor B
TLC	: Thin-layer chromatography
Topo	: Topoisomerase enzyme
ICAD	: Inhibitor of caspase-activated DNase
CAD	: Caspase-activated DNase

FADD : Fas-associated polypeptide with death domain

Apaf-1 : Apoptotic protease activating factor-1

TNF : Tumor necrosis factor-a

HCN : Hydrogen cyanide

NNRT : Non-nucleoside reverse transcriptase inhibitor

SUMMARY

Isolation and Identification of Poisonous Triterpenoids from *Elaeodendron croceum*

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Thembela Yelani

Supervisor: Prof J.J.M. Meyer

Department of Plant Science

Magister Scientiae

Various plant species have been reported traditionally as well as in scientific literature for cytotoxicity against animal species. Isolation of several poisonous compounds from plant species has been reported previously. *Elaeodendron croceum* is a well-known poisonous plant species of which the poisonous compounds have not yet been isolated.

A phytochemical investigation of *E. croceum* leaves guided by cytotoxicity against Vero cells, led to the isolation of five known compounds; 20-hydroxy-20-epi-tingenone (**1**), tingenone (**2**), tingenine B (**3**), 11α-hydroxy-β-amyrin (**4**), and naringenin (**5**). Compounds **1** and **2** showed the highest toxicity against Vero cells (IC_{50}: 2.651 nM and 8.233 μM respectively). Cytotoxicity of the isolated compounds against three human cancer cell lines, HeLa, MCF-7, and SNO was also determined. Compounds **1** and **2** again showed the highest cytotoxicity with IC_{50}

values ranging between 2.478 – 0.427 µM. This is the first report on the isolation, identification, and *in vitro* evaluation of poisonous compounds from *E. croceum*.

Keywords: *Elaeodendron croceum,* cytotoxicity, triterpenes

CHAPTER 1: INTRODUCTION

1.1 Phytotoxicity

1.1.1 Introduction

Plants produce various groups of secondary compounds for protection against environmental stress, competition among members of their community, attraction of pollinators and defence against herbivores (Cotton, 1996). Saponins, alkaloids, volatile oils, waxes, resins and tannins play a big role in defence against herbivory. The same groups of secondary compounds also have poisoning effects on humans. (Van Wyk et al., 2002). The damage is often based on the arrest of various stages of the cell cycle by different compounds (Frohne et al., 2005).

Captopril, a drug used in the treatment of cardiovascular diseases, has been found to inhibit mitosis in a variety of cell types including canine renal epithelial cells, human neuroblastoma cells, human lung fibroblasts, hamster pancreatic carcinoma cells, and a cell line derived from a human salivary gland carcinoma (Small et al., 1999). Streptozotocin (STZ), is an anticancer drug used to treat cancer of the pancreas, which has been shown to inhibit the enzyme *N*-acetyl-β-D-glucosaminidase (O-GlcNAcase), which removes *O*-GlcNAc from protein, and is thus the final enzyme in the pathway of O-glycosylation in the β-cell (Konrad et al., 2001). DNA is an important target for the anthracyclines, with the induction of topo-isomerase II-mediated strand breaks as a cytotoxic consequence (Taatjes et al., 1999). Damage done on DNA and tubulin arrests the cell cycle, the end result

becomes apoptosis, which results in the prevention of the duplication of a mutant cell.

1.1.2 How does poisoning occur?

Children, especially younger ones, in learning about their environment are inquisitive about plants and like to try pieces of them. Conspicuous and attractive parts, such as fruits of various kinds, and also seeds with colourful seed coats (e.g. yew), are the ones preferred and are subject to a child's curiosity. However, other parts of the plants are also not exempted from being tried. Often, not even a horrible taste frightens off a child. Consequently, for example, approximately 140 g of leaf material were found in the stomach of a child who died after ingestion of leaves of hemlock (Apiaceae), (Frohne et al., 2005).

Frohne et al., (2005) reported data of O'Leary according to which, of 1 051 cases of ingestion in the duration of a single year, in Vienna, 454 concerned berries, 60 involved nuts, and 81 were related to seeds and beans, i.e. pods of various kinds. Some 288 cases of mushroom ingestion were included in the total; the remaining 168 cased involved ingestion of flowers, leaves, stems and roots. Fortunately, most of these efforts do not have any serious consequences. It is pertinent here to point out the distinction between cases of ingestion (the swallowing of plant material without the occurrence of symptoms of poisoning, a feeling of being unwell and vomiting once may be experienced) and cases of poisoning (the occurrence of symptoms of poisoning, which may be of a mild or a serious nature and which definitely require medical attention). It must be remembered that the toxic dose of a highly active substance in a child is smaller than in an adult, so that,

in principle, poisoning is more likely to occur in children than in adults (Frohne et al., 2005).

Poisoning by plants in adults is relatively rare. It can come about by eating unknown or incorrectly identified plant material, the collector had probably confused an edible plant with the poisonous plant. Cases of poisoning can also occur as a result of confusion in searches for alternative sources of food, by self-medication with plants or by the misuse of herbal remedies. Problems can also occur from the increasing use of popularly enjoyed drugs of traditional Chinese medicine, e.g. by confusion, unprofessional preparation, and too high contamination with harmful substances. Additionally, in the younger generation, trying so-called 'drugs that expand the consciousness' is also a cause of poisoning. Collectively, it can be said that serious cases of intoxication by plants are very much the exception. In Switzerland, over a period of 27 years there were 111 cases of heavy or fatal poisoning by plants, including 22 children, this is a small number, but it shows that at least with some plants, serious damage to health can be caused (Frohne et al., 2005).

1.1.3 Apoptosis

1.1.3.1 Introduction

Apoptosis is a process by which cells in a multicellular organism commit "suicide", a form of death that the cell itself initiates, and regulates. For this reason, the term apoptosis is often used interchangeably with the term programmed cell death. Major reasons are that, apoptosis may be used by a developing organism to control growth of its tissues and organs. For instance, a human fetus has webbed hands and feet early on its development. Later, apoptosis removes skin cells,

revealing individual fingers and toes (Guerrero et al., 2003). Cells may also commit "suicide" in times of distress. For example, in the case of a viral infection, certain cells of the immune system, called T lymphocytes, bind to infected cells and trigger them to undergo apoptosis. Apoptosis also occurs in cells that are triggered by external signaling molecules, such as death activator proteins, or through molecules that reside within the cell and monitor events that might commit the cell to "suicide", such as damage to DNA. It also occurs when a cell is damaged beyond repair, infected with a virus, or undergoing stress conditions such as starvation. DNA damage from ionizing radiation or toxic chemicals can also induce apoptosis via the actions of the tumour-suppressing gene. The decision for apoptosis to commence can come from the cell itself, from the surrounding tissue, or from a cell that is part of the immune system. In each case apoptosis functions to remove the damaged cell, to prevent it from depleting more nutrients from the organism, or to prevent the spread of viral infection (Nagata, 2000).

There are several biochemical pathways that lead to apoptosis. One of the major pathways involves inducing mitochondria to leak one of their proteins, cytochrome c, into the cystosol. This in turn activates a set of related proteases (enzymes that degrade proteins) called caspases. Ultimately, the caspases degrade proteins in the cell and activate enzymes that degrade other cell constituents. Cells undergoing apoptosis exhibit morphological and biochemical traits, which can be recognized by microscopic examination or biochemical assays. Apoptosis can occur in twenty minutes, after which the cell corpse typically becomes engulfed and completely degraded by neighboring phagocytic cells that are present in the tissue and attracted to the apoptotic cell (Scott and David, 2003).

1.1.3.2 Apoptosis mechanism

Studies in the nematode *Caenorhabditis elegans*, the fly *Drosophila melanogaster*, and mammalian cells have demonstrated that the biochemical mechanisms leading to apoptosis are highly conserved among metazoans. These mechanisms have been extensively reviewed (Adams and Cory, 1998; Metzstein et al., 1998; Hengartner, 2000; Zimmermann et al., 2001; Martin, 2002) and are briefly summarized here.

In all of these organisms, the key effector proteins of apoptosis are a family of cysteine proteases termed caspases. (Earnshaw et al., 1999; Nicholson, 1999). Over 200 caspase substrates, some of which are required for the cell to maintain viability, have been identified in mammalian cells. For example, when an inhibitor of caspase-activated DNase (ICAD) is cleaved by caspases, it releases the constitutively expressed endonuclease caspase-activated DNase (CAD), which then cleaves the nuclear DNA (Liu et al., 1997; Enari et al., 1998).

Although DNA degradation is incompatible with long-term cell survival, and has provided a convenient marker allowing detection of apoptotic mammalian cells, it is important to emphasize that proteolysis (the hydrolytic breakdown of proteins into simpler, soluble substances such as peptides and amino acids), rather than DNA degradation, is the key regulatory event in apoptosis. In addition to nuclease activation, proteolysis results in diminished DNA repair, disruption of cell-cycle progression, inhibition of protein synthesis, cleavage of major structural proteins in the cytoplasm and nucleus, and disruption of signal transduction required for cellular homeostasis (Earnshaw et al., 1999). The caspases responsible for this proteolysis are synthesized as inactive precursors (zymogens) that must be

activated either by cleavage or conformational change before they can cleave their substrates (Earnshaw et al., 1999; Nicholson, 1999).

Caspase 3, a so-called downstream or effector caspase, which is responsible for most of the cleavages that occur during apoptosis (Slee et al., 2001; Kottke et al., 2002), is activated when it is cleaved by upstream or initiator caspases such as caspases 8, 9, or 10. In addition to cleaving vital protein substrates within the cell, effector caspases can cleave and activate initiator caspases in an amplification loop (Slee et al., 1999).

Mechanisms of caspase activation are remarkably conserved from *C. elegans, Drosophila,* and to man (Fig. 1.1). In each case, the initiator caspases are activated by adaptor proteins. For example, in mammalian cells, the adaptor, Fas-associated polypeptide with death domain (FADD), can bind to and activate caspases 8 and 10, and the adaptor apoptotic protease activating factor-1 (Apaf-1) can bind to and activate caspase 9.

These procaspase–adaptor protein interactions involve specialized protein interaction domains called death effector domains and caspase recruitment domains in the prodomains of the initiator caspases (Earnshaw et al., 1999; Nicholson, 1999; Hengartner, 2000). In the case of caspase 9, it is now clear that the activating event is an Apaf-1-induced dimerization that drives a conformational change at the active site of the zymogen (Renatus et al., 2001). Signal-induced activation of other initiator caspases presumably involves similar changes (Boatright, 2003). Many different signal transduction pathways can lead to activation of the initiator caspases. For convenience, these are often grouped together (Budihardjo et al., 1999; Hengartner, 2000). Ligation of certain tumor necrosis factor-a (TNF) receptor family members, for example, TNFR1, Fas, or

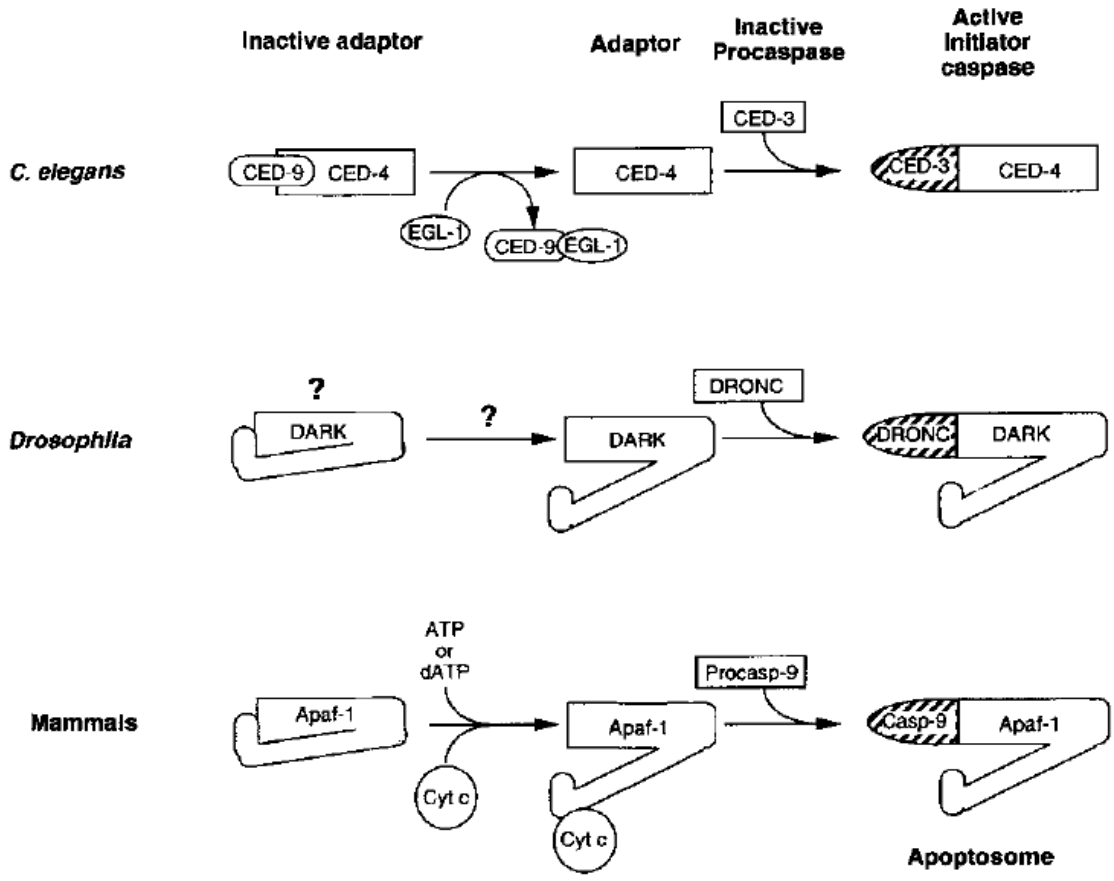

Figure: 1.1 Comparison of initiator caspase activation across species. Conformational changes are indicated by changes in shape, and increased activity is indicated by a cross-hatched fill pattern. Adaptor / active caspase complexes have been shown to be multimers referred to as apoptosomes in *Drosophila* and mammals. Question marks indicate current uncertainty as to whether DARK exists in an inactive conformation that requires activation by binding to cytochrome c or another mitochondrial component (Martin, 2002)

death receptors 4 and 5, often leads to apoptosis involving FADD and caspases 8 and 10 as a consequence of signaling through what is termed the extrinsic pathway or the death receptor pathway (Ashkenazi, 2002).

Apoptosis induced in this way is rarely inhibitable by over-expression of anti-apoptotic Bcl-2 family members (Strasser et al., 1995; Newton and Strasser, 2000). These Bcl-2 family members can be divided into three groups. The first

group, which includes Bcl-2, Bcl-xL and others, inhibits cell death. They contain four conserved regions termed BH (Bcl-2 homology) domains that appear to be involved in various homotypic and heterotypic protein–protein interactions.

The second group includes pro-apoptotic proteins such as Bax and Bak, which also contain multiple BH domains. Family members in the third group, which includes Bim, Bik, Bid, Bad, Hrk, Noxa, Puma, Bmf, and others, are also pro-apoptotic, but they only contain a BH3 domain and are often referred to as BH3-only polypeptides (Adams and Cory, 1998; Huang and Strasser, 2000). On the other hand, apoptosis induced by radiation, p53, glucocorticoids, cytokine deprivation, and most chemotherapeutic drugs usually leads to apoptosis that involves Apaf-1-mediated caspase 9 activation (Kaufmann and Earnshaw, 2000). In mammalian cells, signals upstream of caspase 9 activation involve mitochondria and can be inhibited by Bcl-2, leading to designation of these signals as the Bcl-2-inhibitable pathway, the intrinsic pathway, or the mitochondrial pathway (Zimmermann et al., 2001). Both caspase activation and caspase activity are tightly controlled. In addition, active caspases 3, 7, and 9 can be inhibited by XIAP, a member of the IAP family of proteins (Salvesen and Duckett, 2002).

1.2 Toxicity of secondary compounds

1.2.1 Alkaloids

Alkaloids comprise the largest class of secondary plant substances, at present numbering more that 7 000 (Daniel, 2006). The term ‘alkaloid’ means alkali-like substance. A typical alkaloid is a basic plant product possessing a nitrogen-containing (Carey, 2006) heterocyclic ring system and exhibiting marked pharmacological activity. Alkaloids form a very heterogeneous group. To define

them as just basic, organic nitrogenous plant metabolites seems most indiscriminate, but alkaloids exhibit no basic unity in either their chemical structures, or their biosyntheses.

The alkaline nature of alkaloids is due to nitrogen. Alkaloids may contain a single N atom, as in atropine (Fig. 1.2 k), or five as in ergotamine (Fig. 1.2 l), and more. The N may occur as a primary amine, (RNH_2), as in mescaline (Fig. 1.2 m), a secondary amine (R_2NH) as in cytosine, as a tertiary amine (R_3N), as in physostigmine (Fig. 1.2 n), or as a quaternary ammonium ion ($R_4N^+X^-$), as in tubocurarine (Fig. 1.2 o) chloride. Except in quaternary ammonium compounds, the N atom possesses a pair of unshared electrons, which makes the alkaloids alkaline. The degree of basicity varies from slight to moderate to strong, depending on the position of the N in the skeleton and that of other functional groups. In the cell sap, alkaloids exist as cations of the salts (Shirota et al., 1994) of various organic acids. Apart from C, H and N, most alkaloids also contain O, and are therefore, crystalline solids. Some alkaloids, like coniine, (Fig. 1.2 e) and nicotine (Fig. 1.2 p), lack O, and exist as liquids at room temperature. Almost all are colourless, exceptions being berberine and serpentine, which are yellow and sanguinarine, which is brownish red. Alkaloids are usually insoluble in water, or only sparingly so, but are freely soluble in diethyl ether ($CH_3CH_2OCH_2CH_3$) and other relatively non-polar solvents. This property is made use of in the extraction, purification and quantification of alkaloids. Alkaloidal salts are crystalline often with characteristic crystal forms and habit. Alkaloids can be precipitated out of their aqueous or acid solutions by a number of substances like picric acid and tannic acid (Daniel, 2006).

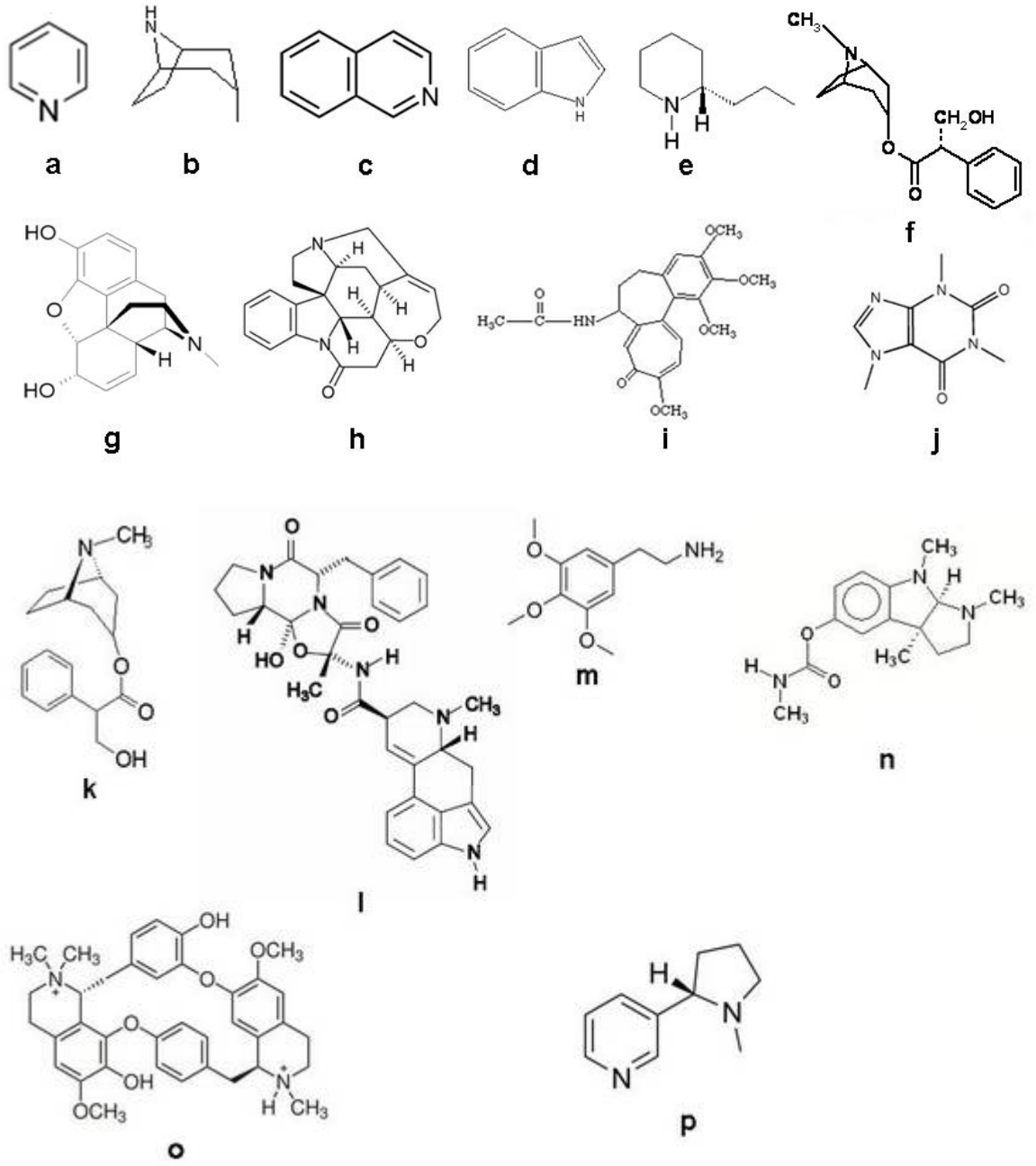

Figure 1.2: (a) Pyridine ring, (b) tropane ring, (c) isoquinoline, (d) indole, (e) coniine (f) hyoscyamine (g) morphine (h) strychnine (I) colchicine (j) caffeine (k) atropine, (l) ergotamine, (m) mescaline, (n) physostigmine, (o) tubocurarine, (p) nicotine (Hour et al., 1999; Van Wyk et al., 2002)

Most alkaloids are active optically and are usually rotatory. Normally, only one of the isomers occurs naturally in a plant, and only occasionally do racemic mixtures occur. In most cases the isomers differ in their physiological activities. Almost all alkaloids absorb UV light, and possess characteristic absorption spectra. This

property is made use of in characterizing the group. Most alkaloids occur in plants and a few of them in animals too. Almost all of them can be synthesized chemically (Carey, 2006).

Present studies indicate a rather restricted dispersion of alkaloids amongst plants. Among Cryptogams, they occur in certain fungi like *Claviceps* and *Amanita*, and also some ferns. Only the Taxaceae and Gnetaceae families, amongst the Gymnosperms contain alkaloids. Monocots generally do not produce alkaloids, but the Amryllidaceae, Liliaceae, Stemonaceae, Dioscoreaceae, Arecaceae, Poaceae and Orhidaceae are exceptions. Among dicots, the Fabaceae, Papaveraceae, Ranunculaceae, Asteraceae, Menispermaceae and Loganiaceae include most of the alkaloid-yielding plants. Usually alkaloids with complex structures are characteristic of specific plant families, e.g. colchicine (Fig. 1.2 i) from *Autumn crocus* of the Liliaceae. Alkaloids do not occur singly in plants. The plant usually produces a series of alkaloids, which may differ only slightly in physical and chemical characters. In plants, alkaloids may be systemic, i.e. distributed throughout, or restricted to specific organs like roots (*Aconite, Belladona*), rhizomes and roots (*Ipecac*, *Hydrastis*), stem bark (*Cinchona, Pomegranate*), leaves (*Hyoscyamus*, *Belladon*), fruits (*Piper nigrum*, *Conium*) or seeds (*Strychnos nux-vomixa*, *Areca*) (Daniel, 2006).

Alkaloids are classified into the following classes: **True alkaloids**, have nitrogen containing heterocyclic ring skeleton derived from a biogenetic amine formed by decarboxylation of an amino acid. **Proto-alkaloids** are derived from amino acids or biogenetic amines, their methylated derivatives, but lack heterocyclic nitrogen. The nitrogen may be present in an aliphatic side chain, as in colchicine. **Pseudo-**

alkaloids are basic compounds that contain nitrogen with the carbon skeleton derived from mono-, di- or tri-terpenes, sterols, or acetate derived polymers. Classification of alkaloids is usually based on their chemical structures. The structural complexity ranges from monocyclic (e.g. coniine Fig. 1.2 e) to heptacyclic (kopsine). The most common skeletons found in alkaloids are indole (Fig. 1.2 d), isoquinoline (Fig. 1.2 c), quinoline, pyridine (Fig. 1.2 a), piperidine, tropane (Fig. 1.2 b), purine, pyrrole, pyrolidine and steroid (Daniel, 2006).

The role of alkaloids in plants is often unclear and varies as listed below (Daniel, 2006).

- They serve as nitrogen reserves.
- They are protective, being poisonous to parasites and herbivores.
- Act as growth regulators, e.g. lupine alkaloids inhibit germination, some alkaloids remove the inhibitory effects of tannins, and a few act as growth stimulators.
- Being basic, they help the plant maintain its ionic balance by replacing mineral bases.

1.2.1.1 Medicinal properties of alkaloids

Many alkaloids (Table 1.1) exhibit marked pharmacological activity, and some find important uses in medicine. Examples include morphine and codeine, which are narcotic (drug which causes drowsiness or unconsciousness,) analgesic (pain relieving), and codeine is also used as an antitussive (capable of relieving or suppressing coughing) agent, it is less toxic and less habit-forming than morphine.

Table 1.1: Pharmacological activity of alkaloids (Daniel, 2006).

Alkaloid	**Pharmacological activity**
Emetine	Emetic (causes vomiting)
Strychnine, Brucine	Central nervous stimulants
Cocaine	Local anaesthetics
Physostigmine, Pilocarpine	Myotics (causes constriction of the pupil of the eye)
Hydrastine	Antihaemorrhagic (controls excessive, uncontrolled bleeding)
Pelletierine	Vermifuges (expels intestinal worms)
Yohimbine	Aphrodisiacs
Ephedriene	Anti-hypotensives
Reserpine	Anti-hypotensives
Quinine	Cardiac repressants
Pilocarpine	Diaphoretics (producing or increasing perspiration)
Tubocurarine	Muscle paralyzers
Camptothecine	Antitumour agents

Morphine (Fig. 1.2 g) is the most well-known isoquinoline alkaloid and occurs in the *Papaver somniferum.* Colchicine (Fig. 1.2 i), from the corms and seeds of the *A. crocus*, is used as a gout suppressant. The poisoning mechanism of colchicine is based on the ability to bind to tubulin, the protein that forms microtubules of the spindle during prophase of mitosis (Van Wyk et al., 2002). Once colchicine binds to

tubulin, formation of microtubules is blocked, the spindle fibers cannot be formed and the cell cannot line up and move its chromosomes to the spindle poles. The cell may copy and complete some or all chromosomes a phenomenon called polyploidy, but cannot arrange them out into new cells. Hence in the presence of colchicine the cell never divides meaning that damaged cells cannot be replaced (Snyder, 1998).

Caffeine (Fig. 1.2 j), also known as trimethylxanthine, theine, mateine, and guaranine (Hour et al., 1999), is an alkaloid found naturally in foods such as coffee beans, tea, kola nuts, and in small amounts of cacao beans. Plants use caffeine as a “pesticide”, to paralyze and kill the insects feeding on them. While caffeine in its pure form is the chemical standard for bitterness (Hour et al., 1999), it is added to some soft drinks such as colas, and Mountain Dew apparently for its taste and stimulation. Such drinks typically contain about 25 to 50 mg of caffeine per serving. Some energy drinks such as Red Bull contain considerably more caffeine per serving, ranging from 100 to 400 mg. Caffeine content varies substantially between *Arabica* and *Robusta* species and to a lesser degree between varieties of each species. One dose of caffeine is generally considered to be 100 mg. In theory, a single serving (150 ml) of drip coffee or one-half caffeine tablet would deliver this dose. In the “real world”, coffee varies considerably in caffeine content per serving, ranging from about 75 to 250 mg. Tea is another common source of caffeine in many cultures. Tea contains less caffeine per serving than coffee, usually about half, depending on the strength of the brew, though certain types of tea, such as Lapsang Sou Chong smoked teas, and Oolong contain less caffeine (Hour et al., 1999).

Caffeine's main pharmacological properties are:

- A stimulant action on the central nervous system with psychotropic effects and stimulation of respiration
- A stimulation of the heart rate, and a mild diuretic effect.

Emetine, the key alkaloid of ipecac root, *Cephaelis ipecacuanha*, is used in the treatment of amebic dysentery and other protozoal infections. Epinephrine or adrenaline, produced in most animal species by the adrenal medulla, is used as a bronchodilator and cardiac stimulant and to counter allergic reactions, anaesthesia, and cardiac arrest (Chao et al., 1991).

Coniine (Fig. 1.2 e) is an extremely toxic compound found in *Conium maculatum*, which belongs to the pyridine alkaloids. The compound was used by Greeks to execute criminals (Van Wyk et al., 2002). Examples of tropane alkaloids include hyoscyamine (Fig. 1.2 f) found in *Atropa belladonna*. The compound is known for having an effect on the autonomic nervous system by increasing the heart rate, decreasing intestinal tone, peristalsis, and secretions such as saliva and gastric. It is also used widely in medicine as an antidote (medicine taken to undo the effect of a poison) to cholinesterase inhibitors such as physostigmine and insecticides of the organophosphate type; and also used in drying cough secretions (Vichova and Jahoda, 2003). Strychnine (Fig. 1.2 h) is a very bitter and extremely poisonous alkaloid obtained form *Strychnos nuxvomica.* The symptoms of strychnine poisoning include anxiety, increased sensitivity to noise and light, followed by convulsive attacks (Van Wyk et al., 2002).

1.2.2 Cyanogenic glycosides

Glycosides are compounds which have a sugar molecule attached to the aglycone. The name of the resulting compound depends on the attached sugar, for instance, if the attached sugar is glucose or fructose the compound is named a glucoside or fructoside, respectively.

The cyanogenic glycosides are defined chemically as glycosides of the α-hydroxynitriles, and belong to the secondary metabolites of plants. They are amino acid-derived plant constituents. The biosynthetic precursors of the cyanogenic glycosides are different L-amino acids, which are hydroxylated, then the N-hydroxylamino acids are converted to aldoximes and these are converted into nitriles and hydroxylated to α-hydroxynitriles and then glycosylated to cyanogenic glycosides (Vetter, 2000). All known cyanogenic glycosides are β-linked, mostly with D-glucose. There are at least 2 650 species of plants that produce cyanogenic glycosides and usually also a corresponding hydrolytic enzyme (beta-glycosidase), which are brought together when the cell structure of the plant is disrupted by a predator, with subsequent breakdown to sugar and a cyanohydrin, that rapidly decomposes to hydrogen cyanide (HCN) and an aldehyde or a ketone (Hosel, 1981; Moller and Seigler, 1999). The glycosides, cyanohydrins and hydrogen cyanide are collectively known as cyanogens. This combination of a cyanogenic compound and hydrolytic enzyme is the means by which cyanogenic plants are protected against predators (Moller and Seigler, 1999).

There are approximately 25 cyanogenic glycosides known with the major cyanogenic glycosides found in the edible parts of plants being: amygdalin (almonds), dhurrin (sorghum), linamarin (cassava, lima beans), lotaustralin

(cassava, lima beans), prunasin (stone fruit), and taxiphyllin (bamboo shoots). The toxicity of a cyanogenic plant depends primarily on the potential concentration of hydrogen cyanide that may be released upon consumption. Prunasin (Fig. 1.3), is an example of a compound that releases the lethal gas hydrogen cyanide after enzymatic breakdown of the glycoside. When the plant cells are damaged through chewing by livestock, the glycosides mix with the enzymes and the released cyanide gas induces an effect known as prussic acid poisoning (Van Wyk et al., 2002).

If the cyanogenic plant is inadequately detoxified during processing or preparation of the food, the potential hydrogen cyanide concentration which may be released can still be high. Upon consumption of a cyanogenic plant, β-glycosidase will be released during digestion and remain active until deactivated by the low pH of the stomach. The enzymes will hydrolyse the cyanogenic glycoside and release at least part of the potential hydrogen cyanide content of the plant (Moller and Seigler, 1999).

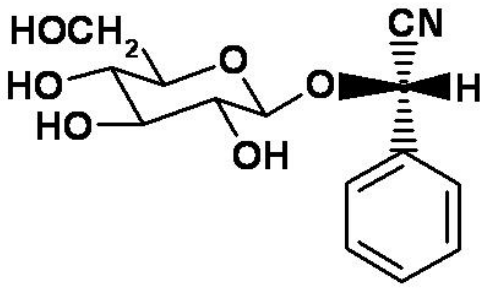

Figure 1.3: Prunasin (Van Wyk et al., 2002)

The actual level of cyanogenic glycosides of a cyanogenic plant is influenced by various factors, both developmental (endogenous) and ecological (exogenous).

The development cycle of cyanogenic plants shows characteristic changes in cyanogenic glycoside and HCN content (Vetter, 2000). In cassava, the major cyanogenic glycoside is linamarin, while a small amount of lotaustralin (methyl linamarin) is also present, as well as an enzyme linamarinase. Catalyzed by linamarinase, linamarin is rapidly hydrolysed to glucose and acetone cyanohydrin and lotaustralin hydrolysed to a related cyanohydrin and glucose. Under neutral conditions, acetone cyanohydrin decomposes to acetone and hydrogen cyanide (Fig. 1.4). The botanical name for cassava is *Manihot esculanta* and it is a member of the Euphorbiaceae (Surge) family. Cassava is also known by the other common names: manioc, manihot, and yucca. Cassava originates in Latin America and was later introduced into Asia and Africa. The plant parts used are the roots and leaves.

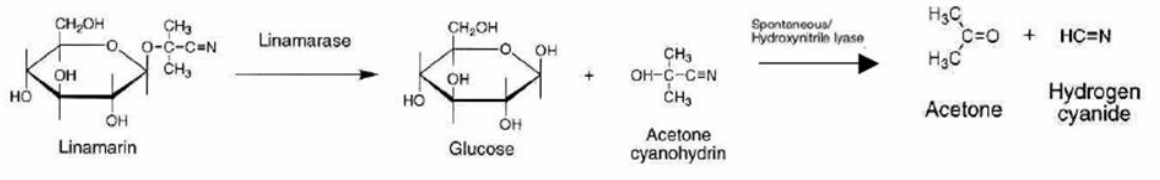

Figure 1.4: Reaction of linamarin with linamarinase in cassava (Brimer and Rosling, 1993).

The starchy tuber is most commonly consumed and very large tubers may reach the size of 0.5 m long and 10 cm in diameter. There are a number of varieties of cassava that range from low cyanide content (referred to as ‘sweet cassava’) to higher cyanide content (referred to as ‘bitter cassava’). Bitter cassava requires more extensive processing (sometimes more than one day) to remove the cyanogenic potential. If the cassava plant is not adequately detoxified during the processing or preparation of the food, it is potentially toxic because of the release of this preformed hydrogen cyanide. The hydrogen cyanide is readily removed

during processing of cassava, however, the presence of residual linamarin and its acetone cyanohydrin in cassava-based food products has the potential to cause adverse health effects. There are many varieties of cassava and the cyanide content differs as well as the suitability for different growing and consumption conditions. Usually higher cyanide content is correlated with higher yields. During periods of drought the cyanide content of both sweet and bitter cassava varieties increases (Bokanga et al., 1994). Bitter cassava varieties are more drought resistant and thus more readily available and cheaper. However, owing to food shortage in times of drought, less time is available for the additional processing required (Akintonwa and Tunwashe, 1992). Values from 15 to 400 mg/kg of hydrocyanic acid in cassava roots on a fresh weight basis have been mentioned in the literature (Carlsson et al., 1999). Sweet varieties of cassava will typically contain approximately 15 to 50 mg/kg of hydrogen cyanide on fresh weight basis. Cassava leaves contain approximately 10% more linamarin than cassava roots.

A part of ingested linamarin in cassava products has been found to pass through the human body unchanged and it is excreted in the urine within 24 hours in both humans (Brimer and Rosling, 1993; Carlsson et al., 1995; Hernandez et al., 1995) and rodents (Barrett et al., 1977). Remaining cyanohydrins are assumed to break down to cyanide in the alkaline environment of the gut (Tylleskar et al., 1992). Carlsson et al. (1999) investigated the metabolic outcome of linamarin in cassava flour when consumed as a stiff porridge, which is one of the 14 most common staple foods in southern Tanzania, and found that less than one-half of orally ingested linamarin is converted to cyanide and hence thiocyanate, about one-quarter is excreted unchanged and another quarter is metabolized into an as yet unknown compound.

Hydrogen cyanide inactivates the enzyme cytochrome oxidase in the mitochondria of cells by binding to the Fe^{3+} / Fe^{2+} contained in the enzyme. This causes a decrease in the utilization of oxygen in the tissues. Cyanide causes an increase in blood glucose and lactic acid levels and a decrease in the ATP/ADP ratio indicating a shift from aerobic to anaerobic metabolism. Cyanide activates glycogenolysis and shunts glucose to the pentose phosphate pathway decreasing the rate of glycolysis and inhibiting the tricarboxylic acid cycle. Cyanide can inhibit several other metalloenzymes most of which contain iron, copper or molybdenum (e.g. alkaline phosphatase) as well as enzymes containing Schiff base intermediates (e.g. 2-keto-4-hydroxyglutarate aldolase). Hydrogen cyanide reduces the energy availability in all cells, but its effect is most immediate on the respiratory system and heart. The lethal dose for an adult depends on body weight and nutritional status and is estimated between 30 and 210 mg of hydrogen cyanide (Tylleskar et al., 1992). If the hydrogen cyanide exceeds the limit an individual is able to detoxify or tolerate, death may occur due to cyanide poisoning.

Hydrogen cyanide is readily absorbed after oral ingestion or administration and rapidly distributed in the body through the blood. It is known to combine with iron in both methaemoglobin and haemoglobin present in erythrocytes. The cyanide level in different human tissues in a fatal case of HCN poisoning has been reported as gastric content, 0.03; blood, 0.5; liver, 0.03; kidney, 0.11; brain, 0.07; and urine, 0.2 (mg/100g) (Lang, 1933). The major defense of the body to counter the toxic effects of cyanide is its conversion to thiocyanate mediated by the enzyme rhodanese (Lang, 1933). The enzyme contains an active disulfide group which reacts with the thiosulphate and cyanide. The enzyme is localized in the mitochondria in different tissues and is relatively abundant, but in sites, which are not readily accessible to

thiosulphate, the limiting factor for the conversion of cyanide is thiosulphate. This detoxification requires sulphur donors, which by different metabolic pathways are provided from dietary sulphur amino acids (Bradbury and Holloway, 1988; Rosling, 1994). If the dietary intake of sulphur amino acids is adequate, the sulphur containing amino acids methionine and cysteine, which are not required for protein synthesis, are degraded to inorganic sulphate and excreted in the urine. There are also several minor reactions that detoxify ingested cyanide. Firstly, cystine may react directly with the cyanide to form 2-imino-thiazolidine-4-carboxylic acid, which is excreted in the saliva and urine. Secondly, a minor amount may be converted into formic acid, which may be excreted in urine. Thirdly, cyanide may combine with hydroxycobalamine (vitamin B_{12}) to form cyanocobalamine, which is excreted in the urine and bile, or may be reabsorbed by the intrinsic factor mechanism in the ileum allowing effective recirculation of vitamin B_{12}. Fourthly, methaemoglobin effectively competes with cytochrome oxidase for cyanide, and its formation from haemoglobin, affected by sodium nitrile or amylnitrite, is exploited in the treatment of cyanide intoxication (Bradbury and Holloway, 1988).

In humans, symptoms of acute cyanide intoxication include, rapid respiration, drop in blood pressure, rapid pulse, dizziness, headache, stomach pains, vomiting, diarrhoea, mental confusion, twitching and convulsions (Rosling, 1987). If the hydrogen cyanide exceeds the limit an individual is able to detoxify or tolerate, death may occur due to cyanide poisoning. The acute oral lethal dose of hydrogen cyanide for human beings is reported to be 0.5 to 3.5 mg/kg body weight. Approximately 50 to 60 mg of free cyanide from cassava and its processed products constitutes a lethal dose for an adult man. Data on the oral lethal dose of cyanide for humans in four cases of suicide, calculated from the amount of

hydrogen cyanide absorbed in the body at the time of death, and from the amount of hydrogen cyanide found in the digestive tract, differed considerably and corresponded to doses of 0.58-22 mg/kg body weight (Rosling, 1987). Studies in African countries such as Zimbabwe have established that goitre and cretinism due to iodine deficiency can be considerably aggravated by a continuous dietary cyanide exposure from insufficiently processed cassava. This effect is caused by thiocyanate, which is similar in size to the iodine molecule and interferes with uptake of iodine into the thyroid gland. High thiocyanate levels, which can occur after exposure to cyanide from cassava, can only affect the gland when the iodine intake is below 100 mg/day, which is regarded minimal for normal function. Populations with very low iodine intake and high thiocyanate levels from consumption of cassava, showed severe endemic goitre, but a decrease was shown with iodine supplementation (Rosling, 1987).

1.2.3 Coumarins

Coumarins are aromatic compounds with a specific ring structure. Many coumarins have been identified from natural sources, especially green plants. There are simple coumarins (Fig. 1.5 a) and furanocoumarins, which have an additional furan ring attached to the first ring of the basic coumarins structure, an example is xanthotoxin (Fig. 1.5 b), which is known to cause severe light induced dermatitis (a severe allergic reaction that may result in painful blistering of the skin) (Van Wyk et al., 2002).

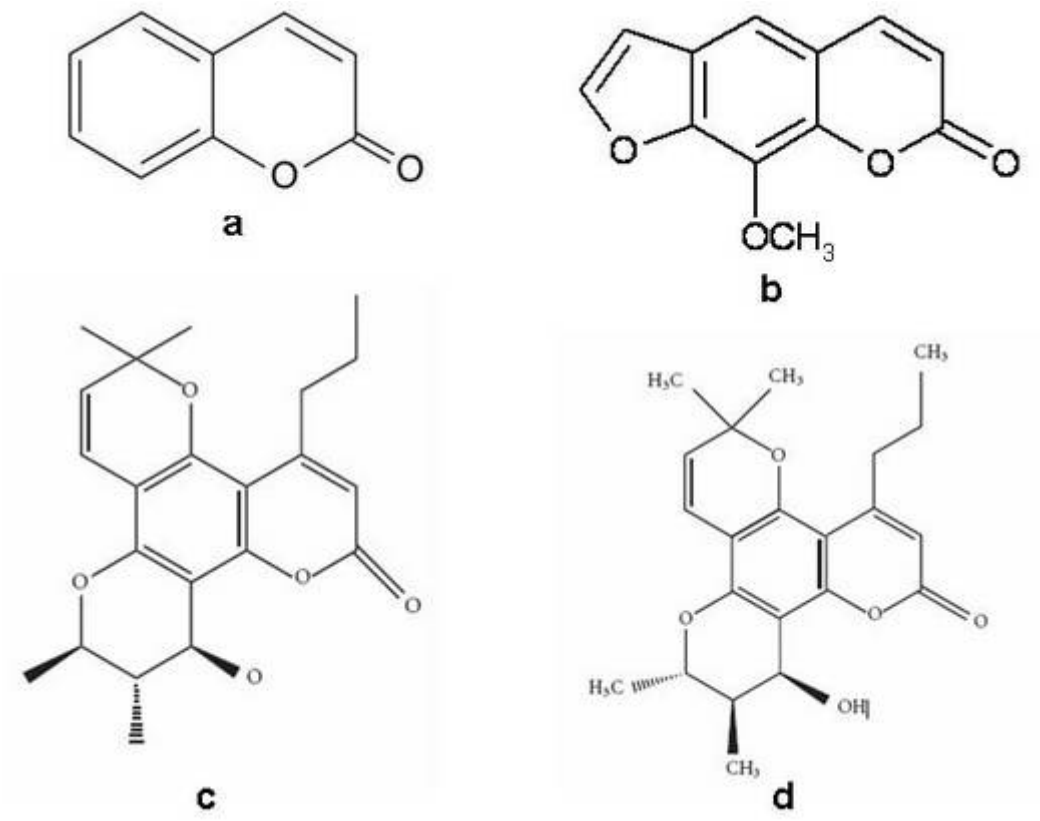

Figure 1.5: (a) Coumarin (b) xanthotoxin (Van Wyk et al., 2002) (c) (+)-calanolide A (d) (−)-calanolide B (Kostova et al., 2006).

The pharmacological and biochemical properties and therapeutic applications of simple coumarins depend upon the pattern of substitution. Coumarins have attracted intense interest in recent years because of their diverse pharmacological properties. Coumarins comprise a group of natural compounds found in a variety of plant sources. The very long association of plant coumarins with various animal species and other organisms throughout evolution may account for the extraordinary range of biochemical and pharmacological activities of these chemicals in mammalian and other biological systems (Kostova et al., 2006). Several biological parameters should be evaluated to increase the understanding of mechanisms by which the coumarins act.

Coumarins have important effects in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors, and precursors of toxic substances. In addition, these compounds are involved in the actions of plant growth hormones and growth regulators, the control of respiration, photosynthesis, as well as defense against infection. The coumarins have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (Kostova et al., 2006). The hydroxycoumarins are typical phenolic compounds and, therefore, act as potent metal chelators and free radical scavengers. They are powerful chain-breaking antioxidants. The coumarins display a remarkable array of biochemical and pharmacological actions, some of which suggest that certain members of this group of compounds may significantly affect the function of various mammalian cellular systems. The coumarins are extremely variable in structure, due to the various types of substitutions in their basic structure, which can influence their biological activity (Kostova et al., 2006).

Calanolides are coumarins classified as pyranocoumarins (Kashman et al., 1992). Several compounds of pyranocoumarins include (+)-calanolide A (Fig. 1.5 c) a novel non-nucleoside reverse transcriptase inhibitor (NNRTI) with potent activity against HIV-1. The compound was first isolated from a tropical tree, *Calophyllum lanigerum* in Malaysia (Kostova et al., 2006). Structural biology studies and enzyme kinetic experiments bear out the unique anti-HIV properties of calanolide A. In particular, calanolide A is active against viral isolates with the Y181C amino acid mutation in the reverse transcriptase of HIV-1. This is a commonly observed mutation identified in both laboratory and clinical viral isolates and is associated with high-level resistance to most other NNRTIs. However, viral isolates that contain multiple AZT-resistant mutations and the Y181C mutation are actually

hypersensitive to the antiviral activity of calanolide A. When tested *in vitro* in combination with a range of nucleoside analogues, protease inhibitors and NNRTIs, calanolide A demonstrated additive to synergistic anti-HIV activity (Kostova et al., 2006). Kashman et al. (1992) reported that (−)-calanolide B (Fig. 1.5 d) inhibited the HIV-1 reverse transcriptase.

1.2.4 Terpenoids

Terpenoids are compounds with basic skeletons derived from mevalonic acid, or a closely related precursor (Daniel, 2006). They are considered to be built up of isopentane or isoprene units linked together in various ways, with different modes of ring closure, unsaturation and different functional groups. The isoprene residues are usually linked in a head-to-tail fashion. However, head-to-head and tail-to-tail linkages also occur. Not all terpenoids comprise carbon atoms in multiples of five. Some compounds have additional or missing carbon atoms, the modifications probably being formed in the course of the long biosynthetic pathways. Sometimes isoprenoid side chains occur attached to non-terpenoid central skeletons (Daniel, 2006). The classification and sources of terpenoids are presented in Table 1.2, and the structures in Fig. 1.6.

Table1. 2: Classification of terpenoids (Daniel, 2006).

Class	**Number of carbon atoms**	**Number of isoprenes**	**Sources**
Hemiterpenoids	5	1	Volatile oils, esters
Monoterpenoids	10	2	Volatile oils, glycosides, mixed terpenoids
Sesquiterpenoids	15	3	Volatile oils, bitter principles
Diterpenoids	20	4	Resins, chlorophyll
Sesterpenoids	25	5	Rare (mostly in animals)
Triterpenoids	30	6	Resins, waxes, steroids, saponins, cardiac glycosides
Tetraterpenoids	40	8	Carotenoids
Polyterpenoids	α	η	Rubber and gutta

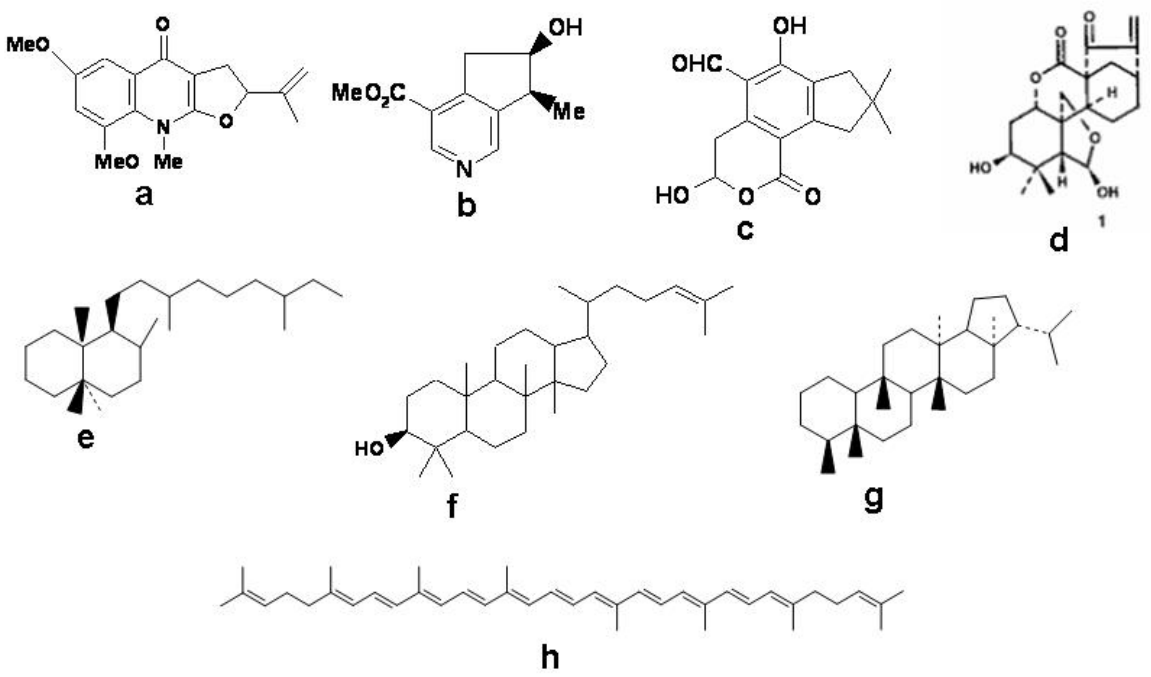

Figure 1.6: Classification of terpenoids; (a) hemiterpenoids (b) monoterpenoids (c) sesquiterpenoids (Manske, 1971) (d) diterpenoids (e) sesterpenoids (f) triterpenoids (g) tetraterpenoids (h) polyterpenoids (Rahman, 1995).

Monoterpenoids are colourless, distillable, water insoluble liquids with a characteristic aroma, and boiling points ranging from 140^0C to 180^0C. These compounds are formed by the head-to-tail, head-to-head or tail-to-tail condensation of two isoprene residues, and exhibit every possible mode of ring closure, various degrees of unsaturation, and substitution of different functional groups (Daniel, 2006). Based on their chemical structures, monoterpenoids are classified into normal, cyclopentanoid, and tropolones. These monoterpenoids include all aliphatic and cyclic steam distillable monoterpenes. These occur usually in their free state in steam distillable oils, and possess a distinct aroma. However, non-steam distillable monoterpene glycosides, e.g. geraniol, neral, citronellol, thymol and carvacrol, have been found to exist naturally. Based on their ring closures, normal monoterpenes are classified as acyclic, monocyclic, and bicyclic.

Functionally, these may be hydrocarbons, aldehydes, alcohols, ketones or oxides (Daniel, 2006).

Sesquiterpenoids occur in distillable volatile oils, and in the bitter principles of many plants. Sesquiterpenoids contribute to the flavour, and are classified into acyclic, monocyclic, and bicyclic. Farnesol is an acyclic sesquiterpenoid widely distributed amongst plants. Its pyrophosphate is a key intermediate in terpenoid biosynthesis. Abscissic acid, a growth regulator, is a sesquiterpenoid carboxylic acid, related structurally to the carotenoid violaxanthin. Sesquiterpenoids are known to exhibit a number of biological and pharmaceutical properties. Examples include guaiazulene and camazulens, which are anti-inflammatory, and bisabolol is antiphlogistic (Daniel, 2006). Many sesquiterpenoids are bitter in taste and are the constituents of many bitter medicines. A large number of sesquiterpenoids, such as gemacranolides, guaianolides, psedoguaianolides, and elemanolids are found to exhibit antitumour activities

Triterpenoids belong to a heterogeneous collection of biochemical substances, which are believed to be derived from the C-30 acyclic compound squalene by ring closures and substitutions (Daniel, 2006). Apart form a few acyclic members, the majority is represented by tetra- or pentacyclic compounds. Alcohols, aldehydes, ketones or carboxylic acids may also be found in triterpenoids. Many are colourless crystalline compounds with high melting points. Optical isomerism is a common phenomenon exhibited by these natural products. Tetracyclic triterpenes are recognized by a cyclopentanoperhydrophenanthrene nucleus and an 8-carbon side chain at C_{17}. Daniel (2006) reported that, the resemblance with the steroids has inaccurately prompted some authors to group them as sterols and name them

accordingly, e.g., lanosterol. Triterpenoids are abundant in laticiferous plants. Cucurbitacins, a group of bitter principles, in which 12 have been identified occurring free or as glycosides, represent a physiologically active set of compounds in this series: Pentacyclic triterpenes are widely distributed in higher plants, occurring free or as glycosides (saponins). The aglycones, found in the excretions and cuticle, have a protective as well as water-proofing function, e.g. β-amyrin. Invariably all the members are oxygenated at the C_3 position. The bitterness exhibited by some of these members are notable, e.g. limonoids and quassinoids. Gymnemic acids, which can destroy the ability of compounds to taste sweet, form another interesting group (Daniel 2006).

Toxicity of many triterpenoids is caused by their ability to link with membrane sterols, which results in pore formation in the membranes and release of the cellular content to the environment (lysis). In erythrocyte membranes, triterpenoids link with cholesterol, causing leakage of haemoglobin (haemolysis of erythrocytes) (Simoes et al., 1999). The minimum concentration of triterpenoids that causes staining by the blood plasma haemoglobin (supernatant liquid resulting from precipitation of blood cell elements), is called the hemolytic index and it constitutes a toxicity indicator (Dyakov et al., 2007). Examples of toxic triterpenoids include lupeol and betulin acid isolated from *Anemone raddeana*. The effect of two triterpenoids on superoxide generation in human neutrophils was investigated using fMLP, PMA and AA as the stimuli. Lupeol and betulin suppress superoxide generation by preventing tyrosyl phosphorylation of a 45.0-kDa protein in human neutrophils, and may have pharmaceutical applications (Yamashita et al., 2002).

1.2.5 Saponins

Saponins are high-molecular-weight glycosides, consisting of a sugar moiety, linked to a triterpene or steroid aglycones. The classical definition of saponins is based on their surface activity. Many saponins have detergent properties, form stable foams in water, show haemolytic attributes, while not common to all saponins, they have frequently been used to characterize this class of natural products. However, because of the numerous exceptions which exist, saponins are more conveniently defined on the basis of their molecular structure, namely as triterpene or steroid glycosides. Some saponin-containing plants have been employed for hundreds of years as soaps and this fact is reflected in their common names: soapwort (*Saponaria offcianalis*), soaproot (*Chlorogalum pemridianum*), soapbark (*Quillaja saponaria*), soapberry (*Sapindus saponaria*), and soapnut (*Sapindus mukurossi*). The name 'saponin' comes from the Latin word *sapo*, which means soap (Hostettmann and Marston, 1995).

The aglycone or non-saccharide portion of the saponin molecule is called the genin or sapogenin. Depending on the type of genin present, the saponins can be divided into three major classes: triterpene glycosides, steroid glycosides, and steroid alkaloid glycosides. The genins of these three classes can be depicted as shown in Fig. 1.7. The aglycones are normally hydoxylated at C-3 and certain methyl groups are frequently oxidized to hyroxymethyl, aldehyde or carboxyl functionalities. When an acid moiety is esterified to the aglycone, the term ester saponin is often used for the respective glycoside. All saponins have in common the attachment of one or more sugar chains to the aglycone. Monodesmosidic saponins (Fig. 1.7 d) have a single sugar chain, normally attached at C-3. Bidesmosidic saponins (Fig. 1.7 e) have two sugar chains, often with one attached

through an ether linkage at C-3 and one attached trough an ester linkage (acyl glycoside) at C-28 (triterpene saponins). Tridesmosidic saponins have three sugar chains and are seldom found (Hostettmann and Marston, 1995).

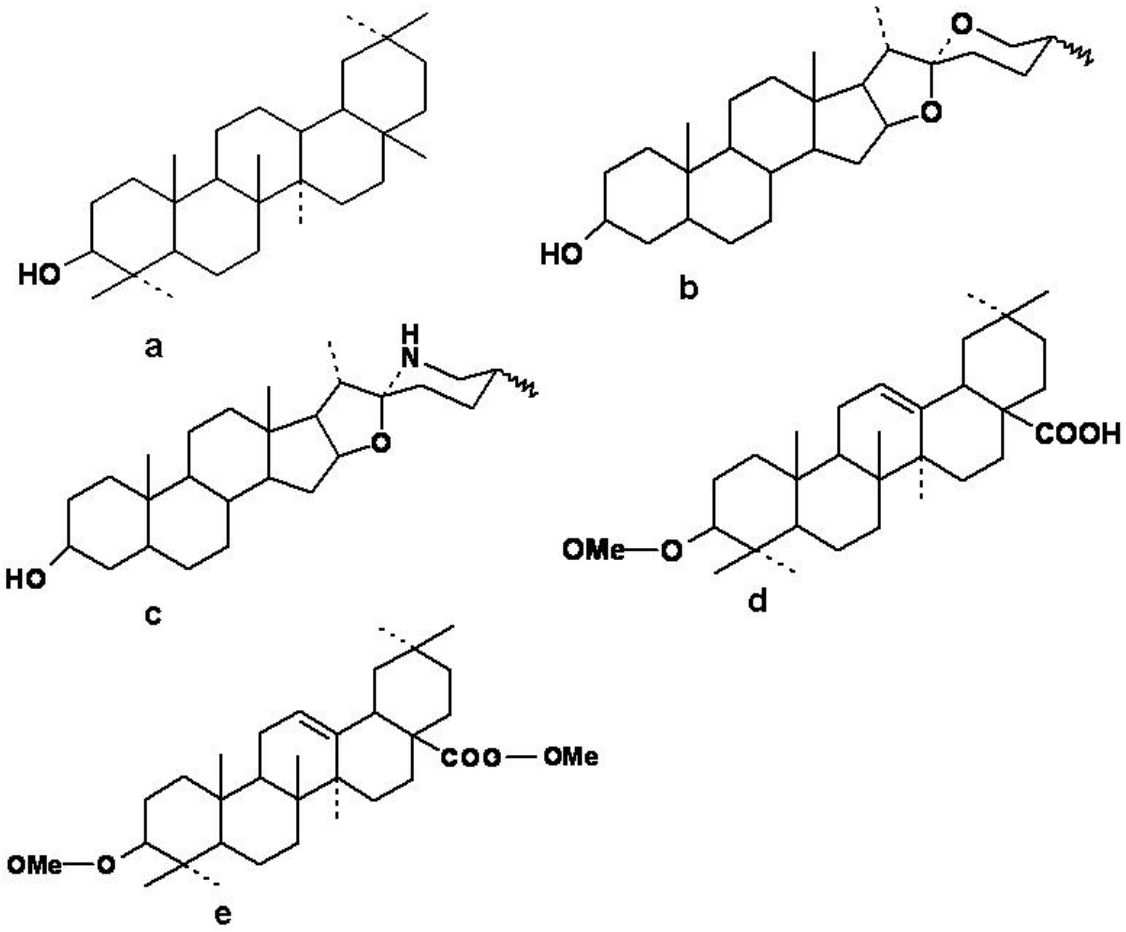

Figure 1.7: Classification of saponins; (a) triterpene class (b) steroid class (c) steroid alkaloid class (d) monodesmosidc (e) bidesmosidic (Hostettmann and Marston, 1995).

Generalizations about the solubility of saponins are difficult but many are soluble in water, particularly if the water contains small amount of alkali, and alcohol; some are soluble in ether, chloroform, benzene, ethyl acetate or glacial acetic acid. One of the most striking features of saponins is the enormous difference between mono- and bidesmosidic saponins, with bidesmisides either lacking or exhibiting a

reduction of the properties of the corresponding monodesmosides. An exception to this phenomenon is surface activity. This is more pronounced in the bidesmosides and increases with length and branching of the saccharide moiety. Despite the large differences, however, the inactive bidesmosidic compounds can often be readily converted into the corresponding biologically active monodesmosidic saponins by simple basic hydrolysis. The most characteristic of saponin properties is to form stable foams, and the phenomenon had been exploited as a test for the presence of saponins. Caution should however, be exercised, because not all saponins foam in aqueous solution (Hostettmann and Marston, 1995).

The list of biological activities associated with saponins is very long. Certain attributes of saponins, such as the fungicidal and piscicidal (fish) effects have been known for many years, while new activities are continually being discovered. The function of saponins in plants has often been questioned and there is not always a satisfactory explanation for their very high content (up to 30%) in some species. One theory is that they protect plants against fungal attack. As there is often an increase in saponin content of the plant part undergoing microbial attack, this supposition would seem to be reasonable. It has been proposed that bidesmosidic saponins exist as a transport form from the organs not at risk (e.g. leaves) to those parts of the plant (e.g. roots, bark, seeds) under attack by various microorganisms. When plant tissue is damage, the released enzymes act on the bidesmosidic saponins. Once transformed into their monodesmosidic derivatives, the saponins can provide defence against microbial invasion at the threatened area. An example is the production of α-hederin from hederasaponin in *Hedera helix* (Araliaceae). The *in vitro* antiviral activity of several glycosides of aclylated-β-amyrin aglycones against influenza virus has been reported. When tested against herpes simplex

type 1, and poliovirus, a plant extract of *Anagallis arvensi* (Primulaceae) was active (Hostettmann and Marston, 1995).

The toxicity of saponins is an extremely important issue as their occurrence in food such as beans, peas, soya beans, peanuts, lentils, spinach and oats, is widespread. Luckily, the oral toxicity of saponins to warm-blooded animals is relatively low. The reason for this low-risk phenomenon is the weak absorption which saponins undergo in the body (Hostettmann and Marston, 1995). The question as to whether consumption of saponins over a long term by humans leads to contra-indications would seem to be answered by the fact that few negative effects are observed after continued intake of saponins from edible plants. Over-consumption, however, poses some risk, as illustrated by the examples of licuorice. Prolonged exposure to excessive amounts has been known to produce hypertension, flaccid quadriplegia, hypokalkaemia, fulminant congestive heart failure and hyperprolactinaemia with amenorrhoea. As a consequence, patients with hypertension or circulatory disorders should avoid licuorice. The safety of alfalfa saponins for human consumption has been extensively investigated. Signs of intoxication by saponins include abundant salivation, vomiting, diarrhoea, loss of appetite and manifestation of paralysis. There are occasional instances when oral intake of saponin-containing plants can be lethal; cases of mortality have been recorded after consumption of *Agrostemma githago* (Caryophyllaceae). Experiments with humans are rare, but, Keppler, in 1878, injected 100 mg of saponin dissolved in water into his thigh, and suffered from terrible pain, accompanied by an anaesthetizing effect around the area of injection. In rats alfalfa saponins at a level of 1% in the diet for up to 6 months showed no ill effects,

although a potentially beneficial reduction in serum cholesterol and triglycerides was observed (Hostettmann and Marston, 1995).

The pharmaceutical applications of saponins are varied as their origins and chemical structures. Just to mention a few: a number of saponins or saponin-rich mixtures have found use as anti-inflammatory, antidiuretic, antipyretic, analgesic agents, central nervous system depressants, and as treatment for ulcers. These effects seem to be related to the stimulatory effects of saponins on the hypothalamus or pituitary gland. Antiulcerogenic activity is presumed to be related to effects of saponin on the synthesis of prostaglandins in the intestinal mucosa. Aescin saponins have been used in France topically to treat hemorrhoids and venous congestion. Saponins from *Buffa operculata*, primrose, and tea seeds act as expectorants and promote secretion of mucus from the respiratory tract. The Chinese ginseng saponins calm the mental condition, stabilize the spirit, stimulate the mind, extend the memory, make the sight clear and remove ill-feeling. Cimicifugocide saponins possess strong immunosuppressive activity which is preferentially directed towards β-cell function, but which in large doses also affects T-cell function. Extracts from *Maytenus diversifolia* have been found to inhibit growth *in vivo* of leukemic lymphocytes. A mixture of saponins isolated from *Achyranthes aspera* acted as a heart stimulant when tested on isolated hearts form frogs, guinea pigs, and rabbits (Cheeke, 1989).

Antispasmogenic effects have been found with a saponin from *Gargenia turgi,* which has also been claimed to have potential as an anti-asthmatic drug. Crude saponin extracts from *Albizzia lebbek* have been shown to protect mast cells against antigenic shock. Saponins possess excellent emollient properties and have

found extensive use in cosmetics. They also have numerous applications as surfactants. This is because they have the advantage of being non-ionic surfactants which are almost unaffected by the presence of salt or acid or alkaline conditions. Shampoos, other hair preparations, and various cleaning agents are manufactured with saponin levels ranging from 0.001 to 10 %. In biological waste-treatment plants, addition of saponins can improve oxygen transfer efficiency resulting in increased growth of bacteria and increased production on bacterial growth (Cheeke, 1989).

1.2.6 Heart glycosides

Cardiac glycosides and similar other glycosides are composed of two portions: the sugar and the non-sugar (aglycone) moiety. The aglycone portion of the cardiac glycosides is a steroid nucleus with a unique set of fused rings, which makes these agents easily distinguished form the other steroids. Rings A-B and C-D are *cis* fused, while rings B-C have a *trans* configuration. The steroid nucleus also carries, in most cases, two angular methyl groups at C-10 and C-13. Hydroxyl groups are located at C-3, the site of the sugar attachment, and at C-14. The C-14 hydroxyl is normally unsubstituted. However, additional hydroxyl groups may be found at C-12 and C-16, the presence or absence of which distinguishes the important genins: digitoxigenin, digoxigenin, and gitoxigenin (Fig. 1.8). These additional hydroxyl groups have significant impact on the partitioning and pharmacokinetics for each glycoside. The lactone ring at C-17 is another major structural feature of the cardiac aglycones. The size and degree of unsaturation of the lactone ring varies with the source of the glycoside. In most cases, the cardiac glycosides of plant origin, the cardenolides, possess a five-membered α,β-unsaturated lactone ring, whereas those derived from animal origin, the bufadienolides, possess a six-

membered lactone ring with two conjugated double bonds, generally referred to as α-pyrone (Williams et al., 2002).

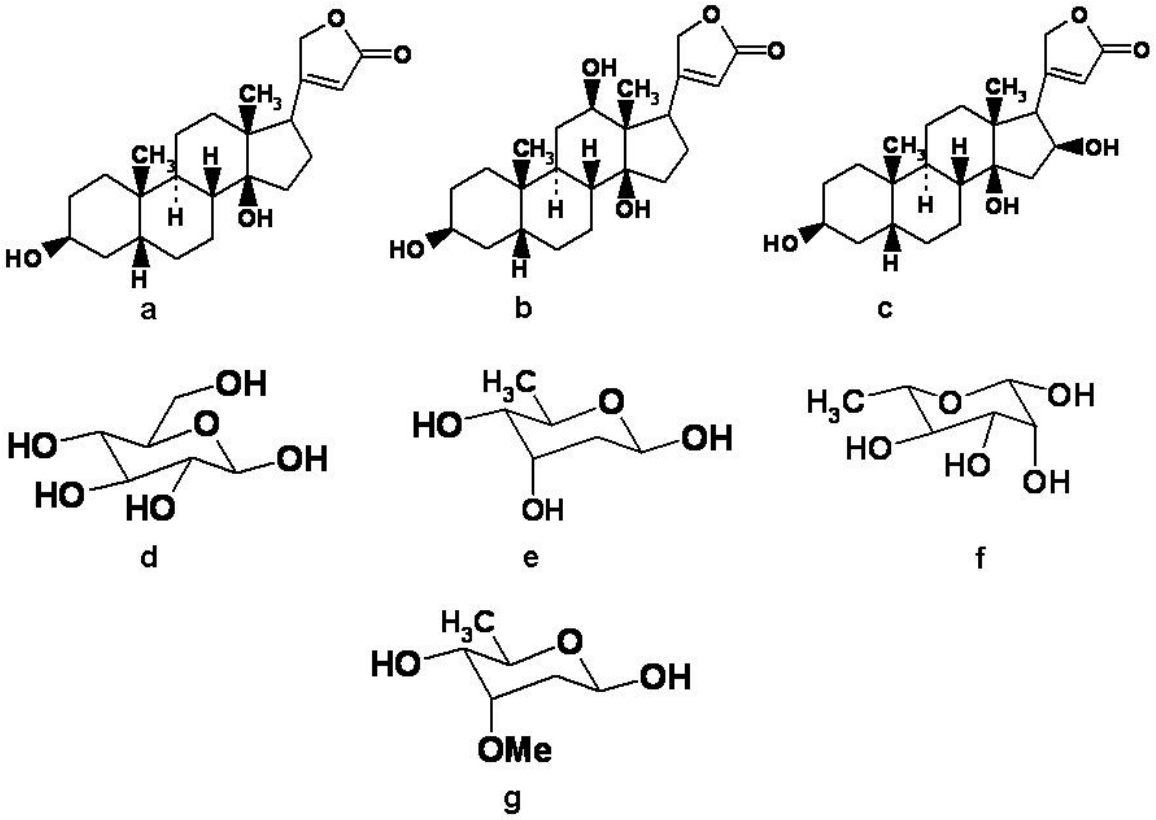

Figure 1.8: Structural examples of cardiac glycosides: (a) digitoxigenin, (b) digoxigenin, (c) gitoxigenin (d) D-glucose, (e) D-digitoxose, (f) L-rhamnose, and (g) D-cymarose (Williams et al., 2002).

The hydroxyl group of the aglycone portion is usually conjugated to a monosaccharide or a polysaccharide with β-1,4-glucosidic linkages. The number and identity of sugars vary from one glycoside to another. The most commonly found sugars in the cardiac glycosides are D-glucose, D-digitoxose, L-rhamnose, and D-cymarose (Fig. 1.8). These sugars predominately exist in the cardiac glycosides in the β-conformation. In some cases, the sugars exist in the acetylated form. The cardiac glycosides occur mainly in plants and in rare cases in animals, such as poisonous toads. *Digitalis purpurea* or the foxglove plant, *D. lanata*,

Strophanthus gratus, and *S. kombe* are the major plant sources of the cardiac glycosides (Williams et al., 2002).

All cardiac glycosides preparations have the potential to cause toxicity. Because the minimal toxic dose of the glycosides is only two to three times the therapeutic dose, intoxication is quite common. In mild to moderate toxicity, the common symptoms are: anorexia, nausea and vomiting, muscular weakness, bradycardia, and ventricular premature contractions. The nausea is a result of excitation of the chemoreceptor trigger zone (CTZ) in the medulla. In severe toxicity, the common symptoms are: blurred vision, disorientation, diarrhoea, ventricular tachycardia, and atriventricular block, which may progress into ventricular fibrillation. It is generally accepted that the toxicity of the cardiac glycosides is due to inhibition of the Na^+ / K^+ -ATPase pump, which results in increased intracellular levels of Ca^{++} and hypokalemia (decreased potassium). A common procedure used in treating cardiac glycosides toxicity is to administer potassium salts to increase extracellular potassium level, which stimulates the Na^+, K^+ -ATPase pump, resulting in decreased intracellular sodium levels and thus decreased intracellular calcium. In treating any cardiac glycoside-induced toxicity, it is important to discontinue administration of the drug, in addition to administering a potassium salt (Williams et al., 2002).

The cardiac glycosides are an important class of naturally occurring drugs whose actions include both beneficial and toxic effects on the heart. Their desirable cardiotonic action is of particular benefit in the treatment of congestive heart failure and associated edema and their preparations have been used as medicinal as well as poisonous agents. This dual application serves to highlight the toxic potential for

this class of life-saving drugs. Today the cardiac glycosides represent one of the most important drug classes available to treat congestive heart failure. Cardiac glycosides affect the heart in a dual fashion, both directly on the cardiac muscle and the specialized conduction system of sinoatrial node, and indirectly on the cardiovascular system mediated by the autonomic nervous reflexes. The combined direct and indirect effects of the cardiac glycosides lead to changes in the electrophysiological properties of the heart, including alteration of the contractility, heart rate, excitability, conductivity, refractory period, and automaticity of the atrium and ventricle. The heart response to the cardiac glycosides is a dose dependent process and varies considerably between normal and the congestive heart failure diseased heart. The effects observed after the administration of low doses differ considerably from those observed at high doses. The effects of cardiac glycosides on the properties of the heart muscle and different sites of the conduction system are summarized in Table 3 (Williams et al., 2002).

Table 3: Effects of cardiac glycosides on the heart (Williams et al., 2002).

Effect	Atrium	Ventricle	Purkinje Fiber	Atrioventricular node	Sinoatrial node
Contractility	↑	↑	–	–	–
Excitability	0	Variable	↑	–	–
Conductivity	↑	↑	↓	↓	–
Refrac. period	↓	↓	↑	↑	–
Automaticity	–	–	↑	–	↓

↑ : increased action; ↓ :decreased action; 0 : no action; – : no data available

1.2.7 Oxalates

Several types of oxalate compounds may be found in plants. Oxalic acid (Fig. 1.9) is a byproduct of cellular metabolism and is ever-present in plant species. Certain

families and species of plants contain relatively large amounts of oxalic acid, mainly as the soluble sodium, potassium, diphenyl, and insoluble calcium oxalates (Fig. 1.9). Some of the common plant foods containing appreciable amounts of oxalates are spinach (0.3 to 1.2 %), rhubarb (0.2 to 1.3 %), beet leaves (0.3 to 0.9 %), tea (0.3 to 2 %), and cocoa (0.5 to 0.9 %). Lettuce, celery, cabbage, cauliflower, turnips, carrots, potatoes, peas, and beans also contain small amounts of oxalates. Soluble salts are a source of toxicity in livestock and domestic animals, which may graze on large quantities and develop systemic poisoning (Deshpande, 2002).

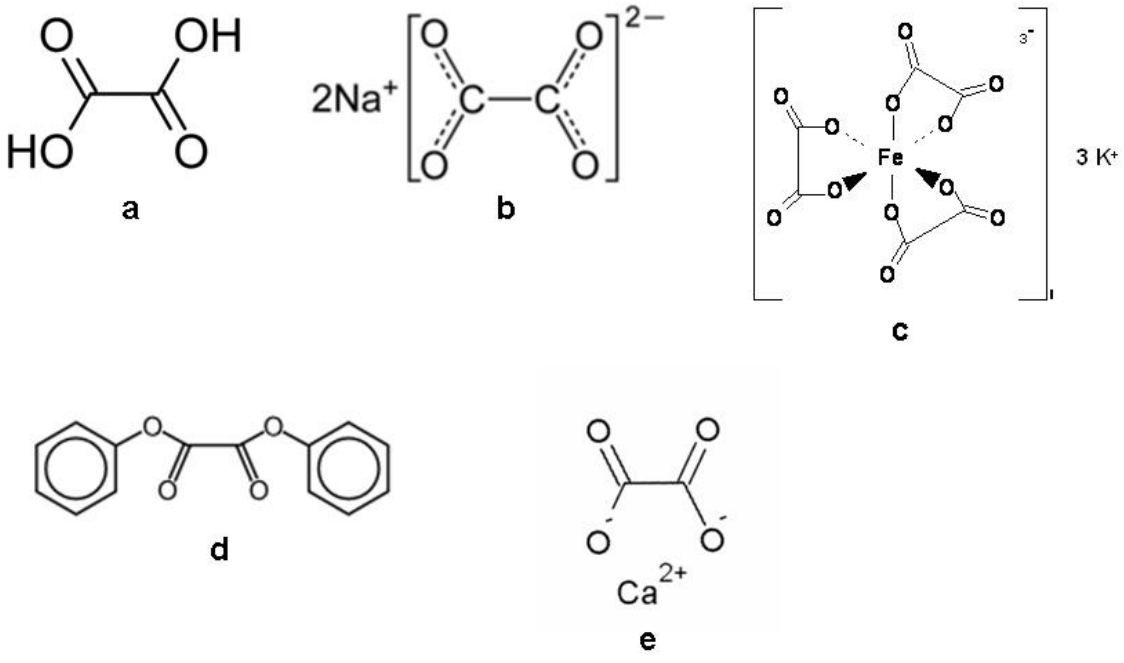

Figure 1.9: Examples of oxalates: (a) oxalic acid, (b) disodium oxalate, (c) potassium oxalate, (d) diphenyl oxalate, and (e) calcium oxalate (Deshpande, 2002).

In biological experiments, sodium oxalate, like citrates, can also be used to remove calcium ions (Ca^{2+}) from blood plasma and to prevent blood from clotting. By removing calcium ions from the blood, sodium oxalate can impair brain function,

and deposit calcium oxalate in the kidneys. Oxalic acid and oxalates are abundantly present in many plants, most notably *Chenopodium album* (fat hen), *Oxalis pes-caprae,* (sour grass), and *Oxalis grandis*, sorrel. The root and leaves of rhubarb and buckwheat are listed as being high in oxalic acid. The affinity of divalent metal ions is sometimes reflected in their tendency to form insoluble precipitates. Thus in the body, oxalic acid also combines with metals ions such as Ca^{2+}, Fe^{2+}, and Mg^{2+} to deposit crystals of the corresponding oxalates, which irritate the gut and kidneys. Because it binds vital nutrients such as calcium, long-term consumption of foods high in oxalic acid can be problematic. Healthy individuals can safely consume such foods in moderation, but those with kidney disorders, gout, rheumatoid arthritis, or certain forms of chronic vulvar pain (vulvodynia) are typically advised to avoid foods high in oxalates. Conversely, calcium supplements taken along with foods high in oxalic acid can cause calcium oxalate to precipitate out in the gut and drastically reduce the levels of oxalate absorbed by the body (by 97% in some cases.). The calcium oxalate precipitate (better known as kidney stones) obstructs the kidney tubules (Pabuccuoglu, 2005.)

Plants with insoluble calcium oxalate crystals are most frequently responsible for human toxicity. The stalk of the *Dieffenbachia* produces the most severe reactions. It is well known that humans excrete varying amounts of calcium oxalate crystals in the urine (6 to 45 mg/day, with a mean of about 20 mg/day in normal subjects). About two thirds of the urinary oxalate is derived from ascorbic acid and the amino acid glycine; the rest is from dietary oxalate and possibly form precursors, such as glycolic and glyoxylic acids. A metabolic disorder resulting in the production of renal stones and deposits of calcium oxalate in other tissues is often attributed to the excessive consumption of oxalates from foods. Under such conditions, urinary levels of oxalates and glycolates are greatly increased. Acute oxhumans is

associated with corrosive gastroenteritis, shock, convulsions, low plasma calcium levels with correspondingly higher levels of oxalates, and renal damage. Acute oxalate poisoning in humans, however is rare (Deshpande, 2002).

The shape of calcium oxalate crystal varies and to some extent determines the degree of local toxicity. Some common shapes include cylindrical or pinpoint needles, rosettes, and druses (star-like clusters of polyhedral crystals). Parallel needle-shaped crystals arranged in bundles are known as raphides. In some plants, the raphides are contained within specialized cells known as idioblasts, which have thick, rigid walls and nozzle apertures at both ends. Some raphides, such as those found in *Alocasia* and *Colocasia*, are coated with a thin film of an acrid material. Other plants contain raphides within thin-walled parenchyma cells. Plants with abundant raphide crystals, but no idioblasts, such as *Impatiens textorii*, are not known to cause local irritation (Spoerke and Smolinske, 1990).

The mechanism of irritation from calcium oxalate-containing plants has been investigated most thoroughly for *Dieffenbachia* species. When examined microscopically, each idioblast is found to contain a bundle of 100 to 200 sharp, pointed needle-like projectiles. When pressure is applied to the idioblast wall, the crystals are fired singly in rapid succession, until the contents are emptied. Conditions responsible for triggering cell firing include mechanical pressure, forceful crushing, and slicing. Dried preparations do not fire; thus ingestion of dried leaves is not likely to produce irritation. Boiling for 5 to 10 minutes also inactivates the idioblasts. Although the mechanism is not completely understood, it is most widely accepted that the raphides penetrate tissue and facilitate entry of other

irritants or inflammatory substances, such as enzymes or free oxalic acid (Spoerke and Smolinske, 1990).

1.2.8 Lectins

Lectins are an important class of proteins or glycoproteins of no-immune origin that bind non-covalently to characteristic carbohydrate structures with specificity or selectivity. There are many ways to classify lectins. Given the fact that a wide range of living organisms, from microbes to mammals, produce lectins, they can be divided according to species origin, such as microbial, plant, invertebrate, and vertebrate lectins. Lectins can be classified according to their binding specificity or selectivity with carbohydrates. Many lectins are able to cross-react with a panel of sugar chains with a common terminal sugar residue. Lectins are functionally classified based on their relative binding reactivities with the structural units of carbohydrate or glycol-epitopes. They are grouped according to their monosaccharide specificities and then further sub-grouped based on their reactivities with more complex structures. Carbohydrate specificities of biomedically important lectins are classified into six groups according to their specificities to monosaccharides. The groups are: *GalNAc*-specific, *Gal*-specific, *Glc*-specific, *4GlcNAcβl*-linked specific, *LFuc*-specific, and *Sialic* acid specific agglutinins (Pusztai, 1991).

Whether extracted from natural sources or expressed in cell cultures, lectins provide models for the study of protein-carbohydrate interactions and exquisite tools for the analysis of carbohydrate, in either free-form or bound to lipids or proteins. The most fully characterized group of lectins are those from the plant kingdom because they are frequently hydrophilic and produced in large amounts

such as is the case with the seed lectins. Secreted plant lectins may be highly toxic, such as in the case of ricin (hemagglutinin) and abrin, (Pusztai, 1991).

Phytohemagglutinin is a lectin found in plants, especially beans. It is found in the highest concentrations in uncooked red kidney beans of *Phaseolus vulgaris*, and in lower quantities in many types of green beans. It has a number of physiological effects and is used in medical research. This lectin has a number of effects on cell metabolism. It induces mitosis, and affects the cell membrane in regard to transport and permeability to proteins. It agglutinates most mammalian red blood cell types. As a toxin it can cause poisoning in monogastric animals, such as humans, through the consumption of raw or improperly prepared kidney beans. Measured in haemagglutinating units (hau), a raw kidney bean can contain up to 70 000 hau. This can be reduced around 200-fold by correct cooking. The bean also contains α-amylase inhibitor. Poisoning can be induced from as few as five raw beans and symptoms occur within three hours, beginning with nausea then vomiting which can be severe and sustained, then diarrhoea. Recovery occurs within four or five hours of onset, usually without the need for any medical intervention. Medically it is used as a mitogen to trigger cell division in T-lymphocytes, and to activate latent HIV-1 from human peripheral lymphocytes (Hamelryck et al., 1996)

Abrin is a natural poison that is found in the seeds of a plant called the rosary pea or jequirity pea. Abrin is a powdered yellowish-white stable substance that can last for a long time in the environment despite extreme conditions such as very hot or very cold temperatures. It works by getting inside the cells of a person's body and preventing the cells from making the proteins they need. Without the proteins, cells

die. Eventually this is harmful to the whole body, and death may occur. Effects of abrin poisoning depend on whether abrin was inhaled, ingested, or injected (Pusztai, 1991).

The major symptoms of abrin poisoning depend on the route of exposure and the dose received, though many organs may be affected in severe cases. Initial symptoms of abrin poisoning by inhalation may occur within 8 hours of exposure. Following ingestion of abrin, initial symptoms may occur in less than 6 hours but usually are delayed for 1 to 3 days. Within a few hours of inhaling significant amounts of abrin, the likely symptoms would be respiratory distress, fever, cough, nausea, and tightness in the chest. Heavy sweating may follow as well as fluid building up in the lungs (pulmonary edema). This would make breathing even more difficult, and the skin might turn blue. Excess fluid in the lungs would be diagnosed by x-ray or by listening to the chest with a stethoscope. Finally, low blood pressure and respiratory failure may occur, leading to death (Hamelryck et al., 1996).

1.3 Anticancer activity of poisonous compounds

1.3.1 Introduction

Cancer is a leading cause of morbidity and death worldwide, and the mortality caused by cancer is increasing with the aging of the population. There are many clinically approved anticancer drugs that have exhibited medicinal potential in a limited array of tumor types, but in most cancers, partial responses occur. There are several unique attributes associated with development of anticancer drugs, by virtue of their cytotoxic or cytostatic mechanisms of action. These drugs are generally highly toxic, affecting rapidly dividing cell populations such as gastrointestinal tract, bone marrow, and lymphoid tissues (Greenlee et al., 2001).

The goal of most current cancer therapies, including radiation, chemotherapy, immunotherapy, and gene therapy, is the reduction or elimination of cancer cells. Although cytostatic effects, if complete and durable, might be acceptable in some situations, the current regulatory climate favours the development of cytotoxic agents. Despite the approval of such agents for the treatment of cancer, however, the age-adjusted death rate from most common epithelial neoplasms has scarcely changed in the past four decades (Greenlee et al., 2001). These observations suggest that cytotoxic agents often fail to selectively eradicate neoplastic cells and provide the impetus for asking whether there is something wrong with the cell death process in cancer cells.

1.3.2 Effects of chemotherapeutic agents

Chemotherapeutic agents can have two distinct but related effects on cells as outlined in Fig. 1.10. They can directly block a vital cellular process, and can stress a cell, indirectly prompting it to kill itself. Either or both of these effects can contribute to cell death (Kaufmann and Vaux, 2003).

The first effect can be adequately described by a lock and key model. For example: Taxol (Fig. 1.11 a) is an anticancer agent originally isolated from the bark of the pacific yew, *Taxus brevifolia* and has been effective against carcinomas of the ovary, lung, and for both early stage breast cancer, and refractory or metastatic breast cancers. Taxol binds to and promotes the assembly of microtubles from tubulin dimers and stabilizes microtubles by preventing depolymerization and

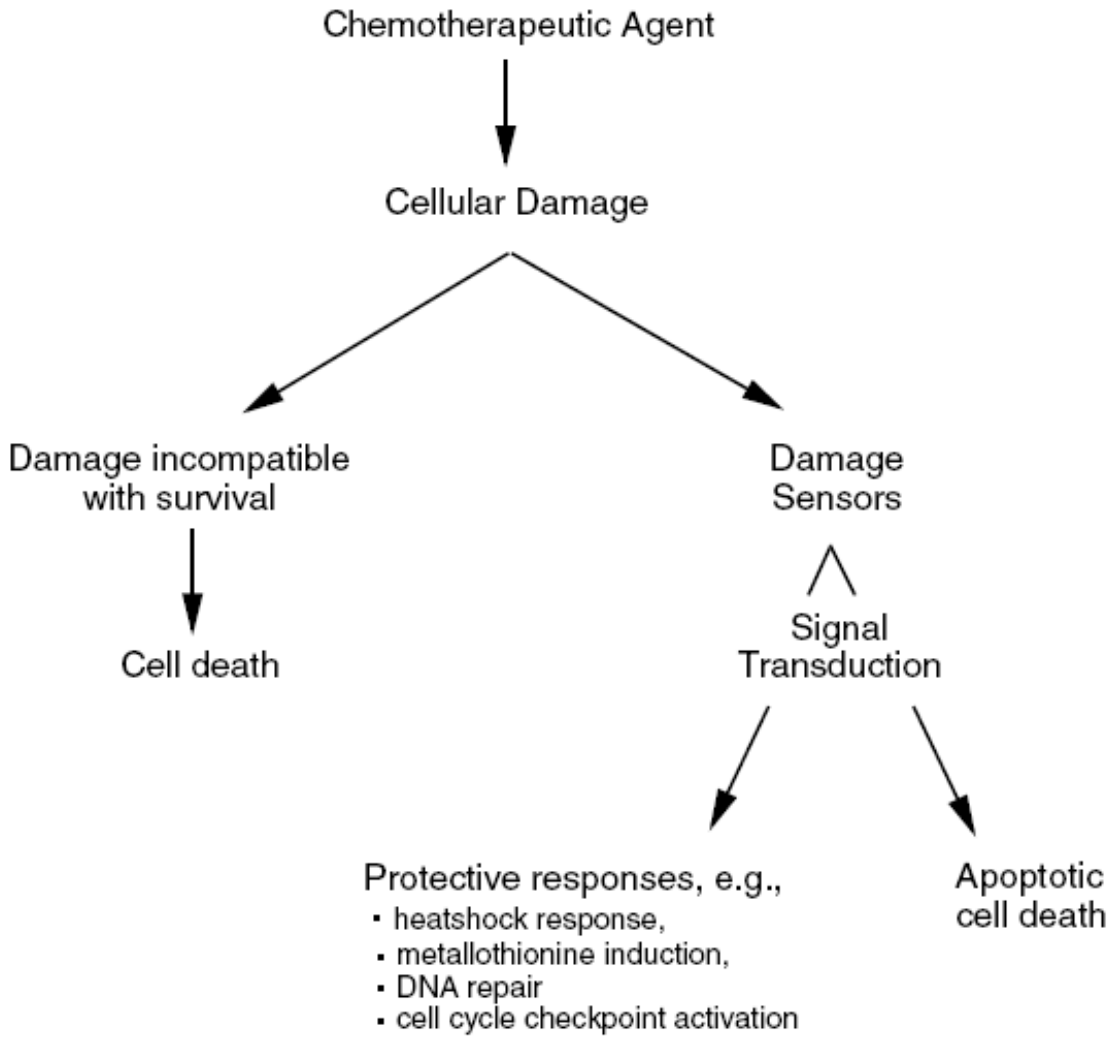

Figure 1.10: Two types of responses to anticancer therapy. Most toxins, radiation, and chemotherapeutic agents can directly damage both normal and cancer cells. If the damage is severe enough and sustained for a sufficient time, it will kill a cell directly as illustrated by examples in the text. If the damage is not as great, or it has not had enough time to kill the cell directly, the cell might detect the damage and respond. Some responses allow the cell to repair the damage, thereby helping protect the cell. Apoptosis is a common stress response in which the cell kills itself. Because of differences in some of the properties described in other reviews in this monograph (e.g. drug uptake, xenobiotic metabolism, and target abundance), normal cells and cancer cells might have different thresholds for direct toxicity. Likewise, because of differences in signals that regulate apoptotic pathways, normal cell and cancer cells can have differences in apoptotic thresholds (Kaufmann and Vaux, 2003).

blocks dihydrofolate reductase. Both events inhibit or destroy the normal dynamic reorganization of microtubule network essential for mitotic apparatus, block the cells at G2/M phase of the cell cycle and eventually lead to cell apoptosis (Yanyuan et al., 2007).

Camptothecin (Fig. 1.11 b), found in the bark of *Camptotheca acuminate,* inhibits the relegation step of DNA topoisomerase I. For these types of agents, the drug–target interaction has been shown to cause cellular changes that could result in cell death. Camptothecin-induced trapping of covalent topoisomerase I–DNA complexes, on the other hand, leads to DNA double-strand breaks and mutations that, if they occur in survival-critical genes, could result in death once the corresponding polypeptides turn over (Ryan et al., 1991; Hashimoto et al., 1995).

An alkaloid, vincristine (Fig. 1.11 c) isolated from the Madagascar periwinkle (*Catharanthus roseus)*, is a drug that blocks the formation of the mitotic spindle required for chromosome migration during mitosis. This compound also activates G2/M phase arrest of a cell cycle and apoptosis in MCF-7 cancer cells. However, the biochemical events occurring between this compound binding to tubulin and apoptosis are not well understood. It has been reported that in CCRF-CEM leukemia cells, proteins such as FKBP59, TCTP, HSP90 or L-plastin are involved in cellular response to vincristine (Casado et al., 2007).

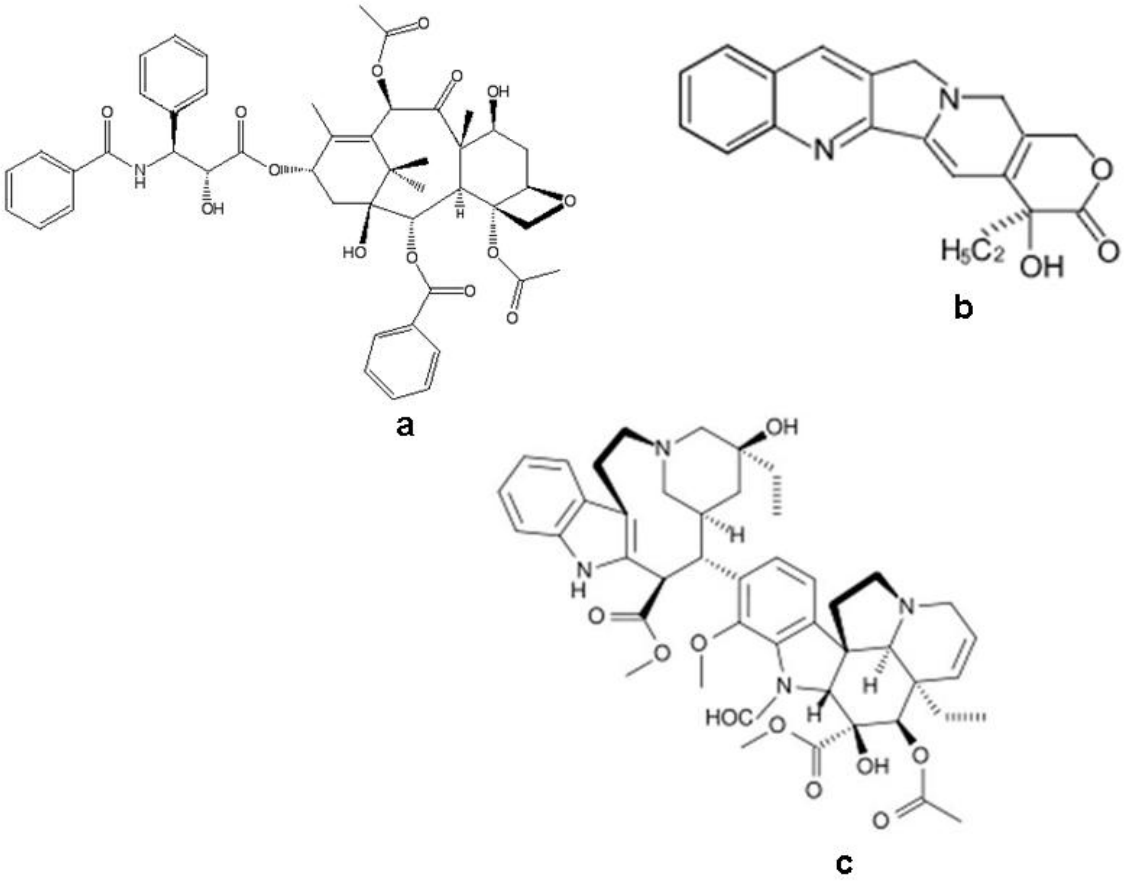

Figure 1.11: Structures of anticancer compounds: (a) taxol (Casado et al., 2007), (b) camptothecin, and (c) vincristine (Jordan et al., 1996)

The second effect of chemotherapeutic agents results in a response in which cells kill themselves by activating an endogenous cell suicide process (Kaufmann and Earnshaw, 2000). This form of cell death, often recognizable by its characteristic appearance known as 'apoptosis,' uses effector mechanisms that are also employed for the removal of unwanted cells during development and to maintain constant cell number by balancing mitosis (Arends and Wyllie, 1991). Stress responses, including cell suicide, are much more complicated than the 'lock and key' processes described above. These are more appropriately thought of as a 'stimulus–response model' in which a cell needs receptors to sense an alteration, signal transduction pathways to pass on the information, and effector mechanisms to mediate a response. For example, exposure of a cell to ionizing radiation leads

to detection of DNA lesions, activation of a series of kinases, and recruitment and/or synthesis of a series of polypeptides that can block the cell cycle and repair the damage or cause the cell to "kill itself" (Zhou and Elledge, 2000).

Inhibition of any vital metabolic process can lead to stress responses, including apoptosis. For some therapies, the detection and signal transduction pathways have been elucidated. Damage to DNA caused by ionizing radiation or drugs, for example, can lead to stabilization of p53 (Giaccia and Kastan, 1998) and consequent transcriptional activation of genes encoding a number of polypeptides, including proapoptotic proteins: Bax (Miyashita and Reed, 1995), Apaf-1 (Fortin et al., 2001), the BH3-only polypeptides Noxa and PUMA, and p53AIP1 (Oda et al., 2000). Each of these polypeptides can potentially activate the Bcl-2- inhibitable pathway. Because of the involvement of other transcriptional activators and repressors in expression of these polypeptides, not all of these polypeptides are regulated to the same extent by p53 in all model systems. The role of Bax transcription in p53-induced death, for example, has been questioned because it is difficult to demonstrate DNA damage induced Bax upregulation in many cells, thymocytes from Bax undergo p53-dependent apoptosis normally, and the putative p53 binding elements in the Bax promoter are not conserved between man and mouse (Knudson et al., 1995). Likewise, the extent and importance of Noxa upregulation has been questioned (Yu et al., 2001).

Because transfecting (infection of a cell with purified viral nucleic acid) p53 cells with bcl-2 causes a further reduction in apoptosis of irradiated cells (Strasser et al., 1995), p53 cannot be the only mediator of DNA damage-induced apoptosis. For example, in mitogen-stimulated T cells, IRF-1 rather than p53 has been implicated

as the mediator of radiation-induced apoptosis by the Bcl-2-inhibitable pathway (Tamura et al., 1995).

In addition to DNA damage, apoptosis can be induced by inhibition of transcription, translation, DNA replication, or microtubule function. Apoptosis in response to these types of stresses is reduced in cells overexpressing Bcl-2 (Strasser et al., 1995) or cells containing targeted deletion of both Bax and Bak (Lindsten et al., 2000). In contrast, apoptosis in response to these agents still occurs in cells expressing the caspase 8 inhibitor CrmA, an inhibitor of FADD, or activated protein kinase C isoforms that inhibit the death receptor pathway (Eischen et al., 1997). Experiments such as these suggest that, with a few possible exceptions such as 5-fluorouracil (Tillman et al., 1999), chemotherapy-induced apoptosis proceeds via the Bcl-2-inhibitable pathway. Nevertheless, increased expression of death receptors and their ligands has often been observed in cells treated with chemotherapeutic agents (Kaufmann and Earnshaw, 2000). This upregulation of death receptor pathway components may reflect part of a generalized stress response.

1.4 Background on *Elaeodendron croceum*

1.4.1 Plant description

The Celastraceae is a large family of herbs, woody lianas, shrubs and trees distributed worldwide in tropical and temperate zones. A report by Simmons et al. (2001) states that the number of genera and species of the family is not known. The report conveys inconsistent estimates partly because little taxonomic work has been done on the family, and because generic delimitations are controversial. The

family has been subject to considerable nomenclatural confusion and name changes. *Elaeodendron croceum* (Thunb.) DC. has been treated by different authors under several names such as *Cassine crocea, C. papillosa, Crocoxylon croceum, E. capense, E. papillosum,* and *Ilex crocea* (Archer and Van Wyk, 1998; Germishuizen and Meyer, 2003).

E. croceum is a medium to tall evergreen tree. The main stem is up to 1.2 m broad in diameter and 3-13 m tall with spreading crown. Bark is often grayish with layers of a powdery yellow pigment in exposed rhytidome, exfoliating in thin scales, surface longitudinally fissured. Branchlets have brown or dark brown lenticels. Leaves (Fig. 1.11 a) are opposite, oblong to elliptic, thick and leathery, dark green above and paler below with dark net vein, and a glandular–denticulate to prominently spinulose-denticulate margin. Flowers are in auxiliary clusters, small and greenish. Sepals are greenish, ovate, 1.5 × 1 mm, and fleshy. Petals are whitish green, widely ovate, 1.5 × 1.5 mm. Stamens are erect and spreading, filaments are 0.5 mm long (Archer and Condy, 1995). Fruit (Fig. 1.11 b) is an oval drupe up to 25 mm long, cream to pale yellow with a single stony endocarp. Seeds are light brown, narrowly ellipsoid, 15-20 × 4-5 mm, and embryo with fleshy cotyledons (Van Wyk and Van Wyk, 1997).

1.4.2 Plant distribution

This species usually grows on the margins of coastal forest, dry forest and dune forest (Pooley, 1997; Archer and Condy, 1995; Van Wyk and Van Wyk, 1997), and also found in the middle of the forest. The species is distributed along the coast (Fig. 1.12), including different provinces such as Limpopo, KwaZulu-Natal, Eastern

Cape, and Western Cape (Van Wyk and Wan Wyk, 1997), and in Swaziland and Mount Chirinda in Zimbabwe (Archer and Condy, 1995; Pooley, 1997).

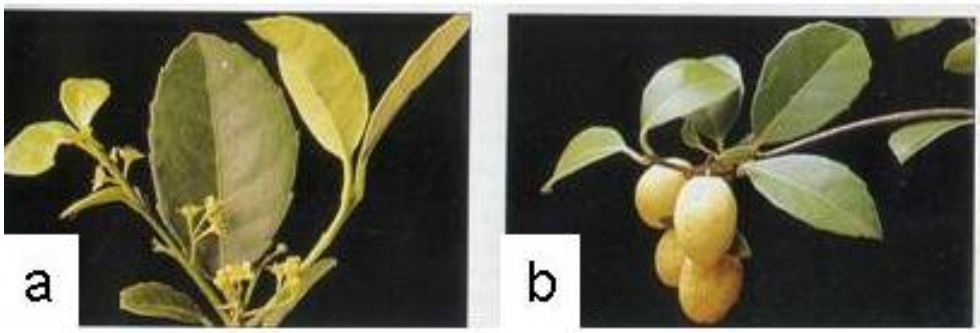

Figure1.11: *E. croceum* (a) leaves (b) fruit, (Van Wyk and Van Wyk, 1997).

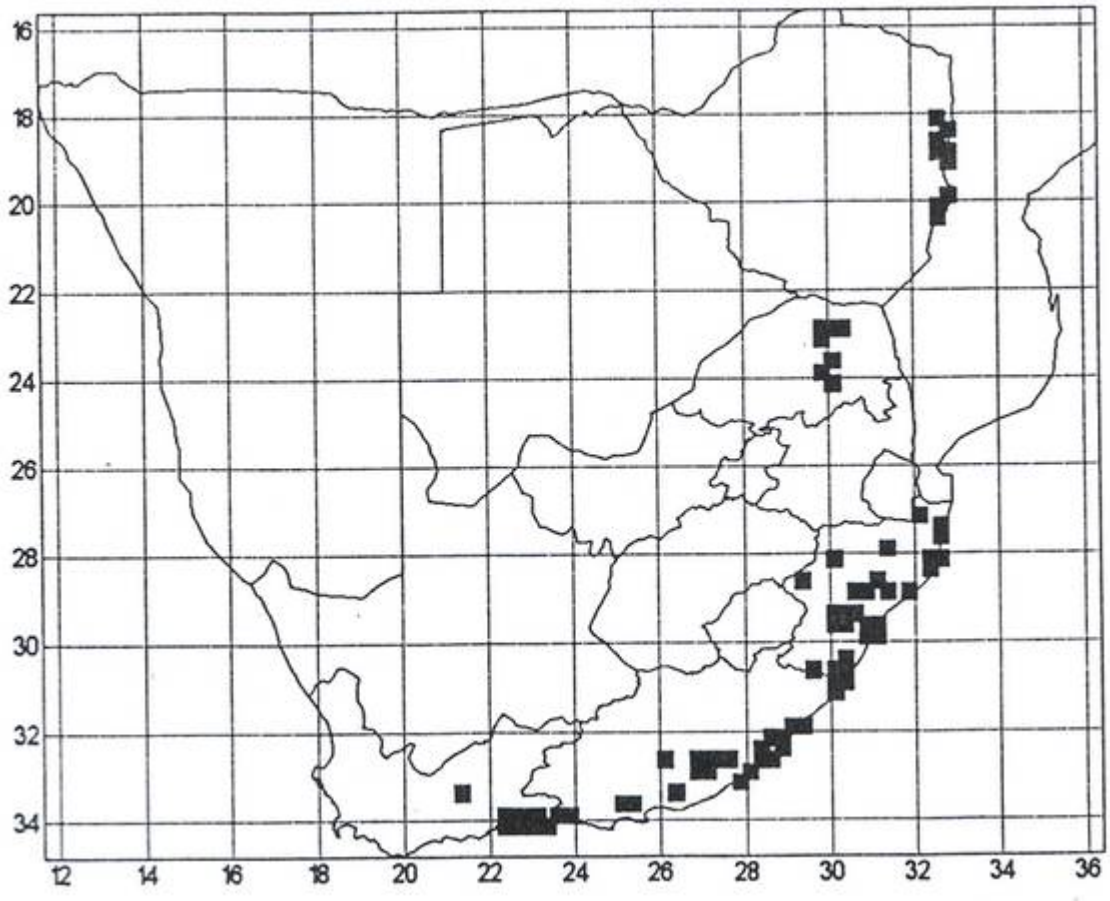

Figure 1.12: Distribution map of *E. croceum* (Van Wyk and Van Wyk, 1997).

1.4.3 Medicinal uses

Several authors reported that most parts of *E. croceum* are poisonous (Van Wyk and Gericke, 2000; Van Wyk and Van Wyk, 1997). The plant is used in curing TB-related symptoms such as cough, fever, blood in the sputum (Lall and Meyer, 1999). The roots were apparently previously used to cause death by traditional healers (Palmer and Pitman, 1973; Watt and Breyer-Brandwijk, 1962). The bark is used to clean the digestive tract (Schmidt et al., 2002), as a purgative, and to clear a congested chest (Van Wyk et al., 2002) and has been reported to be fatal to humans, presumably due to the presence of alkaloid (Archer and Condy, 1995).

1.5 Objectives

- Isolation and identification, of poisonous compounds from *Elaeodendron croceum.*
- *In vitro* cytotoxicity evaluation of isolated compounds.

1.6 Scope of thesis

A description of *Elaeodendron croceum* and the nature of poisonous compounds are portrayed in chapter 1. The Vero cell line bioassay guided isolation and identification of isolated compounds are described in chapter 2. Chapter 3 reports on the toxicity of isolated compounds on Vero, HeLa, SNO, and MCF-7 cell lines. Chapter 4 contains the general discussion and conclusion.

1.7 Hypothesis

E. croceum contains poisonous compounds which have *in vitro* anticancer activity.

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CHAPTER 2: ISOLATION AND IDENTIFICATION OF COMPOUNDS FROM *E. CROCEUM*

2.1 Introduction

It has been reported that most parts of *E. croceum* are poisonous (Van Wyk, 2000; Van Wyk and Van Wyk, 1997). The roots are poisonous, and in some cases they are believed to have caused death. Traditional healers once used it for trial by 'ordeal' and perhaps still use it (Palmer and Pitman, 1973; Watt and Breyer-Brandwijk, 1962). The bark has proved to be fatal to humans, presumably due to the presence of alkaloids (Archer and Condy, 1995). On two occasions the plant has come under suspected cause of human death (Watt and Breyer-Brandwijk, 1962). Experiments upon rabbits found that 2.5 g per kg body weight, and 10 g of fresh leaves per kg body weight caused death fifteen minutes after administration (Steyn, 1934). Traditional healers reported that a goat bleated repeatedly for a couple of hours before dying, and pieces of fresh leaves of *E. croceum* were found in its stomach. The bark is widely used for medicinal and magical purposes (Van Wyk and Van Wyk, 1997; Pooley, 1997), dyeing and tanning (Van Wyk and Gericke, 2000; Palmer and Pitman, 1973). The wood is used for manufacture of all kinds of furniture and wagons (Archer and Condy, 1995; Palmer and Pitman, 1973).

2.1.1 Previously isolated compounds from *E. croceum*

Several compounds (Fig. 2.1) have been isolated by Drewes and Mashimbye (1993) from the stem bark of *E. croceum,* previously known as *Cassine papillosa*. No analysis of the biological activity of the compounds was reported in their study. No other paper reporting the isolation of compounds form this species could be found.

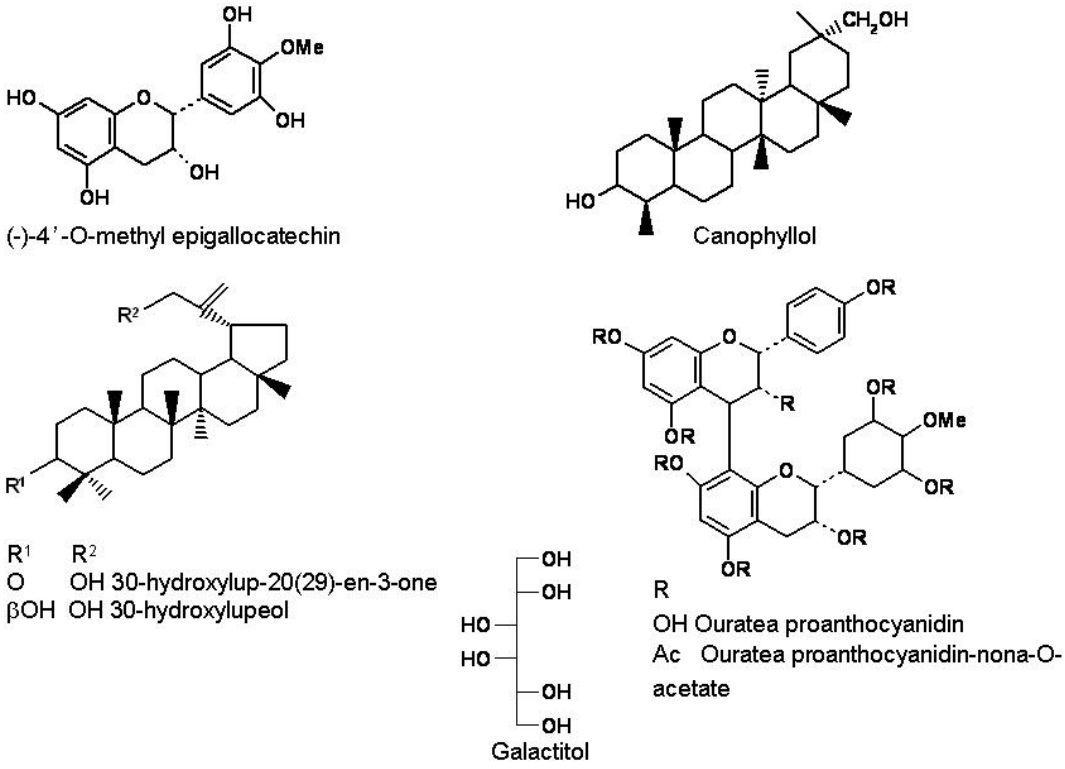

Figure 2.1: Structures of previously isolated compounds from *E. croceum* (Drewes and Mashimbye, 1993)

2.1.2 Compounds previously isolated from other *Elaeodendron* species

Cao et al. (2007) isolated six compounds from an EtOH extract of the bark of *E. ilicifolium*: elaeodendroside B, elaeodendroside F, elaeodendroside G, (2α,3β,14β)-trihydroxy-3-O-(4-deoxy-3-O-methyl-R-L-erythropentopyranosyl)-card-4,20(22)-dienolide, elaeodendroside T, and elaeodendroside U (Fig. 2.2). The

report identified the most active compounds on an ovarian cancer cell line as elaeodendroside T and elaeodendroside U.

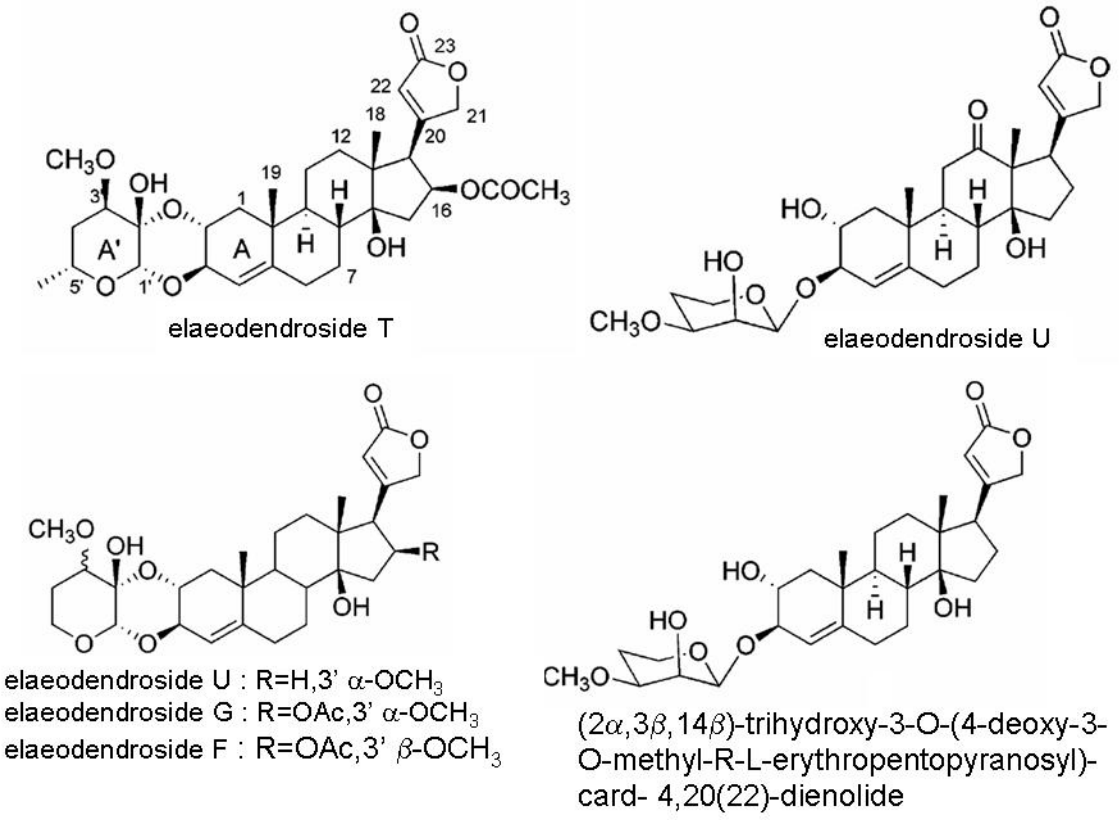

Figure 2.2: Compounds isolated from other *Elaeodendron* species (Cao et al., 2007).

Elaeodendron buchananii, is a tropical tree that grows in east Africa and is poisonous to animal stock and human beings. Ingestion of its leaves, fruits or bark is said to cause sudden death. On the other hand, the roots can be dried and powdered for use in the treatment of wounds and in the primary symptoms of syphilis. Chewing of the plant has been said to cure diarrhoea. The chloroform extract of the unripe fruit of *E. buchananii* led to the isolation of a glycoside: 2α,3β-14-trihydroxy- 16α,-acetoxy- 14β-carda-4,20 (22)-dienolide-7β,8β-epoxide (Fig. 2.3), which acts as an antifeedant substance for *Spodoptera* exempta (Tsujino et al., 1995).

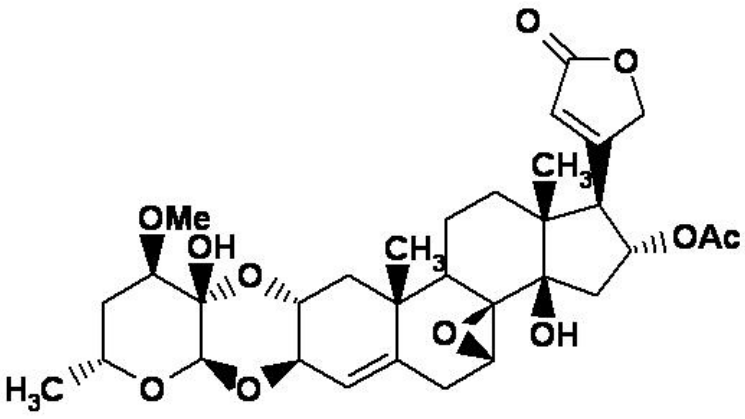

Figure 2.3: 2α,3β-14-trihydroxy-16α,-acetoxy-14β-carda-4,20 (22)-dienolide-7β,8β-epoxide isolated form *E. buchananii* (Tsujino et al., 1995).

2.1.3 Materials and methods

2.1.3.1 Plant material

E. croceum leaves were collected from the Eastern Cape Province (Butterworth, Centane) of South Africa on 20 February 2006, identified and preserved at the HGWJ Schweickerdt Herbarium of the University of Pretoria, (voucher specimen number: Yelani 094021).

2.1.3.2 Preparation of plant extracts

E. croceum dried leaves (3.7 kg) were successively extracted with n-hexane, dichloromethane and then acetone, two times each. All three extracts were filtered and rotor-evaporated at 40 ^{0}C to dryness to yield 53.0 g (n-hexane), 43.0 g (dichloromethane), and 104.0 g (acetone).

2.2 Isolation and identification of compounds

2.2.1 Isolation of compounds

The n-hexane extract (53.0 g), which had the highest cytotoxicity (method described in chapter 3), was applied to a silica gel column (Merck, 230-400 mesh) (10 × 80 cm) (Fig. 2.4). The column was eluted with a solvent system of n-hexane : ethyl acetate in order of increasing polarity (100:0 to 0:100). The chemical similar fractions of the 43 (Fig. 2.5) collected fractions (100 ml each) were combined according to thin layer chromatography (TLC) to form nine main-fractions (A to I, Fig. 2.5). Fraction I (3.0 g) was the most toxic to Vero cells and further purified on a silica gel column (5 × 60 cm) eluted with n-hexane : ethyl acetate (8:2). Collected fractions were combined according to the TLC profiles resulting in ten sub-fractions (I_1 to I_{10}). Crystals of a red compound (**1**, 14.0 mg, Fig. 2.4) were formed when the I_{10} fraction was dissolved in methanol.

According to a TLC profile, sub-fractions I_4 (43.0 mg) and I_5 (83.0 mg) contained similar constituents, which were collected in small amounts and therefore combined to obtain fraction I_4I_5 (126.0 mg) and applied to a Sephadex LH-20 (Sigma) column. The column was eluted with absolute ethanol. Three fractions (Fig. 2.6 a) were collected from the column. The third fraction was shown to contain only one pure compound (**3**, 10.0 mg, 2.9).

Fraction G (1.0 g) of the hexane extract's silica column was combined with fraction H (2.0 g) to form fraction GH (3.0 g), and applied to a Sephadex column, which was eluted with ethanol. Two sub-fractions GH_1 (2.4 g) and GH_2 (94.0 mg, Fig. 2.7) were collected. Fraction GH_2 was crystallized from methanol to form white crystals

of compound **4** (21.0 mg, Fig. 2.4). Fraction GH_1 was shown to be poisonous and re-chromatographed on a Sephadex column and eluted with ethanol to obtain two sub-fractions, $GH_{1.1}$ (1.7 g) and $GH_{1.2}$ (210.0 mg, Fig. 2.8). Preparative TLC developed in methanol : dichloromethane (3:97) of fraction $GH_{1.2}$ yielded a reddish compound, **2** (9.0 mg, Fig. 2.4).

The dichloromethane extract (43.0 g) was also found to be poisonous and applied to a silica gel column (10 × 60 cm), and eluted with a gradient of n-hexane : ethyl acetate (8:2 to 0:100) to yield eleven fractions (DA to DK, Fig. 2.9). Fraction DF (2.0 g) was poisonous and applied to a silica gel column (4 × 40 cm) and eluted with a gradient of ethyl acetate : n-hexane, (2:8 to 1:1). Out of five collected fractions, fraction DF_2 (35.0 mg) was applied to a Sephadex column. The column was eluted with absolute ethanol and compound **5** (18.0 mg, Fig. 2.4) was collected in a pure form. The schematic presentation of the isolation steps and structures of compounds are shown in Fig. 2.4.

2.2.2 Identification of isolated compounds

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry were used for structure determination of the isolated compounds. Proton (^{1}H NMR) and carbon (^{13}C NMR) spectra were compared with those already reported in the literature.

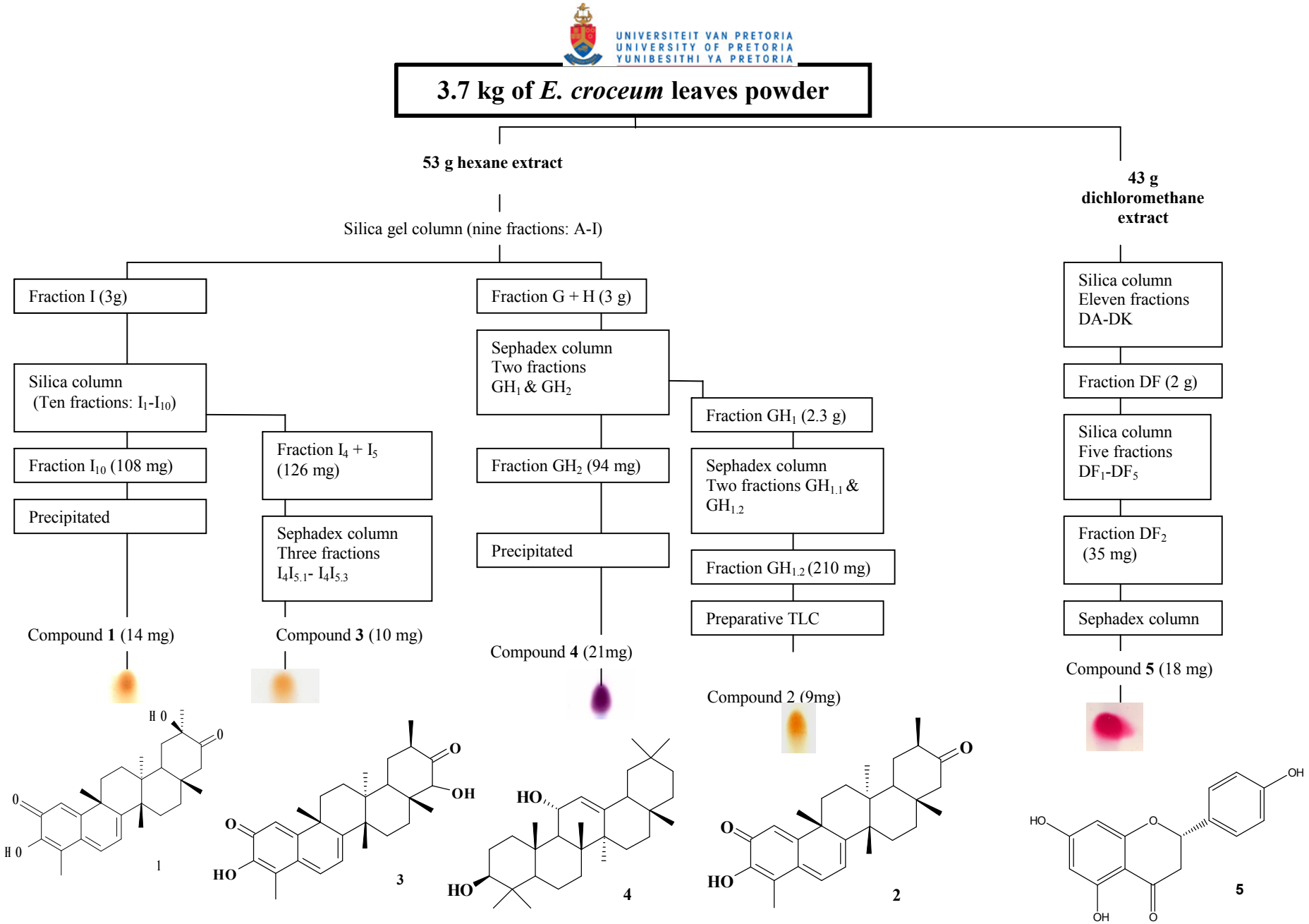

Figure 2.4: Schematic display of isolation steps and structures of the isolated compounds**:** 20-hydroxy-20-epi-tingenone (**1**), tingenone (**2**), tingenine B (**3**), 11α-hydroxy-β-amyrin (**4**), and naringenin (**5**).

2.3 Results

Dichloromethane, acetone, and n-hexane were the three solvents used during the extraction of 3.7 kg *E. croceum* dry leaves. Five compounds, **1** to **5** were collected from a fractionation of the three extracts. The percentage mass of the extracts and isolated compounds are given in Table 2.1. TLC plates of fractions collected from columns are shown in Figs. 2.5 to 2.9.

Table 2.1: Percentage mass of fractions and compounds extracted from 3.7 kg dry leaves of *E. croceum*

Extract / compound	Mass (%) extracted
Hexane	1.43
Dichloromethane	1.16
Acetone	2.81
20-hydroxy-20-epi-tingenone (**1**)	3.78×10^{-4}
Tingenone (**2**)	2.43×10^{-4}
Tingenine B (**3**)	2.70×10^{-4}
11α-hydroxy-β-amyrin (**4**)	5.68×10^{-4}
Naringenin (**5**)	4.86×10^{-4}

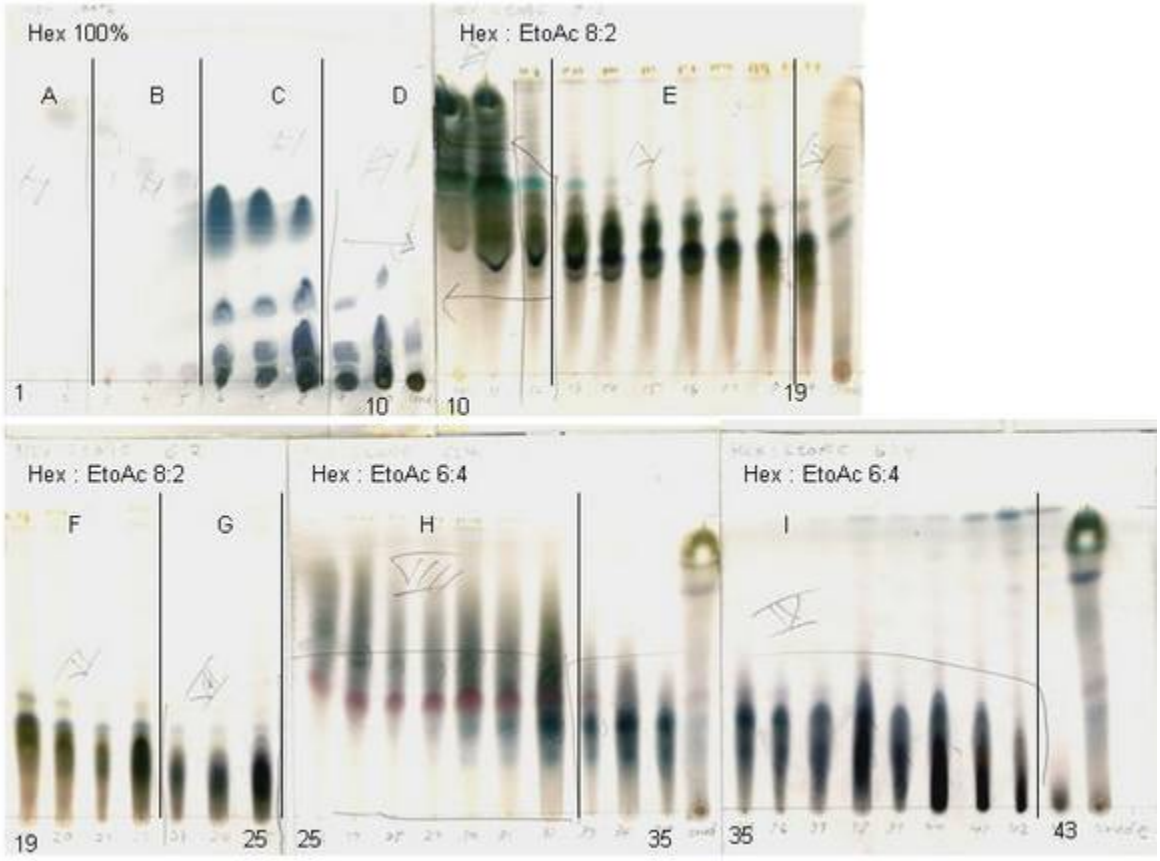

Figure 2.5: TLC plate of 43 fractions collected from hexane extract, were combined to form fractions: A to I.

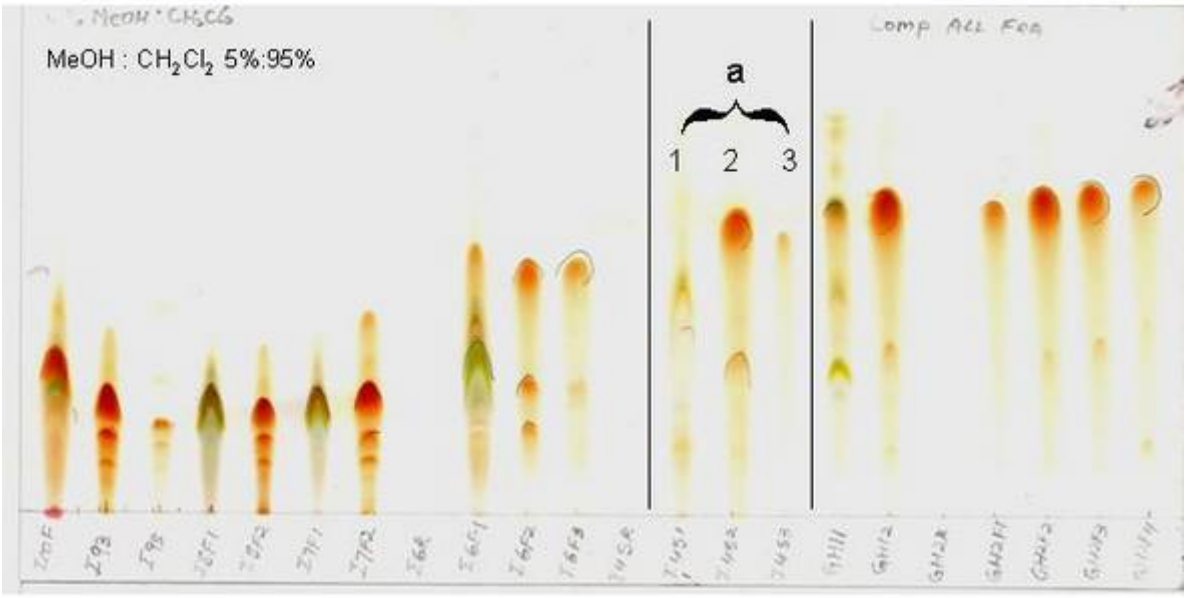

Figure 2.6: (a) Three fractions were collected from a column of fraction I_4I_5.

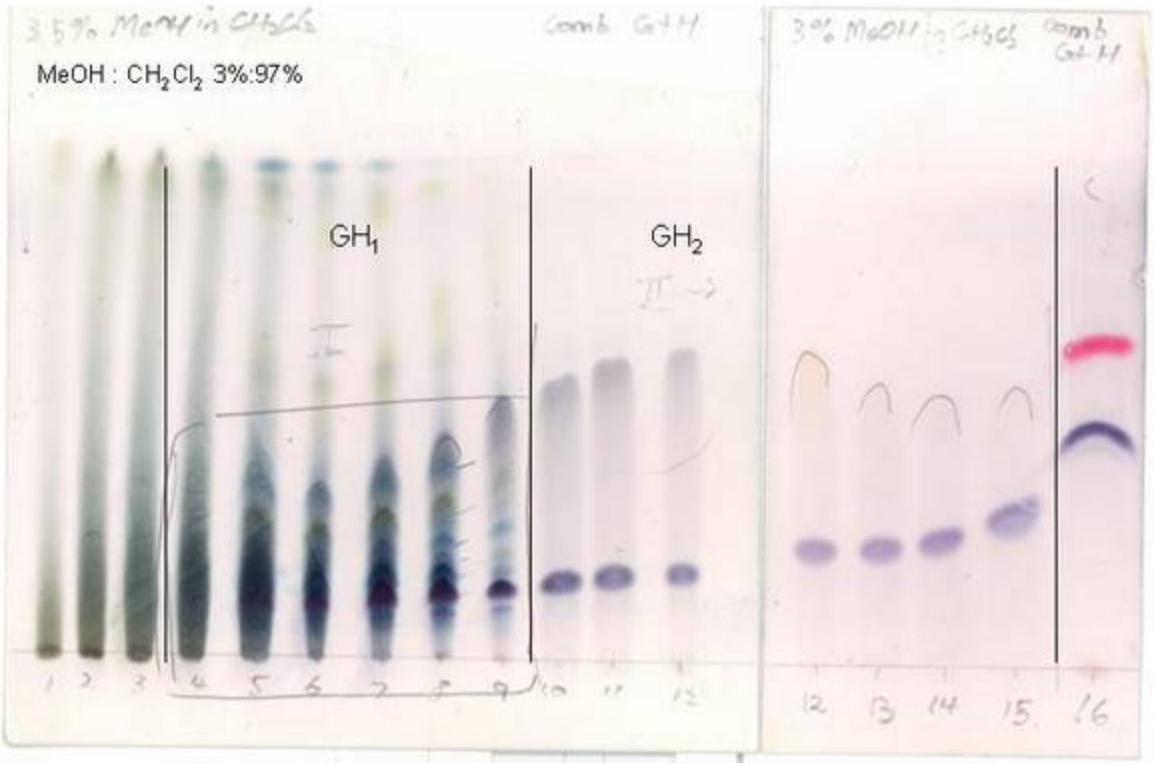

Figure 2.7: Sixteen fractions collected from a column of fraction GH, were combined to form fractions: GH_1 and GH_2.

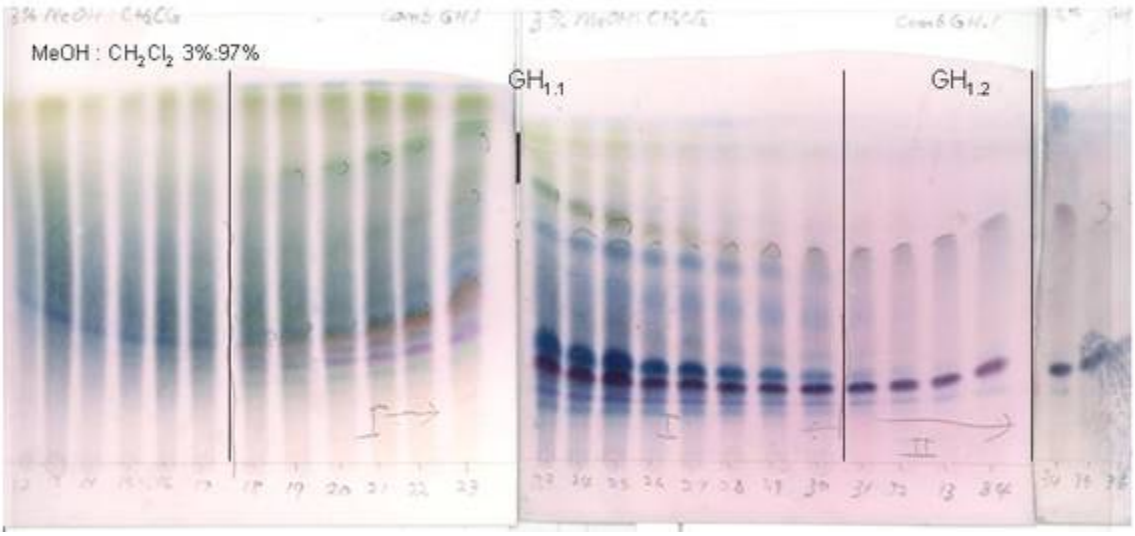

Figure 2.8: Thirty-six fractions colleted from a column of fraction GH_1 were combined to form fractions: $GH_{1.1}$ and $GH_{1.2}$.

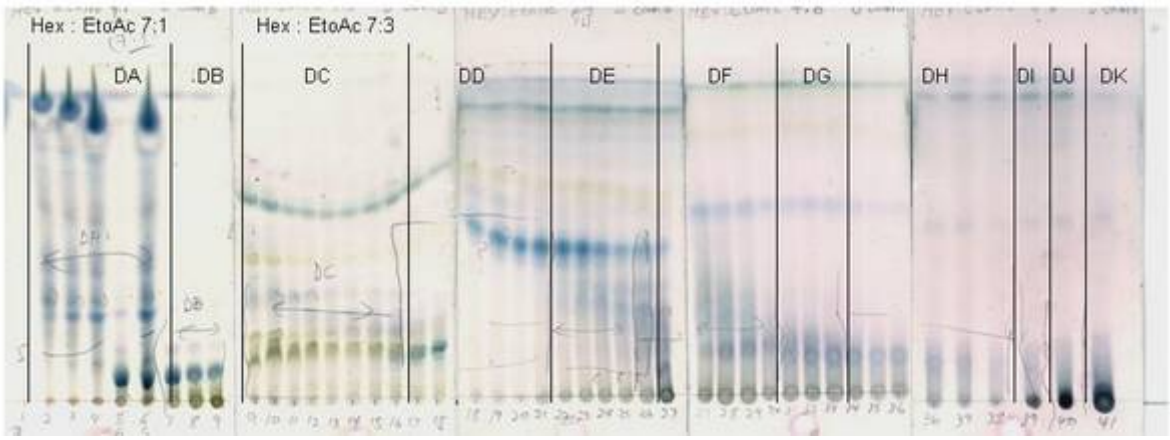

Figure 2.9: Forty-one fractions collected from the dichloromethane extract were combined to form 11 fractions (DA to DK).

2.3.1 20-hydroxy-20-epi-tingenone (**1**)

Compound **1** was identified as 20-hydroxy-20-epi-tingenone (De Oliveira et al., 2006). The compound has a molecular formula of $C_{28}H_{36}O_4$, which is deduced from LRMS (m/z β6.2) and a d - 115 Dept experiment. The ^{1}H NMR spectrum of **1** (Fig. 2.10) showed five methyl group singlet signals at 2.22, 1.48, 1.36 (2Me), 1.13, and 1.00, three olefinic protons appear at δ_H 7.01 (br d, J = 6.9 Hz), 6.36 (d, J = 6.9 Hz) and 6.53 (br s). The ^{13}C NMR spectrum, (Fig. 2.11) demonstrated 28 carbons, 8 olefinic (168.6, 164.3, 146.4, 133.6, 127.1, 119.1, 118.3 and 117.1) two carbonyl groups (215.7 and 178.4) one of them is conjugated with olefinic bonds and a hydroxylated bearing carbon at 73.7. Careful literature review on the chemistry of *E. croceum* showed that the forgoing data are only compatible with 20-hydroxy-20 epi-tingenone (**1**), which was isolated before from *Euonymus tingens* (Likhitwitayawuid et al., 1993; Brown et al., 1973) and *Glyptopetalum sclerocarpum* (Sotanaphun et al., 2005), both species belong in the Celastraceae family but not *Elaeodendron* genus.

The compound has been reported as highly toxic *in vitro* against: Vero cells (Sotanaphun et al., 2005), P-388 (murine lymphocytic leukemia), human fibrosarcoma (HT-1080), human oral epidermoid carcinoma (KB), human epidermoid carcinoma (A431), human gliobastoma (U373), human breast (BC-1), human colon (COL-2), human lung (LU-1), human melanoma (MEL-2), multidrug-resistant (KB-V1), human hormone-dependent breast (ZR-75-1), and prostate (LNCaP) cell lines (Ngassapa et al., 1994; Setzer and Setzer, 2003). This quinone-methide triterpenoid has shown a variety of biological activities such as antitumoral, antimicrobial, antibiotic, antimalarial, spermicidal (Corsino et al., 2000), and anti-inflammatory activities (Sotanaphun et al., 1998).

2.3.2 Tingenone (**2**)

Compound **2** was isolated as a reddish amorphous powder and identified as a tingenone (Furbacher and Gunatilaka, 2001). It demonstrated in ^{1}H NMR (Fig. 2.12) signals of six methyl groups at 2.21 (s, Me–23), 1.46 (s, Me-25), 1.32 (s, Me-26), 1.30 (d, 6.3Hz, Me-30), 0.96 (s, Me-27), 0.95 (s, Me-28); three olefinic protons at 7.03 (br d, 6.7Hz, H-6), 6.53 (br s, H-1), 6.36 (d, 6.7 Hz, H-7) and a signal of a proton at 5.27 (s, H-22). The NMR signals are similar to those of compound **1** except that ring E showed methyl (carbon 30) as a doublet signal and a proton at C-22 adjacent to the hydroxyl group, and the disappearance of the typical Hβ-22. Compound **2** can only fit the structure given in Fig. 2.4, which has identical data with the published data (Furbacher and Gunatilaka, 2001).

Tingenone (**2**) is a quinone-methide triterpene previously isolated from plants of the family Celastraceae, such as: the root epidermis of *Maytenus buchananii* (De Oliveira et al., 2006), stem bark of *M. chuchuhuasca* (Shirota et al., 1994), and *Kokoona ochraeu* (Ngassapa et al., 1994), the roots of *Acanthothamnus aphyllus* (Estrada et al., 1994), and *M. umbellata* (Gonzalez et al., 1992). It has been reported for cytotoxicity against L-1210 (Shirota et al., 1994), BC-1, HT-1080, LU-1, MEL-2, COL-2, A431, LNCAP, ZR-75-1, U373 (Ngassapa et al., 1994), and KB cells (human epidermoid carcinoma of the nasopharynx) (Kutney et al., 1981). Consequently, the compound may be of high priority for development as a cancer chemotherapeutic agent. It showed antibacterial activity against *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus* (Moujir et al., 1990), *Sarcinalutea sp, Microsporum gypseum* and against the Gram-negative bacterium, *Klebsiella pneumoniae* (Sotanaphun et al., 1999). The quinone-methide system at rings A / B of this compound is important for its bioactivity whereas the oxygenated substitution on

ring E is associated with potency (Sotanaphun et al., 2005). Under acidic conditions, the quinone-methide chromophore can easily rearrange to phenolic systems (Sotanaphun et al., 2005). The antibiotic activity of the compound may be associated with the presence of free hydroxyl groups in ring A (Gonzalez et al., 1996).

2.3.3 Tingenine B (**3**)

Tingenine B is also known as 22β-hydroxytingenone, (Kishi et al., 2003). It showed similar ^{1}H NMR signals (Fig. 2.13) to those of compounds **1** and **2,** six methyl groups 2.23, 1.51, 1.35, 1.22, 1.00, and 0.85, three olefinic protons at 7.05, 6.55, and 6.39 except for ring E which showed only a methyl group at 1.00 (d, 6.1) and a typical H-22β at 2.92 (d, 14.3 Hz). The forgoing data indicated that compound **3** is the well known tingenone which was isolated from *Salacia chinensis* (Kishi et al., 2003).

Tingenine B (**3**), a quinoid triterpene, has been previously isolated from the stems of *Salacia chinensis* (Hippocrateaceae) (Morikawa et al., 2003), stem bark of *M. chuchuhuasca* (Shirota et al., 1994), and *Tripterygium wilfordii*, (Takaishi et al., 1997), the last two mentioned species belong to the Celastraceae. Sotanaphun et al. (2005) reported that tingenine B (as 22b-hydroxytingenone) is highly toxic against Vero cells, it displayed inhibitory effects on rat lens aldose reductase (Morikawa et al., 2003), and cytotoxic activity against cultured P-388 tumor cell lines (Shirota et al., 1994).

2.3.4 11α-hydroxy-β-amyrin (**4**)

Compound **4** was identified as 11α-hydroxy- β-amyrin (Ikuta and Morikawa, 1992). The compound showed in its ^{1}H NMR (Fig. 2.14) spectrum eight methyl singlet signals at δ_H (1.20, 1.04, 0.98 (2Me), 0.87 (2Me), 0.82, 0.79) and an olefinic proton at 5.44 (d, J = 3.5 Hz), two hydroxymethine groups at δ 4.50 (dd, J = 3.5, 9.0 Hz) and 3.25 (dd, J = 4.9, 11.4 Hz). The ^{1}H NMR spectra was similar to that reported for the same compound by Ikuta and Morikawa (1992). Compound **4** (11α-hydroxy-β-amyrin) has been isolated from callus tissues of *Stauntonia hcxrrpbylka,* of Lardizabalacea, and not tested for biological activity (Ikuta and Morikawa, 1992).

2.3.5 Naringenin (**5**)

The ^{1}H NMR (Fig. 2.15) spectrum exhibited signals for three aromatic hydrogens at δ 6.93 (d, J = 8.9 Hz), 8.11 (dd, J = 8.9 and 2.4 Hz), and 8.56 (d, J = 2.4 Hz), indicative of a 3,4-disubstituted benzoic acid derivative. Additional signals included two doublets of doublets at δ 2.77 (1H, J = 17.4, 3.4 Hz) and 3.05 (1H, J = 17.4, 12.0 Hz), characteristic of an isolated R-carbonyl methylene group, and a doublet of doublet at δ 5.35 (1H, J = 12.0, 3.4 Hz). The compound (**5**) has three hydroxyl groups at C-7 in the A-ring, at C-4' in the B-ring, and at C-5 in A-rings (Ohmura et al., 2000; Lee et al., 2001).

The flavonoid, naringenin (**5**) has been isolated from twigs and leaves of *Eriodictyon califmnicum* (Hydrophyllaceae) (Liu et al., 1992), *Piper crassinervium* (Piperaceae) (Lago et al., 1992) and many other species. It has been reported that the compound inhibited the metabolism of the carcinogen, benzo-*a*-pyrene (Liu et al., 1992), and has antifungal activity against *Cladosporium cladosporioides* (Lago et al., 1992).

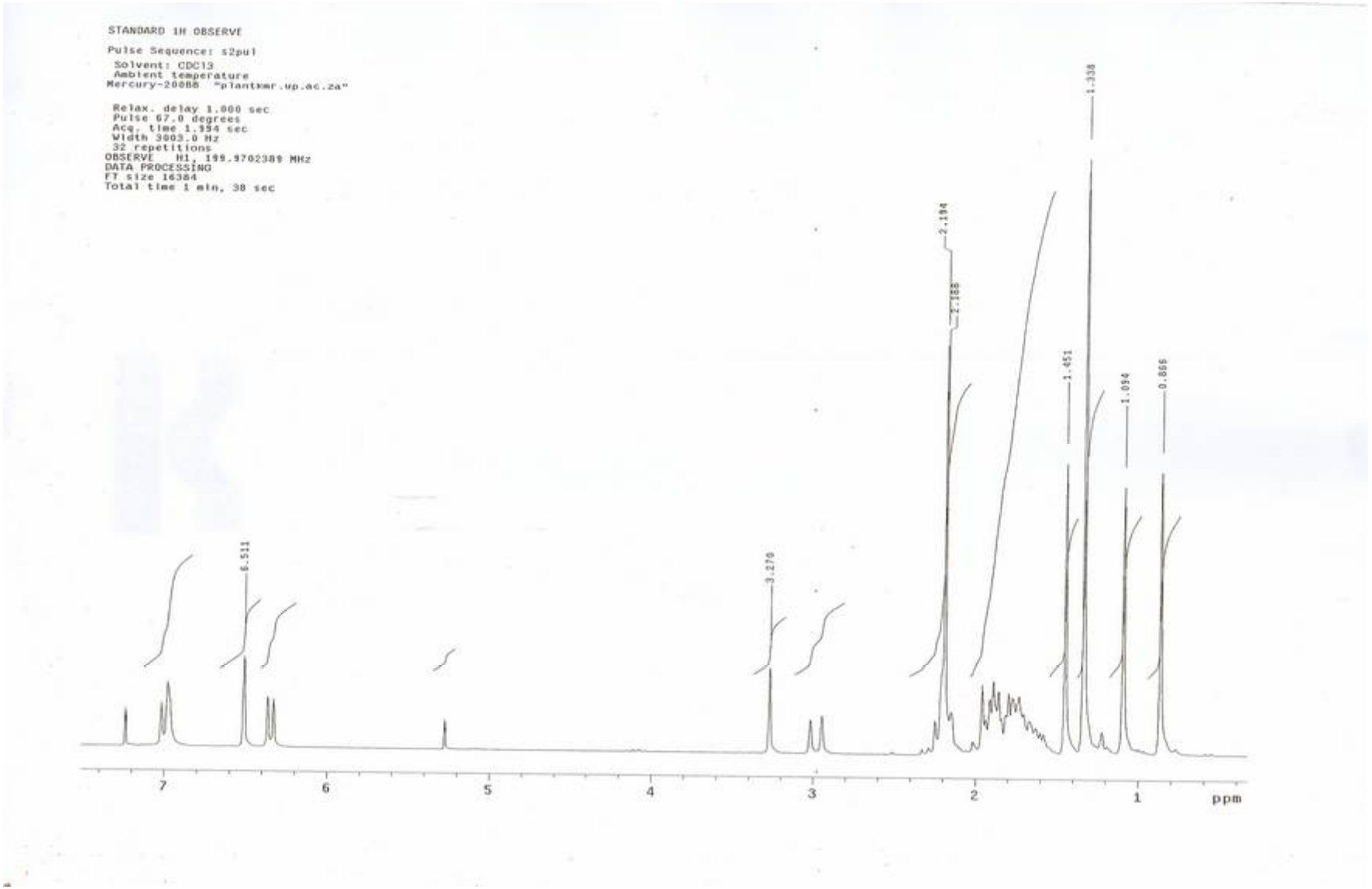

Figure 2.10: Proton NMR spectrum of 20-hydroxy-20-epi-tingenone (**1)**

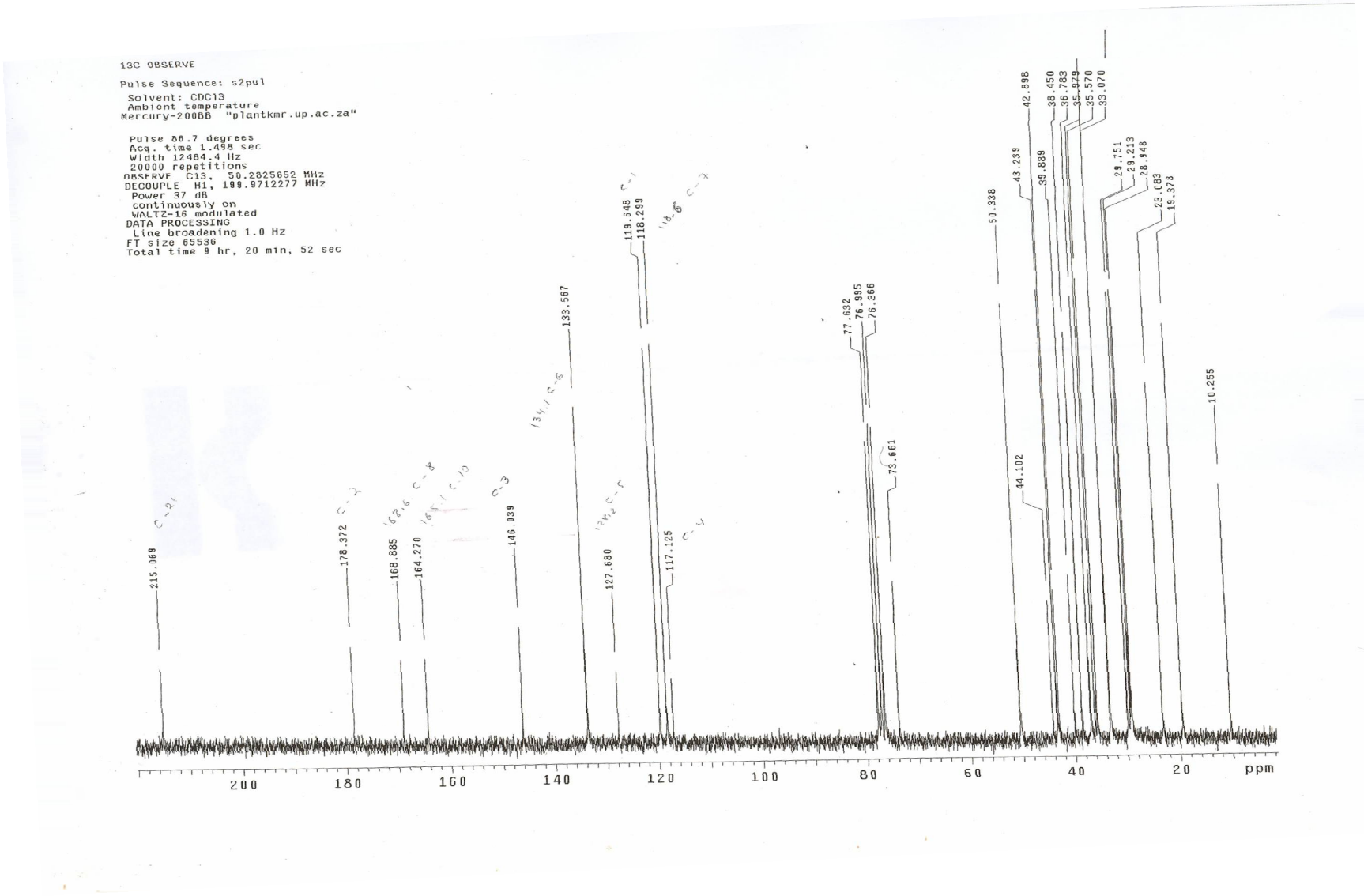

Figure 2.11: Carbon NMR spectrum of 20-hydroxy-20-epi-tingenone (**1**)

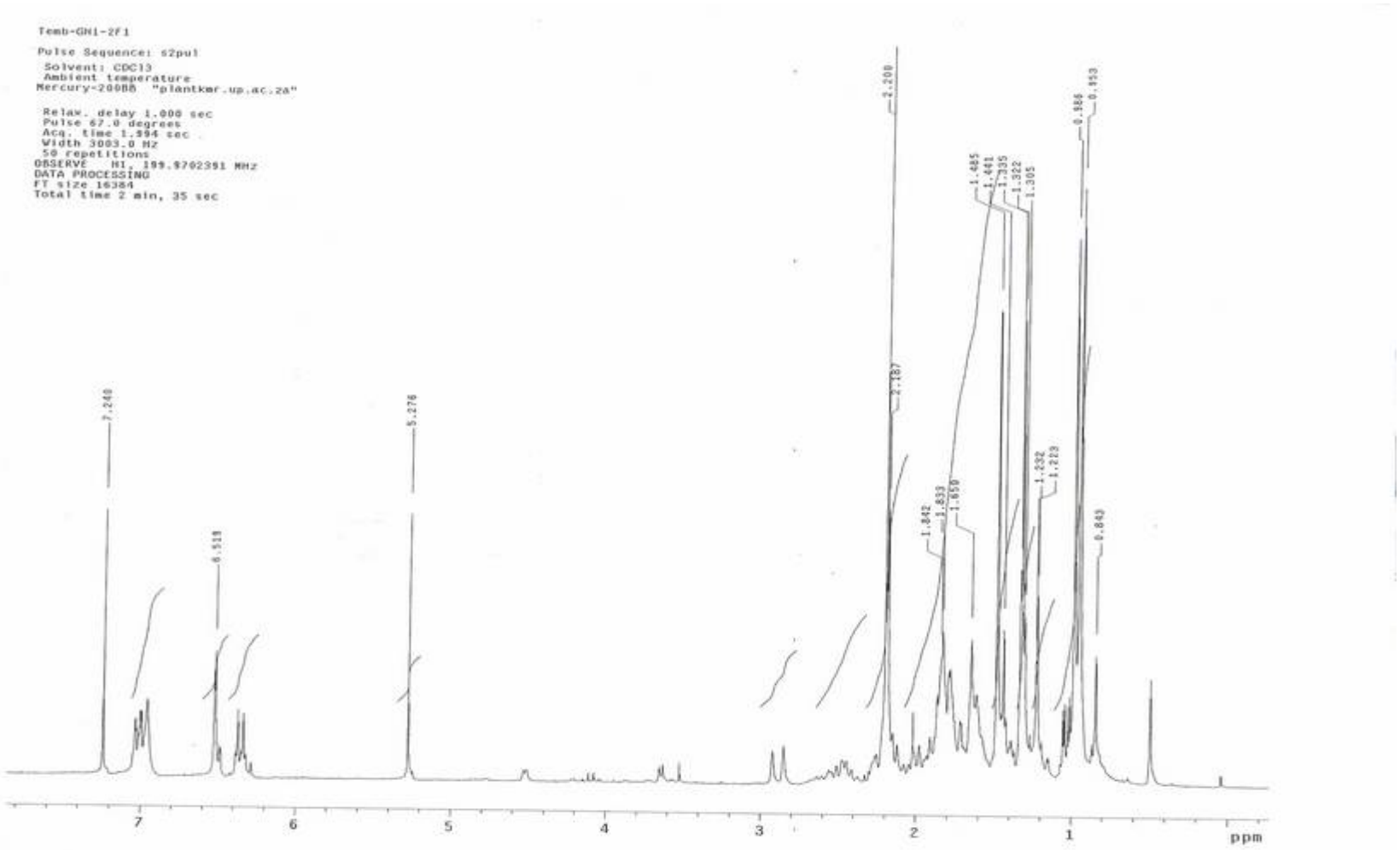

Figure 2.12: Proton NMR spectrum of tingenone (**2)**

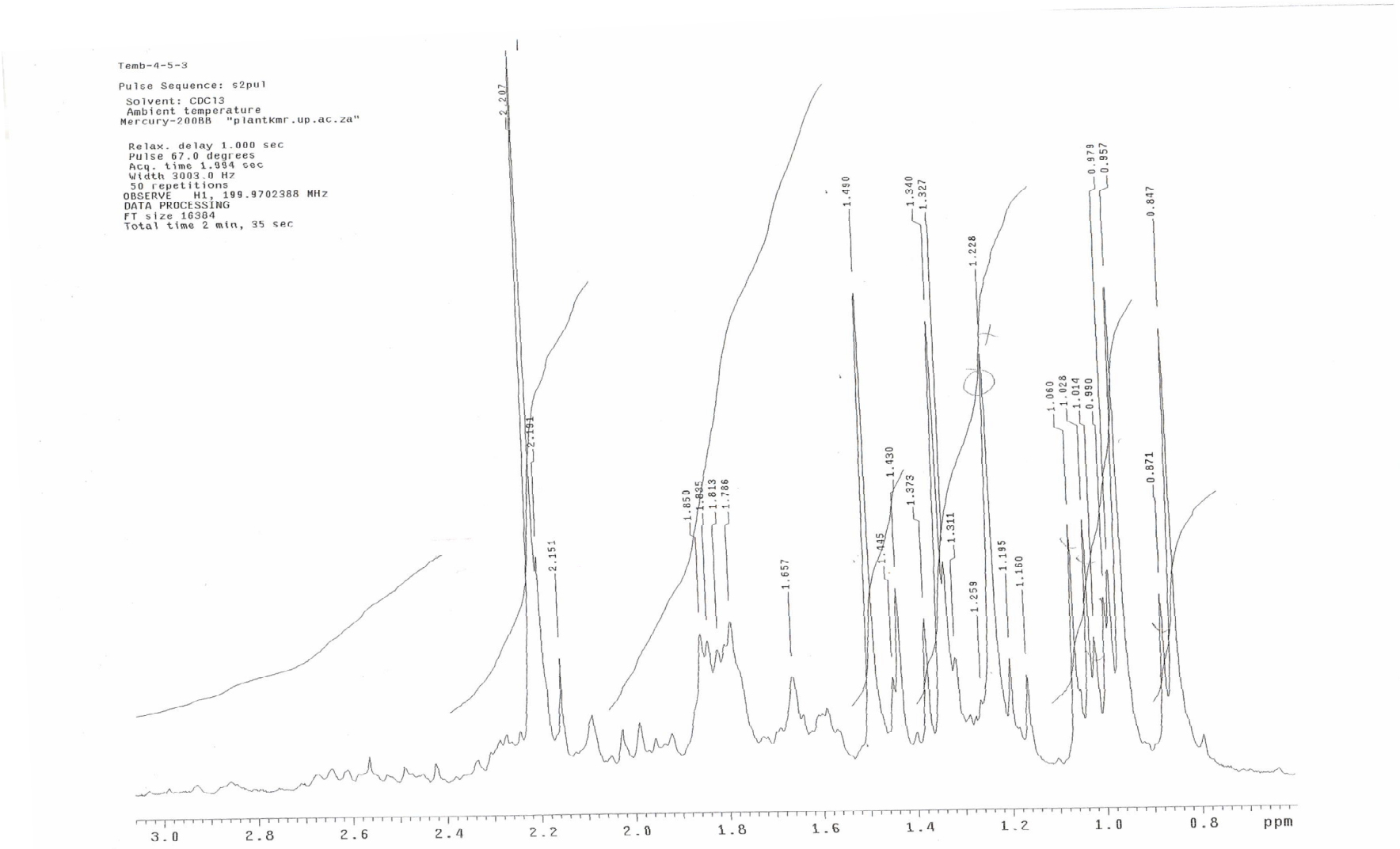

Figure 2.13: Proton NMR spectrum of tingenine B **(3)**

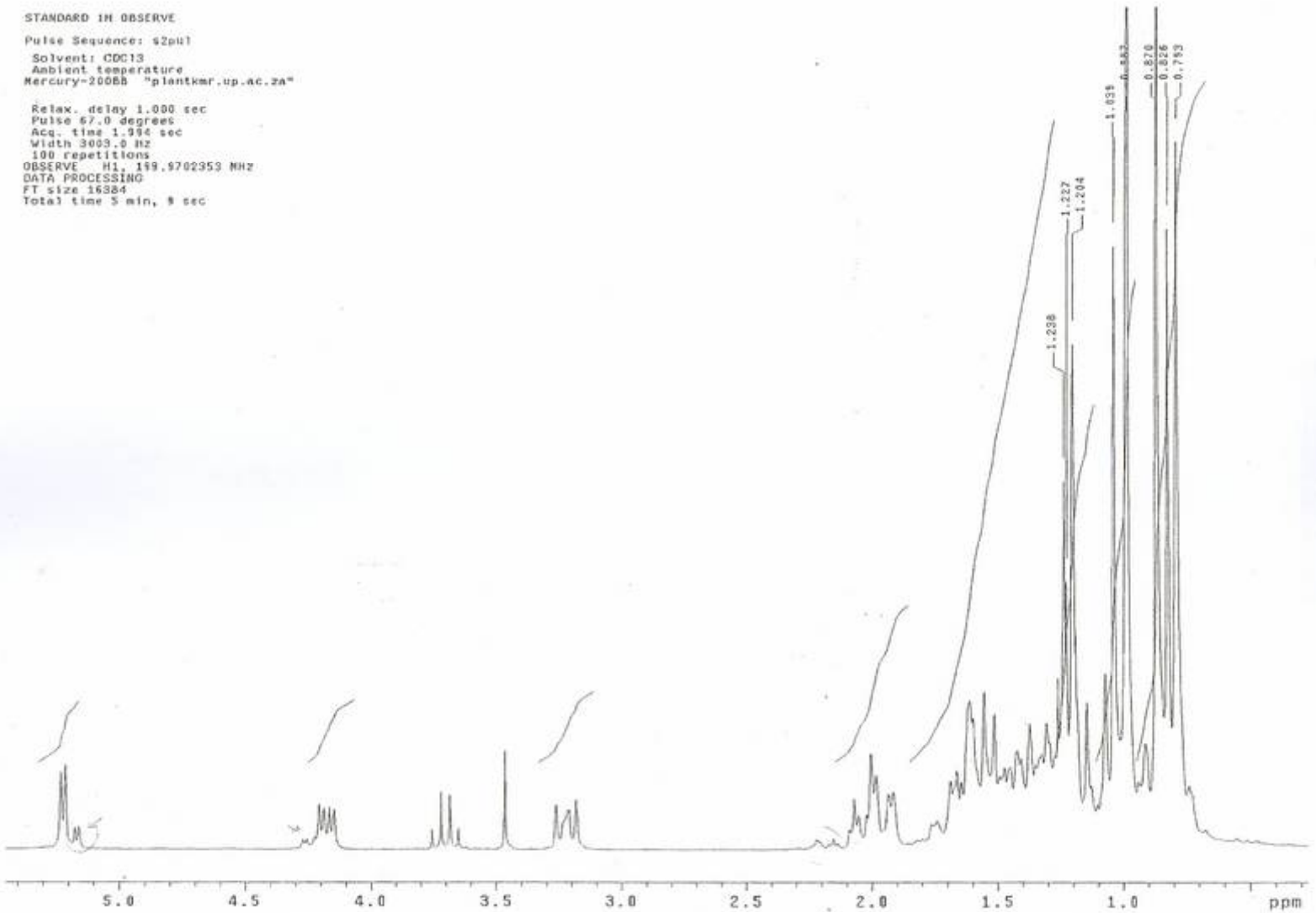

Figure 2.14: Proton NMR spectrum of 11α-hydroxy-β-amyrin (**4)**

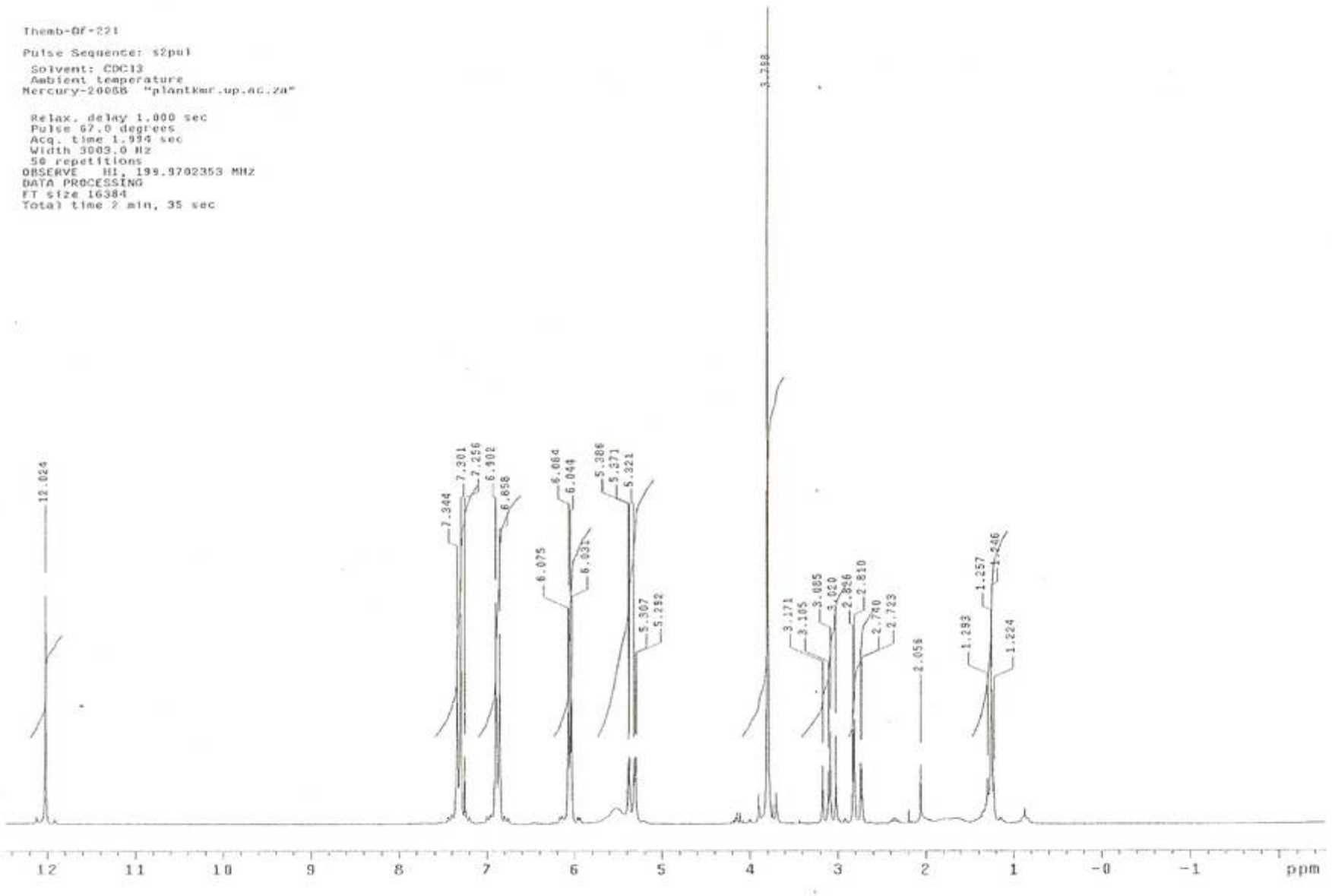

Figure 2.15: Proton NMR spectrum of naringenin (**5)**

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CHAPTER 3: CYTOTOXICITY OF *ELAEODENDRON CROCEUM* CRUDE EXTRACTS AND ISOLATED COMPOUNDS

3.1 Introduction

Plants synthesize various primary compounds for their establishment and reproduction. Being members of the ecosystem, plants are faced with a challenge to defend themselves against herbivory and competition with other plant species. Some plants use a physical defence strategy (Ingrouille 1992) while other plants use a chemical defence system to hinder plant-eating animals and sometimes even kill them. Chemical defence is based on the synthesis of secondary compounds (Van Wyk et al. 2002). Tropical rainforest plants are known to have higher concentrations and a greater diversity of chemical defence than plants from any other biome, and are also a potential source of new medicines (Ajaiyeoba et al. 2005). The harmful chemicals can either cause irritation or discomfort through skin contact or serious poisoning when ingested in sufficient quantities. According to statistics of the Poison Unit of the Johannesburg General hospital, plants are responsible for about 6.5% of all poisoning cases (Van Wyk et al. 2002).

The true incidence of human poisoning in South Africa is not well documented. The largest number of acute poisoning occurs in the age group 1-5 years. Sometimes adults are poisoned by confusing a toxic plant with something thought to be edible. Symptoms of human poisoning may vary depending on the type of plant and

quantity eaten. Human responses to plant poisoning might be pains, cramps, diarrhea, difficult breathing, quick irregular pulse and many more (Van Wyk et al. 2002).

Small amounts of toxins eaten by herbivores can be broken down by microorganisms in the rumen. Hence monogastric animals such as pigs and birds are often more sensitive than ruminants such as cattle and goats. Animal poisoning increases when animals are moved from one place to another, where they come into contact with toxic plants they have not yet learnt to avoid (Van Wyk et al. 2002).

Plants can be subdivided into those which cause sudden death and those which cause sub-acute to chronic poisoning. Poisonous plants are generally categorized according to the body system damaged, such as circulatory, digestive, reproductive, nervous, and lymphatic (Tokarnia et al. 2002).

E. croceum was one of the selected poisonous plants screened during a previous study (not published), which showed interesting toxicity results. Literature reports and traditional healers confirmed the poisonous nature of *E. croceum.* Astonishingly, no literature report had any details about the isolation of toxic compounds from this species.

Cytotoxicity analysis of *E. croceum* leaf extracts and the five isolated compounds; 20-hydroxy-20-epi-tingenone (**1**), tingenone (**2**), tingenine B (**3**), 11α-hydroxy-β-amyrin (**4**), and naringenin (**5**) was done *in vitro* against Vero cells and three human cancer cell lines, HeLa, MCF-7, and SNO for anticancer activity.

3.2 Methods

3.2.1 XTT toxicity assay

The XTT assay is suitable for measuring cell proliferation, cell viability or cytotoxicity. The tetrazolium salts are converted into a coloured formazan product by cellular enzymes present in the mitochondria of a metabolically active cell. The formazan dye is soluble in aqueous solutions, and is quantified using a scanning multiwell spectrophotometer (ELISA reader). These enzymes are rapidly inactivated when a cell dies, and hence the activity of these enzymes can be used to monitor the viability of a cell (Wenk and Fernandis, 2007).

Cells were grown in a 96 well microtitre plate and incubated with the yellow XTT solution for 1 to 1.5 hours. After this incubation period, the orange formazan solution is formed. An increase in the number of living cells results in an increase in the overall activity of mitochondrial dehydrogenase in the sample. This increase directly correlates to the amount of formazan formed, and is monitored by the absorbance at a wavelength of 450 nm (Williams et al., 2003).

3.2.2 Cell culture

Four cell lines, Vero (monkey kidney), HeLa (human cervix cancer), SNO (human oesophagus cancer), and MCF-7 (human breast cancer) were cultured separately in minimal essential medium (Eagle), (MEM), containing 1.50 g/L sodium bicarbonate, 2.00 mM L-glutamine, 0.10 mM non-essential amino acids, 1.00 mM sodium pyruvate, 10.00 µg/ml penicillin, 10.00 µg/ml streptomycin, 0.25 µg/ml fungizone, and 10 % fetal bovine serum. Cells were grown in a humified incubator

at 37 ^{0}C under 5 % CO_2, and then harvested by trypsinization (Williams et al., 2003).

3.2.3 Preparation of cells for toxicity screening

Confluent cells were trypsinized and diluted in complete MEM to a concentration of 1×10^5 cells/ml. In the outer wells of a 96 well plate, 200.00 µl of medium was dispensed. All inner wells received 100.00 µl of cell suspension. The plates were incubated overnight at 37 ^{0}C in a humidified atmosphere with 5 % CO_2.

3.2.4 Preparation of final concentrations of compounds

Stock solutions of compounds and extracts were prepared in dimethyl sulphoxide (DMSO) at 20.00 mg/ml. For compounds, the solutions were diluted 100 times in complete medium to 200.00 µg/ml. This was then serially diluted to obtain eight different concentrations. For extracts, the solutions were diluted 50 times in complete medium to 400.00 µg/ml. This was then serially diluted to obtain eight different concentrations.

3.2.5 Toxicity screening

On day one, 100.00 µl of each compound was dispensed into cell-containing wells of the sample plates in triplicate. The final concentrations of crude extracts in the wells were 3.13, 6.26, 12.50, 25.00, 50.00, 100.00, 200.00 and 400.00 µg/ml. Compounds had final concentrations of 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 µg/ml. Control wells received a final concentration of 0.5 % (for compounds) and 1 % (for extracts) DMSO in complete medium. Reference plates contained 100.00 µl of medium and 100.00 µl of dilute compound and were prepared in duplicate. Doxorubicin and Zearalenone were used as positive toxicity

controls. Plates were returned to 37 ^{0}C in humidified atmosphere with 5 % CO_2 for three days incubation. On day four, 50.00 µl of XTT reagent was added to the wells and incubated for four hours. The optical densities of the wells were measured at 450 nm (690 nm reference wavelength).

3.3 Results

Successive extraction of the dried leaves showed that hexane, dichloromethane, and acetone extracts were all toxic. Phytochemical studies of the three extracts (Fig. 3.1 and 3.2) resulted in the isolation of five toxic compounds. Significant toxicity of the isolated compounds against Vero cells (IC_{50} values presented in Table 3.1, and graphs in Fig. 3.3 to 3.7) was demonstrated by 20-hydroxy-20-epi-tingenone (**1**) at 2.651 nM and tingenone at 8.233 µM. The toxicity demonstrated by the isolated compounds on Vero cells prompted us to evaluate the compounds on different human cancer cell lines.

Compounds **1** and **2** (tingenone) showed potent activities against the tested cancer cell lines. Compound **2** was 3.4 times more toxic to HeLa and SNO cells and about 8 times more toxic to MCF-7 cells than to Vero cells, indicating its potential as an anticancer drug. Compound **2,** has a similar level of toxicity (IC_{50} 2.435 µM) as the potent anticancer drug, vincristine, on HeLa cells (IC_{50} 4.56 µM) (Lu et al., 2005). Consequently further studies on the toxicity mechanism of compound **2** may lead to the development of an anticancer drug.

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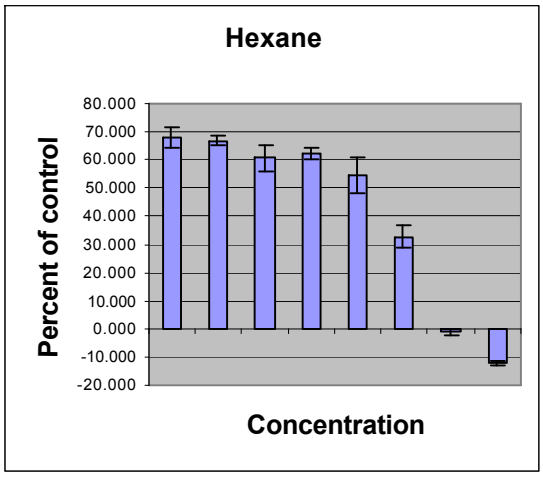
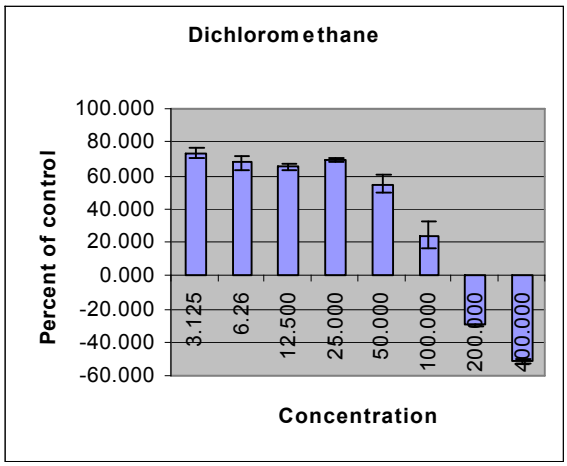
Table 3.1: IC_{50} toxicity values of *E. croceum* extracts, compounds: 20-hydroxy-20-epi-tingenone (**1**), tingenone (**2**), tingenine B (**3**), 11α-hydroxy-β-amyrin (**4**), and naringenin (**5**).

Extract / Fraction / Compound	IC_{50} ±SD			
	Vero	HeLa	SNO	MCF-7
Hexane	46.60 µg/ml ± 2.66	nt	nt	75.44 µg/ml ± 1.63
Dichloromethane	51.28 µg/ml ± 3.73	nt	nt	43.71 µg/ml ± 3.89
Acetone	56.11 µg/ml ± 4.22	nt	nt	126.4 µg/ml ± 3.24
Fraction I	37.44 µg/ml ± 2.30	nt	nt	23.06 µg/ml ± 0.27
Fraction GH	28.53 µg/ml ± 2.45	nt	nt	3.05 µg/ml ± 0.25
Fraction E	61.79 µg/ml ± 0.51	nt	nt	26.78 µg/ml ± 1.43
Compound **1**	2.651 nM ± 0.747	2.011 µM ± 1.511	0.427 µM ± 0.910	0.600 µM ± 1.827
Compound **2**	8.233 µM ± 4.169	2.435 µM ± 0.476	2.478 µM ± 6.669	< 1.859 µM
Compound **3**	0.130 mM ± 0.003	0.036 mM ± 0.006	0.046 mM ± 0.006	0.075 mM ± 0.003
Compound **4**	0.192 mM ± 0.009	0.042 mM ± 0.010	0.057 mM ± 0.007	0.056 mM ± 0.005
Compound **5**	0.188 mM ± 0.002	0.447 mM ± 0.017	0.452 mM ± 0.024	0.057 mM ± 0.004
Doxorubicin	2.277 µM ± 0.170	0.014 µM ± 0.196	0.008 µM ± 0.894	0.015 µM ± 0.312
Zearalenone	8.223 µM ± 1.158	7.422 µM ± 2.345	7.221 µM ± 4.501	7.422 µM ± 1.532

nt = not tested



(a)



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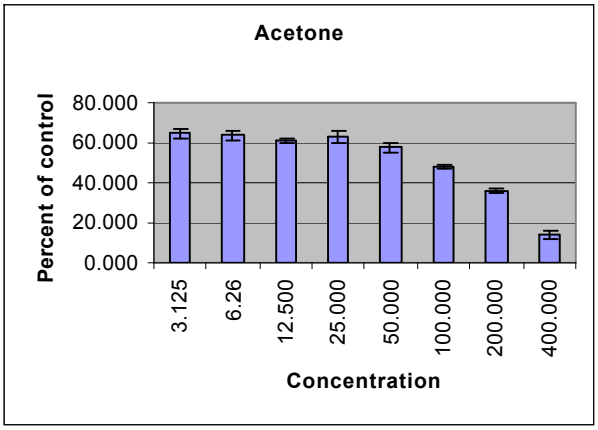
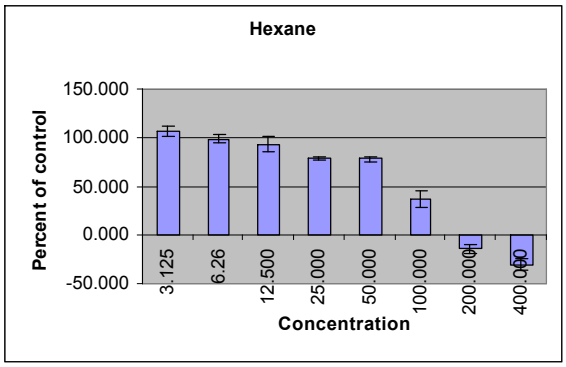
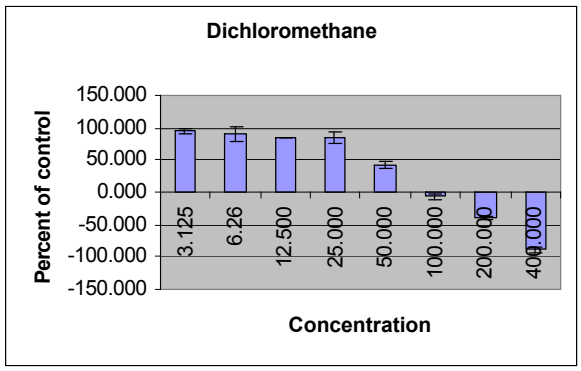


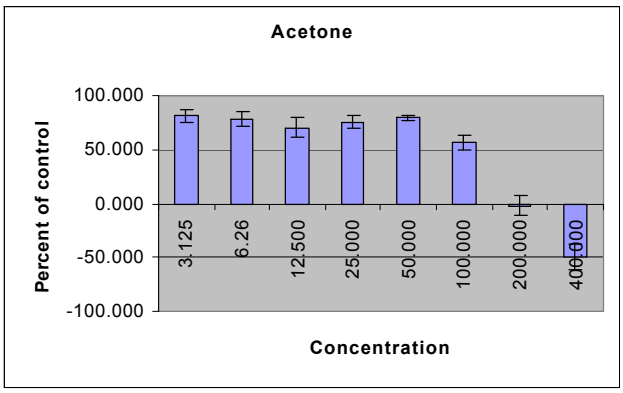
(c)

Figure 3.1: Toxicity of (a) Hexane, (b) Dichloromethane, and (c) Acetone extracts in µg/ml on Vero cells.



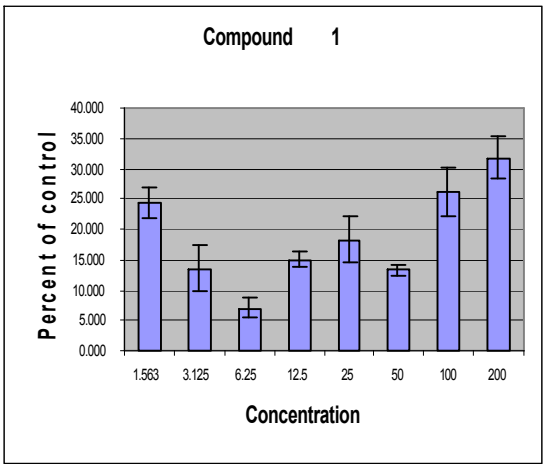


(a) (b)

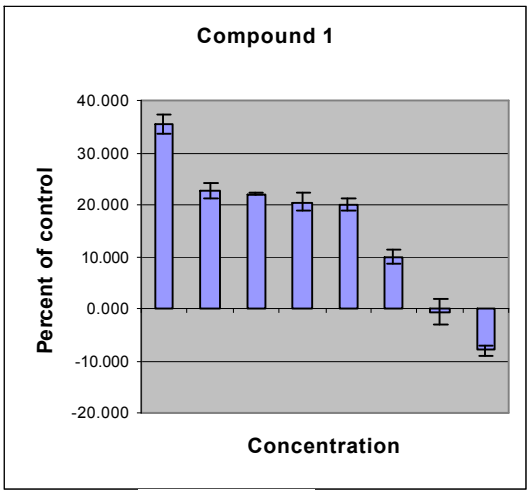


(c)

Figure 3.2: Toxicity of (a) Hexane, (b) Dichloromethane, and (c) Acetone extracts in µg/ml on MCF-7 cells



(a)



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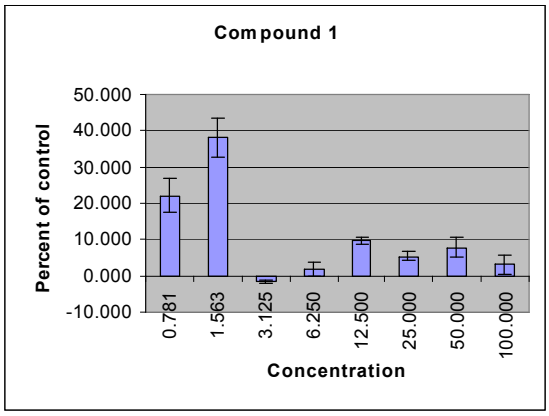

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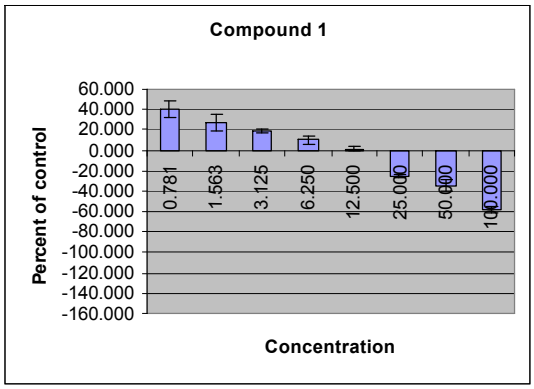
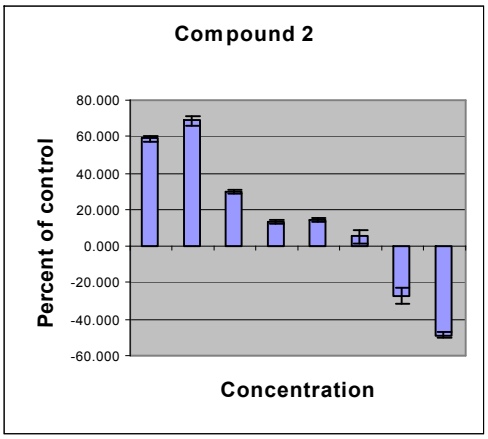


(d)

Figure 3.3: Toxicity of 20-hydroxy-20-epi-tingenone on: (a) Vero cells, (b) HeLa cells, (c) SNO cells, and (d) MCF-7 cells in µg/ml.



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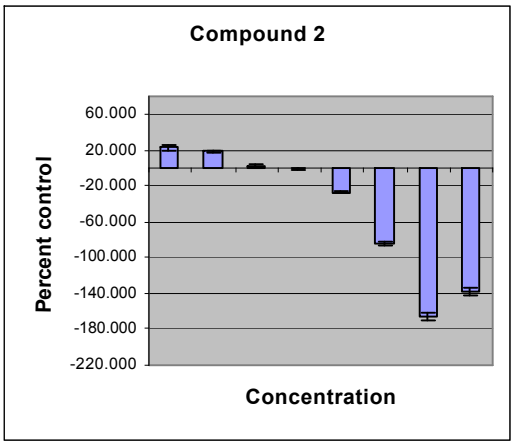

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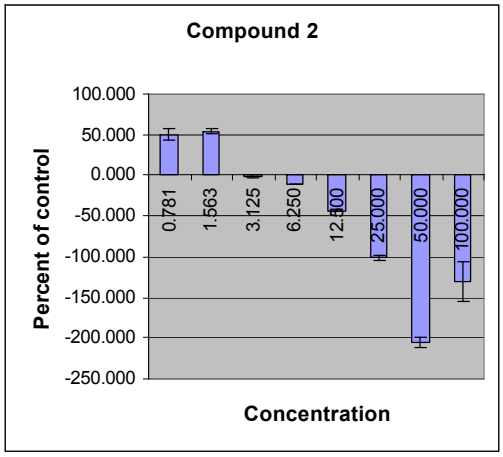

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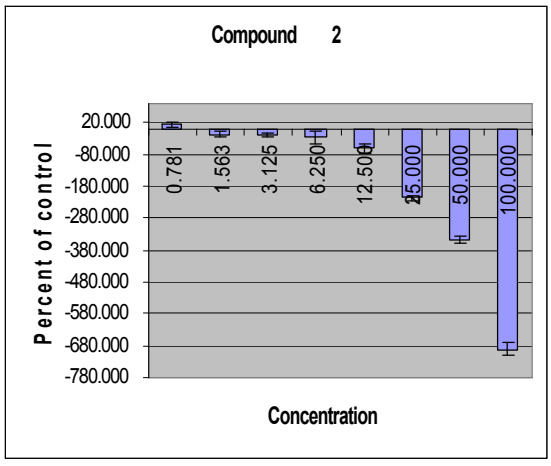
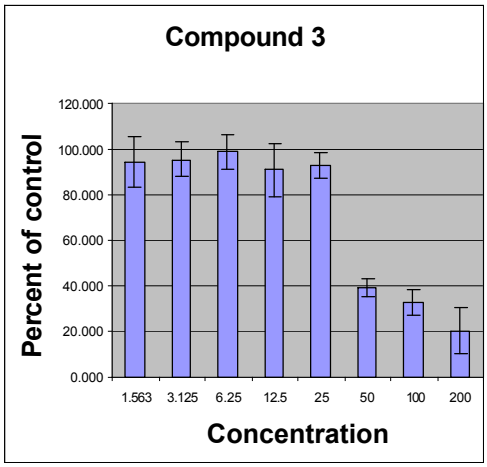


(d)

Figure 3.4: Toxicity of tingenone on: (a) Vero cells, (b) HeLa cells, (c) SNO cells, and (d) MCF-7 cells in µg/ml.



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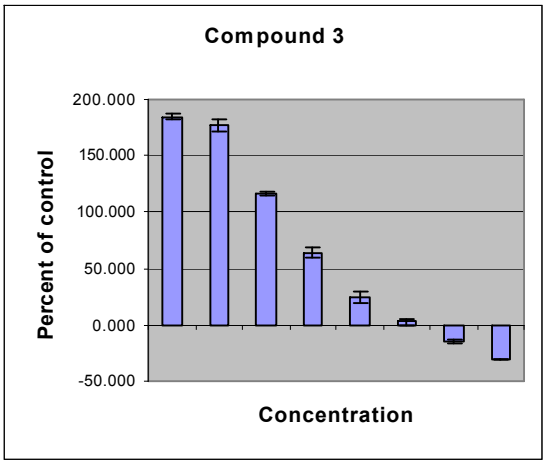

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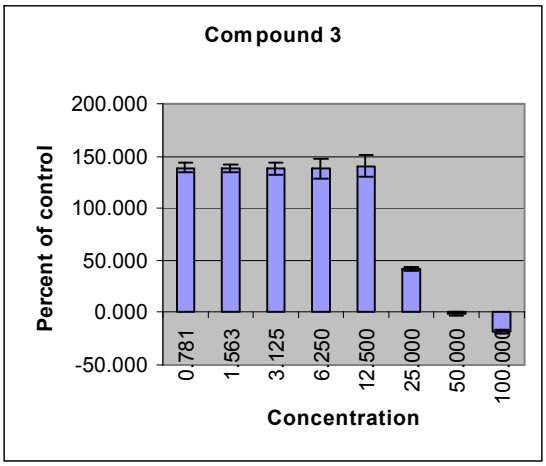

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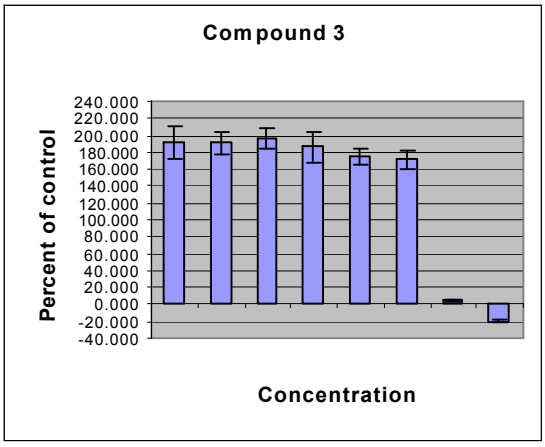
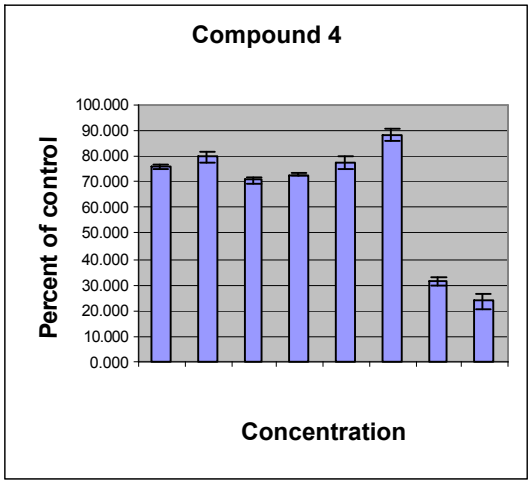


(d)

Figure 3.5: Toxicity of tingenine B on: (a) Vero cells, (b) HeLa cells, (c) SNO cells, and (d) MCF-7 cells in µg/ml.



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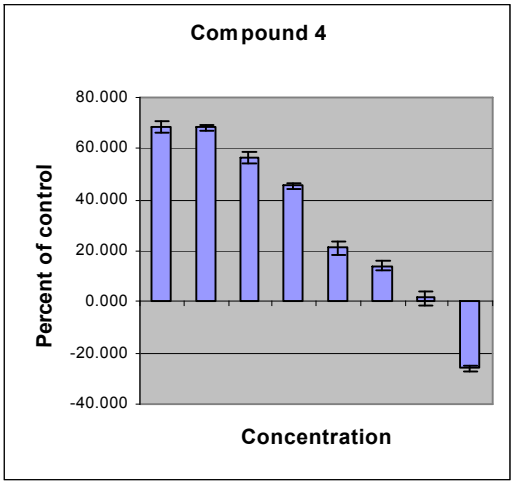

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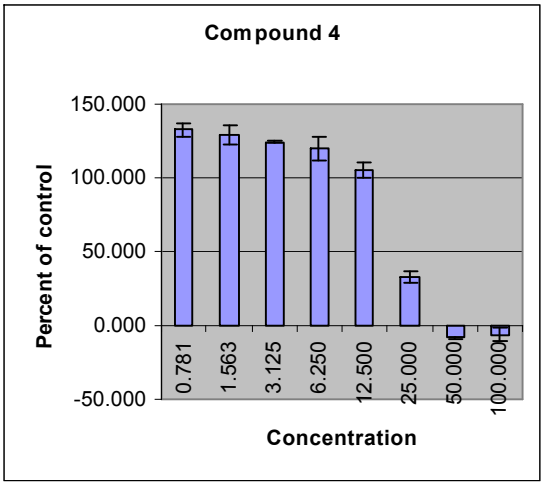

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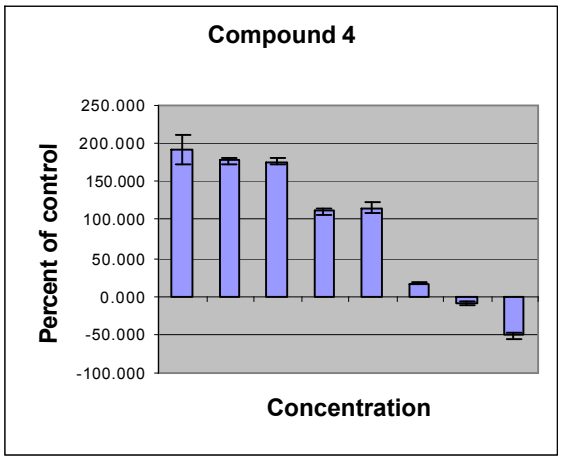

(d)

Figure 3.6: Results for 11α-hydroxy-β-amyrin on: (a) Vero cells, (b) HeLa cells, (c) SNO cells, and (d) MCF-7 cells in µg/ml.

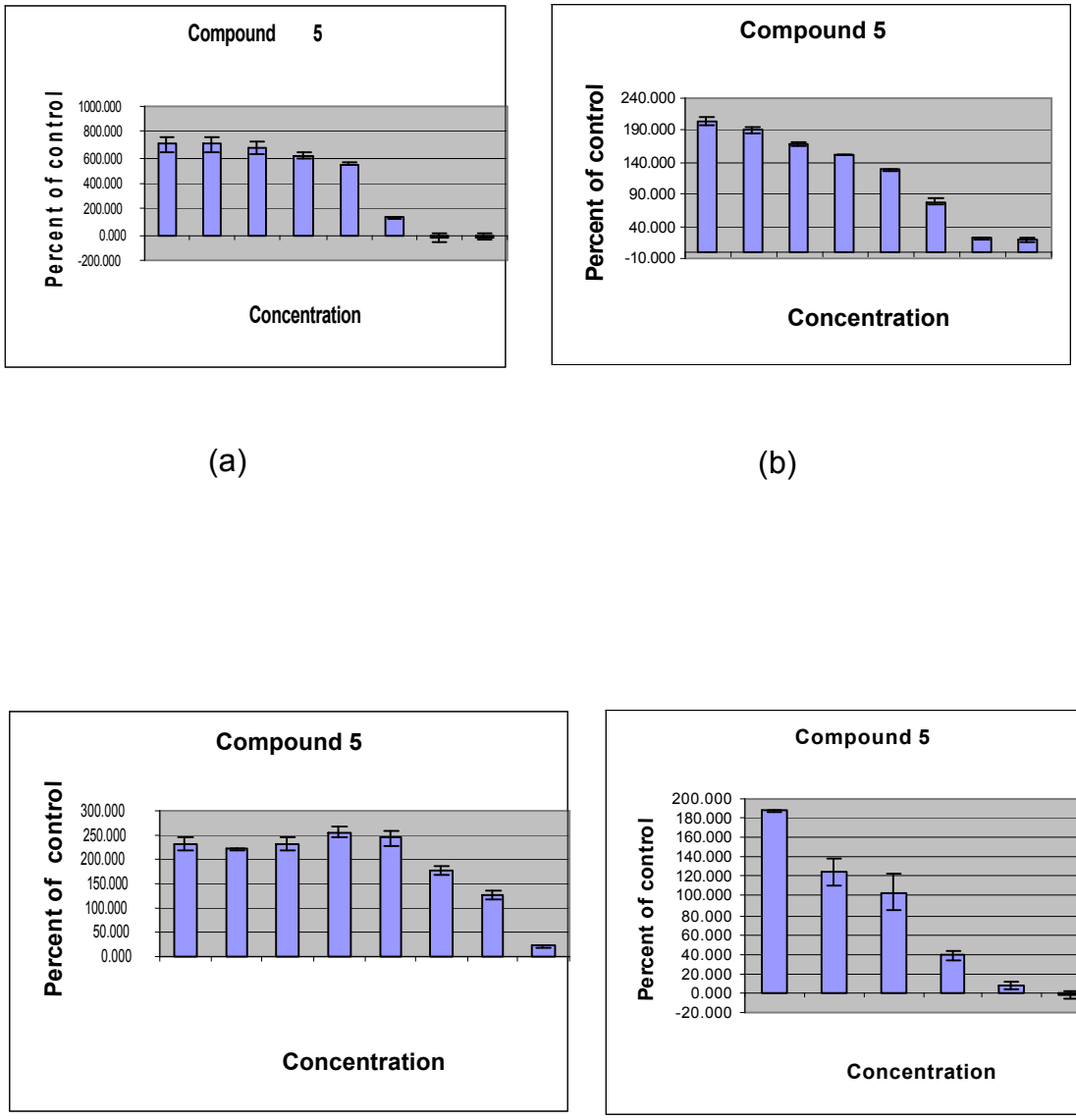

Figure 3.7: Results for naringenin on: (a) Vero cells, (b) HeLa cells, (c) SNO cells, and (d) MCF-7 cells in µg/ml.

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CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

4.1 Introduction

Many plants produce compounds that interfere with the metabolism of living organisms and can exert toxic actions directly or indirectly. Only a small group of plants can give rise to serious poisoning after the ingestion of a limited amount of plant material. Other plants are considered toxic only under bulk consumption. There are plants that do not have documented cases of serious poisoning but are accepted as poisonous (Frohne et al., 2005).

The long known principle '*dosis sola facit venenum*' (Latin: all things are poison and nothing is without poison; only the dose makes a thing not a poison) is as true for 'poisonous plants' as it is for 'poisons'. However, it also has to be realized that the content of active compounds, and hence the toxicity, in individual plants of a particular species can be subject to qualitative and quantitative fluctuations. These are, in part, determined genetically or are dependent on the locality or other environmental conditions, or on the age of the plant or particular plant part, i.e. the degree of maturity. Thus, for example, roots of perennials as organs of storage are often more dangerous in winter or early spring than in summer or autumn in certain plants such as *Aconitum* and *Cicuta* (Frohne et al., 2005).

In fruits, the content of toxic principles often decreases during the ripening process. In other respects the contradictory statements on the toxicity of fruits can also be explained when no distinction is made between the seeds and the pericarp. Thus, cherry laurel, mezereon and yew contain toxic substances in their seed, whereas the fruit pulp (and the testa) is largely free of them. On the other hand, in the opium poppy the opium alkaloids (as components of the latex) can be found in a high concentration in the wall of the unripe capsule, but the seeds contain hardly detectable quantities (Frohne et al., 2005).

Preconditions for poisoning are that:

- There should be a sufficiently high dose of toxic constituents to exert their activity in or on the body,
- The defence mechanisms or detoxification processes of the body should be overcome.

Many plants have only became known poisonous plants in a given region after they have taken their place as a garden or ornamental plant in the human environment, e.g. *Dieffenbachia* and *Prinada obconica* (Frohne et al., 2005).

4.2 Toxicity of Celastraceae and isolated compounds

Since time immemorial, humans have primarily depended on herbs in the treatment of diseases. It is estimated that less than 1% of the plants in developing countries are utilized as medicine, although one third of all world pharmaceuticals are of plant origin. About 80% of the 5 200 million people in the world live in under-developed countries (Rahman, 2000). The World Health Organization estimates that these people rely almost exclusively on traditional medicine for

their primary health-care needs. Since medicinal plants are the 'backbone' of traditional medicine, this means that more than 3 300 million people in the under-developed countries utilize medicinal plants on a regular basis. Therefore, for this segment of the world population, who are generally unable to afford the costs of 'Western' drugs, there is a need to study these plants for safety and efficacy and to develop Galenical (medicinal preparation composed mainly of herbal or vegetable matter) products that are standardized and stable (Rahman, 2000). On the other hand, from a developed country's point of view; plants are chemical factories that produce a vast array of unusual chemical structures that display a variety of biological activities (Rahman, 2000).

The Celastraceae are known for their toxic compounds and toxic triterpenes from the Celastraceae include:

- Lantadenes A and B, and triterpene acids found in *Lantana.*
- Loterogenins A, B, and C found in *Lippia*.
- Cucurbitacins, tetracyclic triterpenes found in *Cucumis africannus* and *C. myriocarpus.*
- Meliatoxins A and B, tetranortirterpenes, derived form the berries of *Melia azederach.*
- Colocynthin, a glucoside found in the fruit of the vine *Citrullus colocynthis*. (Radostits et al., 2000).

Loterogenins and Lantadenes cause liver damage and nephrosis, neither of which is specific, but the lantadenes cause damage to bile canaliculi, gallbladder paralysis, and intrahepatic cholestasis. Cucurbitacins are a group of tetracyclic triterpenes found in the fruits of the vines of *Cucumis africanus*. The ripe fruits are

most toxic, and in cattle, sheep, and horses cause a syndrome of lethargy, dehydration, abdominal pain, diarrhoea, dyspnea, and death. Meliatoxin administered to pigs also causes a syndrome of gastroenteritis manifested by diarrhoea, melena and vomiting, plus dyspnea due to pulmonary edema (Radostits et al., 2000).

The most toxic compound '20-hydroxy-20-epi-tingenone' isolated from *E. croceum*, during this work has no previous report of being isolated from the same genus. Tingenone is the second toxic compound isolated from *E. croceum*, which has been reported as previously isolated from the same genus. This compound may be considered as a compound that can be developed as an anticancer drug. Other isolated compounds; tingenine B, 11α-hydroxy-β-amyrin, and naringenin, which have not been previously isolated from the genus, also displayed toxicity.

4.3 Conclusion

The poisoning statistics of the Poison Information and Control Centres (Vienna) reported that about 19% of the alleged poisoning cases during the year 2004 were due to plant material (Frohne et al., 2005). This number underlines the necessity and importance of maintaining toxicological information centres. Admittedly, these cases were reported as accidents of children due to plants with conspicuous fruits, and suicides or abuse by adults.

The toxicity of *E. croceum* is probably mainly based on the synergistic effect of the above reported toxic compounds isolated from the species. The results displayed by compound **1** signify the toxicity of *E. croceum*. Most of the isolated

compounds are found in other species of the Celastraceae, a family well-known for its toxicity in several genera. Although toxic, the species is used for medicinal purposes by traditional healers, (discussed under the section 'medicinal uses'). Tingenone (**2**) is three times more toxic to cancer cells than to Vero cells. This indicates that further research of the mechanism of the toxicity of it may possibly lead to an anticancer drug.

There is a critical need for new, effective drugs to be used in cancer chemotherapy. Since cancer chemotherapeutic agents are generally administered to the point of toxicity in patients, the toxicologist needs to establish the reversibility (or not) of the toxic effects identified in animals. Reversibility is established through observation of animals during a period after drug treatment (Welling and Iglesia, 1993).

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CHAPTER 5: ACKNOWLEDGEMENTS

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APPENDIX 1: PAPER ACCEPTED FOR PUBLICATION IN NATURAL PRODUCTS RESEARCH

Isolation and identification of poisonous triterpenoids from *Elaeodendron croceum*

Thembela Yelani, Ahmed A. Hussein[a], J.J. Marion Meyer[*]

Department of Plant Science, University of Pretoria, Pretoria 0002, South Africa.

[*] *Corresponding author*. Tel.: +27 (0)12 4202224; fax: +27 (0)12 3625099
Email address: marion.meyer@up.ac.za
[a] Present address: Chemistry of Medicinal Plants Department, National Research Centre, Dokki, Cairo, Egypt.

Abstract

A phytochemical investigation of the poisonous *Elaeodendron croceum* leaves guided by cytotoxicity against Vero cells led to the isolation of five known compounds; 20-hydroxy-20-epi-tingenone (**1**), tingenone (**2**), tingenine B (**3**), 11α-hydroxy-β-amyrin (**4**), and naringenin (**5**). Compounds **1** and **2** showed the highest toxicity against Vero cells (IC_{50}: 2.65 nM and 8.23 µM respectively). Cytotoxicity of the isolated compounds against three human cancer cell lines, HeLa, MCF-7, and SNO was also determined. Compounds **1** and **2** again showed the highest cytotoxicity with IC_{50} values ranging between 2.47 – 0.43 µM. This is the first report on the isolation of poisonous compounds from *E. croceum,* a species well known for its toxicity.

Keywords: *Elaeodendron croceum,* toxicity, triterpenes

1. Introduction

Plants produce various groups of secondary compounds for protection against environmental stress and defense against herbivores (Cotton, 1996). Various groups of secondary compounds including saponins, alkaloids, waxes, resins, tannins, glycosides and terpenoids have been reported to be causes of poisoning of herbivores (Van Wyk et al., 2002).

The Celastraceae is a large family of herbs, woody lianas, shrubs and trees distributed worldwide in tropical and temperate zones. A report by Simmons et al. (2001) states that the number of genera and species of the family is not known. The report conveys inconsistent estimates partly because little taxonomic work has been done on the family, and because generic delimitations are controversial. The family has been subject to considerable nomenclatural confusion and name changes. *Elaeodendron croceum* (Thunb.) DC. has been treated by different authors under several names such as *Cassine crocea, C. papillosa, Crocoxylon croceum, E. capense, E. papillosum,* and *Ilex crocea* (Archer & and Wyk, 1998; Germishuizen and Meyer, 2003).

Several authors have reported that most parts of *E. croceum* are poisonous (Van Wyk and Gericke, 2000; Van Wyk and Van Wyk, 1997). The roots were apparently previously used to cause death by "witchdoctors" and are perhaps still being used (Palmer and Pitman, 1973; Watt and Breyer-Brandwijk, 1962); the bark has been reported to be fatal to humans, presumably due to the presence of alkaloids (Archer and Condy, 1995). On two occasions the plant has come under official suspected cause of human death (Watt and Breyer-Brandwijk, 1962). The leaves are toxic to rabbits, 2.5 g per kg of the fresh material being the minimum lethal dose (Watt and Breyer-Brandwijk, 1962), and 10.0 g per kg of fresh leaves caused death fifteen minutes after administration (Steyn, 1934). The

bark is widely used for medicinal and magical purposes (Van Wyk and Van Wyk, 1997; Pooley 1997), to clean the digestive tracts and for chest congestion (Lall and Meyer, 1999; Schmidt et al., 2002). Several traditional healers confirmed the poisonous nature of this species. We report here for the first time on the isolation, identification, and *in vitro* evaluation of highly poisonous compounds from *E. croceum*.

2. Materials and methods

2.1 Plant material

E. croceum leaves were collected from the Eastern Cape Province (Butterworth, Centane) of South Africa on 20 February 2006, identified and preserved at the HGWJ Schweickerdt Herbarium of the University of Pretoria, (voucher specimen number: Yelani 094021).

2.2 Isolation and identification of compounds

E. croceum dry leaves (3.7 kg) were successively extracted with *n*-hexane, dichloromethane and then acetone, two times each. All three extracts were filtered and rotor-evaporated at 40 ^{0}C to dryness to yield 53.0 g (*n*-hexane), 43.0 g (dichloromethane), and 104.0 g (acetone). The *n*-hexane extract, which had the highest cytotoxicity, was applied to a silica gel column (Merck, 230-400 mesh). The column was eluted with a solvent system of *n*-hexane : ethyl acetate in order of increasing polarity (100:0 to 0:100). Similar fractions were combined according to thin layer chromatography (TLC) to form nine main-fractions (A to I). Fraction I was the most toxic to Vero cells and further purified on a silica gel column eluted with *n*-hexane : ethyl acetate (8:2). Collected fractions were combined according to the TLC profiles resulting in ten sub-fractions (I_1 to I_{10}). Crystals of a red compound (**1,** 14.0 mg) were formed when the I_{10} fraction was dissolved in methanol. According to a TLC profile, sub-fractions I_4 (43.0 mg) and I_5 (83.0 mg) contained similar constituents, which were collected in small amounts and therefore

combined to obtain fraction I_4I_5 (126.0 mg) and applied to a Sephadex LH-20 (sigma) column. The column was eluted with absolute ethanol. Three fractions were collected from the column. The third fraction was shown to contain only one pure compound (**3,** 10.0 mg). Main-fraction G (1.0 g) of the hexane extract's silica column was combined with main-fraction H (2.0 g) to form fraction GH (3.0 g), and applied to a Sephadex column, which was eluted with ethanol. Two sub-fractions GH_1 and GH_2 were collected. Fraction GH_2 contained only one compound, which was crystallized from methanol to form white crystals of compound **4** (21.0 mg). Fraction GH_1 was shown to be poisonous and re-chromatographed on a Sephadex column and eluted with ethanol to obtain two sub-fractions, $GH_{1.1}$ and $GH_{1.2}$. Preparative TLC developed in methanol : dichloromethane (3:97) of fraction $GH_{1.2}$ yielded a reddish compound, **2** (9.0 mg).

The dichloromethane extract was also found to be poisonous and applied to a silica gel column, which was eluted with a gradient of *n*-hexane : ethyl acetate (8:2 to 0:100) to yield eleven fractions (DA to DK). Fraction DF was poisonous and applied to a silica gel column and eluted with a gradient of ethyl acetate : *n*-hexane, (2:8 to 1:1). Out of five collected fractions, fraction DF_2 was applied to a Sephadex column. The column was eluted with absolute ethanol and compound **5** (18.0 mg) was collected in a pure form.

1D and 2D NMR spectroscopic data were obtained on 500 Bruker NMR instrument. The structure of the isolated compounds was determined by comparing the obtained spectroscopic data with published values.

2.3 Determination of cytotoxicity

2.3.1 XTT toxicity assay

The assay is based on the conversion of the yellow tetrazolium salt XTT to the orange formazan dye by metabolic active cells (Williams et al., 2003). The formazan dye is soluble in aqueous solutions, and is quantified using a scanning multiwell

spectrophotometer (ELISA reader). Cells were grown in a 96 well microtitre plate and incubated with the yellow XTT solution for 1 to 1 ½ hours. After this incubation period, the orange formazan solution formed. An increase in the number of living cells results in an increase in the overall activity of mitochondrial dehydrogenase in the sample. This increase directly correlates to the amount of formazan formed, as monitored by the absorbance at a wavelength of 450 nm.

2.3.2 Cell culture

Four cell lines, Vero (monkey kidney), HeLa (human cervix cancer), SNO (human oesophagus cancer), and MCF-7 (human breast cancer) were cultured separately in minimal essential medium (Eagle), (MEM), containing 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 µg/ml penicillin, 10 µg/ml streptomycin, 0.25 µg/ml fungizone, and 10 % fetal bovine serum. Cells were grown in a humified incubator at 37 ^{o}C under 5 % CO_2, and then harvested by trypsinization (Williams et al., 2003).

2.3.3 Preparation of cells for toxicity screening

Confluent cells were trypsinized and diluted in complete MEM to a concentration of 1 × 10^5 cells/ml. In the outer wells of a 96 well plate, 200 µl of medium was dispensed. All inner wells received 100 µl of cell suspension. The plates were incubated overnight at 37 ^{o}C in a humidified atmosphere with 5 % CO_2.

2.3.4 Preparation of final concentrations of compounds and extracts

Stock solutions of compounds and extracts were prepared in dimethyl sulphoxide (DMSO) at 20.00 mg/ml. For compounds, the solutions were diluted 100 times in complete medium to 200.00 µg/ml. This was then serially diluted to obtain eight different

concentrations. For extracts, the solutions were diluted 50 times in complete medium to 400.00 µg/ml. This was then serially diluted to obtain eight different concentrations.

2.3.5 Toxicity screening

On day one, 100 µl of each compound was dispensed into cell-containing wells of the sample plates in triplicate. The final concentration of crude extracts in the wells were 3.13, 6.26, 12.50, 25.00, 50.00, 100.00, 200.00 and 400.00 µg/ml. Compounds had final concentrations of 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 and 200.00 µg/ml. Control wells received a final concentration of 0.5 % (for compounds) and 1 % (for extracts) DMSO in complete medium. Reference plates contained 100 µl of medium and 100 µl of dilute compound and were prepared in duplicate. Doxorubicin and Zearalenone were used as positive toxicity controls. Plates were returned to 37 ^{o}C in humidified atmosphere with 5 % CO_2 for three days incubation. On day four, 50 µl of XTT reagent was added to the wells and incubated for four hours. The optical densities of the wells were measured at 450 nm (690 nm reference wavelength).

3. Results and discussion

Successive extraction of the dried leaves showed that hexane, dichloromethane, and acetone extracts were all toxic. Phytochemical studies guided by in-vitro toxicity against Vero cells of the three extracts resulted in the isolation of five toxic compounds; 20-hydroxy-20-epi-tingenone (**1,** De Oliveira et al., 2006), tingenone (**2,** Furbacher and Gunatilaka, 2001), tingenine B (**3,** Kishi et al., 2003), 11α-hydroxy-β-amyrin (**4,** Ikuta and Morikawa, 1992), and naringenin (**5,** Lee et al., 2001). Structural formulas of the isolated compounds are displayed in Figure 1. Significant toxicity against Vero cells (Table 1) was demonstrated by 20-hydroxy-20-epi-tingenone (**1)** (2.65 nM). The toxicity demonstrated by the isolated compounds on Vero cells prompted us to evaluate the compounds on

different human cancer cell lines. Compounds **1** and **2** (tingenone) showed potent activities against the tested cancer cell lines. Compound **2** was 3.4 times more toxic to HeLa and SNO cells and about 8 times more toxic to MCF-7 cells than to Vero cells, indicating its potential as an anticancer drug. Compound **2**, has a similar level of toxicity (IC_{50} 2.43 µM) as the potent anticancer drug, vincristine, on HeLa cells (IC_{50} 4.56 µM) (Lu et al., 2005).

It has been reported that compound **1**, which was previously isolated from the stem bark of *Glyptopetalum sclerocarpum* (Celastraceae), is highly toxic *in vitro* against the following cell lines; Vero (Sotanaphun et al., 2005), P-388 (murine lymphocytic leukemia), human fibrosarcoma (HT-1080), human oral epidermoid carcinoma (KB), human epidermoid carcinoma (A431), human gliobastoma (U373), human breast (BC-1), human colon (COL-2), human lung (LU-1), human melanoma (MEL-2), multidrug-resistant (KB-V1), human hormone-dependent breast (ZR-75-1), and prostate (LNCaP) (Ngassapa et al., 1994). This quinone-methide triterpenoid has shown a variety of biological activities such as antitumoral, antimicrobial, antibiotic, antimalarial, spermicidal (Corsino et al., 2000), and anti-inflammatory activities (Sotanaphun et al., 1998). Tingenone (**2**), was previously isolated from *Maytenus buchananii* (Celastraceae), and has been reported for toxicity against KB cells (human epidermoid carcinoma of the nasopharynx) (Kutney et al., 1981). It showed antibacterial activity against *Bacillus cereus, B. subtilis*, *Sarcinalutea sp, Microsporum gypseum*, against the Gram-negative bacterium, *Klebsiella pneumoniae* (Sotanaphun et al., 1999), and also activity against *Staphylococcus aureus* (Moujir et al., 1990). Sotanaphun et al. (2005) reported that tingenine B (**3)** is highly toxic against Vero cells, it displayed inhibitory effects on rat lens aldose reductase (Morikawa et al., 2003), and cytotoxic activity against cultured P-388 tumor cell lines (Shirota et al., 1994). Naringenin (**5**) has not been reported for significant

toxicity previously. The isolation of these highly toxic compounds most probably explains the previous reports on the toxicity of *E. croceum*.

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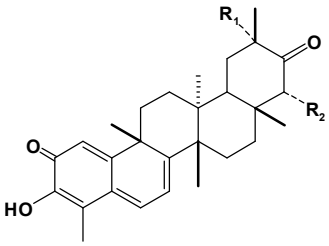
Table 1: IC_{50} toxicity values of *E. croceum* extracts, fractions and compounds: 20-hydroxy-20-epi-tingenone (1), tingenone (2), tingenine B (3), 11α-hydroxy-β-amyrin (4), and naringenin (5).

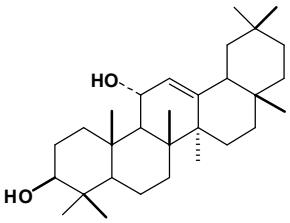
Extract / Fraction / Compound	IC_{50} ±SD			
	Vero	HeLa	SNO	MCF-7
Hexane	46.60 µg/ml ± 2.66	nt	nt	75.44 µg/ml ± 1.63
Dichloromethane	51.28 µg/ml ± 3.73	nt	nt	43.71 µg/ml ± 3.89
Acetone	56.11 µg/ml ± 4.22	nt	nt	126.4 µg/ml ± 3.24
Fraction I	37.44 µg/ml ± 2.30	nt	nt	23.06 µg/ml ± 0.27
Fraction GH	28.53 µg/ml ± 2.45	nt	nt	3.05 µg/ml ± 0.25
Fraction E	61.79 µg/ml ± 0.51	nt	nt	26.78 µg/ml ± 1.43
Compound **1**	2.651 nM ± 0.747	2.011 µM ± 1.511	0.427 µM ± 0.910	0.600 µM ± 1.827
Compound **2**	8.233 µM ± 4.169	2.435 µM ± 0.476	2.478 µM ± 6.669	< 1.859 µM
Compound **3**	0.130 mM ± 0.003	0.036 mM ± 0.006	0.046 mM ± 0.006	0.075 mM ± 0.003
Compound **4**	0.192 mM ± 0.009	0.042 mM ± 0.010	0.057 mM ± 0.007	0.056 mM ± 0.005
Compound **5**	0.188 mM ± 0.002	0.447 mM ± 0.017	0.452 mM ± 0.024	0.057 mM ± 0.004
Doxorubicin	2.277 µM ± 0.170	0.014 µM ± 0.196	0.008 µM ± 0.894	0.015 µM ± 0.312
Zearalenone	8.223 µM ± 1.158	7.422 µM ± 2.345	7.221 µM ± 4.501	7.422 µM ± 1.532

nt = not tested

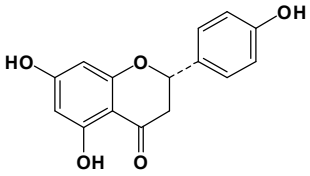
Figure 1: Chemical structures of compounds isolated from *E. croceum*.



1 $R_1 = R_2 = H$ (20-hydroxy-20-epi-tingenone)
2 $R_1 = OH$, $R_2 = H$ (tingenone)
3 $R_1 = H$, $R_2 = OH$ (tingenine B)



4 11α-hydroxy-β-amyrin



5 naringenin