IN-VITRO PROPAGATION, BIOAUGMENTATION AND SCREENING OF *Ceratoteca triloba* FOR THE PRODUCTION OF SECONDARY METABOLITES

Viresh Mohanlall

Submitted in complete fulfillment for the Degree of Doctor of Technology (Biotechnology) in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

***SUBMISSION APPROVED FOR EXAMINATION**

_____ _____

SUPERVISOR **DATE**
Professor B. Odhav (PhD)

_____ _____

CO-SUPERVISOR **DATE**
Dr B. Okole (PhD)

REFERENCE DECLARATION

I, **Mr V. Mohanlall- (Student number: 19351878)** and **Prof Bharti Odhav** (full name of supervisor) do hereby declare that in respect of the following dissertation:

Title: *In-vitro* propagation, Bioaugmentation and Screening of *Ceratoteca triloba* for the production of secondary metabolites.

1. As far as we ascertain:
   (a) no other similar dissertation exists;
   (b) the only similar dissertation(s) that exist(s) is/are referenced in my dissertation as follows:

_____

_____

_____

2. All references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

_____ _____

Signature of student Date

_____ _____

Signature of supervisor Date

_____ _____

Signature of supervisor Date

AUTHORS DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Bharti Odhav** and **Dr Blessed Okole.**

_____

Students signature

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks and gratitude to:

- My supervisor and co-supervisor, Prof. B. Odhav and Dr. B. Okole for their patience, guidance, constructive criticism and words of encouragement throughout the course of this project,

- My wife Ravika and sons, Kiash and Yash for their undying love, support, encouragement and assistance throughout the project,

- Prof. H. Baijnath for his assistance and guidance throughout the course of this project,

- The Department of Chemistry, Durban University of Technology, MLS Campus for their technical assistance,

- Prof. Francis Shode and Mr Dilip Jugjivan, Department of Organic Chemistry, University of Kwazulu Natal, Westville Campus for their technical assistance,

- Dr P. Steenkamp, Centre for Scientific and Industrial Research for his technical assistance,

- Staff and students at the Department of Biotechnology, Durban University of Technology for their general support and encouragement,

- My family, for all their support and encouragement,

- The Centre for Research Capacity Development (CRCD) and National Research Foundation (NRF) for funding this project.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AC	Affinity Chromatography
AQ4N	1,4-bis-{[2-(dimethylamino-*N*-oxide)ethyl]amino}5,8-dihydroxyanthracene-9, 10-dione
AQ4	1,4-bis-[{2-(dimethylamino)ethyl}amino]5,8-dihydroxyanthracene-9, 10-dione
CDPK	Calcium-dependent protein kinase
CFU	Colony forming unit
CH_2Cl2	Dichloromethane
COSY	Correlation spectroscopy
COX-1	Cyclooxygenase 1
CSIR	Centre for Scientific and Industrial Research
DBT	Durban University of Technology Culture Collection
DCM	Dichloromethane
DEHP	Di-(2- ethylhexyl) phthalate
DEPT	Distortionless enhancement by polarization transfer
DHNA	dihydroxynaphthalene-2-caroxylic acid
DMAPP	dimethylallyl diphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1.1-diphenyl-2-picrylhydrazyl
ECI	Electron capture ionization
EtOAc	Ethyl acetate
EtOH	Ethanol
GCMS	Gas chromatography/mass spectrometry
GLC	Gas-liquid Chromatography
H_2O	Water
HMBC	^{1}H-13C-Heteronuclear Multiple Bond Connectivity
HMQC	^{1}H-13C-Heteronuclear Multiple Quantum Correlation
HPLC	High Performance Liquid Chromatography
HREILC-MS	High Resolution Electron Impact liquid Chromatography Mass Spectrometry
HTS	High throughput screening
ICS	isochorismate synthase
INT	p-Iodonitrotetrazolium violet

IPP	isopenthyl diphosphate
IR	Infra-red spectroscopy
LCMS	Liquid chromatography/mass spectrometry
LDH	Lactate dehydrogenase
Maldi-TOF-MS	Time of Flight Mass Spectrometry
AMSA	Amsacrine
MeOH	Methanol
MHB	Mueller Hilton Broth
MIC	Minimum Inhibitory Concentration
MS	Murashige-Skoog medium
NADPH	Nicotinamide adenine dinucleotide phosphate
NCCLS	National Committee for Clinical Laboratory Standards
NDGA	Nordihydroguaiaretic acid
NH_4Ac	Ammonium acetate
NH_4OH	Ammonium hydroxide
NMR	Nuclear magnetic resonance
NSAID	Non steroidal anti-inflammatory drugs
OSB CoA	o-succinylbenzoic acid-Co-enzyme A ester
OSB	O-succinylbenzoic acid
PDA	photodiode array
PE	Petroluem Ether
P-HPLC	Preparative High Performance Liquid Chromatography
PTLC	Preparative Thin Layer Chromatography
R*f*	Resolution factor
SEC	Size Exclusion Chromatography
TDH	Threonine dehydrogenase
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UV-Vis	Ultravoilet-Visible light spectroscopy

SUBMITTED PUBLICATIONS

Mohanlall, V and Odhav, B. (2010) Novel anthraquinones from *Ceratotheca triloba* (Bernh.) E.Mey. ex Hook. f. *Phytochemistry*.

Mohanlall, V and Odhav, B. (2010) Topoisomerase II α inhibition by novel anthraquinones from *Ceratotheca triloba*. *Journal of Ethnopharmacology*

Mohanlall, V and Odhav, B. (2010) Methyl jasmonate induced overproduction of anthraquinones from *Ceratotheca triloba*. *Journal of Agricultural and Food Chemistry.*

INTERNATIONAL CONFERENCE PRESENTATIONS

Mohanlall, V and Odhav. B. (2008) Novel antraquinones from *Ceratotheca triloba* (Bernh.) E. Mey. ex Hook. f. Biennial Congress of the South African Society for Microbiology, Grahamstown, January 2008.

Mohanlall, V and Odhav. B. (2009) Novel antraquinones from *Ceratotheca triloba* (Bernh.) E. Mey. ex Hook. f. Joint Bio2Biz and South African Society for Microbiology Congress, ICC Durban, September 2009.

ABSTRACT

Ceratotheca triloba (Bernh.) E. Mey. ex Hook. f. is one of four species that is common to the summer rainfall areas of South Africa, especially the grasslands. It is used in traditional medicine to treat stomach cramps, nausea, fever and diarrhea. Like many other plants used in the traditional medicine system, these uses are not justified through scientific investigations. This study was undertaken to characterize the functionality of the main bioactive compounds from *Ceratotheca triloba.* This was achieved by isolating and identifying predominant chemicals from the non polar extracts using conventional chromatography techniques. Once identified the crude extracts and identified compounds were tested for their antimicrobial activity, anti-oxidant activity, anti-inflammatory activity, and anticancer activity. This was followed by investigating the safety of the crude extracts and the purified compounds by the Brine shrimp lethality assay, and its toxicity to $HepG_2$ cells and the Salmonella mutagenecity test. For large scale production, we set up a protocol to produce 9, 10 anthracenedione in a cell suspension culture system.

Following a complete chemical profile of the roots, stems, flowers and leaves the predominant compounds were isolated, characterized and identified by UV-Vis, IR, EI-LCMS and NMR (COSY, HMQC, HMBC and DEPT). Three anthraquinone derivatives and one steroid, 9, 10 anthracenedione, 1-hydroxy-4-methylanthraquinone, 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H-phenanthrene-4, 9-dione and androst-5-ene-3, 17, 19-triol were isolated from the roots of *C. triloba*. The structures of these compounds as 9, 10 anthracenedione, 1-hydroxy-4-methylanthraquinone, 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H-phenanthrene-4, 9-dione and androst-5-ene-3,17,19-triol were determined by analysis of spectral data (UV, ^{1}H NMR, ^{13}C NMR and EI-LC-MS)

9, 10 anthracenedione and 1 hydroxy -4-methylanthraquinone showed antibacterial activity against *S. aureus, M. luteus, B. cereus* and *E. coli*. Due to the synergistic effect of the individual compounds, the crude extract exhibited good potency (>500) against *S. aureus* and *M. luteus,* medium potency against *E. coli* and *S. typhimurium* (<100) and very low potency against *B. cereus* (<10). Although a similar trend was observed for 9, 10 anthracenedione and 1-hydroxy -4-methyl anthraquinone unlike the crude extract. A very low potency against *S. aureus* was observed for 9, 10 anthracenedione and a high potency for 1-hydroxy-4-methylanthraquinone. Thus 9, 10 anthracenedione is an effective drug against *E. coli* and *S. typhimurium* and 1-

hydroxy-4-methylanthraquinone is effective against *S. aureus* and *M. luteus*. The crude root extract and 9, 10 anthacenedione, 1-hydroxy-4-methylanthraquinone and 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H-phenanthrene-4 showed a ± 50 % reduction of the free radicals. No anti-inflammatory activity was observed. The purified extracts showed moderate toxicity against $HepG_2$ cells at high concentrations and no toxicity was observed against brine shrimp larvae. No mutagenicity was observed with the crude extracts using the Ames test. All purified and crude extracts showed potent inhibition of the human topoisomerase II enzyme.

In conclusion, although this study does not indicate any relationship to its traditional usage it provides valuable information that paves a way for commercial exploitation of *C. triloba*. 9, 10 anthracenedione and 1-hydroxy-4-methylanthraquinone can be used as antibacterial agents. Their antioxidative potential can be exploited for anti-cancer treatment as in many cancers reactive oxygen species are implicated in the aetiology of these cancers. Furthermore, in this study 9, 10 anthracenedione was produced from both callus cultures and cell suspension cultures. This compound demonstrates potent anti-topoisomerase II activity which is vital to cancer treatment. Thus, the synergistic effect of 9, 10 anthracenedione and 1-hydroxy-4-methylanthraquinone as antibacterial, anti-oxidative and anti-cancer compounds demonstrate the importance of *C. triloba*.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Ceratotheca triloba (Bernh.) E. Mey. ex. Hook.f. is one of four species found in South Africa. *C. triloba* is used in traditional medicine to treat stomach cramps, nausea, fever and diarrhea (Hutchings, 1996). There is no scientific evidence for the traditional use of this plant. This study represents the first attempt to identify and isolate secondary metabolites from *C. triloba* and also culture *C. triloba in vitro* and elicit the secondary metabolites of interest by chemical elicitation.

To achieve this, our objectives are:

(i) To extract, isolate and identify the major groups of secondary metabolites

(ii) To evaluate the biological activity of the crude extract and the major phytochemicals of *C. triloba*

(iii) To establish callus and suspension cultures of *C. triloba* and elicit the production of 9, 10 antracenedione and 1-hydroxy-4-methyl anthraquinone

The thesis is covered in four chapters and chapter one is a general literature review that describes *C. triloba* and the major groups of compounds, their biosynthesis and methods that are used to isolate the compounds. Each of the chapters following will give a brief overview of this study; outline the objectives, followed by the methodology and finally the results and discussions. The last chapter is dedicated to a comprehensive summary of this work.

1.2. *Ceratotheca triloba*

In this study, we examined *C. triloba,* an indigenous widespread annual. There are only four known species of Ceratotheca that is found in Southern Africa (Smithies, 2000). Ceratotheca means having horned capsules, from the Greek word kerato-, horned and theke, a case. The species name triloba means three-lobed (Latin), alluding to the leaves. It is used in traditional medicine to treat painful menstruation, stomach cramps, nausea, fever and diarrhea (Hutchings, 1996). The plant was first described in 1842 as *Sporledera triloba* Bernh. and since then has undergone several name changes (Table 1). In the current classification system, it belongs to the family Pedaliaceae which is characterized by having mucilaginous hairs, which often give the stems and leaves a slimy or clammy feel, and often have fruits with hooks or horns (Table 2). It is commonly called South African Foxglove; Wild foxglove; Vingerhoedblom; ludvonca (Swazi); udonqa (Swazi, Zulu); undoncalwabathwa, udonqabathwa (Zulu). *C. triloba* is found

in the summer rainfall areas of South Africa, especially the grasslands. Being an opportunistic annual, they germinate best in disturbed areas like roadsides where they manage to grow, flower and seed before the onset of the dry, frosty winter. The wild foxglove varies in height, depending on the amount of water it receives during summer. The soft, green leaves are about 50 mm long and divided into three lobes with a bluntly serrated margin. The leaves are carried on long thin stalks up the stems. Plants with pink flowers usually have dark red stems (Fig. 1A) while white flowering plants have yellow-green stems. The white or mauve foxglove-like flowers are carried in pairs up the stems in tall, sparsely flowered spikes. The bottom flowers open first and form fruits while new buds are still developing at the tip of the stem. Each flower is about 50 mm long with 5 lobes, the bottom lobe longer than the others and streaked with delicate lines running into the throat. The small black seeds are formed in the 30 mm long fruits (Fig. 1B) which have two very prominent horns at their tips. Within a few weeks of flowering, the green fruits turn brown and dry, splitting open to release the flat pear shaped seeds. The leaves, stems and flowers are covered in fine white hairs. The plants are slightly sticky and when crushed give off a strong unpleasant smell (van der Walt, 2001).

There are no publications of the phytochemicals or the biological properties of *C. triloba* (Pedaliaceae), except for the recent work of Ramesar *et al*. (2008) where they evaluated sixteen nutritive plants from Kwa-Zulu Natal for angiotensin 1-converting enzymes. However, this activity was not observed in the extracts from *C. triloba*. Previous nutritional, chemical and antioxidant studies were conducted on the *C. triloba* plant. In terms of traditional leafy vegetables, *C. triloba* serves as a good source of energy and magnesium (Odhav *et al*., 2007).

Figure 1: **A - Wild Foxglove - *Ceratotheca triloba* (Bernh.) E. Mey. ex Hook. f**
B - Seed pods of *Ceratotheca triloba* (Bernh.) E. Mey. ex Hook.f

1.2.1. Classification & Taxonomy

Table 1: History of *C. triloba* (Bernh.) E. Mey. ex Hook.f

SYNONYM	FULL CITATION
Ceratotheca lamiifolia (Engl.) Engl.	*Ceratotheca lamiifolia* (Engler) Engler, Bot. Jahrb. Syst. 19: 156. 1894. BASIONYM: *Sesamum lamiifolium* Engler 1888.
Sesamum lamiifolium Engl.	*Sesamum lamiifolium* Engler, Bot. Jahrb. Syst. 10: 256, t. 8. 1888. TYPE: SOUTH AFRICA:
Sporledera kraussiana Bernh.	*Sporledera kraussiana* Bernhardi, Linnaea 16: 41. 1842. TYPE: SOUTH AFRICA:
Sporledera triloba Bernh.	*Sporledera triloba* Bernhardi, Linnaea 16: 42. 1842. TYPE: SOUTH AFRICA:
Volkameria lamiifolia (Engl.) Kuntze	*Volkameria lamiifolia* (Engler) Kuntze, Revis. Gen. Pl. 2: 482. 1891. BASIONYM: *Sesamum lamiifolium* Engler 1888.

Table 2: Classification for Kingdom Plantae down to species *C. triloba*

Kingdom Plantae –Plants
Subkingdom Tracheobionta -Vascular plants
Superdivision Spermatophyta -Seed plants
Division Magnoliophyta -Flowering plants
Class Magnoliopsida -Dicotyledons
Subclass Asteridae -Daisy superorder
Order Scrophulariales -figwort order
Family Pedaliaceae - Sesame family
Genus Ceratotheca Endl. - ceratotheca P
Species - *Ceratotheca triloba* (Bernh.) E. Mey. ex Hook. f.

1.3. SECONDARY PLANT METABOLITES

Plants contain two classes of compounds; primary metabolites that are required for the livelihood of the plant i.e. the plants machinery, and secondary metabolites that are not a necessity for the plants survival but are produced to protect the plant against fungi, bacteria, insects and viruses. These have been used as food flavourants, colour dyes, poisons, perfumes, industrial products and drugs (Wink, 2000). These secondary metabolites are the biggest source of pharmaceutical drugs and they serve as templates for many medicinal derivatives (O'Neill, 2004) currently used. The major groups of secondary metabolites and their physiological use and effects are summarized in Table 3. These are: (i) Flavonoids and allied Phenolics; (ii) Alkaloids; and (iii) Terpenoids. Only anthraquinones are discussed in detail as they form a major part of this research.

Table 3: Major groups of secondary metabolites isolated from plants. (Adapted from Rosenthal and Berenbaum, 1991)

		Example	Some Effects and Uses
ALKALOIDS		Nicotine, cocaine theobromine	interfere with neurotransmission, block enzyme action
TERPENOIDS	Monoterpenes	Menthol, linalool	interfere with neurotransmission, block ion transport, anesthetic
	Sesquiterpenes	parthenolid	contact dermatitis
	Diterpenes	gossypol	block phosphorylation, toxic
	Triterpenes, cardiac glycosides	digitogenin	stimulate heart muscle, alter ion transport
	Sterols	spinasterol	interfere with animal hormones
PHENOLICS	Phenolic acids	caffeic, chlorogenic	cause oxidative damage, browning in fruits and wine
	Coumarins	umbelliferone	cross-link DNA, block cell division
	Lignans	Podophyllin, urushiol	cathartic, vomiting, allergic dermatitis
	Flavonoids	anthocyanin, catechin	flower, leaf color, inhibit enzymes, anti- and pro-oxidants, estrogenic
	Tannins	gallotannin, condensed tannin	bind to proteins, enzymes, block digestion, antioxidants
	Lignin	lignin	structure, toughness, fiber

1.3.1. Anthraquinones

Anthraquinones are a class of natural compounds that consists of several hundreds of compounds that differ in the nature and positions of substituent groups (Schripsema *et al.,* 1999). This class of compounds contains derivatives that consist of the basic structure of 9,10 anthraquinone (Bajaj, 1999).

Anthraquinones can be divided into alizarin and emodin types based on two main biosynthetic pathways. The alizarin types are formed via chorismate/δ-succinylbenzoic acid pathway and only have one of the rings unsubstituted (Koblitz, 1988; Van der Berg and Labadie, 1989). These anthraquinones are found in the family of plants known as Rubiaceae (*Rubia, Morinda, Galium, Cinchona*) (Korunaglo *et al.,* 1992). The emodin types are formed via the polyketide pathway (acetate-malonate pathway) and have both rings substituted. These anthraquinones are present in the following plant families: Fabaceae (*Cassia, Araroba*), Rhamnaceae (*Rhamnus, Frangula*) and Polygonaceae (*Rheum, Rumex, Fagopyrum*) (Bajaj, 1999). Anthraquinones are widely applied in medicine, food and the dye industry. In the pharmaceutical industry, the natural and synthetic derivatives of 9,10 anthraquinone are beneficial to mammals and humans as they can display antibacterial, antitrypanosomal and antineoplastic activities.

Anthracycline antibiotics are also 9,10 anthraquinones and are key substances that have been known to be used for therapy of several cancers (Dzierzbicka and Kolodziejczyk, 2005). Anthraquinone glycosides are used to produce Pyralvex, to treat gingivitis, stomatitis, mouth ulcers, inflammatory oral mucosa and periodontal conditions. Senna is another member of the anthraquinone class which is used in the preparation of stimulant laxative drug, Senokot. This drug is used to treat constipation or bowel evacuation prior to abdominal radiological procedures (Drug Information for Malaysia and Singapore, 1992; British National Formulary, 1994).The quinones are compounds with either a 1,4-diketocyclohexa-2,5-denoid or a 1,2-diketocyclohexa-3,5-denoid. The former types are p-quinones and the latter o-quinones (Fig. 4). There are widespread examples of pigmented quinones in plants, in particular in the Rubiaceae. They may occur with one, two or three rings (benzo-, naphtho- and anthraquinones, respectively; Fig. 4), or as larger polycyclic quinones (Thomson, 1976; Leistner, 1980; 1981). A large variety of quinine structures are known to be formed by secondary modifications such as hydroxylation and glycosylation.

1.3.2. Secondary Metabolites and their Biosynthesis

The discovery of plant derived drugs epitomizes the importance of using natural products and they continue to provide new target molecules for drug development (Taniguchi and Kubo, 1993; Thatte *et al.*, 2000). These secondary metabolites are structurally diverse and belong to three major groups' i.e. phenolic acids, alkaloids and terpenoids. Despite their structural diversity, these compounds are all produced by photosynthesis via similar pathways. Phenolic compounds and alkaloids are derived via the shikimic acid pathway whilst the terpenoids and the polyketides are produced via the mevalonic and malonic acid pathways respectively. Both the mevalonic and malonic acid pathways are derived via the glycolysis pathway.

Despite enormous structural diversity of the secondary compounds produced by plants, nature uses a few building blocks to create this diversity i.e. Shikimic acid and Shikimate pathway (Fig. 2 and Fig. 3). The basic building blocks for these unique compounds are the acetate (C_2), isoprenoid (C_5) and phenylpropanoid (C_9) units. The acetate unit is considered as the starter unit for polyketide biosynthesis. Polyketides, as the name implies, are synthesized from repetitive condensation reactions that link small carbon precursors typically, 2- and 3-carbon acyl groups derived from coenzyme A (CoA) thioesters. The isoprenoid pathways lead to all terpenoids by coupling two or more C_5 units. The terpenoids are found in all organisms.

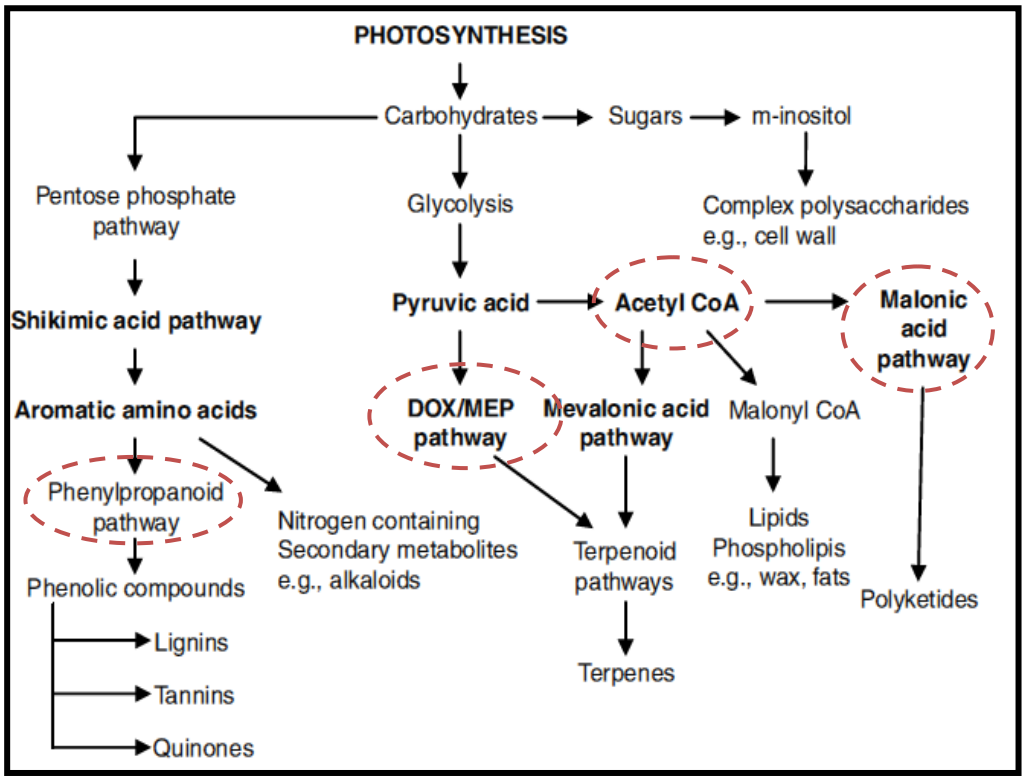


Figure 2: Principal biosynthetic pathways leading to synthesis of secondary metabolite

Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups: (i) flavonoids and allied phenolic and polyphenolic compounds (Fig. 3 [red]), (ii) terpenoids and polyketides (Fig. 3 [blue], Fig. 4), and (iii) nitrogen-containing alkaloids and sulphur-containing compounds (Fig. 3[red]).

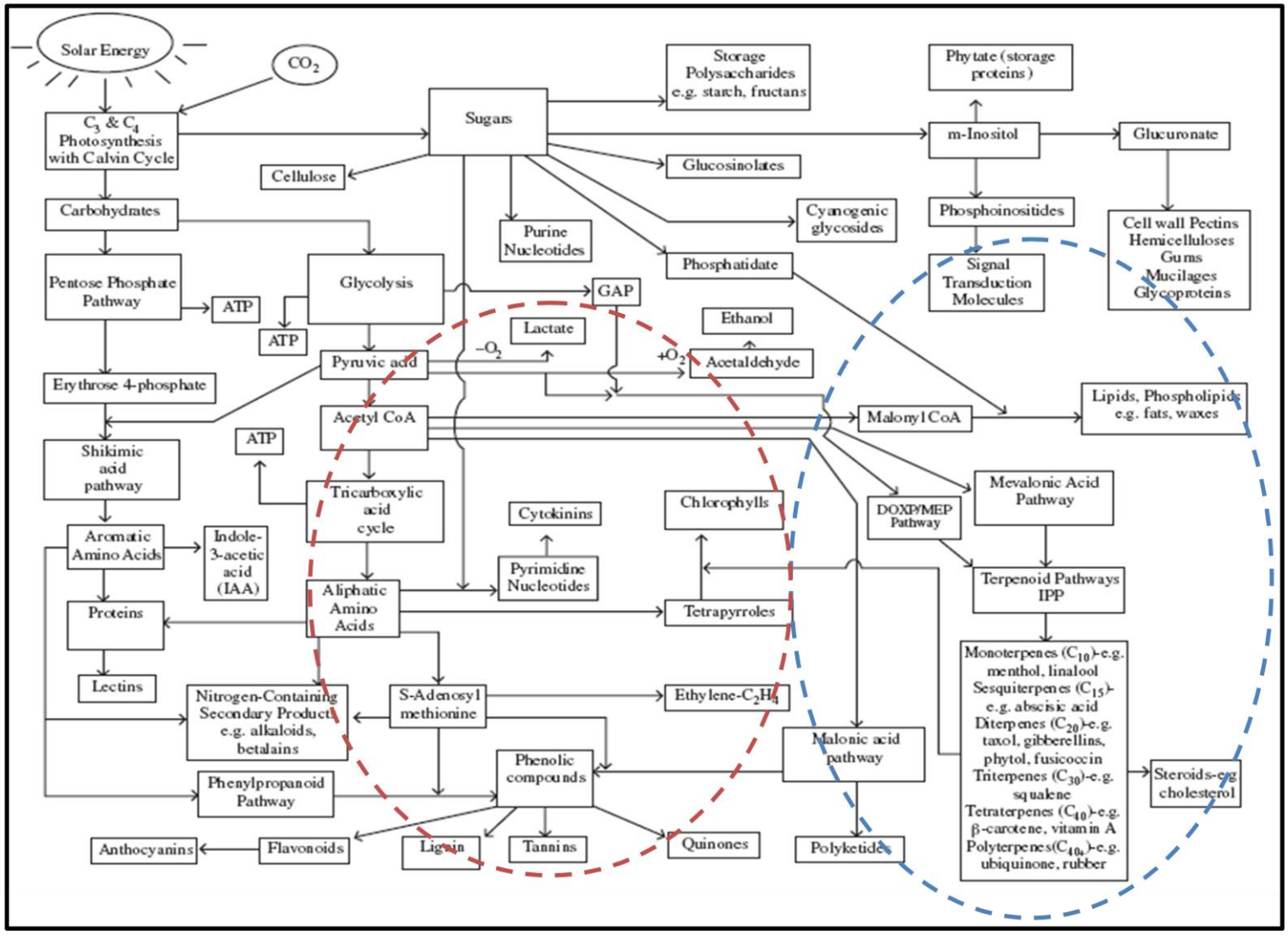


Figure 3: Primary and secondary metabolic pathways in plants.

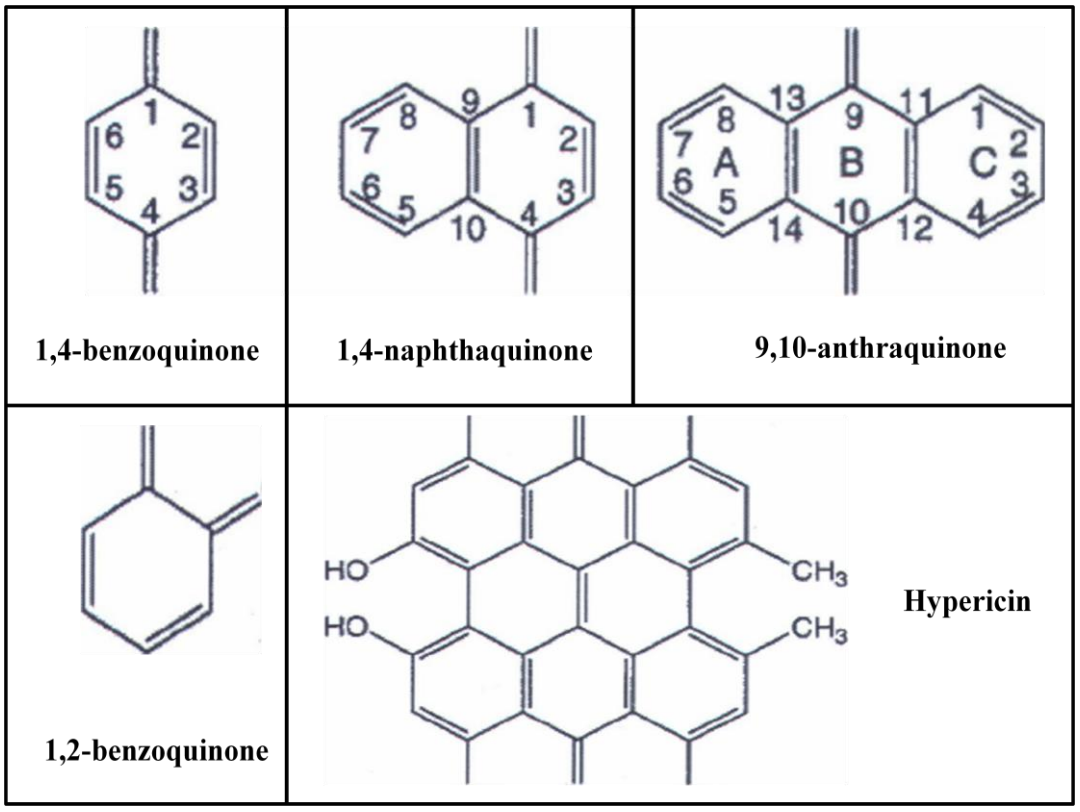


Figure 4: **Benzo-, Naphtho- and anthraquinones illustrating the ring conformation and carbon numbering and a highly polycyclic quinone, hypericin, isolated form *Hypericum* species.**

1.3.3. Pathways for the Production of Anthraquinones

A striking feature of quinones is that they can be derived from several biosynthetic routes, including a number of alternative pathways in plants. Leistner (1980) identified at least six possible biosynthetic routes to benzoquinones. For naphthoquinones, two main routes have been characterised, involving contribution of a phenolic ring from the shikimate pathway and formation of the quinone ring with a mechanism involving either a-ketoglutarate or isopentenyl diphosphate derivatives. At least two biosynthetic routes have also been suggested for the anthraquinones, either starting from a similar pathway to that for naphthoquinones or arising from acetyl-CoA and malonyl-CoA in the polyketide pathway (Leistner, 1981; Han *et al.*, 2001).

The polyketide pathway that is common in fungi may also operate in Leguminosae, Rhamnaceae and Polygonaceae plant species. It involves one acetyl-CoA unit being extended by seven malonyl-CoA units, by undefined enzyme systems. The formation of anthraquinones via the chorismate pathway is well understood.

The A- and B-rings of anthraquinones are formed by joining of isochorismate with a-ketoglutarate in the presence of thiamine diphosphate to form an o-succinylbenzoic acid intermediate, catalysed by the enzyme o-succinylbenzoic acid synthase. Isochorismate is formed from chorismate by the enzyme isochorismate synthase (ICS). Elicitation of anthraquinone formation in cell cultures causes a marked increase in isochorismate synthase activity. In *M. citrifolia* cultures, this increase in isochorismate synthase activity was not accompanied by induction of other enzymes of the shikimate pathway that direct chorismate into other biosynthetic pathways, specifically deoxy-D-arabinoheptulosante 7-phosphate synthase and chorismate mutase (Stalman *et al.*, 2003).

The biosynthetic pathway for the production of anthraquinones via the shikimic acid route is regulated by the isochorismate synthase enzyme, thus providing a key regulatory target. The theory of isochorismate synthase being a key regulatory target is further supported by transgenic experiments using bacterial isochorismate synthase gene (Lodhi *et al.,* 1996). The recent isolation of the isochorismate synthase gene from *M. citrifolia* (Han *et al.*, 2001) should allow genetic approaches to test the role of the endogenous isochorismate synthase genes in controlling the rate of anthraquinone biosynthesis. O-succinylbenzoic acid (OSB) is activated at the aliphatic carboxyl group to produce o-succinylbenzoic acid-Co-enzyme A ester (OSB CoA), a reaction carried out by the enzyme o-succinylbenzoic acid: Co-enzyme A -ligase (Simantiras and Leistner, 1992). Ring closure then produces the A- and B-rings as the intermediate 1,4- dihydroxynaphthalene-2-caroxylic acid (DHNA). Subsequent prenylation of DHNA yields a prenylated naphthoquinone intermediate and finally the formation of the C-ring (Fig. 5).

The source of the prenyl groups is either isopenthyl diphosphate (IPP) or 3,3-dimethylallyl diphosphate (DMAPP), which are interconverted by the enzyme isopenthyl diphosphate isomerase (Ramos-Valdivia *et al*., 1997a). Changes in isopenthyl diphosphate isomerase activity accompany induction of coloured anthraquinone production in Rubiaceae cell cultures (Ramos-Valdivia *et al.,* 1997b, Ramos-Valdivia *et al.,* 1998). Induction of isopenthyl diphosphate isomerase activity at the same time as a reduction in the activity of the enzyme farnesyl diphosphate synthase (which converts IPP and DMAPP into farnesyl diphosphate) may assist in channeling more isopenthyl diphosphate into anthraquinone biosynthesis.

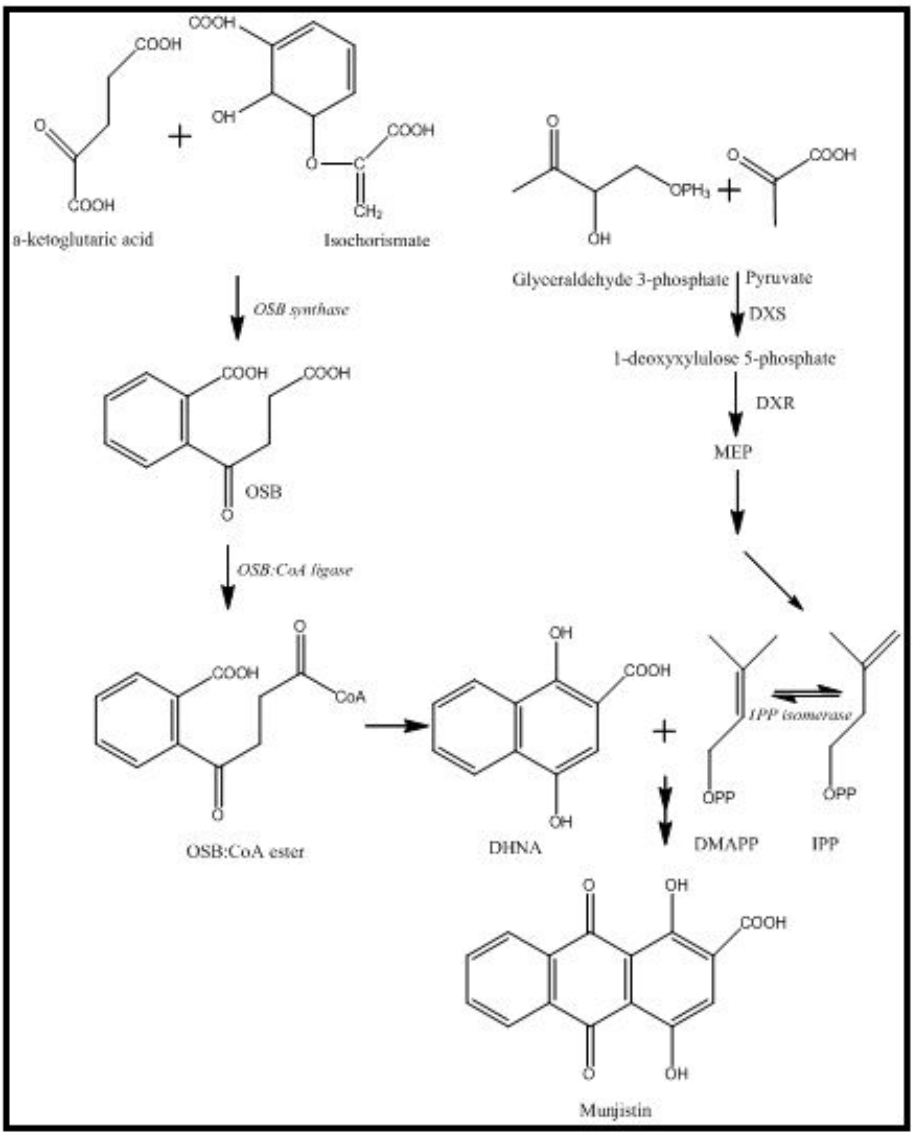


Figure 5: Proposed biosynthetic pathway for the anthraquinone pigments of the Rubiaceae. DXS (1-deoxy-D-xylulose 5-phosphate synthase), DXR (1-deoxy-D-xylulose 5-phosphate reductase).

1.3.4. Biosynthesis via Acetate pathway

A number of natural anthraquinone derivatives are also excellent examples of acetate-derived structures. Endocrocin (Fig. 6), found in species of *Penicillium* and *Aspergillus* fungi are formed by folding a polyketide containing eight C_2 units to form the periphery of the carbon skeleton. Three aldol-type condensations would give a hypothetical intermediate 1, and, except for a crucial carbonyl oxygen in the centre ring, endocrocin results by enolization reactions. Emodin, a metabolite of some *Penicillium* species, but also found in higher plants, e.g. *Rhamnus* and *Rumex* species, would appear to be formed from endocrocin by a simple decarboxylation reaction. This is facilitated by the adjacent phenol function. *O*-Methylation of emodin would then lead to physcion. Islandicin is another anthraquinone pigment produced by

Penicillium islandicum, and differs from emodin in two ways. One hydroxyl is missing, and a new hydroxyl has been incorporated adjacent to the methyl group. Without any evidence for the sequence of such reactions, the structure of intermediate 2 shows the result of three aldol condensations and reduction of a carbonyl.

A dehydration reaction, two oxidations and a decarboxylation are necessary to attain the islandicin structure. In chrysophanol, aloe-emodin and rhein, the same oxygen function is lost by reduction as in islandicin and decarboxylation also occurs. The three compounds are interrelated by a sequential oxidation of the methyl in chrysophanol to a hydroxymethyl in aloe-emodin and a carboxyl in rhein. The pathway outlined for the biosynthesis of endocrocin and emodin is shown in Fig. 6. The only difference between the speculative pathway (Fig. 7) and mechanistically correct pathway is the alteration of the sequence of reactions, but the sequence of reactions is altered. Decarboxylation appears to take place before aromatization of the last-formed ring system and tetrahydroanthracene intermediates such as atrochrysone carboxylic acid and atrochrysone are involved. These dehydrate to the anthrones endocrocin anthrone and emodin anthrone, respectively, prior to introduction of the extra carbonyl oxygen as a last transformation in the production of anthraquinones.

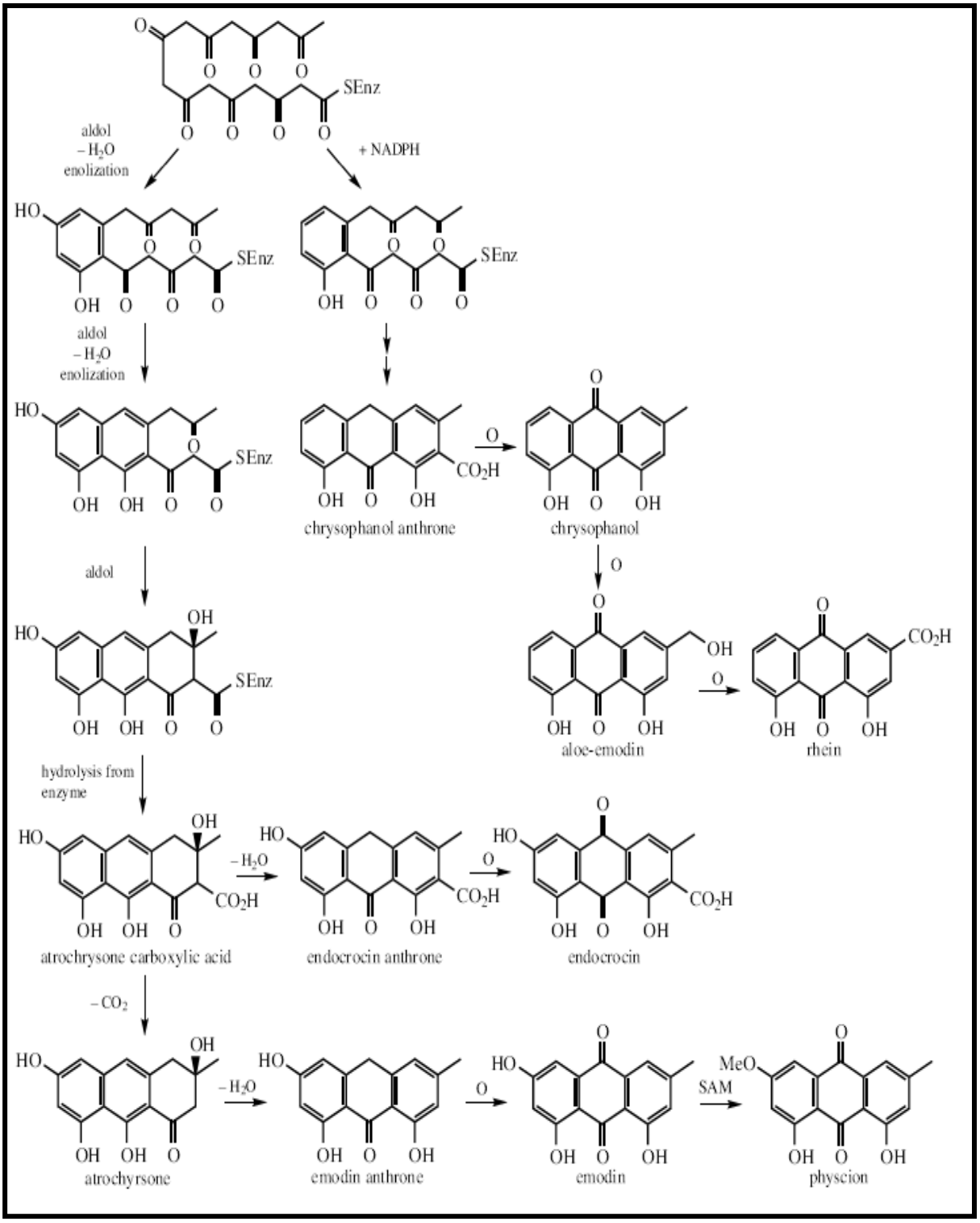


Figure 6: Biosynthesis of endocrocin and emodin via the acetate pathway.

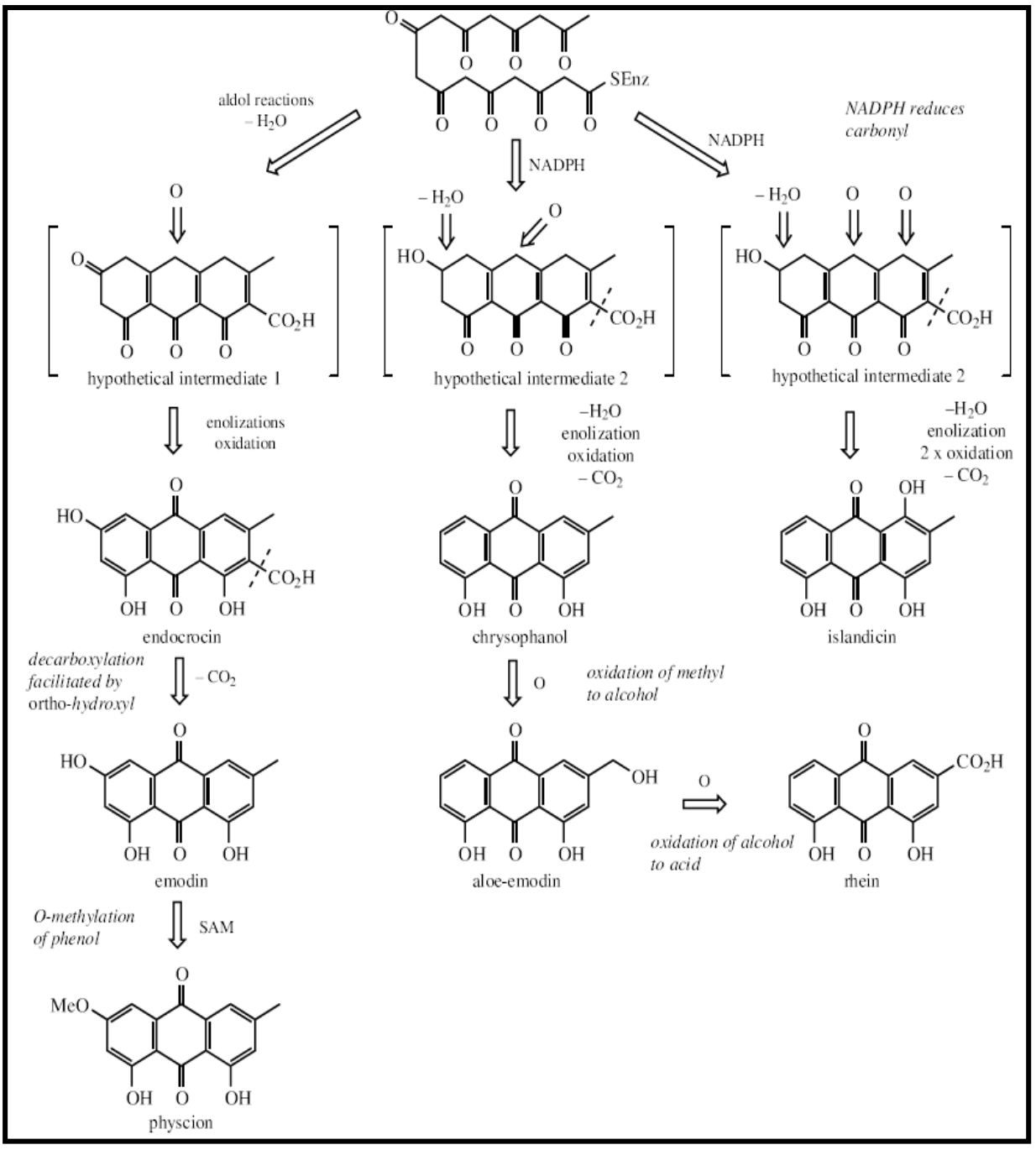


Figure 7: Acetate pathways for the production of polyketides

Many other natural anthraquinone structures are not formed via the acetate pathway, but by a more elaborate sequence involving shikimate and an isoprene unit. Such structures do not contain the characteristic *meta* oxygenation pattern and often have oxygenation in only one aromatic ring. Emodin, physcion, chrysophanol, aloe-emodin and rhein form the basis of a range of purgative anthraquinone derivatives found in long-established laxatives such as Senna, Cascara, Frangula, Rhubarb and Aloes. The free anthraquinones themselves have little therapeutic activity and need to be in the form of water-soluble glycosides to exert their action.

1.3.5. Gene Regulation

Little information regarding the genes encoding the biosynthetic enzymes of the polyketide or acetate pathway, or pathway regulatory factors has been published. Furthermore, it is not known which signals regulate the anthraquinone biosynthetic genes, although fungal elicitors, methyl jasmonate, salicylic acid and the protein phosphatase inhibitor cantharidin, all induce anthraquinone accumulation in transgenic cell cultures. On the other hand, light generally inhibits anthraquinone accumulation and the impact of auxins is variable (Mantrova *et al.,* 1999; Han *et al.,* 2001; Bulgakov *et al.,* 2002, 2003). These gaps in the knowledge of anthraquinone biosynthesis may start to be filled as data emerges from proteomic and gene studies (Han *et al.,* 2001).

1.4. Structural Elucidation of Phytochemicals

Spectroscopic methods coupled with good extraction techniques like chromatography, have contributed to the phenomenal success of natural product chemistry over the past 50 years. A sound isolation strategy (Table 4) has helped in the isolation and characterization of many bioactive molecules. Nowadays, bioassay-guided fractionation of medicinal plants is a feature of routine isolation in the attempt to isolate bioactive components from natural sources. Once the extract has been obtained, the activity within can be demonstrated by bioassay methods using both the crude extract or by using the fractionated extracts. Fractionation has the added advantage of getting to the biologically active material faster. One of the simplest separation methods is partitioning which is a widely used method as an initial extract purification step. A combination of solvents-miscible and immiscible ones are used to separate the phytochemicals making up the extract. This method relies on the ability or the components to be either soluble in water or in the organic phase. These techniques are not only being restricted to plant sources but they are also being applied to microbial and even fungal sources of metabolites.

Table 4: Isolation strategy employed for the isolation and characterization of secondary metabolites from plants.

Gathering Of Plant Materials	• Collection • Storage, and • Vouchering of plants
Extraction Protocols	• Grinding • Cell lysis • Polarity based extractions – Water or Organic solvent extraction
Analytical Protocols (Separation)	• adsorption chromatography • partition chromatography • Gel permeation chromatography. • TLC, gas-liquid chromatography (GLC), affinity chromatography (AC), High Performance Liquid Chromatography (HPLC)
Structural Identification protocols	• UV-Vis Spectroscopy • IR spectroscopy • NMR (carbon and proton) spectrometry • Mass Spectroscopy (EI-LC-MS), Maldi-TOF MS

1.4.1. Sampling, Extraction and Isolation

In practice as soon as the material is collected, in the case of plants, it needs to be identified by a taxonomist so as to ascertain the correct identity of the material. Voucher specimens for herbarium specimens are kept. Various parts of the plant are collected separately (leaves, flowers, stem, wood, bark, root, root bark etc.) and are dried quickly in drying cabinets. Quick drying avoids degradation of the components by air or by microbes. Sometimes the plant samples are lyophilized at high vacuum, but again care must be exercised so as to avoid the excessive loss of volatile components as the latter may also exhibit interesting biological activities.

Once the material has been dried to constant weight, it is ground up to smaller particles and extracted usually using a gradient solvent extraction. Once the extraction is complete, the extractant is usually concentrated under vacuum, for large volumes or solvents and dried under nitrogen for small volumes, ensuring at the same time that volatiles are not lost. Aqueous extracts are generally freeze-dried and stored at −20 °C as this low temperature reduces the degradation of the bioactive natural product. Extraction protocols may sometimes be modified depending on the type of molecules being extracted, e.g. sometimes acids may be added to extract alkaloids as their salts (Fig. 8). The activity within the extract is shown by bioassay

methods using crude or fractionated extracts. The fractions from the extract are separated based on whether they are soluble in water or organic solvent.

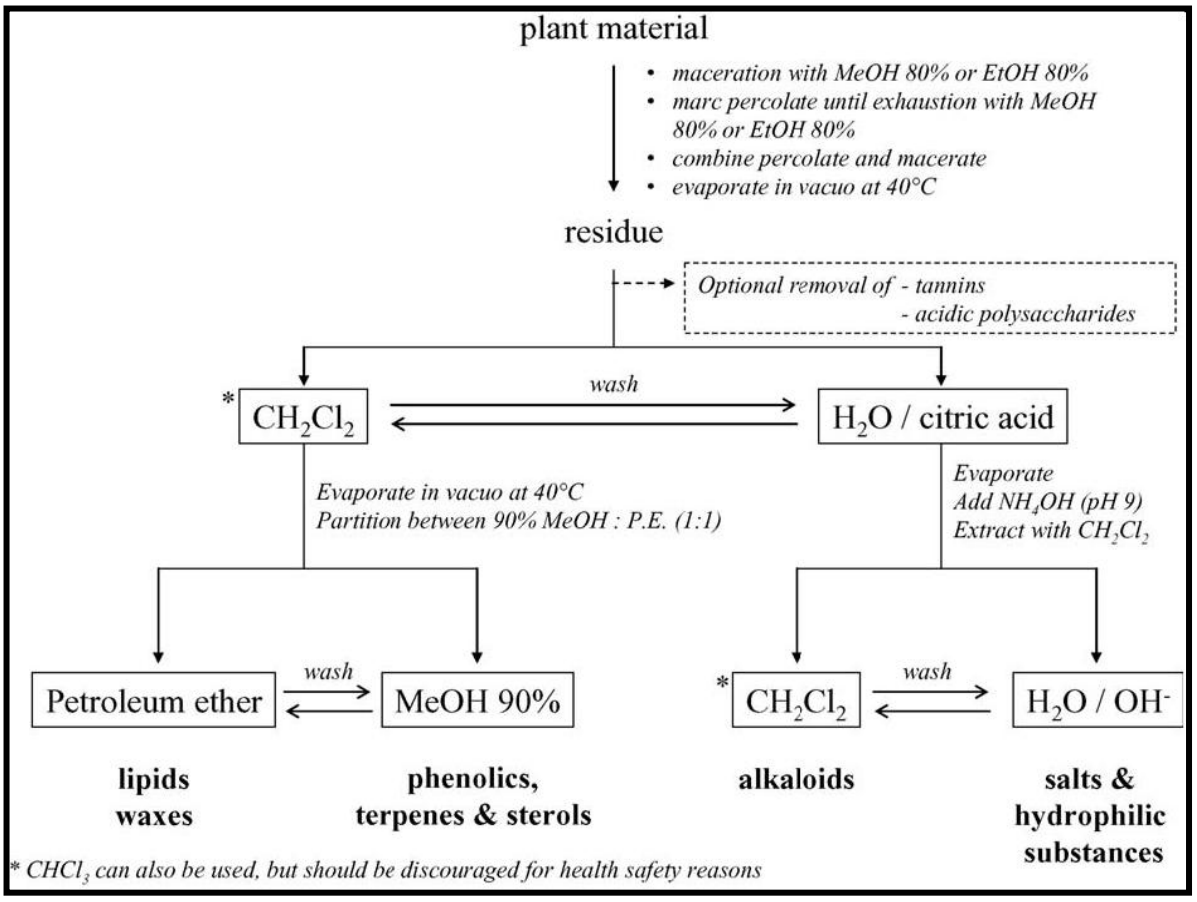


Figure 8: Standard scheme for preparation of plant extracts for biological screening (Mitscher scheme adapted from Ieven *et al*., 1979).

1.4.2. Purification

Chromatographic techniques have been instrumental in the separation of natural products. Some of the techniques are discussed here. One of the fastest and most widely used chromatographic techniques is Thin Layer Chromatography (TLC).

TLC: This method employs glass or aluminum plates pre-coated with the sorbent (e.g. Silica gel) to varying thickness depending on the amount to be loaded. The compound mixture is loaded both in the preparative or analytical plates at around 2 cm from the bottom and lowered in a tank containing the solvent. The latter migrates up the plates and separates the compound mixture according to the polarity of the components. Several reagents are available for visualization of the separated materials. TLC has the advantage of being a highly cost-effective

qualitative technique in as much as a large number or samples can be analyzed or separated simultaneously. The few drawbacks include poor detection and control or elution compared to High Performance Liquid Chromatography (HPLC).

HPLC is very popular and widely used for the analysis and isolation of bioactive natural products. The analytical sensitivity is further enhanced depending on the detector that is being used. The detectors can be based on UV detection such as a photodiode array (PDA), which enables the acquisition of UV spectra of eluting peaks between 190 nm to 800 nm. PDA UV detection has the advantage of detecting even compounds with poor UV characteristics and this is particularly useful in the analysis of natural products such as terpenoids or polyketides, which may not necessarily have chromophores that will rise to a characteristic UV signature. However, the one drawback of HPLC is that it is expensive both from the machine and consumable view-points.

Another 'clean up' chromatographic technique that has become increasingly popular and useful is Gel Chromatography or sometimes referred to as Size Exclusion Chromatography (SEC). This technique employs cross-linked dextran which upon contact with a suitable solvent swells up to form a gel matrix. The latter contains pores of a finite size allowing smaller molecules to be retained and excluding larger molecules. This method is excellent for separating out fatty acids, chlorophyll etc prior to biological assays. This is a non-destructive method for the recovery of a high quantity or extract. It is a method of choice for large molecules such as proteins, polypeptides, carbohydrates etc.

1.4.3. Tools for Structural Elucidation

Structure elucidation is crucial in assessing the biological activity of the molecule as it is a well-known fact that biological activity depends to a large extent on the 3-D arrangement of functional groups on the molecule.

Structure elucidation depends on classical spectroscopic techniques such as: Nuclear Magnetic Resonance (NMR) 1-D and 2-D Proton NMR as well as C^{13} NMR, Infra Red (IR), Mass Spectrometry (MS) and X-Ray analysis.

1.4.3.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is a valuable structure elucidation tool for organic and biological molecules. Besides qualitative information, NMR can provide valuable quantitative information about a sample. A normal liquid state 1D ^{1}H NMR spectrum is commonly recognized as a reliable method for quantification. Other nuclei have also been utilized with one-dimensional experiments, both in liquids and solid state (Martin *et al*., 1980 and Harris, 1985).

The major limitation of NMR spectroscopy is the rather low detection sensitivity, rendering the experiments time-consuming compared to other methods used for molecular structure determination or verification such as X-ray crystallography or mass spectrometry. This is because the sensitivity of the NMR signal depends on the small difference in the populations of the Zeeman energy levels. The separations between the nuclear spin states are small, corresponding to energies in the radiofrequency range. The population difference is given by the Boltzman distribution. For ^{1}H nuclei at room temperature and magnetic field of 10 T the difference in the population is in the order of 1 in 10^{5} which means that most of the nuclei do not contribute to the NMR signal. This is in contrast to optical spectroscopic methods such as, for instance, infrared (IR) spectroscopy where basically a single photon can be detected.

For a high-resolution NMR investigation using a conventional probe operating at ambient temperature the required amount of substance is often milligrams. In many applications the available amount of sample is limited, or the inherent solubility of the substance of interest may be low, or a dilute solution is required because the sample may tend to aggregate at higher concentrations. In such cases, the cryogenic probe technology moves the lower limit of the feasible sample concentration to the microgram and micromolar range. For biological macromolecules, the change in the sample requirement from the millimolar to the micromolar sample concentration range greatly increases the number of compounds that can be studied by NMR. The strength of NMR spectroscopy is given by its multifarious applications, which range from statistical analysis of mixtures to the determination of three-dimensional structures for molecules of biological interest. The information content of NMR at the atomic level is both comprehensive and diverse. To fully elucidate a molecular structure, distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum coherence (HMQC) and correlation spectroscopy (COSY) are also done.

1.4.3.1.1. Distortionless enhancement by polarization transfer (DEPT)

DEPT is a technique that gives information about the number of protons bonded to each carbon.

1.4.3.1.2. Heteronuclear multiple bond correlation (HMBC)

HMBC gives information about weak proton-carbon J-coupling. A weak proton-carbon J-coupling indicates that the proton is two, three, or four bonds away from the carbon. This experiment gives information about which protons are near to (but not directly bonded to) different carbons. HMBC can give an enormous amount of information about molecular structure, since the long-range proton-carbon correlations can include quaternary carbons, in addition to protonated carbons.

1.4.3.1.3. Heteronuclear multiple quantum coherence (HMQC)

HMQC gives information about strong proton-carbon J-couplings. A strong proton carbon J-coupling indicates that the proton is directly bonded to the carbon. HMQC is selective for direct C-H coupling.

1.4.3.1.4. Correlation Spectroscopy (COSY)

Correlation Spectroscopy (COSY) gives information about pairs of protons that are J- coupled. This usually indicates that the protons are on adjacent carbons, e.g. 3-bonds away (though protons further apart may in some cases be J-coupled).

1.4.3.2. Mass spectrometry (MS)

Mass spectrometry is the most sensitive and selective method for molecular analysis and can yield information on the molecular weight as well as the structure of the molecule. Combining chromatography with mass spectrometry provides the advantage of both chromatography as a separation method and mass spectrometry as an identification method. In mass spectrometry, there is a range of methods to ionize compounds and then separate the ions (Gong *et al.*, 2001a). Common methods of ionization used in conjunction with gas chromatography are electron impact (EI) and electron capture ionization (ECI). EI is primarily configured to select positive ions, whereas ECI is usually configured for negative ions (ECNI). EI is particularly useful for routine analysis and provides reproducible mass spectra with structural information, which

allows library searching. GC–MS was the first successful online combination of chromatography with mass spectrometry, and is widely used in the analysis of essential oil in herbal medicines (Guetens *et al*., 2002).

With the GC–MS, one can produce a chromatographic fingerprint of the essential oil of the herbal medicine and also the information related to its most qualitative and relative quantitative composition (Li *et al*., 2001). Used in the analysis of the herbal medicines, there are at least two significant advantages for GC–MS, that is: (1) with the capillary column, GC–MS has in general very good separation ability, which can produce a chemical fingerprint of high quality; (2) with the coupled mass spectrometry and the corresponding mass spectral database, the qualitative and relatively quantitative composition information of the herb investigated could be provided by GC–MS, which is extremely useful for further research in elucidating the relationship between chemical constituents in herbal medicine and its pharmacological applications (Gong *et al*., 2001 b).

For exploring nature's chemodiversity, the situation has changed dramatically in recent years by the introduction of high throughput screening (HTS) methods. By using molecular targets, a large number of samples (up to 100,000 in 24 h) can be screened for a single activity. Obviously synthetic chemists are not able to produce such numbers or new compounds. Their answer was the development of combinatorial chemistry and testing mixtures of compounds obtained through these novel solid-phase chemical synthetic methods. (Keefer and Zock, 2008). HTS offers new possibilities for natural products. It allows rapid screening of large number of extracts and it is very suitable for bioassay-guided fractionation, which in the past was the major bottleneck in studies of active compounds in plant extracts. Powerful chromatographic methods in combination with HTS are now a very efficient way to new leads for drug development. Searching data bases such as Chemical Abstracts, NAPRALERT as well as the Dictionary of Natural Products ensure that no time is wasted on re-investigating existing and known molecules. These databases lead to primary literature information ranging from molecular weights to spectrometry data, which help to recognize common metabolites at an early stage.

1.5. Biological Activity of Secondary Metabolites

The driving force behind much phytochemical research is the discovery of new biologically active compounds for medicinal or agricultural uses. Biological assays, need, to be carried out to identify promising plant extracts, to guide the separation and isolation, and to evaluate lead compounds. The bioassays used in this research will be dicussed below.

1.5.1. Antimicrobial activity

During the last decade, infectious diseases have threatened the life of millions of people around the world (Ashbolt, 2004). Statistics for the next decade appear even worse because of the increase in the development of antimicrobial resistance by different microorganisms. The development of novel, efficient and inexpensive drugs is thus of great importance. The antibacterial and antifungal test methods are classified into three main groups, i.e. diffusion, dilution and bioautographic methods. A fourth and upcoming test method is the conductimetric assay, detecting microbial growth as a change in the electrical conductivity or impedance of the growth medium (Sawai *et al*., 2002). Many South African plants have been tested for antimicrobial activity (Rabe and van Staden, 1997; Lin *et al*., 1999; Luyt *et al*., 1999; Shale *et al*., 1999; Kelmanson *et al*., 2000; Lall and Meyer, 2001 and Tetyana *et al*., 2002). In these publications, majority of the plants extracts display better activity against Gram-positive strains of bacteria (Rasoanaivo and Ratsimamanga-Urverg, 1993; Rabe and van Staden, 1997; Kelmanson *et al*., 2000; Rabe and van Staden, 2000; Pillay *et al*., 2001; Rabe *et al*., 2002; and Grace *et al*., 2002; Reid *et al*., 2005; Heyman *et al*., 2009). Some of the compounds showing antibacterial properties have been chemically characterised and some of these are shown in Fig. 9 (1) muzigadial; (2) vernodalin; (3) wighteone; (4) palmitic acid; (5) sibyllenone; (6) cathinone, (7) ibogaine; (8) lobeline; (9) kirkinine; (10) galanthamine; (11) mesembrine; and (12) 1-O-acetyllycorine.

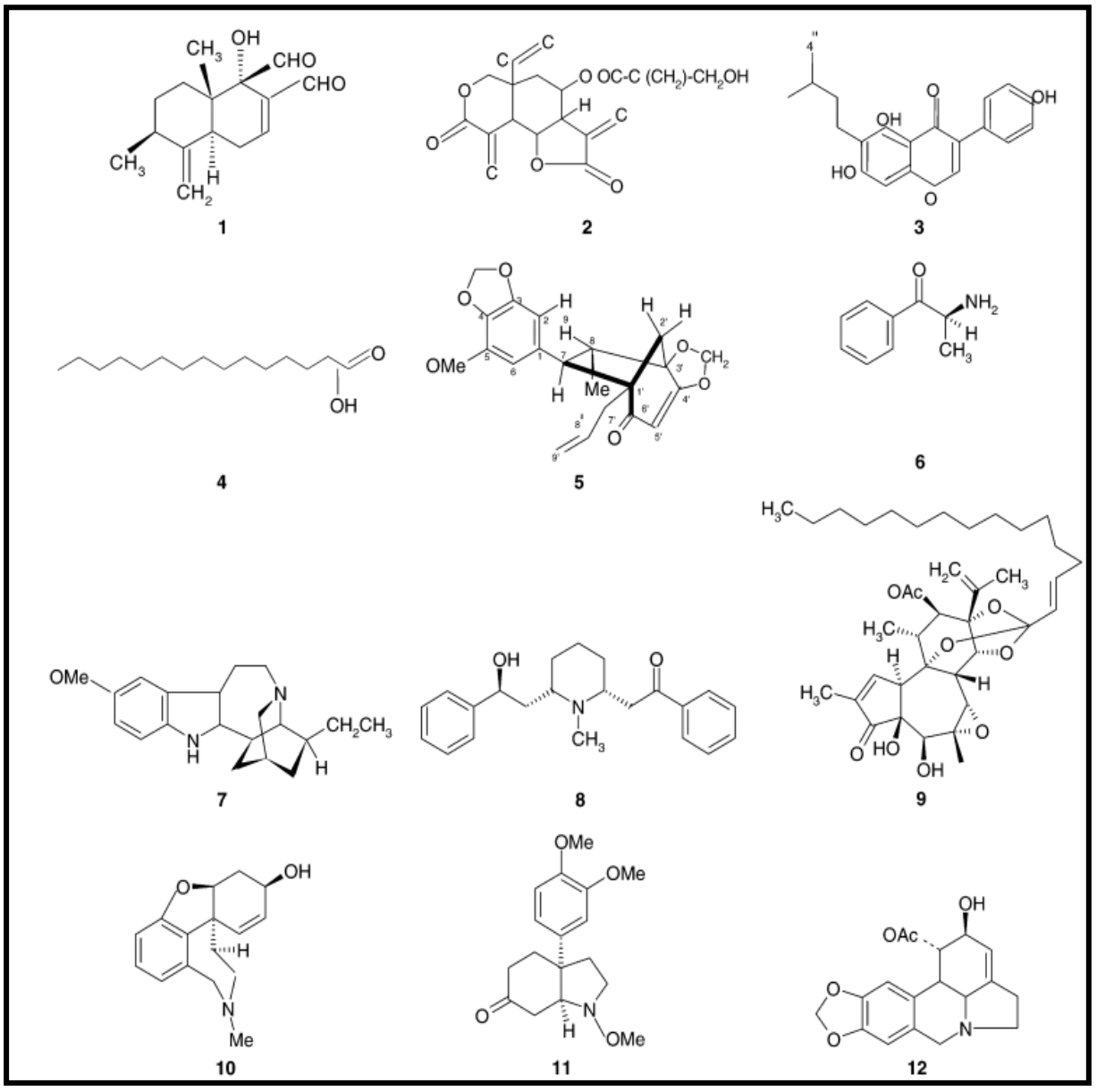


Figure 9: **Biologically active compounds identified in African medicinal plants: (1) muzigadial, (2) vernodalin, (3) wighteone, (4) palmitic acid, (5) sibyllenone, (6) cathinone, (7) ibogaine, (8) lobeline, (9) kirkinine, (10) galanthamine, (11) mesembrine, (12) 1-O-acetyllycorine.**

1.5.2. Antioxidant Compounds

Many plants are also known as sources of natural antioxidants for the scavenging of free radicles. The latter are known to initiate a series of chain reactions resulting in oxidative tissue damage and a wide range of degenerative diseases such as cancer, premature aging, diabetes, and host of cardiovascular diseases (Lindsey *et al*., 2002). The rising, awareness and consumer concern on issues of food preservation and chemical preservatives necessitates a search for natural anti-oxidants that could not only be used to preserve food, but also in the treatment of

some diseases. Some of plants that yield a high level of anti-oxidant activity include the *Amaranthu*s sp. (Amaranthaceae), *Sisymbrium thellungii* O.E. Schulz (Brassicaceae) and *Urtica dioica* L. (Urticaceae). Traditionally, these plants are used in the preparation of 'imfino' which forms an important part of the diet. High activity (greater than 70%) was also shown by the tuberous *Colocasia esculanta* Schott (Araceae) and the teas *Galium aparine* L. (Rubiaceae) and *Aspalathus linearis* (Burm.f.) R. Dahlgren (Fabaceae) (rooibos) (Fennell *et al*., 2004).

1.5.3. Anti-inflammatory Compounds

Many plant derived compounds inhibit the formation of pro-inflammatory signaling molecules such as prostaglandins (made via cyclooxygenase) or leukotrienes (made via lip-oxygenase). Prostaglandins are involved in the complex process of inflammation and are responsible for the sensation of pain. To evaluate the efficacy of a plant in reducing pain and inflammation, extracts can be tested for prostaglandin synthesis inhibitory activity in an *in vitro* assay (White and Glassman, 1974). This assay measures the degree of inhibition of the cyclooxygenase enzyme active in prostaglandin synthesis. Cyclooxygenase exists in two isoforms: COX-1 and COX-2 (Mantri and Witiak, 1994 and Vane, 2004). In recent years attention has focused on finding non steroidal anti-inflammatory drugs (NSAIDs) which selectively inhibit COX-2 with little interference of COX-1. Compounds which are selective inhibitors of COX-2 are potentially anti-inflammatory and nonulcerogenic, and would therefore be of considerable interest for therapeutic use (Mantri and Witiak, 1994). Some of the South African plants used in the treatment of pain and inflammation have been studied. (Jagër *et al.*, 1996; McGaw *et al*., 1997; Lindsey *et al*., 1999 and Shale *et al.*, 1999). *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt (Zingiberaceae), *Ocotea bullata* (Burch.) Baill. (Lauraceae) and *Eucomis autumnalis* (Mill.) Chitt. (Hyacinthaceae) were identified as having good anti-inflammatory activity (McGaw *et al*., 1997; Lindsey *et al*., 1999 and Zschocke *et al*., 2000). *Ocotea bullata* is one of the top ten traditional medicinal plants used in South Africa (Mander, 1997).

Two compounds sibyllenone and ocobullenone were found to contribute to inhibitory activity of *Ocotea bullata* bark extracts (Zschocke *et al*., 2000). Taylor and van Staden (2002) also found significant COX-2 inhibitory activity with extracts from *Eucomis autumnalis*. A review by Iwalewa *et al* (2007) reviewed 123 South African plants species for anti-inflammatory activity. In most of these there is no indication of the chemical compounds present. In many other cases the chemicals present are based on group tests and only on rare cases have the active compounds been isolated and characterized.

1.5.4. Anti-Cancer compounds

Cancer is one of the predominant killers in the world today. Thus, there continues to be great interest in the search for new and better treatments. In order to understand phytochemical-cell interactions it is first important to understand a little about the life cycle of human cells, including proliferation, differentiation and cell death. The cell reproductive life cycle has four phases: G_1, S, G_2, and M. G_0 is a stage of quiescence which can be of variable length (Fig. 10). Many enzymes must work together to reproduce an accurate replication of DNA for the new cell. One enzyme of this system that seems to be particularly vulnerable to exogenous plant chemicals is topoisomerases. DNA topoisomerases act by opening transient, protein-bridged, single- or double-stranded breaks through which the DNA strand can be passed in order to solve the topological problems and to relieve the torsion stress accumulated in all cellular transactions of the DNA molecule. In regard to their ability to cleave single- or double- strand DNA molecules, these enzymes have been classified as type I and type II DNA topoisomerases (Topo I and II), respectively. (Caron and Wang, 1994).

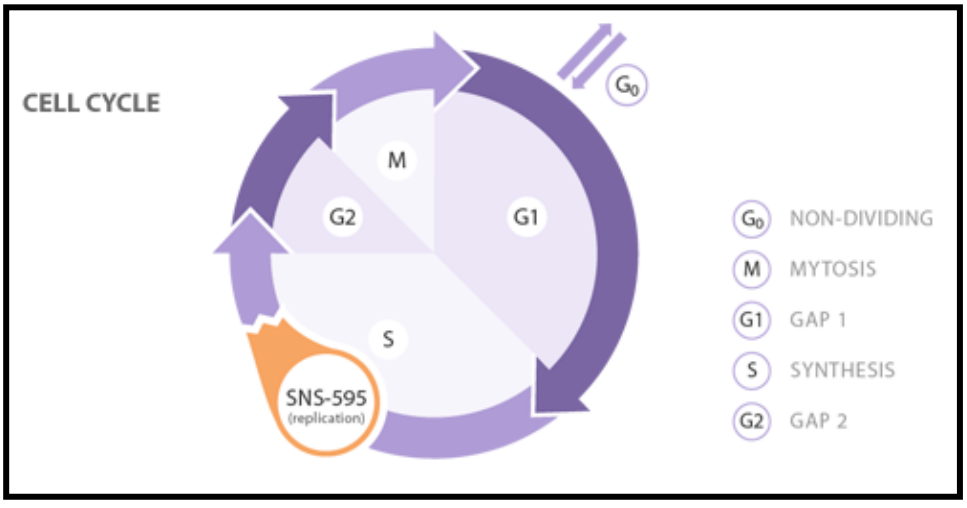


Figure 10: Cell life cycle

Type II topoisomerases are highly conserved proteins working as dimeric and ATP-dependent enzymes. In eukaryotes, DNA Topo II are homodimeric proteins while in prokaryotes and phages they consist of heterodimeric structures. The prokaryotic DNA Topoisomerase II is defined as DNA gyrases: they are the only type of topoisomerases capable of introducing negative supercoiling in DNA coupled to ATP hydrolysis. Topoisomerase II is a protein dimer with a heart-shaped form and large holes (Fig. 11).

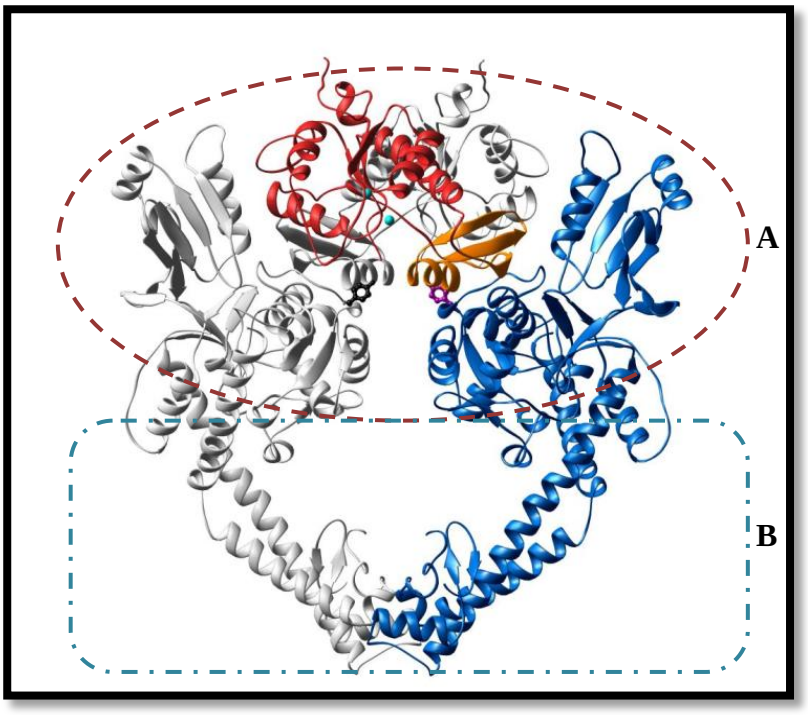


Figure 11: **High resolution 3-D crystallography image of the binding (A) and cleavage core (B) of Type II topoisomerase (topo II) from *Saccharomyces cerevisae***

Preventing topoisomerase II from disentangling a cell's DNA is fatal to the cell, which is why drugs that target topoisomerase II serve as agents against bacterial infections and some forms of cancer. Topoisomerase IIα and IIβ are the targets of a diverse group of natural and synthetic compounds (Fortune and Osheroff, 2000; Wilsterman and Osheroff, 2003: Velez Cruz and Osheroff, 2004) some of which are depicted in Table 5. Although the compounds shown vary in their ring structures, all are potent topoisomerase II poisons *in vitro* and in human cells. Many of these agents are in wide clinical use as anticancer agents and represent some of the most successful chemotherapeutic drugs currently used for the treatment of human malignancies.

Table 5: Anti-topoisomerase drugs

Drug class	Example	Topoisomerase Inhibited	Effects	References
Acridines	Amsacrine (m-AMSA)	II	Stabilize cleavable complex	Louie and Issel 1985
Actinomycins	Actinomycin D	main effect on Topo II	stabilize cleavable complex	DeMarini *et al.*, 1987
Anthracenediones	Mitoxantrone	II	stabilize cleavable complex	Harker *et al.* 1991
Anthracyclines	Doxorubicin	II	stabilize cleavable complex	Au *et al.* 1981
Ellipticines	2-methyl-9-OH-ellipticinicum acetate	II	stabilize cleavable complex	Aimova *et al.*, 2007
Coumarine	Novobiocin	bacterial gyrase (sub B)	interferes with ATPase activity of Topo II	Sugino *et al.* 1978
Isoflavonoids	Genistein	II	PTK inhibitor and cleavable-complex blocker	Markovits *et al.* 1989 Adlerkreutz 1995
Alkaloid	Camptothecin and its derivatives	I	stabilize cleavable complex	Hsiang *et al.* 1985
bis-piperazinediones	ICRF-159, 193	II	inhibits DNA relaxation and cleavable complex formation	Jensen *et al.* 2000
Anthracenyl peptides	Merbarone	II	inhibits cleavable complex formation	Khelifa and Beck 1999

1.6. Biosafety and Plant Compounds

Although plants may possess pharmacological properties, they may also be toxic or mutagenic. The toxic effects of most widely used medicinal plants are not well documented in the literature although one might expect plants used in traditional medicine over a long period to be safe (Elgorashi *et al.*, 2003). Hence, an evaluation of the toxicity, cytotoxicity and mutagenicity (Schimmer *et al.*, 1988; Schimmer *et al.*, 1994; Higashimoto *et al.*, 1993; Kassie *et al.*, 1996; De Sa Ferrira and Ferrao Vargas, 1999) are an important part of all scientific studies.

There are many toxicity and cytotoxicity tests available to test for the safety of plant extracts or compounds. Cytotoxicity is the measurement of the ability of a chemical to damage or kill cells. Toxicity is evaluated using two different criteria, firstly by its capacity to cause injury to a living organism and secondly as any diverse effects of a chemical on a living system (Verschaeve *et al.,* 2004). The severity of toxicity produced by any chemical is directly proportional to the concentration and the exposure time. This relationship depends on the developmental stage of an organism and its physiological status (Meyer *et al.,* 1982). The MTT assay using a tetrazolium salt has been used to develop a quantitative colorimetric assay for mammalian cell to evaluate survival and proliferation. The assay detects living but not dead cells. Hence by evaluating the cell survival one can deduce the LC_{50} and the cytotoxicity of the plant extract.

The brine shrimp lethality bioassay is another simple bioassay for natural products and known to be rapid, reliable, inexpensive and convenient, as a general bioassay for screening of toxicity (Meyer *et al.,* 1982). The brine shrimp *Artemia salina* Leach, from the family Artemiidae is an invertebrate (crustacean) component of saline aquatic and marine ecosystems (Ayo *et al.,* 2007). The procedure determines LD_{50} values in µg/ml of plant extract. It is known that a positive correlation exists between brine shrimp lethality and cytotoxicity (Ayo *et al.,* 2007).

Many plants also have compounds that are mutagenic which are used in traditional medicine , and have also been found to cause damage to genetic material. In a study by Elgorashi *et al*. (2003) potential genotoxic effects were studied using *in vitro* bacterial and mammalian cell assays such as Ames test (tester strains TA98 and TA100), VITOTOX test, micronucleus test and comet assay, most of the plant species investigated caused either DNA damage or chromosomal aberrations, and only a few plants showed frame shift mutations in the *Salmonella* / microsome assay using strain TA98. Among the plants that had mutagenic effects in the *Salmonella*/microsome assay were *Crinum macowanii*, *Chaetacme aristata*, *Plumbago auriculata*, *Catharanthus roseus* and *Ziziphus mucronata* (Elgorashi *et al*., 2003). Of the 51 plant species tested using human white blood cells, *Kigelia africana*, *Merwilla plumbea*, *Boophane disticha*, *Celtis africana*, *Crinum macowanii*, *Erythrina caffra*, *Ochna serrulata*, *Sclerocarya birrea* and *Tulbaghia violacea* showed genotoxicity in the micronucleus test, in the form of structural and numerical chromosome aberrations. *Acokanthera oblongifolia*, *Afzelia quanzensis*, *Bersama lucens*, *Ocotea bullata*, *Siphonochilus aethiopicus* and *Tetradenia riparia* caused DNA damage as detected in the comet assay (Taylor *et al.,* 2003).

Some plants listed below are reported to be highly toxic because they cause both DNA damage and chromosomal aberrations: *Antidesma venosum*, *Balanities maughamii*, *Catharanthus roseus*, *Catunaregam spinosa*, *Chaetacme aristata*, *Diospyros whyteana*, *Euclea divinorum*, *Gardenia volkensii*, *Heteromorpha trifoliata*, *Hypoxis colchicifolia*, *Plumbago auriculata*, *Prunus africana*, *Rhamnus prinoides*, *Ricinus communis*, *Spirostachys africana*, *Trichilia emetica*, *Turraea floribunda*, *Vernonia colorata* and *Ziziphus mucronata* (Taylor *et al.*, 2003).

1.7. Plant Cell Culture Systems

1.7.1. Plant cell cultures for the production of secondary metabolites

Many medicinal plants are agronomically cultivated for the production of clinical drugs. The major draw back of field cultivation of these plants is that these do not withstand field cultivation due to the impact of biological pathogens and environmental influences (pathogen sensitivity and insects) in nature (Mulabagal and Tsay, 2004). An alternative route to producing the potential anticancer anthraquinones from *C. triloba* is via plant cell culture technology. Although, the utilization of plant cells has had limited commercial success and low productivity levels of secondary metabolites in culture (Ramachandra Rao and Ravishankar, 2002). Various strategies have been developed for stimulation and improvement of biosynthetic activities for increasing product yields, to enable commercialization of metabolites produced by plant cell cultures (Mulabagal and Tsay, 2004; Ramachandra Rao, 2000). An outstanding example is the commercial production of paclitaxel, an anticancer drug isolated from T*axus spp* (Zhong, 2002).

Plant cell culture technology offers an attractive alternative for production of high-value secondary metabolites over whole plant cultivation due to the following reasons:

- Plant cells are biosynthetically totipotent which means plant cells in culture retain the complete genetic information and therefore have the ability to produce a range of secondary metabolites that are found the parent plant (Ramachandra Rao and Ravishankar, 2002).
- Product profiles of the *in vitro* cell culture system and parent plant can differ, thus novel metabolites can be produced (Ramachandra Rao and Ravishankar, 2002).
- Plant cell culture biotransformation systems can allow for the conversion of inexpensive precursors to novel or valuable compounds (Ramachandra Rao and Ravishankar, 2002).

- Plant cell cultures can accumulate higher levels of the plant derived product through optimization of cultural conditions (Ramachandra Rao and Ravishankar, 1998).
- In addition the metabolites of genetically modified plants can be produced under the appropriate biological containment to avoid contamination of other field grown crops (Jelaska *et al*., 1981).

Plant cell propagation methods were developed in the 1950's, when it was realized that plant cells possessed the potential to synthesize a variety of useful, low molecular weight molecules. Plant cell cultures have gained a reputation of being safe, flexible and an efficient production platform. A definite advantage of plant cells for metabolite production is its ability to outweigh whole plant production systems by by-passing the long development times, variations in product yield and quality and it eliminates contamination with fertilizers and pesticides. Good Manufacturing Practice (GMP) is easily implemented at all stages of metabolite production *in vitro* (Hellwig *et al*, 2004).

1.7.1.1. Induction of callus cultures from explant material

The first step in the process of establishing a cell culture system involves choosing the explant plant material (Dixon and Gonzales, 1994). Explants (plant tissue) are obtained from a wide range of plant organs (stems, leaves, roots) for induction of callus. The source of the explant is a critical factor to be considered by plant biotechnologists in terms of secondary metabolite synthesis, example: the roots of *Medicago sativa L.* retain their biosynthetic ability to produce most of the secondary metabolites; even in undifferentiated cell suspension cultures (Kessmann *et al*., 1990). Explant material used to establish callus cultures is usually washed with several sterilization agents to prevent bacterial and fungal growth which adversely affect culture growth. Disinfectants are commonly used to destroy microbial contaminants and preserve the explant tissue. The exposure time and concentration of the disinfectant are critical factors that should be considered during the sterilization process (Table 6) (www.sigmaaldrich.com, 2008).

Table 6: Disinfectants commonly used to sterilize explant tissue, with concentrations and exposures times

Disinfectant	Concentration (%)	Exposure (min)
Calcium hypochlorite	9-10	5-30
Sodium hypochlorite	0.5-5	5-30
Hydrogen peroxide	3-12	5-15
Ethyl alcohol	70-95	0.1-5.0
Silver nitrate	1	5-30
Mercuric chloride	0.1-1.0	2-10
Benzalkonium chloride	0.01-0.1	5-20

1.7.1.2. Cultivation of plant cell cultures

The plant tissue culture technique enables for the production of callus cultures i.e. the undifferentiated tissue that develops on or around an injured or cut plant surface or in tissue culture. It is these undifferentiated cell masses, in friable form, that are integral for the production of homogenous cell suspension cultures. When cultured *in vitro*, all the needs, both chemical and physical, of the plant cells have to be met by the culture vessel, the growth medium and the external environment (light, temperature, etc.). The growth medium has to supply all the essential mineral ions required for growth and development as well as additional organic supplements such as amino acids and vitamins. There are nine basic media formulations and these contain elements such as nitrogen, potassium, calcium, magnesium, phosphorus, sulphur, iron etc which all play a role in the growth of healthy cultures *in vitro.* One of the most commonly used media for plant tissue cultures is that developed by Murashige and Skoog (MS), for tobacco tissue culture (Murshige and Skoog, 1962). The significant feature of the MS medium is its very high concentration of nitrate, potassium and ammonia.

The culture medium for *in vitro* cultivation of callus cultures consists of four components: the essential elements which can be divided into three categories: micronutrients, macronutrients and iron source; organic supplements to supply vitamins and/or amino acids; a fixed carbon source which is usually sucrose and plant growth hormones (Dixon and Gonzales, 1994). Most plant tissue culture media contain two classes of plant growth hormones, cytokines and auxins. These are usually used together in the culture medium. The ratio of auxin to cytokine plays an important role in determining the type of culture that will be established. An intermediate ratio favors callus induction and continued growth of the callus tissue without differentiation. An

auxin promotes callus induction from explant tissue, cell elongation and maintains callus tissue in an undifferentiated state while the cytokine allows for stimulation of plant cell division (Dixon and Gonzales, 1994). Therefore plant growth hormones function synergistically to promote culture growth. Fig. 12 below illustrates the results obtained from a study which evaluated the effects of different hormones in callus and cell suspension cultures of *Gymnema sylvestre*. This plant originates from India and is used to treat diabetes as it is also known to produce gymnemic acid based bioactives (Gopi and Vatsala, 2006).

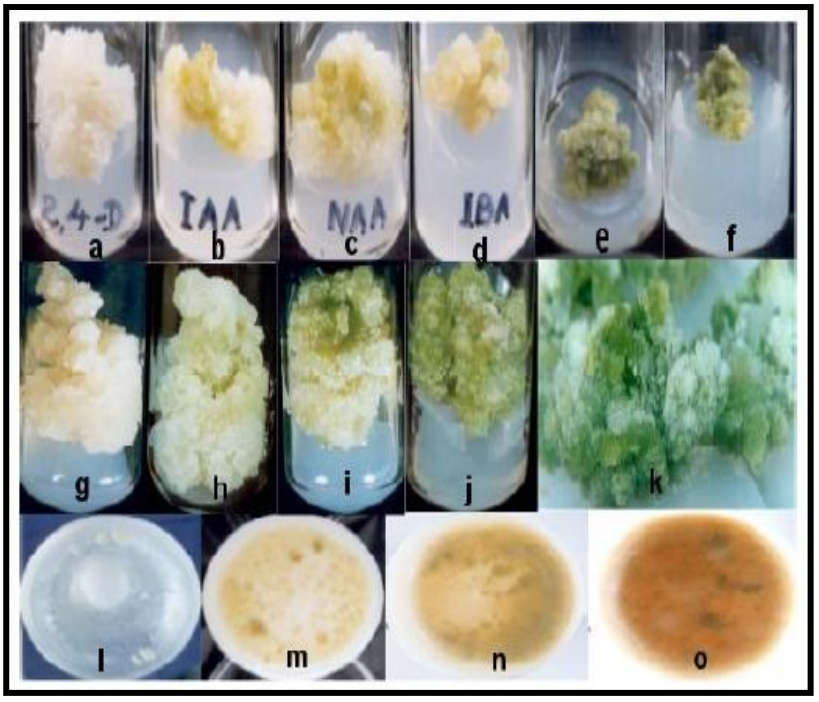


Figure 12: Callus (a-k) and suspension culture (l-o) developments from *Gymnema sylvestre*.

1.7.1.3. Plant cell suspension cultures

Plant cell suspension cultures are complex and heterogenic systems composed of a mixture of single cells and aggregates with different shapes and sizes (Trejo-Tapia *et al*., 2001). Aggregation occurs with the secretion of extracellular polysaccharides or in most instances there is a lack of cell separation after cell division (Chattopadhyay *et al*., 2002; Taticek *et al*., 1991). Plant cell suspension cultures are prepared by agitating friable callus tissue in shake flasks for the formation of single cells and smaller aggregates (Hellwig *et al*., 2004). Suspension cultures are the preferred mode of cultivation of plant cells due to the rapid growth cycles and higher secondary metabolite yields as nutrient uptake is enhanced in submerged culture conditions (Tripathi and Tripathi, 2003). In addition, secondary metabolites are intracellular based, liquid culture allow for cultivation of high concentration of the cell mass

before biosynthesis of secondary metabolites are induced. The production of the metabolite in liquid culture is important for scale up of the process from a shake flask to a bioreactor. The cultivation of plant cell cultures in bioreactors is the key step towards development of processes for the commercial production of cell-culture derived metabolites as a controlled environment independent of season or weather is ensured, as opposed to crop cultivation (Doran, 2000; Tripathi and Tripathi, 2003).

1.7.2. Elicitation of Secondary Metabolites

1.7.2.1. Manipulation of secondary metabolite production by elicitation

Plant cell culture systems have been established from a variety of plants but in many cases high production levels of the secondary metabolite have not been achieved. The enhancement of secondary metabolite production through elicitation has created a new research area that will economically benefit the pharmaceutical industry (Namdeo, 2007). Various manipulative strategies such as screening and selecting high productive cell lines, altering nutrient conditions and optimization of environmental conditions are available for promoting productivity of secondary metabolites in plant cell cultures, but elicitation promises to almost guarantee a dramatic increase in product yield (Ramachandra Rao and Ravishankar, 2002; Yu *et al*., 2002; Yuan *et al*., 2002).

1.7.2.2. The Elicitation Mechanism

Elicitation is the induction of secondary metabolite production by applying molecules or treatments called elicitors, thereby reducing the process time taken to attain high concentrations of the product (Zhong, 2002; Ramachandra Rao and Ravishankar, 2002). Generally, elicitors are utilized to mimic stresses such pathogen defense or wound responses which lead to the production of secondary metabolite. Therefore, production of secondary metabolites that are involved in protective functions (phytoalexins) increases when the plant is damaged by pathogens. Elicitors can be biotic (glucan polymers, glycoproteins, low molecular organic acids or fungal cell materials) or abiotic (UV irradiation, salts of heavy metals and many chemicals) (Zhong, 2002). Activation of plants defense mechanisms through the most general and well studied pathway (Fig. 13) consists of: **1)** detection of the pathogenic signal, **2)** activation of H^+ - ATPase, **3)** increase of the calcium infux in the cells, **4)** the activation of calcium-dependent protein kinase (CDPK) and **5)** activation of NADPH-oxidase. Active oxygen radicals are generated by NADPH- oxidase which activates MAP-kinases, this leads to an increase in the

expression level of certain protective genes that are involved in the biosynthesis of secondary metabolites.

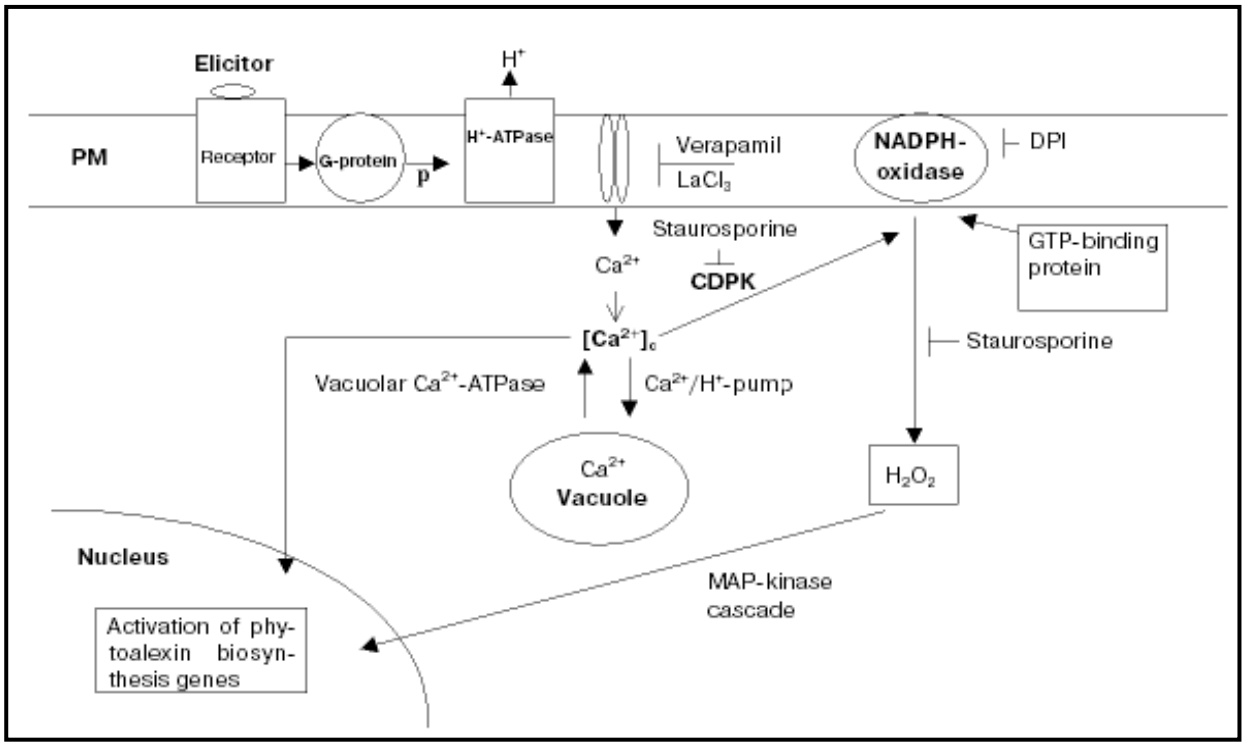
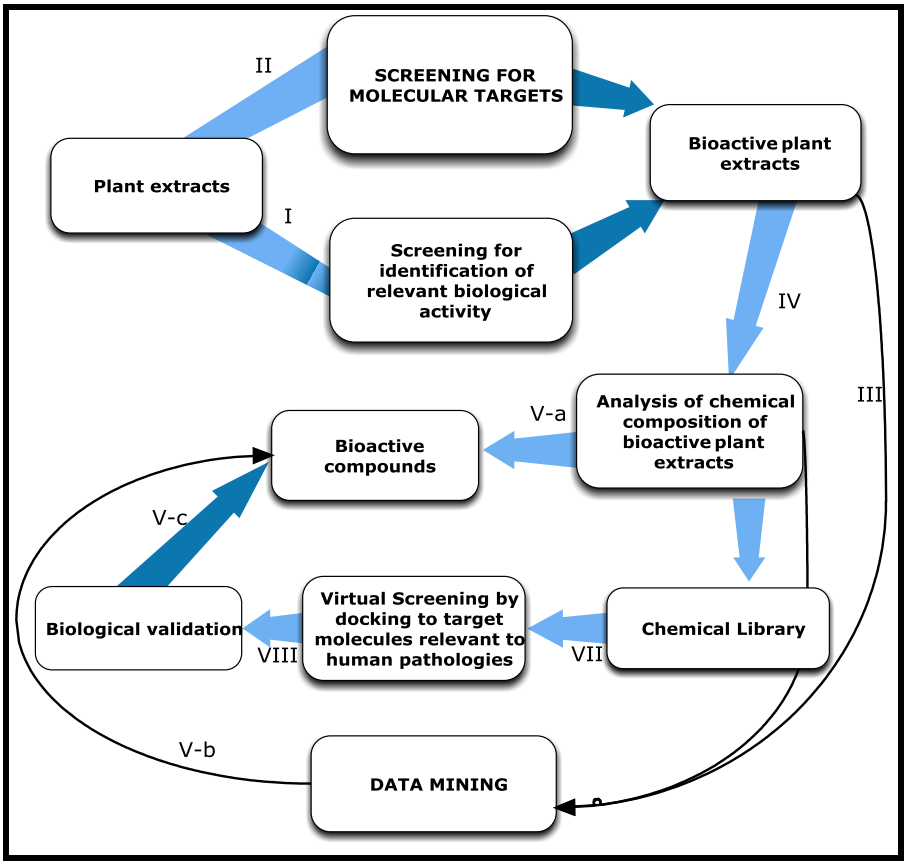


Figure 13: Diagram of the signaling pathway that result to the activation of phytoalexin biosynthesis genes

CHAPTER 2: PHYTOCHEMISTRY OF *C. TRILOBA*

2.1. INTRODUCTION

The discovery and characterization of the biological chemicals from plants with therapeutic activities, including antibacterial, antitumor, antiviral**,** anti-inflammatory and pro-apoptotic is a multidisciplinary activity which requires different experimental strategies shown in Fig. 14. A first level of screening that is followed by many research groups allows the identification of plant extracts and essential oils exhibiting relevant biomedical effects (action I of the flow chart shown in Fig. 14). A complementary approach focusing on mechanism(s) of action instead of bio-medical effects identifies possible molecular targets of plant extracts (action II of Fig. 14). After the formal demonstration of a relevant biological activity, including possible effects on specific molecular targets, two complementary approaches can be followed for identification of putative lead compounds. The first (action IV of Fig. 14) is a direct chemical analysis of plant extracts, based on several methods such as gas chromatography/mass spectrometry (GC–MS) and high-performance liquid chromatography/MS (HPLC–MS). The second (action III of Fig. 14) is an activity of data mining focusing on what is available in the literature concerning bioactive plant extracts. Both these approaches can generate sets of molecules that are possibly responsible for biological activity found in analyzed extracts (actions V-a and V-b of Fig. 14) When the identification of a molecular target is available, then novel approaches might be undertaken to characterize both mechanisms of action, binding modes and novel bioactive molecules (actions VI–VIII of Fig. 14). Integrated computer-assisted strategies may help to process the huge amount of available structural and biological information in a reasonably short time for a straightforward search of bioactive natural products (action V-c of the flow chart shown in Fig. 14).



***** Numbering (I–VIII) facilitates the presentation of the different activities leading to the final identification of bioactive compounds starting from plant extracts, through intermediate products (databases from bioactive plant extracts, chemical libraries).

Figure 14: **The interplay between several actions contributing to the characterization of biological activity of natural products from plant extracts.**

In this study the initial approach of data mining did not give any information related to the chemical constituents or the biological actions of *C. triloba*, therefore we took the chemical approach to identify the predominant compound/s. The extraction approach was based on a scheme described by Harborne and Harborne (1998) that is the separation of plant metabolites including phenolics, terpenoids and alkaloids based on their polarity. TLC results showed that the roots had the highest number of compounds. After column chromatography and HPLC three predominant compounds were identified. These were bulked up by preparative TLC, HPLC and identified by the various spectroscopic methods. The compounds were separated and purified by various chromatographic techniques. They were then characterized by extensive spectroscopic techniques including UV-Vis, IR, EI-LCMS and NMR (COSY, HMQC, HMBC and DEPT).

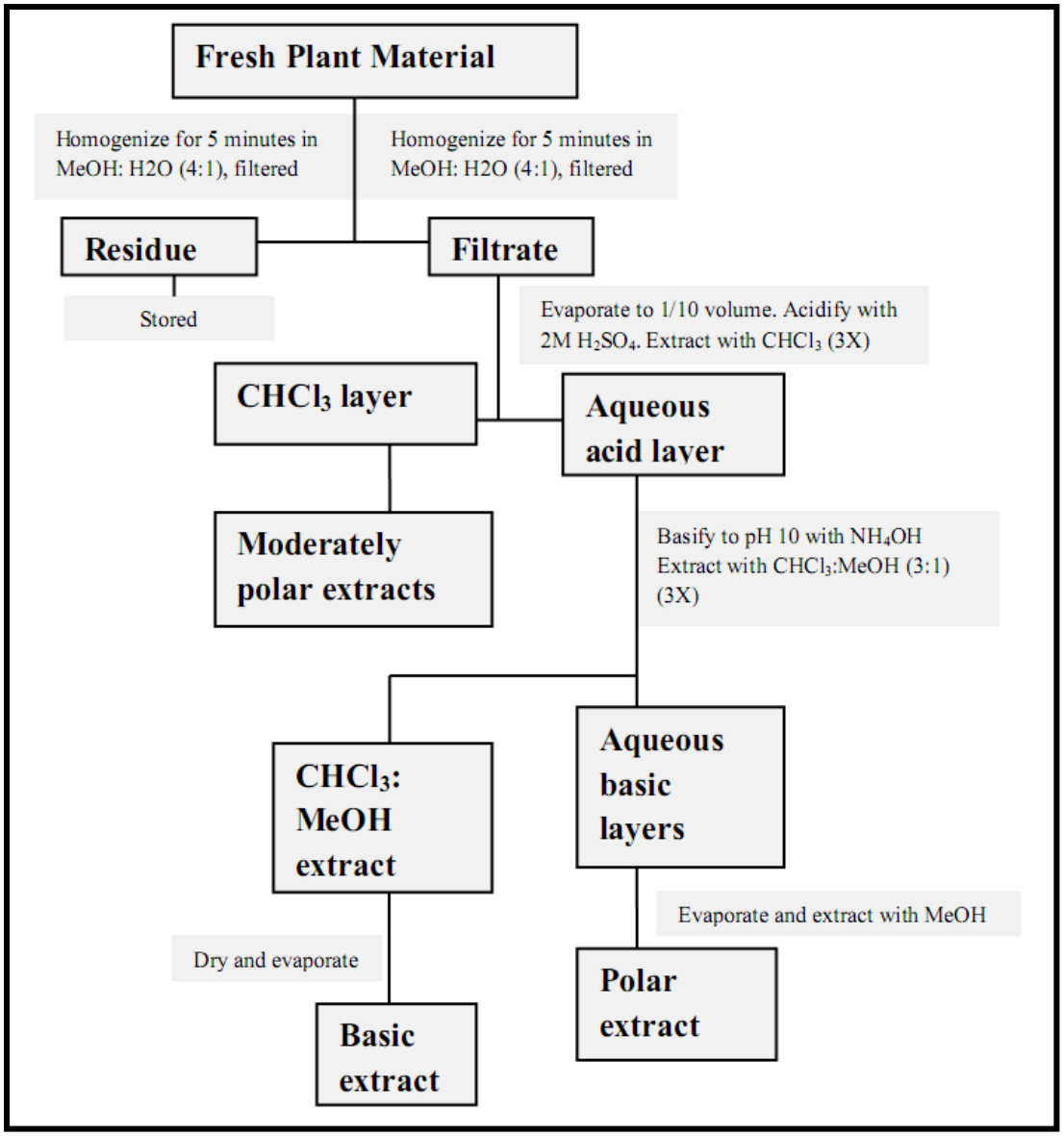


Figure 15: **Modified procedures for extracting fresh plant material and fractionating into different classes.**

2.2. MATERIALS AND METHODS

2.2.1. Preparation of Plant Material

Ceratotheca triloba (Bernh.) E. Mey. ex. Hook.f. was collected in Durban, Kwazulu Natal, South Africa, and identified by using available floral keys. A voucher specimen was deposited in the Ward Herbarium, University of Kwazulu Natal (Westville Campus). The plant portals were carefully examined and old, insect-damaged, fungus-infected roots were removed. Healthy roots, stems, leaves, flowers and seed pods were spread out and dried in the laboratory at room temperature for 5-8 days or until they broke easily by hand. Once completely dry, plant material was ground to a fine powder using a Wareing blender. Larger quantities were crushed to a fine powder of 1.0 mm diameter using a Retsch Mühle mill at the Department of Biotechnology and Food Technology (DUT). Material was stored in a closed container at room temperature until required.

2.2.2. Extraction

The extraction protocol of Harborne and Harborne (1998) was modified and used in this research. This method separates the plant metabolites into different groups based on their polarity. The fresh plant material was homogenized for 5 minutes in methanol/water (4:1) and then filtered. This resulted in the filtrate and residue. The residue was discarded and the filtrate was concentrated to 1/10 volume and acidified with 2M H_2SO_4. The acidified filtrate was extracted with chloroform (3X) and this resulted in the chloroform partition and the aqueous acid layer. The chloroform partition would contain all the moderately polar compounds (terpenoids and phenolics). The aqueous acid layer was then basified to pH 10 with NH_4OH and further extracted with chloroform: methanol (3:1) to yield the polar and basic extract (mostly alkaloids). This extraction protocol is represented schematically in Fig. 15. Extraction was initially performed using a method prescribed by Harborne and Harborne, 1998. The principle of separation of bioactive molecules was based on the dissolution of these molecules in solvents of differing polarities. This method for extracting fresh plant material and fractionating into different classes according to polarity is outlined in Fig. 16.

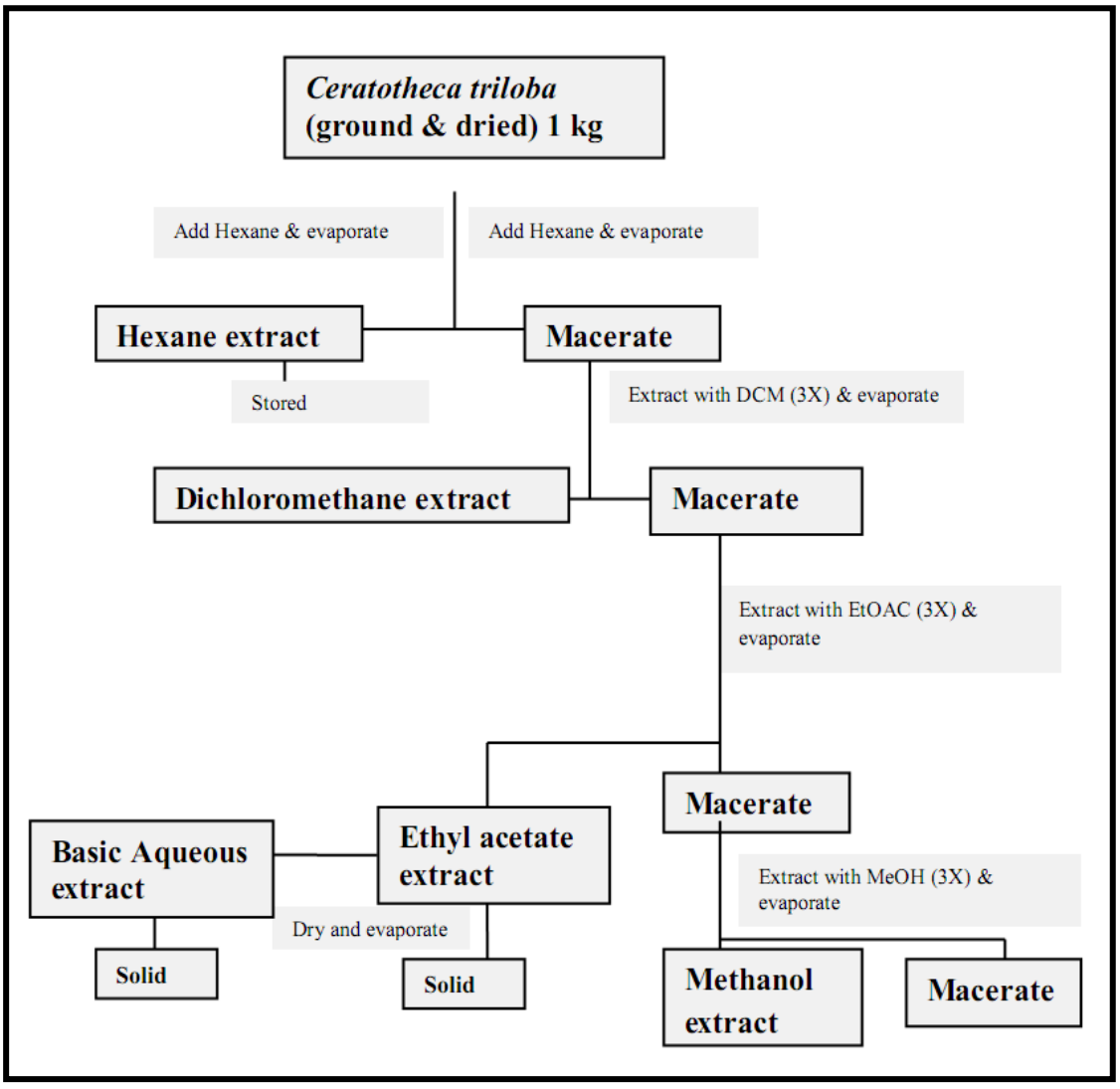


Figure 16: **Extraction protocol used to extract the hexane, dichloromethane, ethyl acetate and methanol components from *C. triloba* with modifications.**

2.2.3. Phytochemical screening of plant extracts

Phytochemical screens for tannins, phlobatannins, saponins, flavanoids, steroids and terpenoids were carried out on the aqueous extract and on the powdered specimens using standard procedures to identify the constituents as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973).

2.2.3.1. Test for tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

2.2.3.2. Test for phlobatannins

An aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid. Phlobatannins were indicated by the presence of a red precipitate at the base of the test tubes.

2.2.3.3. Test for saponin

About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion. Presence of saponins was indicated by the formation of a heavy emulsion.

2.2.3.4. Test for flavonoids

A portion of the powdered plant sample was in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration indicated a positive test for flavonoids.

2.2.3.5. Test for steroids

Two millilitre of acetic anhydride was added to 0.5 g powdered extract in ethanol of each sample. This was followed by the addition of 2 ml H_2S0_4. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

2.2.3.6. Test for terpenoids (Salkowski test)

Five millilitre of each extract was mixed in 2 ml of chloroform, and concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids. Steroids and terpenoids were determined by the presence of blue-green and red-brown interfaces between layers respectively.

2.2.4. Separation of compounds in the different plant portals by Thin Layer Chromatography

Thin layer chromatography [5-10 µl of a 100 mg extract/ml solution] was on Merck TLC F_{254} or Silica gel 60 plates using Hexane: ethyl acetate [9:1] as eluent. Streaking of polar components was minimized by the addition of 1% ammonium chloride to the mobile phase solution. Samples were spotted and run without delay to minimize the possibility of oxidative or photo-oxidative change. Separated components were visualized under visible and ultraviolet light (254 and 360 nm, Camag Universal UV lamp TL-600). Plates were also sprayed with ρ-anisaldehyde (5% anisaldehyde in 5% sulphuric acid in ethanol) and heated for 2-5 minutes at 100°C to allow for development of colour changes (Carr and Rogers, 1986).

2.2.5. Purification of Crude Extracts

2.2.5.1. Column Chromatography

Silica gel (Kieselgel 60, 0.015-0.04 mm) was suspended in required solvent and left for approximately 2 hours to swell after which it was poured into the column. The fraction obtained during solvent/solvent extraction was suspended in the minimum amount of the particular solvent (30g silica/1g sample) in which it would dissolve and filtered to remove impurities and any large particles which could cause diffusion problems whilst developing the column. This fraction was applied to the top of the column using a pipette with great care as not to disturb the top of the column. After application, the solvent flask was raised to facilitate solvent flow into the column and was run using gravitational force. A concentration gradient of eluents was used. With the hexane fraction, a 500 ml mixture of hexane: ethyl acetate (1:1) was used, gradually introducing a mixture of hexane: ethyl acetate (2:1). 100% hexane was introduced next and finally methanol was added to elute any components that could not be moved with the other solvents. The column was left to run overnight at a flow rate of 0.5 ml/min.

2.2.5.2. Analysis and Concentration of fractions

As soon as column chromatography was completed, test-tubes were placed under a stream of nitrogen to facilitate concentration of the fractions for TLC analysis and bioassay. After approximately 40% of the volume of the eluent had evaporated, every second fraction was analyzed by TLC using 0.1% of each fraction. In some cases test-tubes were not pre-weighed and the fractions were not dried, therefore it was not possible to determine the exact concentration applied to the TLC plates. Fractions were analyzed using Hexane: ethyl acetate [9:1]. Ethyl acetate/Methanol/Water (45:5:4.4) was also attempted but with poor results. Separated components were sprayed with ρ-anisaldehyde reagent.

From TLC results, fractions were combined according to their separation profile. Combined fractions were placed under an air current to facilitate drying. Once dry, the components were weighed to calculate the total mass extracted, dissolved in the minimum amount of acetone possible and transferred to a glass vial with a screw top to prevent evaporation.

2.2.5.3. Preparative TLC

Fractions were dissolved in the smallest amount of solvent (usually acetone) in which it would dissolve (1 ml) and applied in a band across the preparative TLC plate (Silica gel 60 F_{254}) starting and ending at least 1 cm from either side. The plate was developed repetitively (at least three times) in the mobile phase and the bands visualized under ultraviolet light (254 and 360 nm). A small section on the side of the plate was sprayed with vanillin and heated with a heating gun, whilst protecting the rest of the plate with foil. Components were easily visualized and marked using a soft 2B pencil. After spraying the plate with water to facilitate easier removal of components, the bands were scraped off the glass plate. The components were collected into separate beakers and crushed to a fine powder using a glass rod. The adsorbent powder was eluted with 5 ml acetone, depending on the quantity and pigment recovered by filtration using a sintered glass funnel. The process was repeated at least twice or until the powder regained its original white colour. This was followed by 1% acetic acid in methanol for a final rinse to remove any polar components not removed with acetone. Each component was collected into a separate vial and concentrated under a stream of cold air.

2.2.6. Melting Point Determination

Melting points were determined on Kofler hot-stage apparatus and are uncorrected.

2.2.7. UV/Vis and EI-LC-MS

The analysis was done on a Waters Thermabeam (TMD) system comprising of a 2695 Solvent Delivery System, a 2996 photodiode array (PDA) detector, column heater and Thermabeam (TMD) Electron Ionization Mass spectrometry detector. Chromatographic separation was done on a Waters Xbridge C_{18} column (150 x 2.1 mm, 3.5 µm) maintained at 40^0C. The starting eluent consisted of water (containing 10 mM Formic acid) and Acetonitrile (70:30) at 0.2 ml/min. The gradient table of the chromatographic method is summarised in Table 7. The Photodiode array (PDA) detector was placed first in line and full scan spectra were collected between 200 and 600 nm at a sample rate of 1 spectrum per second and a resolution of 1.2 nm. The TMD detector was placed after the PDA detector and operated in positive scan mode (50 – 650 amu) with a gain of 10 collecting 1 spectrum per second. The nebuliser temperature was set at 90^0C, the expansion region temperature at 80^0C and the source temperature at 225^0C. The total volume of post-column eluent was sent to the TMD detector and helium was used as nebulisation gas at 30 L/h. The TMD detector was tuned every day prior to starting an analysis run and caffeine was injected as test compound to ensure functionality of the total system.

Table 7: Gradient conditions on the Waters 2695 solvent delivery system

Time	Flow	%C	%D	Curve
0.0	0.20	70	30	6
1.0	0.20	70	30	6
40.0	0.20	0	100	6
48.0	0.20	0	100	4
50.0	0.20	70	30	3
60.0	0.20	70	30	6

Injection volumes ranged between 1 and 10 µL depending on the concentration of the sample. For samples CTREM01 to CTREM03 the injection volume was 2 µL while the injection volume for CTREh was 10 µL.

2.2.8. IR, ^{1}H NMR and ^{13}C NMR spectroscopy (COSY, DEPT, HSBC, and HMQC)

Infrared spectroscopy was carried out on a Varian 800 FTIR Scimitar series utilizing a PIKE MIracle™ cell with KBr loaded lenses. Samples were run in ATR (attenuated total reflectance) mode. The clean samples were weighed and dissolved in maximum 2 ml deuterated solvents used for NMR [Merck]. In the studies, $CDCl_3$ was used as the solvent of choice, although other solvents were also attempted, because of its good ability to dissolve a wide range of compounds. The samples were then pipetted into NMR tubes with the aid of a Pasteur pipette and sent to Mr Dilip Jugjivan of the Chemistry Department, University of Kwazulu Natal (Westville). ^{1}H NMR was run at 400 MHz and ^{13}C at 75 MHz using the solvent signal tetramethylsilane (TMS), $(CH_3)_4Si$ as reference on a BRUKER Avance III NMR system. The spectra were interpreted by the aid of the COSY, DEPT, HSBC, and HMQC techniques.

2.3. RESULTS AND DISCUSSION

2.3.1. Yields

From 250 g of fresh material the amount of dried material obtained yielded the highest yield from flowers, followed by the roots, seed pods, stems and leaves (Table 8). Although fresh or dried plant material can be used as a source for secondary plant components in these experiments dried material was used since; (i) traditional healers frequently use dried plant material; (ii) the time delay between collecting plant material and processing makes it difficult to work with fresh material; and iii) there are fewer problems associated with the large scale extraction of dried plant material.

Table 8: Dry weight and percentage yield extracted from plant material using hexane solvent system.

Plant Portal	Initial material weight	Dry weight	Percentage Yield Dry weight
Stems	250 g	78.8 mg	0.0312
Roots	250 g	109.8 mg	0.0436
Flowers	250 g	219.1 mg	0.0876
Leaves	250 g	46.2 mg	0.0184
Seed Pods	250 g	96 mg	0.0384

2.3.2. Optimization of TLC conditions for the resolution of compounds from roots

2.3.2.1. Optimum mobile phase

Fig. 17 shows the separation of compounds from the *C. triloba* roots. Hexane which is non-polar combined with a solvent with medium polarity; ethyl acetate, resulted in the best separation. The combination of the two solvents at differing ratios resulted in the selection of an ideal combination for the separation of the plant components from the root extract of *C. triloba* (Fig. 17). TLC with hexane as a mobile phase resulted in the compounds not eluting from the baseline, however, when hexane was combined with ethyl acetate the components resolved to a certain degree and several attempts using various ratios of hexane and ethyl acetate the ideal

ratio of hexane: ethyl acetate was found to be 90:10. Fig. 18 (A) shows poor separation with the compounds not eluting from the baseline. Increasing the polarity ratio by the addition of ethyl acetate resulted in the compounds migrating from the baseline but the separation was still poor (B). Only six compounds were visible in Fig. 18 (B). The ratio of ethyl acetate was increased to 10% and the separation was improved, nine compounds were visible with hexane: ethyl acetate (90:10) Fig. 18 (C). A further combination was attempted by increasing the ethyl acetate concentration to 20% but resulted in the bands being poorly separated and only seven bands were visible (Fig. 18 D). Maximum resolution was obtained with ρ-anisaldehyde in 100ml acid alcohol and UV light (254 and 366nm).

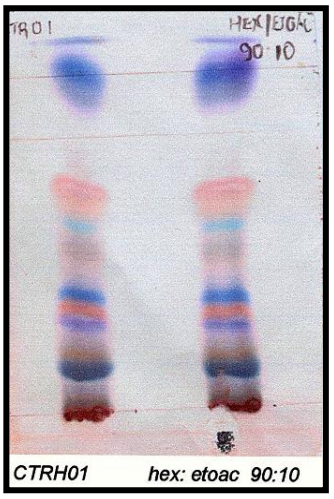


Figure 17: **Separation of compounds isolated from *C. triloba* roots using hexane extraction. Mobile Phase: Hexane: ethyl acetate (90:10) and ρ –anisaldehyde in 100ml acid alcohol.**

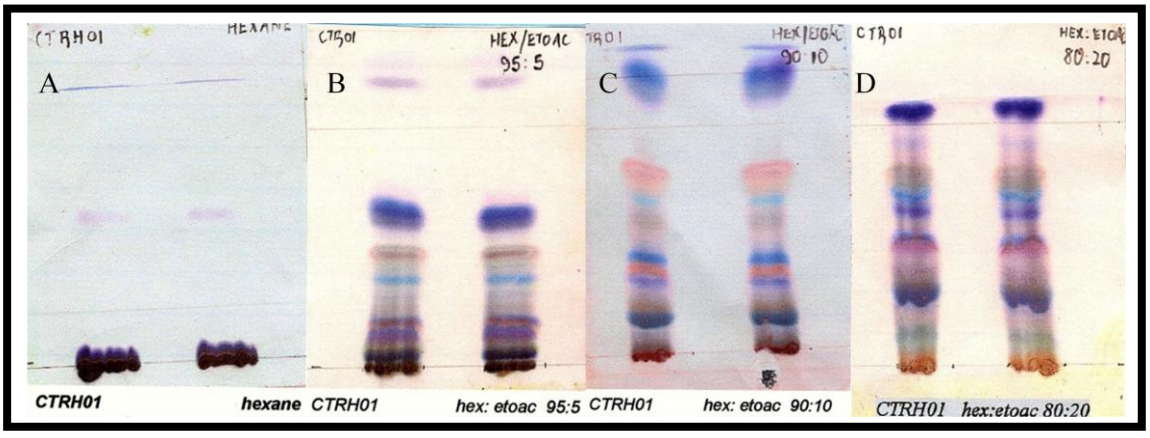


Figure 18: **Optimization of mobile phase for the separation of compounds isolated from *C. triloba* roots. A- Hexane: ethyl acetate (100:0), B- Hexane: ethyl acetate (95:5), C- Hexane: ethyl acetate (90:10) and D- Hexane: ethyl acetate (80:20).**

2.3.3. Profile of compounds in plant portals

Using the optimised seperation and detection parameters, the components in the different portals of the plant were compared. The compounds varied from six in the stems and seed pods to 13 in the roots (Fig. 19). A profiling of the R*f* values from roots, flowers, leaves, seed pods and stems showed three common compounds at R*f* values of 0.21, 0.34 and 0.38 represented as 1, 2 and 3 in Figure 19.

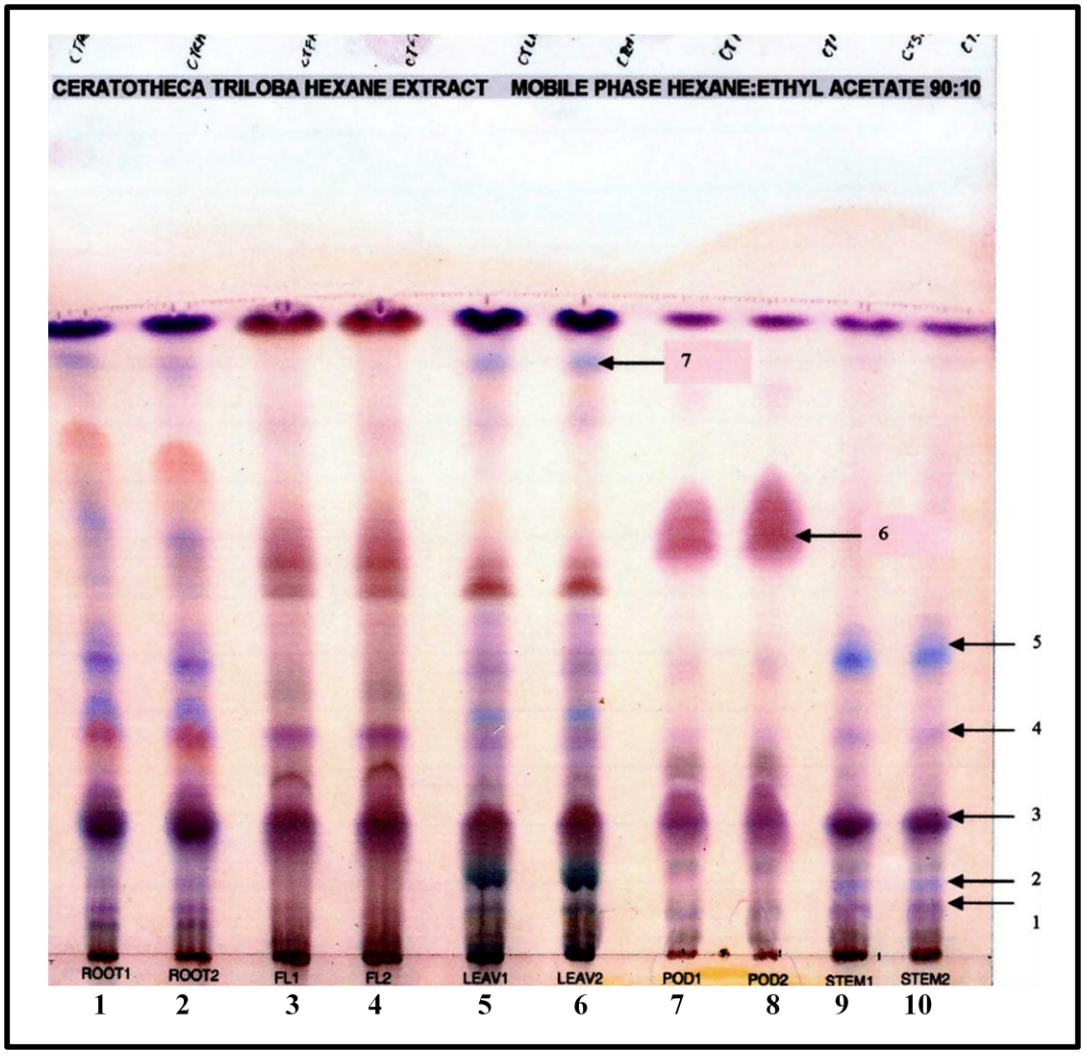


Figure 19: **Separation of components from the different portals of *Ceratotheca triloba*. Lane 1, 2 – root extract; Lane 3, 4 –flower extract; Lane 5, 6 –leaf extract; Lane 7, 8- seed pod extract and Lanes 9 and 10- stem extracts.**

2.3.4. Major phytochemical classes in *C. triloba*

Major groups of the phytochemical from the crude extracts showed the presence of phlobatannins, saponins, steroids and terpenoids. No flavonoids, tannins and cardiac glycosides were detected. These results are shown in Table 9.

Table 9: Major phytochemical compounds from crude extracts of *C. triloba*

Phytochemical constituents	Aqueous Root Extraction preparation 1	Aqueous Root Extraction preparation 2
Tannins	-	-
Phlobatannins	+ red precipitate at base of test tube	+ red precipitate at base of test tube
Saponins	+ Formation of heavy emulsion	+ Formation of heavy emulsion
Flavonoids	- no yellow colour change	- no yellow colour change
Steroids	+ Formation of a blue-green interface between layers	+ Formation of a blue-green interface between layers
Terpenoids (Salkowski test)	+ Formation of a red-brown interface between layers	+ Formation of a red-brown interface between layers
Cardiac glycosides (Keller-Killani test)	-	-

2.3.5. Column Chromatography

The root extract was passed through a Silica Gel column using Hexane-ethyl acetate (90:10) as the mobile phase. Twenty fractions were collected and analyzed by TLC. The colour and R*f* values were recorded. This enabled us to combine fractions with similar TLC profiles. Fraction number E6-E20 were combined and this resulted in 3 compounds (A-C), which were then further analyzed by TLC (Fig. 20 and 21 respectively)

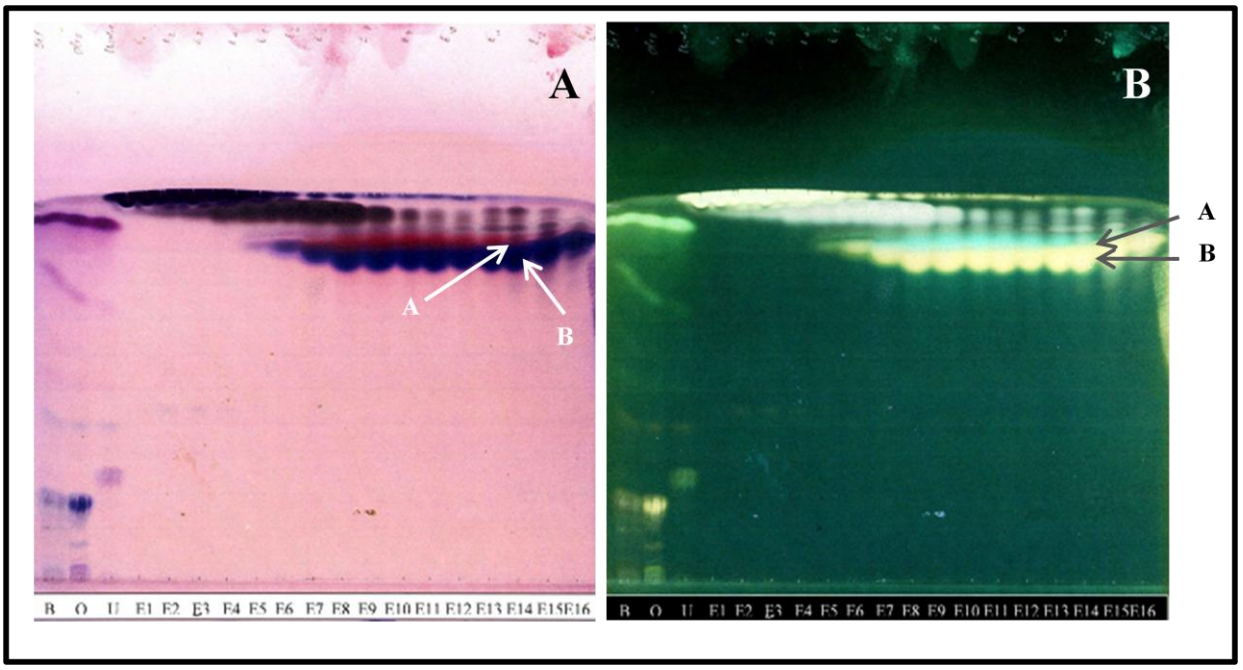


Figure 20: **A- TLC of the fractions (E1-E16). B- Inverted or Negative image TLC of the fractions (E1-E16). The fractions were eluted with Hexane: ethyl acetate (90:10) and developed with ρ-anisaldehyde.**

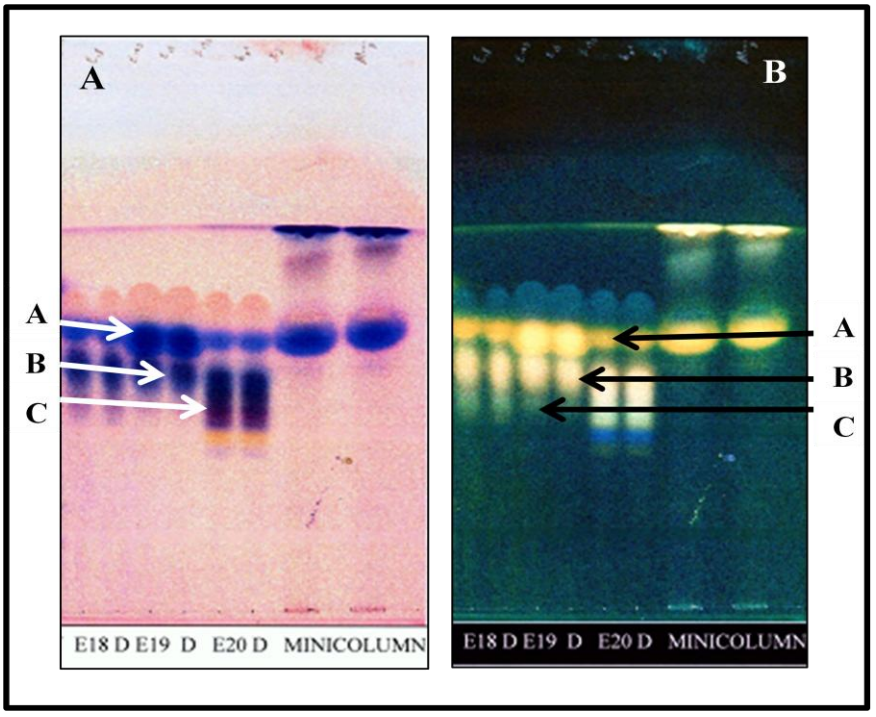


Figure 21: **TLC (A) and inverted image (B) of the fractions (E18-E20). The fractions were eluted with Hexane: ethyl acetate (90:10) and sprayed with ρ-anisaldehyde visualization spray reagent.**

2.3.6. Preparative TLC of fractions A, B and C

Fractions A, B and C were dissolved in as little hexane as possible and applied to a preparative TLC plate. The three bands (I-III) were marked off using UV and visible light as a visual aid and then scraped off and extracted first with acetone, chloroform and then 1% acetic acid in methanol to remove polar components. Products were dried under a stream of cold air to avoid heat decomposition and stored in the fridge. Only the acetone fractions were subjected to the qualitative analysis (LC-MS) as the quantities extracted with chloroform and 1% acetic acid/methanol were insignificant.

Fig. 22 illustrates the separation between the three compounds isolated from *C. triloba.* The R*f* value of the three compounds did not vary by more than 0.10 and the overlapping of compounds was always a difficult task to overcome. Compound 1 had an R*f* value of 0.53, compound 2- 0.43 and compound 3- 0.44. Two different mobile phases were used to separate these compounds based on their polar nature [(Petroleum ether: ethyl acetate, 90:10) and (Dichloromethane: methanol, 98:2)]. These results are shown in Fig. 23. Both mobile phases showed poor separation of the isolated compounds with all compounds migrating to a similar solvent distance. Fig. 23 (A) showed the compounds migrated to an R*f* value of 0.50 and Fig. 23(B) showed all compounds migrating to an R*f* value of 0.90.

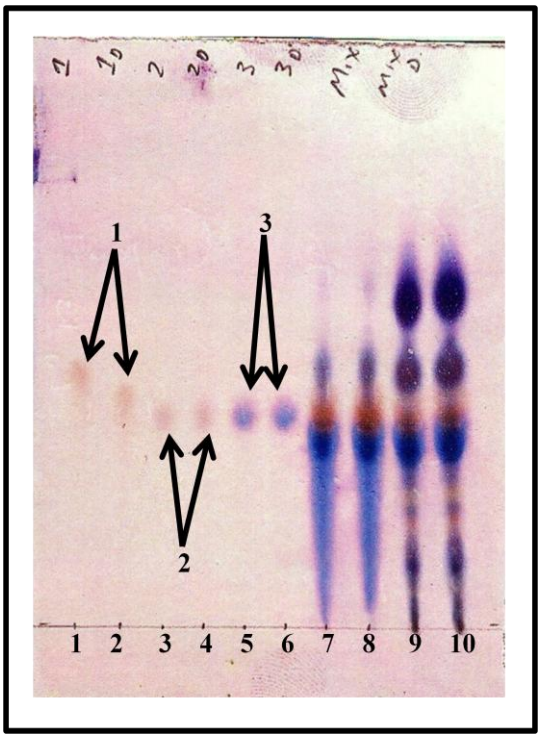


Figure 22: **Preparative TLC of the combined extracts. A- Preparative TLC template. B- Three compounds were identified, Lanes 1-6: purified extracts from *C. triloba*. Lanes 7-10: combined extracts with no purification. The fractions were eluted with Hexane: ethyl acetate (90:10) and sprayed with ρ-anisaldehyde visualization spray reagent.**

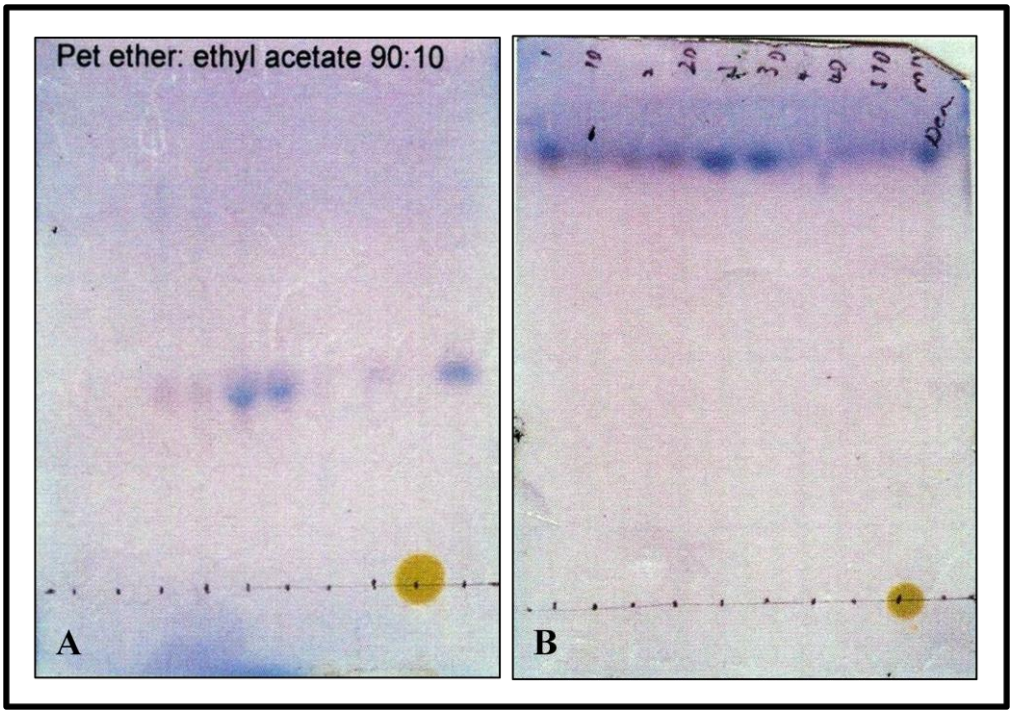


Figure 23: **Separation of the isolated compounds from *C. triloba* using two different eluents. A- Petroleum ether: ethyl acetate 90:10, B - Dichloromethane: methanol, 98:2.**

2.3.7. Preparative High Performance Liquid Chromatography and UV-Vis spectroscopy of compounds isolated from *C. triloba.*

Partially purified extracts were coded as CTREh01, CTREh02 and CTREh03 and further purified using Preparative High Performance Liquid Chromatography. Figures 24, 25 and 26 illustrate the separation of pure compounds from CTREh01, CTREh02 and CTREh03 respectively. The compounds that were isolated from PTLC were not pure enough to determine their structures using LC-MS. Table 10 shows a distinct relationship of the three extracts from *C. triloba*. All three extracts contained similar compounds at retention times of 22.133 and 24.500 (Table 10). This indicates the difficulty in the separation of the structurally identical compounds using preparative thin layer chromatography and further purification was necessary using a much more sensitive technique like preparative high performance liquid chromatography. By utilizing preparative high performance liquid chromatography the isolated fractions containing the pure compounds can be isolated and chemically elucidated by Liquid Chromatography-Mass Spectrometry (LC-MS). The individual compounds that were separated by Preparative High Performance Liquid Chromatography were further assayed by scanning the maximum absorption wavelength in the Ultraviolet light range (200-600 nm). These results are illustrated in Fig 27, 28 and 29.

Table 10: Summary of the peaks of interest of CTREh01, CTREh02 and CTREh03 extracted from *C. triloba* roots.

Extract	Retention time in P-HPLC	Wavelength of Peaks of interest (nm) in UV-Vis spectral analysis
CTREh-01		
CTREh-01A	22.133	205.2, 256.9, 329.2
CTREh-01B	24.500	256.9, 329.2, 411.5
CTREh-01C	30.367	274.6, 447.7, 474.3, 491.3 508.2, 547.1, 565.4
CTREh-01F	30.894	-
CTREh-02		
CTREh-02D	17.350	205.2, 272.2, 412.7
CTREh-02A	22.133	205.2, 256.9, 329.2
CTREh-02B	24.500	256.9, 329.2, 411.5
CTREh-03		
	13.200	239.2, 310.2, 499.7
	16.600	205.2, 256.9, 482.8
CTREh-03D	17.350	206.4, 272.2, 412.7
CTREh-03E	20.717	219.3, 287.6, 373.1
CTREh-03A	22.167	206.4, 261.6, 329.2
CTREh-03B	24.500	256.9, 329.2, 411.5

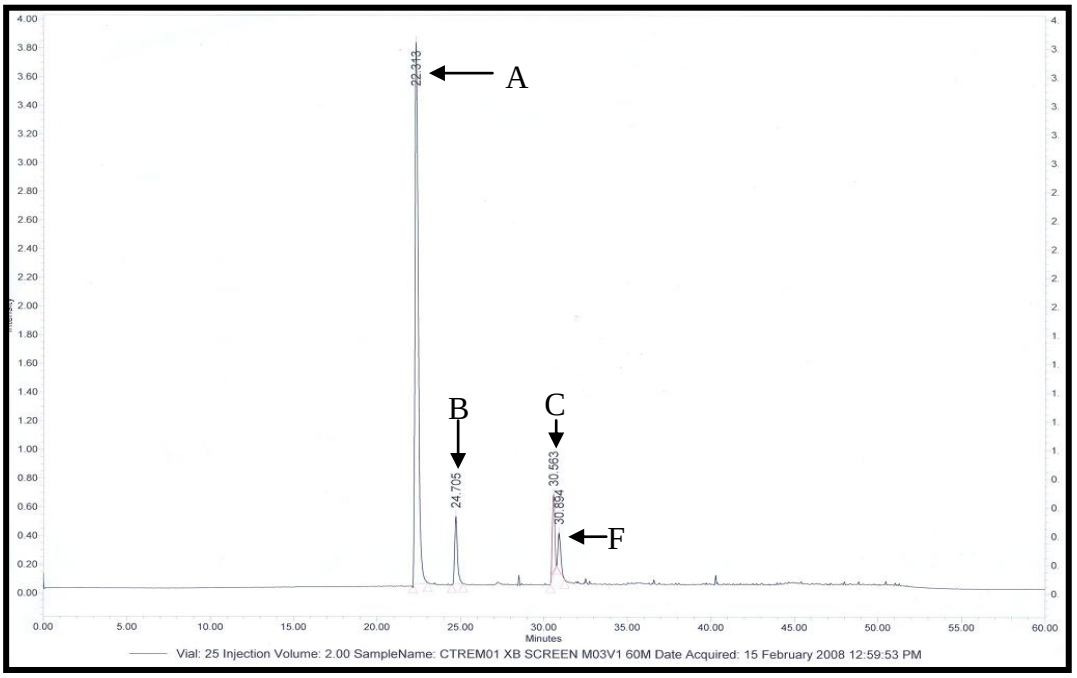


Figure 24: **Preparative High Performance Liquid Chromatography (P-HPLC) of CTREh01 showing the presence of four distinct compounds at varying retention times. A- Retention time = 22.136, B- Rt = 24.705, C- Rt = 30.563 and F- Rt=30.894.**

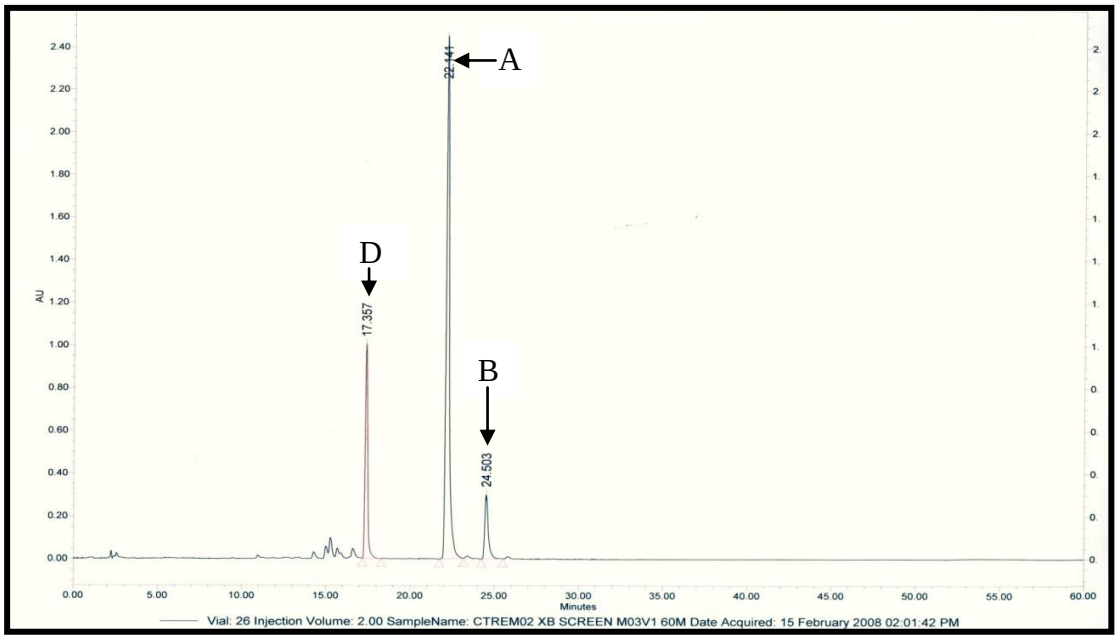


Figure 25: **Preparative High Performance Liquid Chromatography of CTREh02 showing the presence of three distinct compounds at varying retention times. D-Retention time = 17.357, A- Rt = 22.141 and B- Rt = 24.503.**

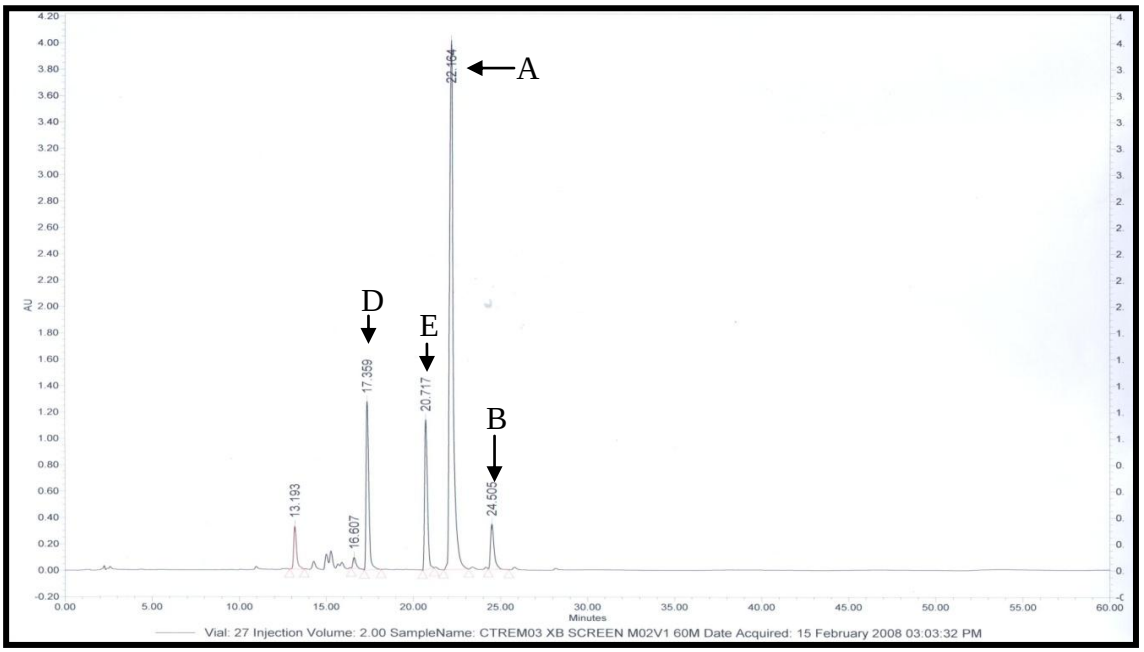


Figure 26: **Preparative High Performance Liquid Chromatography of CTREh03 showing the presence of four distinct compounds at varying retention times. D-Retention time = 17.359, E- Rt = 20.717, A- Rt = 22.164 and B- Rt = 24.505.**

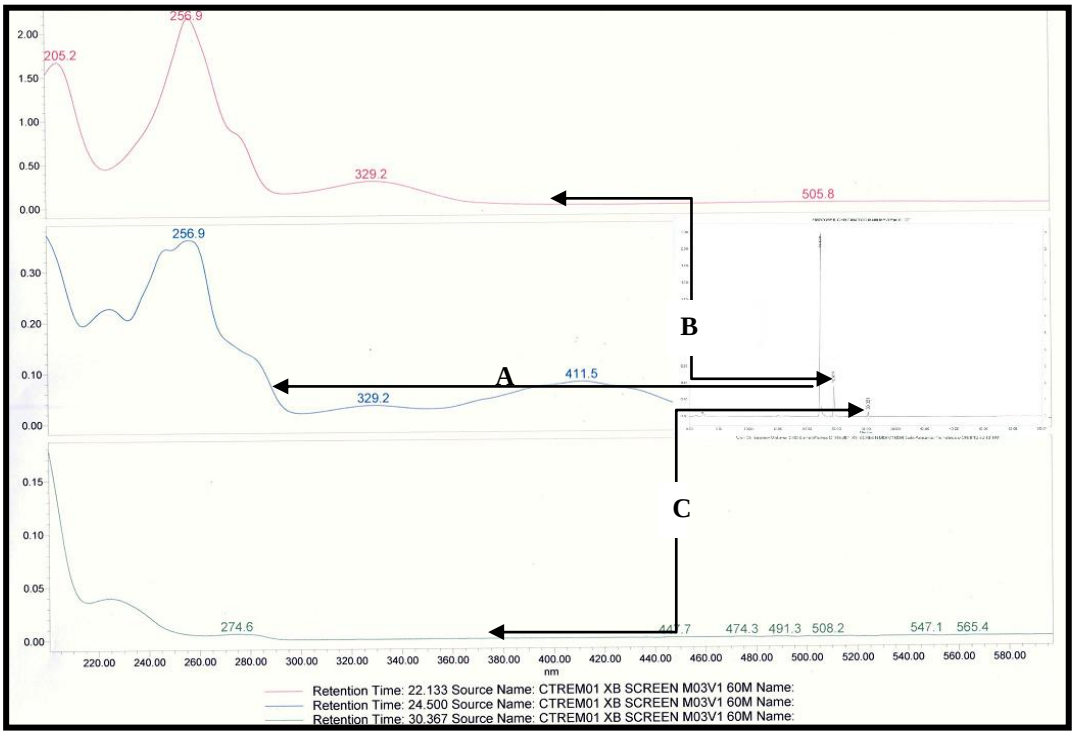


Figure 27: **UV scan of *C. triloba* root extract 1 (CTREh01) showing peaks of interest, A- Retention time = 22.136, B- Rt = 24.503 and C- Rt = 30.361 isolated from P-HPLC and their maximum absorption wavelengths.**

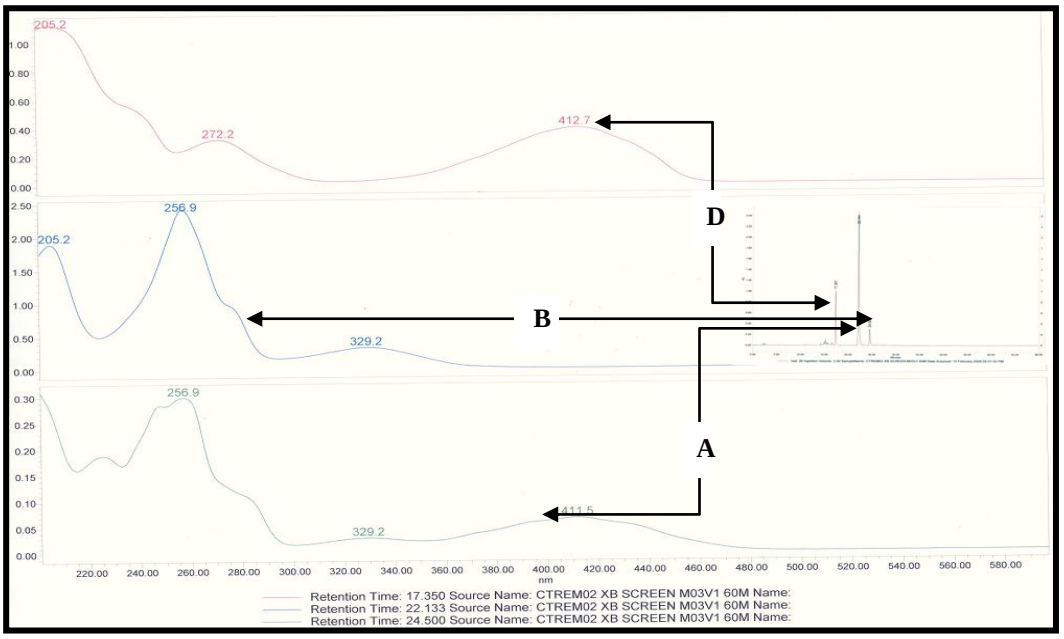


Figure 28: **UV scan of *C. triloba* root extract 2 (CTREh02) showing peaks of interest, D- Retention time = 17.357, A- Rt = 22.141 and B- Rt = 24.503 isolated from P-HPLC and their maximum absorption wavelengths.**

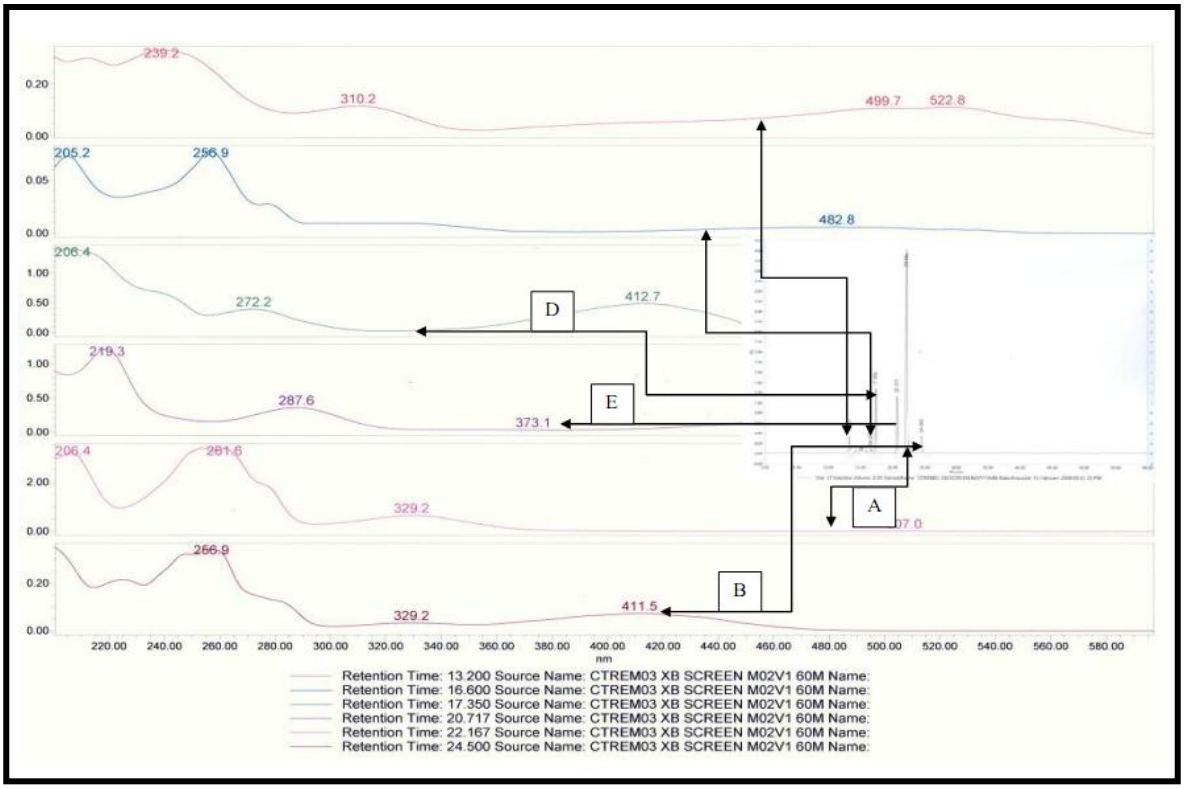


Figure 29: **UV Scan of *C. triloba* root extract 3 (CTREh03) showing peaks of interest, D- Retention time = 17.359, A- Rt = 22.164, B- Rt = 24.505 and E- Rt = 20.717 isolated from P-HPLC and their maximum absorption wavelengths.**

2.3.8. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis of purified components of *Ceratotheca triloba*.

LC-MS indicated six different compounds with varying structural differences representing anthraquinones and a steroid. Prevalent compounds belonged to the anthraquinones which included 9, 10 anthracenedione (Fig. 30A), 1-hydroxy-4-methylanthraquinone (Fig. 30B) and 5, 8- dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione (Fig. 30C). The steroid isolated was identified as androst-5-ene-3, 17, 19-triol (Fig. 30D). Two other compounds were also isolated 1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester (Fig. 30E) and octadecanoic acid (Fig. 30F).

In our study we used preparative thin layer chromatography to seperate the the individual anthraquinones and then subjected the semi-purified extracts to liquid chromatography coupled to a mass spectrometer. The HPLC method was identical to the method employed by Peng *et al*., 1982 in that both methods employed a C_{18} stationary phase based system. The Chromatographic separation for our study utilized a Waters Xbridge C_{18} column (150 x 2.1 mm, 3.5 µm) maintained at 40^0C. A similar method was also used by Lachâtre *et al*. (2000) which consisted of Symmetry C_{18}, 3.5 µm reverse phase column (Waters). The C_{18} stationary phase, mobile phase and mode of detection are comparable to other published HPLC methods for the detection of differently substituted anthraquinones.

AQ4N (1, 4-bis-{[2-(dimethylamino-*N*-oxide) ethyl] amino} 5, 8-dihydroxyanthracene-9, 10-dione) is also closely related to mitoxantrone and is a lead compound from a new series of anti-cancer agents proposed for clinical evaluation in the UK in early 2001 (Patterson and McKeown, 2000). Swaine *et al.* (2000) has developed a sensitive and reproducible reversed-phase HPLC method for the analysis of AQ4N and its reduction metabolite AQ4 (1, 4-bis-[{2-(dimethylamino) ethyl} amino] 5, 8-dihydroxyanthracene-9, 10-dione) in plasma, using mitoxantrone as an internal standard. The method of Swaine *et al.* (2000) was based originally on a paper by Raleigh *et al*. 1998, and similar methods for the analysis of mitoxantrone (Van Belle *et al*., 1985). For the analysis of AQ4N, Raleigh *et al*. used a Waters C_8 reversed-phase column with a mobile phase consisting of 80% aqueous ammonium formate (0.5 *M*, pH 4.2)–20% acetonitrile. Whilst for the determination of the structurally related mitoxantrone Van Belle *et al*. (1985) employed a C_{18} Bondapak reversed-phase column with a mobile phase consisting of acetonitrile–ammonium formate buffer (0.16 *M*) (30:70, v/v) (pH 2.7) with hexane

sulphonic acid added as an ion-pair reagent. The use of ammonium formate buffer (pH 3.6) and a Hichrom HIRPB column by Swaine *et al*. (2000) gave excellent peak shape and separation of AQ4N from its metabolites. Also the use of PDA detection in the visible region at 612 nm confers a high degree of selectivity for the blue coloured AQ4N and its metabolites, which simplifies identification when extracting from biological samples. These methods also compare well with the mobile phase and the type of detection employed in our study. The starting eluent consisted of water (containing 10 mM Formic acid) and Acetonitrile (70:30) at 0.2 ml/min used under gradient conditions (Table 7) and the photodiode array detector was used at 600 nm.

The consistency of the methods was due to the structural similarity between the commercially available mitoxanthrone and these anthraquinones isolated in this study. The anthracenedione ring structure is a predominant component of mitoxanthrone, doxorubicin, daunorubicin, 9, 10 anthracenedione (Fig. 30A) and 1-hydroxy-4-methylanthraquinone (Fig. 30B). Synthetic variants of anthraquinones show a number of factors that appear to be absolute requirements for activity (Johnson *et al*., 1979). These are a protonable nitrogen in the side chain, a $(CH_2)_2$ spacer between the ring and side chain nitrogens and a 1, 4 location for the two side chains. In addition, hydroxyl groups at positions 5 and 8 (as in mitoxantrone) gave compounds with higher biological potency and efficacy. In the clinic, mitoxantrone has shown activity against breast cancer, acute leukaemias and non-Hodgkin's lymphoma with marginal activity observed versus non-small cell lung cancer, Hodgkin's lymphoma, myeloma and cancer of the liver, prostate, bladder, head and neck (Poirier *et al*., 1986, Smith, 1983 and Young and Raymond, 1986).

1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester is also known as phthalates which have been shown to elute at a constant rate from plastic products to the environment (Jaeger and Rubin, 1973). Consequently they are widely distributed in the ecosystem and have been described as being among the most abundant man-made environmental pollutants. In particular, di-(2- ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer. Octadecanoic acid is also known as stearic acid which is useful as an ingredient in making candles, soaps, plastics, dietary supplement, oil pastels and cosmetics and for softening rubber. Stearic acid is used to harden soaps, particularly those made with vegetable oil. These two compounds are quite common and were discarded for the biological assays.

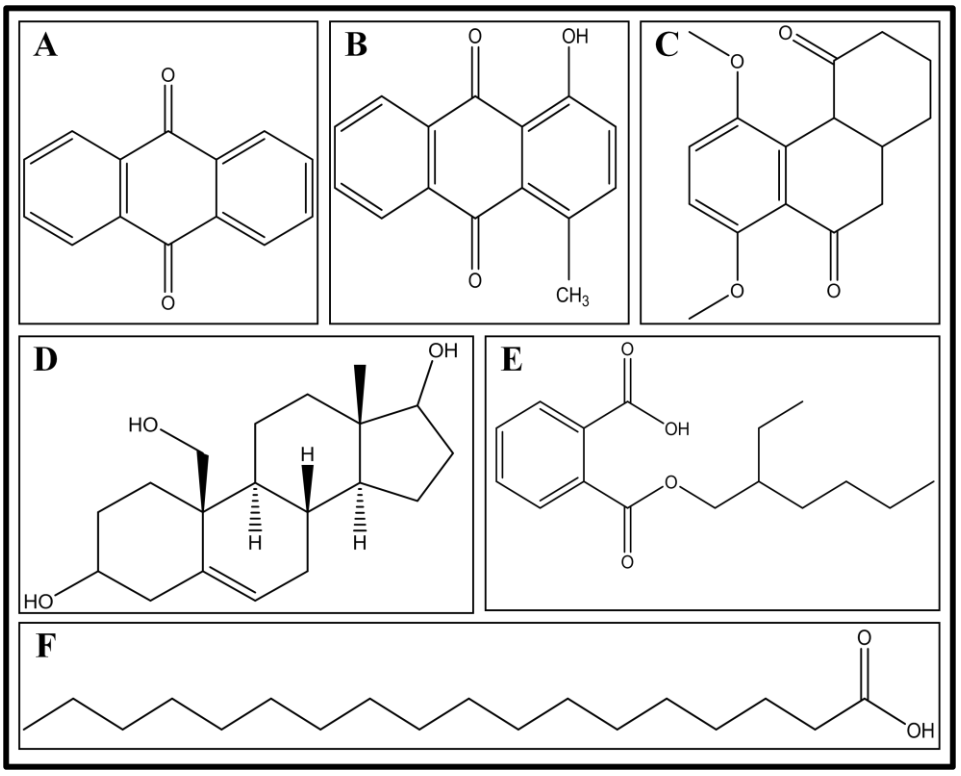


Figure 30: **Six compounds isolated from *C. triloba* roots. Structures were confirmed with EI-LCMS. A- 9, 10 anthracenedione, B- 1-hydroxy-4-methylanthraquinone, C- 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione, D- androst-5-ene-3, 17, 19-triol, E- 1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester and F- Octadecanoic acid.**

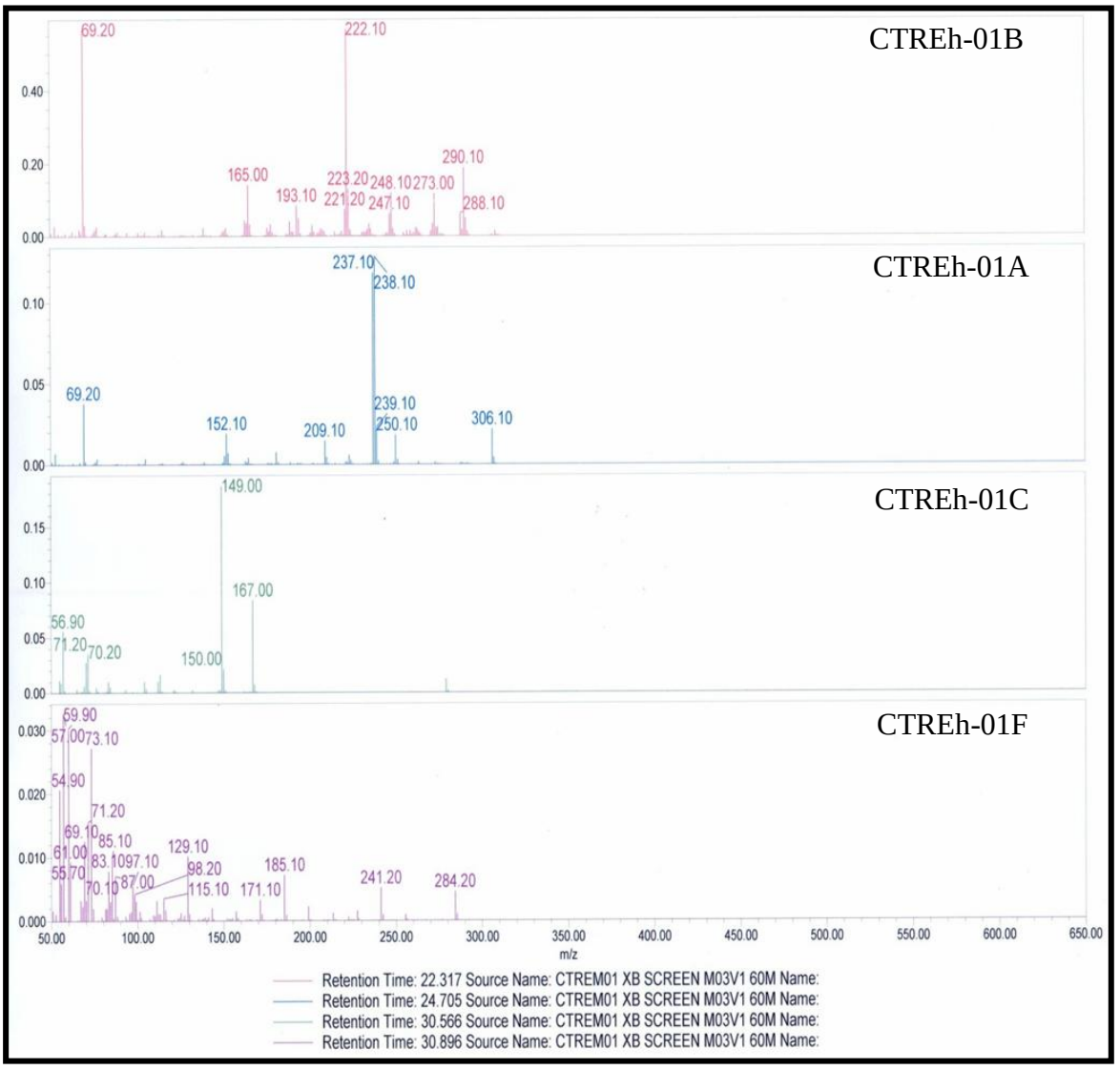


Figure 31: LC-MS scan of the active fractions of interest from P-HPLC of *C. triloba* roots (CTREh01). B- Rt = 22.317, A- Rt = 24.705, C- Rt = 30.566 and F- Rt = 30.896.

Compound structure was verified by comparison of the isolated fraction mass spectrum with a library match. Fraction B showed an excellent match with the commercial compound 9, 10 anthracenedione. Fraction A was verified as 1-hydroxy-4-methylanthraquinone and compounds C and F were determined as 1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester and octadecanoic acid respectively. Further characterization was carried out on the pure fractions isolated from PHPLC.

2.3.9. Identification and Verification of Compound CTREh01B

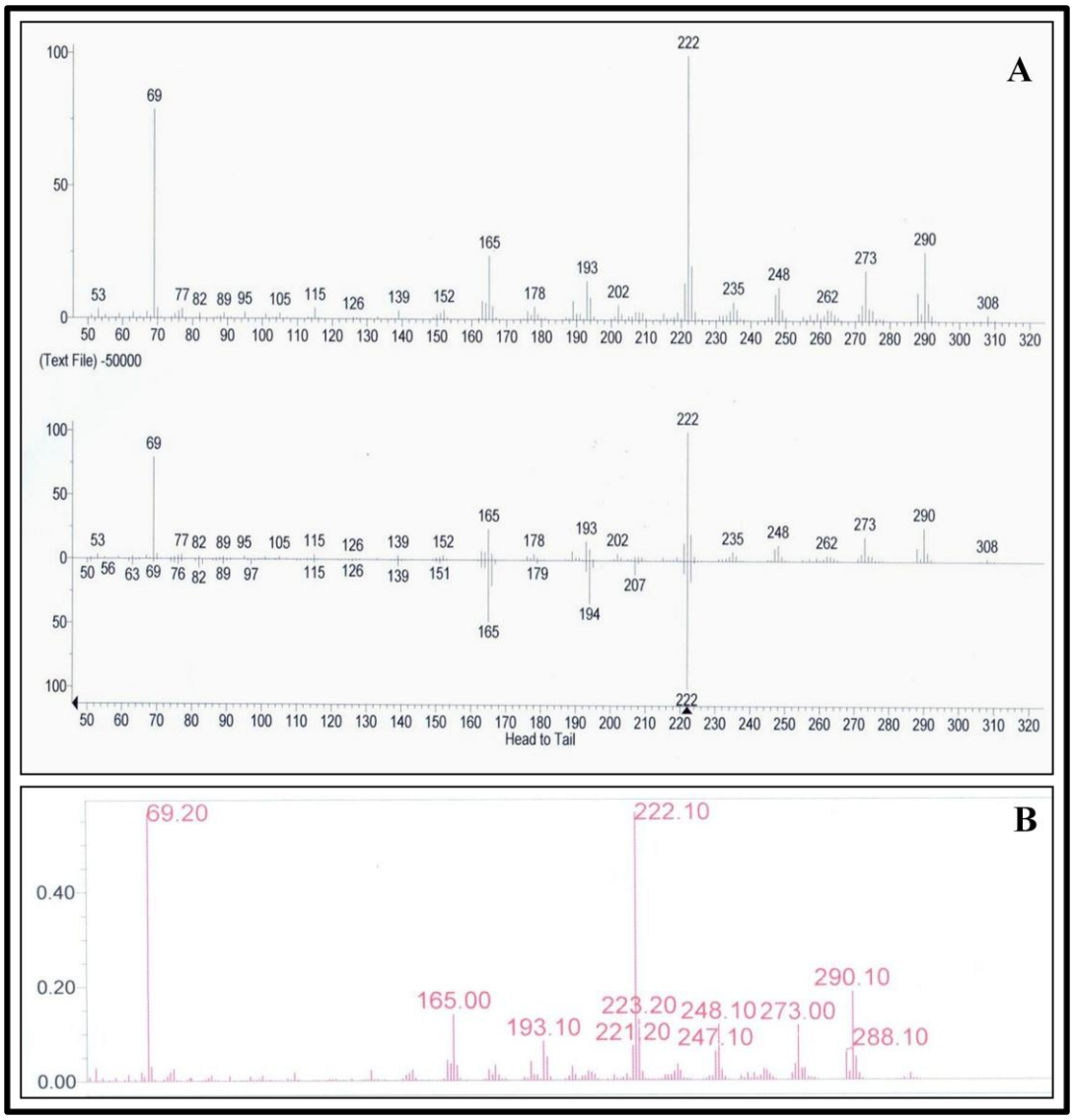


Figure 32: **Library match of 9, 10 anthraquinone isolated from *C. triloba* roots. A-Library template of 9, 10 anthracenedione and B- isolated 9, 10 anthracenedione from *C. triloba* roots.**

Final confirmation of the isolated compound as 9, 10 anthracenedione was achieved using EI-LCMS as well as a range of NMR experiments. The H^1 NMR, HSQC, HSBC and DEPT spectra of 9, 10 anthracenedione was consistent with the molecular formula $C_{14}H_8O_2$. The IR spectra (Fig. A1 - appendices) showed absorption bands for the conjugated carbonyls (υ 1590 cm^{-1}) and the aromatic rings (υ 1590 and 1480 cm^{-1}). H^1 and ^{13}C NMR results are listed in Table 11. The H^1 NMR spectrum revealed four sets of overlapping multiplets integrating two protons at 7.7833-7.7988 and 8.275-8.3102, indicating a typical aromatic ring (Fig. 33A). The H^1 NMR spectrum also revealed the presence of one set of a meta-coupled doublets at δH 7.79 (d, J=3.30 Hz, H-4) and at δH 8.30 (d, J=3.36 Hz, H-3).

The HMQC, HMBC and DEPT analysis revealed the 9, 10 anthracenedione as a symmetrical structure with the absence of chelated hydroxyl and methyl groups (Fig. 34 and 35). ^{13}C NMR revealed 14 carbon signals (Fig. 33B) which were sorted by HSQC techniques into 12 quartenary carbons and two carbonyl groups detected at δ_C 183.17. The NMR results were in agreement with previously published work on anthraquinones (Marques *et al*., 2000).

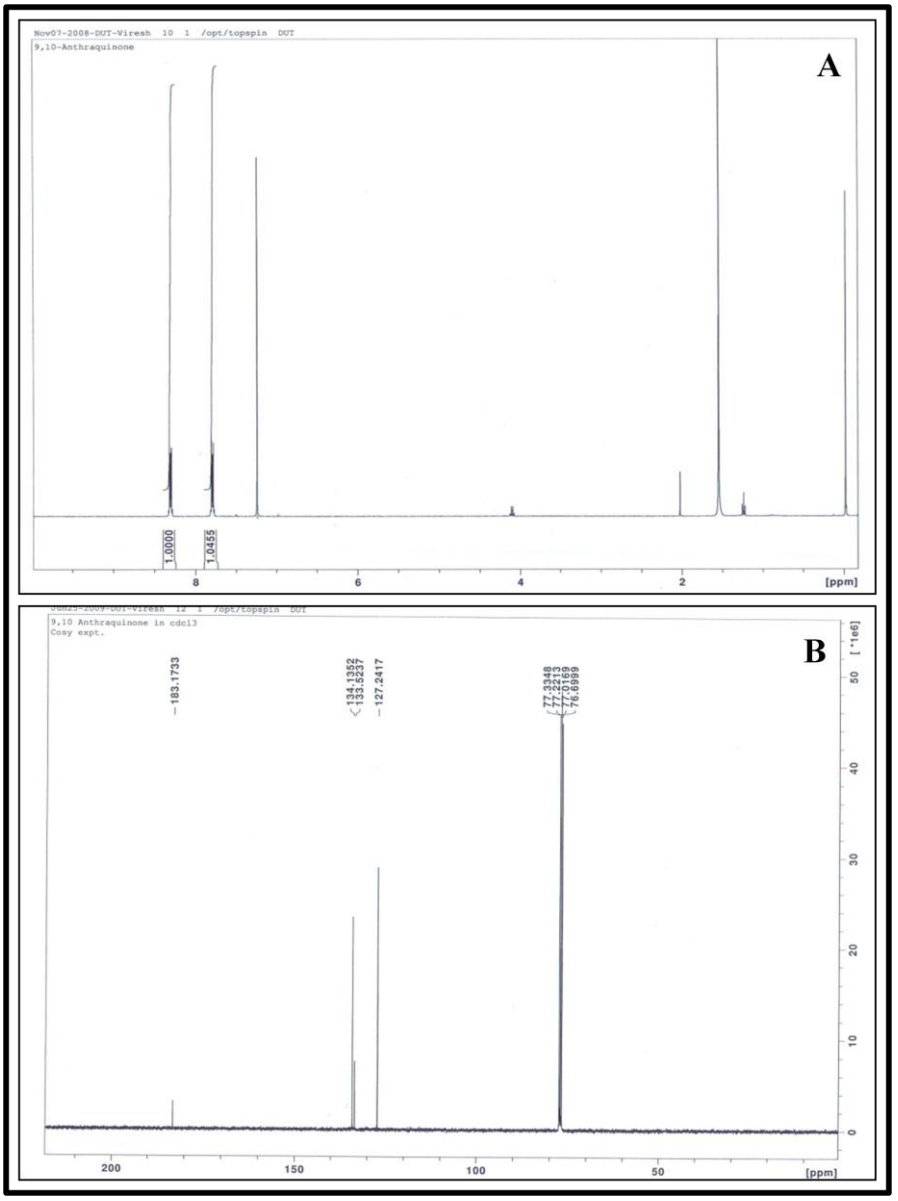


Figure 33: H^1 (A) and ^{13}C NMR (B) spectrum of purified 9.10 anthracenedione.

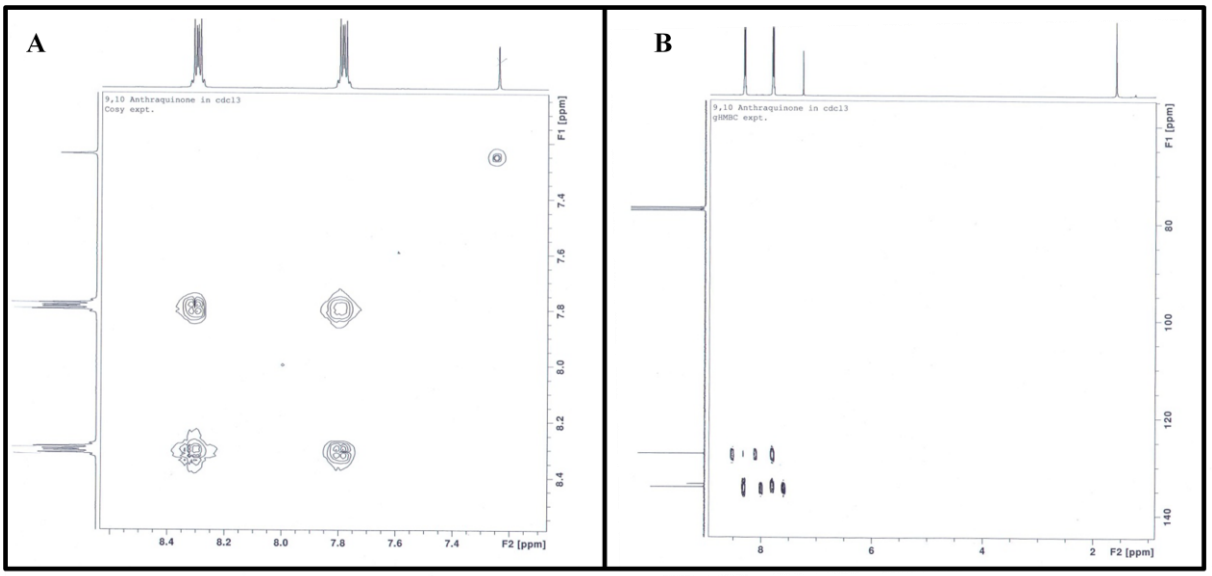


Figure 34: gCOSY (A) and gHMBC (B) NMR spectrum experiments of 9.10 anthracenedione

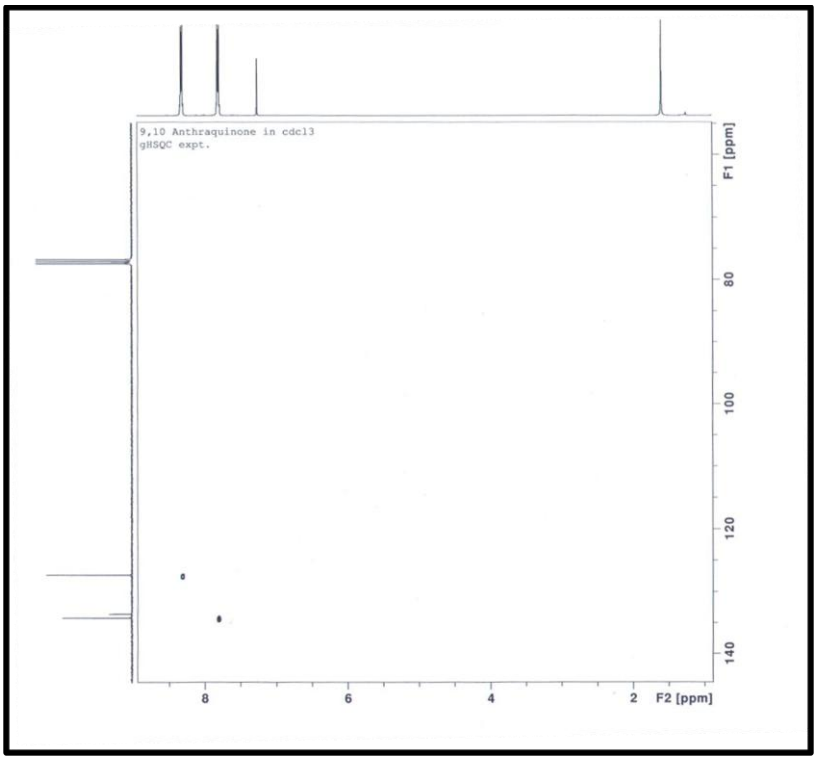


Figure 35: gHMQC experiments of 9, 10 anthracenedione.

9, 10 anthracenedione (CTREH-01B): Obtained as Yellow needles (hexane); mp: 213.50 - 214.10°C; Chemical formula: $C_{14}H_8O_2$; Molecular weight: 208.2 g/mol; UV ($CHCl_3$) λ_{max} (log ε – molar absorption coefficient): 205.2 (1.63), 256.9 (1.72), 329.2 (1.83) HPLC- elution time (H_2O: Acetonitrile) 70:30 – 24.503-24.505; Mass Spectral Data: *m/z* – 62.90, 165.00, 193.10, 221.20, 222.10, 223.20, 247.10, 248.10, 273.00, 290.10 and 288.10.

Table 11: **^{13}C and H^{1} NMR spectral data for 9, 10 anthracenedione.**

Atom Index	^{1}H NMR shifts/ δ_H	^{13}C NMR shifts/ δ_C
1	7.7978 **(1, 1H, *m*)**	127.24
2	8.3102 **(2, 1H, *m*,)**	133.52
3	8.3109 **(3, 1H, *m*)**	133.52
4	7.7895 **(4, 1H,*m*),**	127.24
5	-	134.13
6	-	183.17
7	-	134.13
8	7.7833 **(8, 1H,*m*)**	127.24
9	8.2958 **(10, 1H,*m*)**	133.52
10	8.2875 (**10, 1H,*m*)**	133.52
11	7.7750 **(11, 1H,*m*)**	127.24
12	-	134.13
13	-	183.17
14	-	134.13

***m*- multiplets**

2.3.10. Identification and Verification of Compound CTREh01A

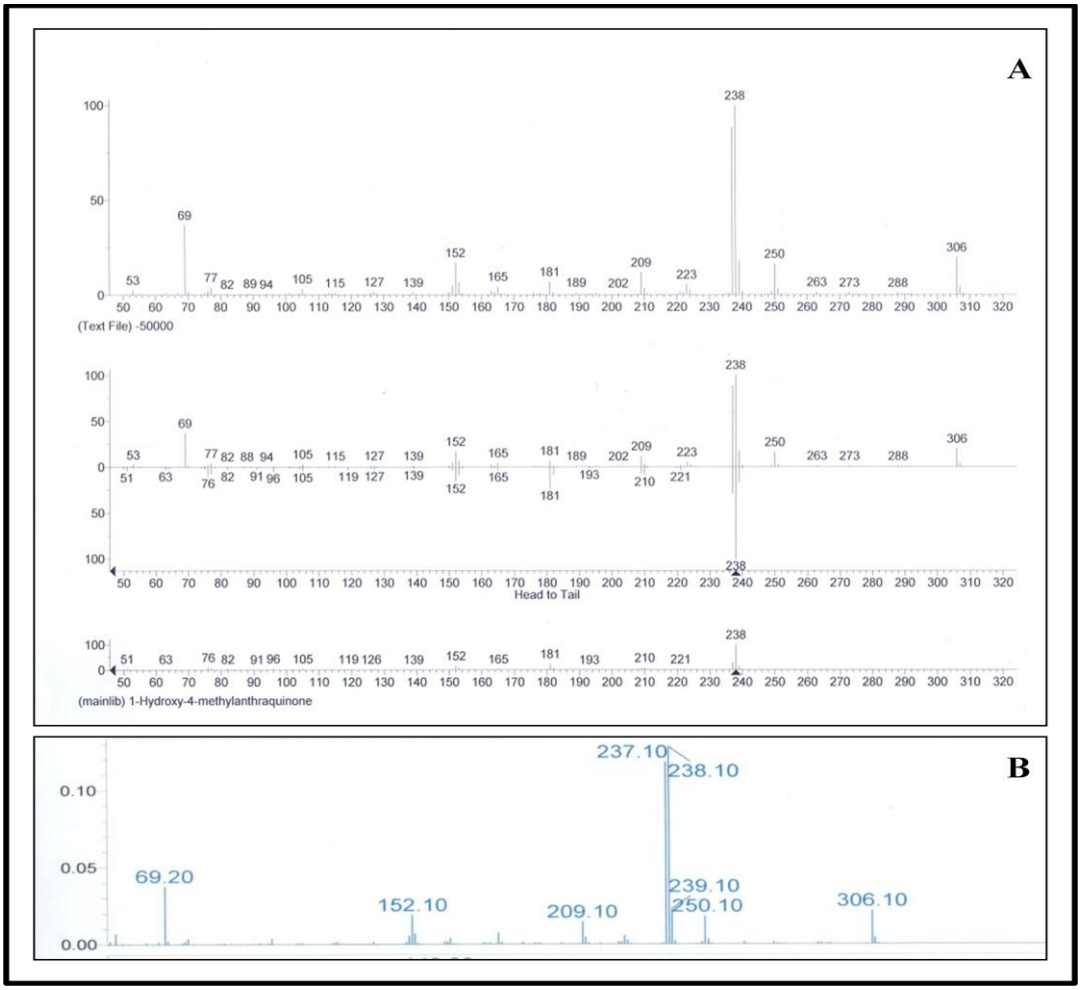


Figure 36: **Library match of 1-hydroxy-4-methylanthraquinone isolated from *C. triloba* roots. A- Library template of 1-hydroxy-4-methylanthraquinone and B- isolated 1-hydroxy-4-methylanthraquinone from *C. triloba* roots.**

The H^1 NMR, HSQC, HSBC and DEPT spectra of 1-hydroxy-4-methyl anthraquinone was consistent with the molecular formula $C_{15}H_{10}O_3$. The IR spectra (Fig. A2- appendices) showed absorption bands for a hydroxyl group (υ 3450 cm^{-1}), the conjugated carbonyls (υ 1590 cm^{-1}) and the aromatic rings (υ 1590 and 1480 cm^{-1}). H^1 and ^{13}C NMR results are listed in Table 2.6. The hydroxyl proton at δ_H 5.35 exhibited heteronuclear interactions (Fig. 37A) with the carbon atoms at δ_C 145.29 (C-1, $^2J_{CH}$), 127.49 (C-14, $^3J_{CH}$) and 127.14 (C-2, δ_H 7.24, $^2J_{CH}$). These data indicated the location of the hydroxyl and methyl groups at carbon atoms C-1 and C-4 respectively. The HMQC, HMBC and DEPT analysis revealed the 1-hydroxy-4-methyl anthraquinone as a non-symmetrical structure with the presence of chelated hydroxyl and methyl groups (Fig. 38 and 39A, B, C). ^{13}C NMR revealed 16 carbon signals which were sorted by HSQC techniques into 15 quaternary deshielded carbons and two carbonyl groups detected at δ_C 183.00 and 183.44 respectively (Fig. 37B).

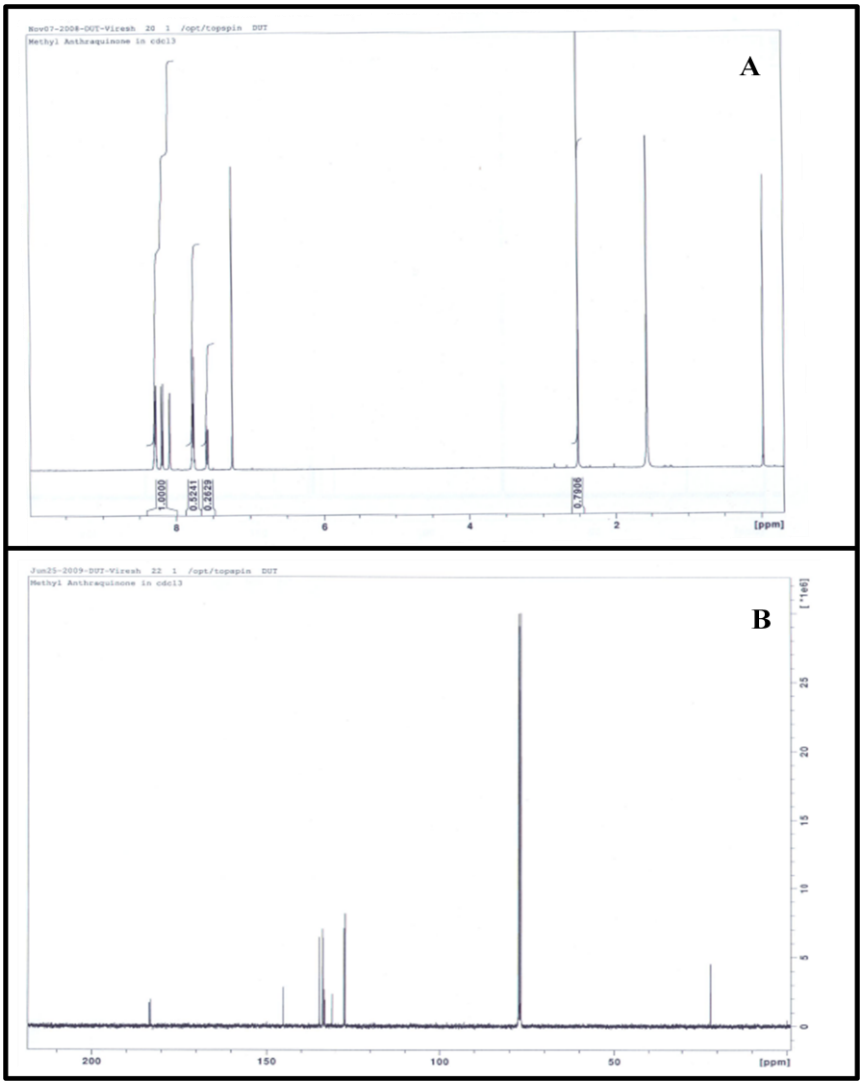


Figure 37: H^1 (A) and ^{13}C NMR (B) spectrum of purified 1-hydroxy-4-methyl anthraquinone.

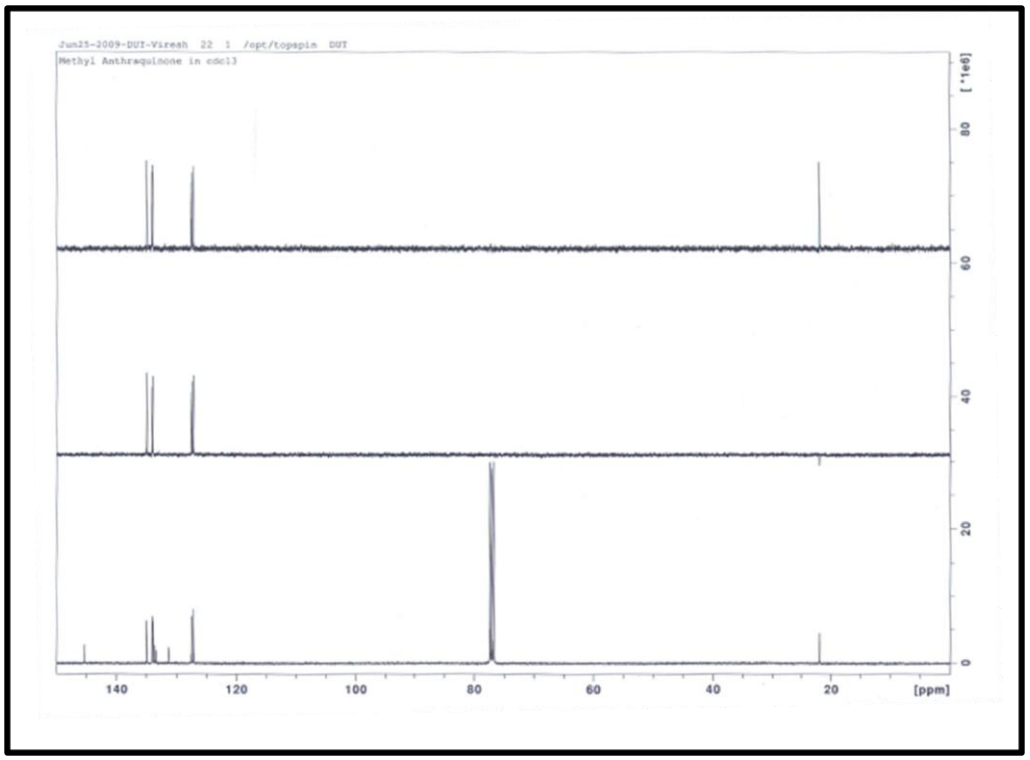


Figure 38: **DEPT (Distortionless Enhancement by Polarization Transfer) spectrum of purified 1-hydroxy-4-methyl anthraquinone.**

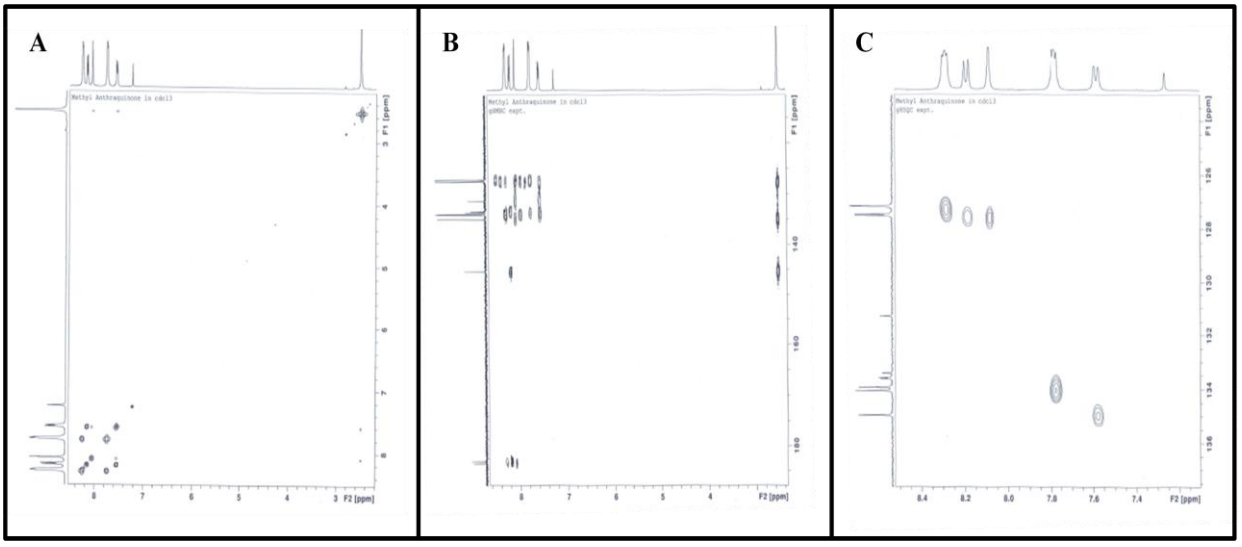


Figure 39: **gCOSY (A), gHMBC (B) and HSQC (C) NMR spectrum experiments of 1-hydroxy-4-methyl anthraquinone.**

1-hydroxy-4-methyl anthraquinone (CTREH-01A); Appearance: Orange needles (hexane); m p: 349.05-350.00°C; Chemical formula: $C_{15}H_{10}O_3$; Molecular weight: 238.23 g/mol; UV ($CHCl_3$) λ_{max} (log ε – molar absorption coefficient): 256.9, 329.2, 411.5; HPLC- Elution time (H_2O: Acetonitrile) 70:30 – 22.136; Mass Spectral Data: *m/z* – 62.90, 152.10, 209.10, 237.10, 238.10, 239.10, 250.10 and 306.10 (Fig. 36).

Table 12: ^{13}C and H^{1} NMR spectral data for 1-hydroxy-4-methyl anthraquinone

Atom Index	^{1}H NMR shifts/ δ_H	^{13}C NMR shifts/ δ_C
1	-	145.29
1a	5.025	-
2	7.24	127.14
3	7.55	134.94
4	-	133.92
4a	2.5086	21.91
5	-	133.60
6	-	183.00 (carbonyl groups)
7	-	133.39
8	7.77	127.44
9	8.27	133.39
10	8.26	134.04
11	7.76	127.16
12	-	131.28
13	-	183.44 (carbonyl groups)
14	-	127.49

NMR spectral data for 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone showed good correlation to studies by Marques *et al*., 2000 and Permana *et al*., 1999. These studies showed the presence of four sets of overlapping multiplets integrating two protons between 7.7-7.8 and 8.2-8.3, indicating a typical aromatic ring which is the structural backbone of the isolated anthraquinones. Data varied for the novel compounds isolated by Marques *et al*., 2000 and Permana *et al*., 1999 due to the presence of differently substituted side chains.

2.3.11. Summary

Three anthraquinone derivatives and one steroid, 9, 10 anthracenedione, 1-hydroxy-4-methylanthraquinone, 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H-phenanthrene-4, 9-dione and androst-5-ene-3, 17, 19-triol were isolated from the roots of *C. triloba*. The structures of these compounds as 9, 10 anthracenedione, 1-hydroxy-4-methylanthraquinone, 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H-phenanthrene-4,9-dione and androst-5-ene-3,17,19-triol were determined by analysis of spectral data (UV, ^{1}H NMR, ^{13}C NMR and EI-LC-MS)

CHAPTER 3: SCREENING FOR BIOLOGICAL ACTIVITY OF *C. TRILOBA*

3.1. INTRODUCTION

Despite the extensive traditional use of *C. triloba*, scientific data confirming its biological activity is lacking. In the previous chapter six compounds were isolated from *C. triloba* roots. Structures were confirmed with EI-LCMS. as; (i) 9, 10 anthracenedione; (ii)1-hydroxy-4-methylanthraquinone; (iii) 5, 8- dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione; (iv) 1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester; (v) Octadecanoic acid and (vi) androst-5-ene-3, 17, 19-triol.

Species belonging to the Pedaliaceae family show that compounds from anthracenedione group are known to have a broad spectrum of biological activities, including effects on bacteria and fungi, on inflammatory responses, on cancer and are also potent antioxidants.

9, 10 anthracenedione derivatives are known to exhibit a quite potent anticancer activity. It has also been reported that these compounds can be effectively employed in both antibacterial and antitrypanosomal therapy. 9, 10 anthracenedione, 1,4-bis[[2-[(2-hydroxyethyl)amino]-ethyl]amino]-diacetate at concentrations ranging from 0.05 to 10.0 µg/ml, altered the cell cycle kinetics of Chinese hamster, Friend murine leukemia and SK-L7 cells cultivated *in vitro*; the drug had little effect on cycle progression of normal phytohemagglutinin-stimulated lymphocytes. The major effect, following a 2 to 18 hour exposure to drug, was a block of cell cycle progression at the G_2-M phases as determined by flow cytometry (Evenson *et al*., 1979).

Anthracenedione-based drugs are characterized by prominent anticancer properties and are widely used in clinical practice (Hortobagyi, 1997; Thomas and Archimbaud, 1997; Wiseman and Spencer, 1997; Arcamone, 1998). Their mechanism of action has been principally correlated to their ability to stimulate DNA cleavage mediated by the enzyme topoisomerase II (Malonne and Atassi, 1997). Nonetheless, recent studies have shown that the telomeric G-quadruplex structure, which interferes with the function of telomerase, can be efficiently targeted by anthraquinones (Perry *et al*., 1998, 1999). In addition, it is well known that the redox cycling of the anthracenedione ring system may generate reactive radical species that are believed to be responsible for a number of toxic effects, including cardiac toxicity (Frishman *et al.*, 1997; Giantris *et al*., 1998).

1-hydroxy-4-methylanthraquinone is an excellent source of synthetic dyes for the textile industry. These compounds have long been known to be useful as dyes (Fig. 40) but, in recent years, many of these compounds have found utility as dyes for synthetic fibers (French Pat. No. 2,002,124 and U.S. Pat. No. 2,533,178).

Excellent dyeing properties for synthetic fibers such as washing fastness and durable press have been discovered in new compounds of the generic structural formula:

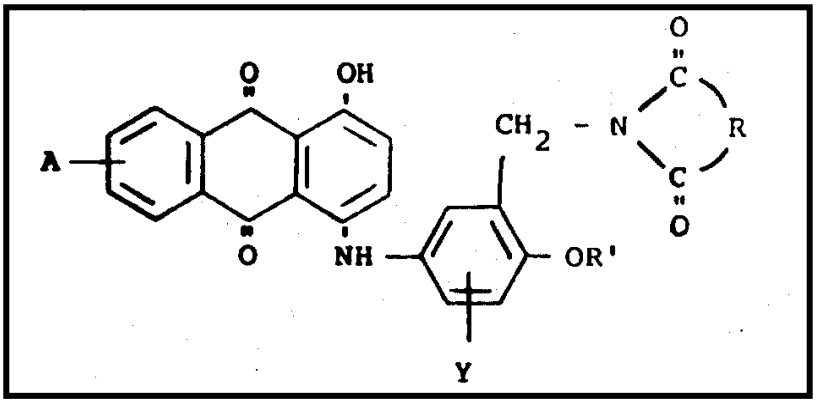


Figure 40: Generic structural formula for anthraquinone compounds with excellent dyeing properties.

in which A represents hydrogen, chlorine, bromine or lower alkyl; R represents alkylene of from 2 to 4 carbon atoms, vinylene, 1,2-benzene, 1,2-cyclohexane, 1,8-napthalene or 1,2-cyclohex-4-ene, all of which may be substituted by chlorine, bromine or lower alkyl; R' is lower alkyl; and Y represents hydrogen, hydroxy, chlorine, bromine, lower alkyl or lower alkoxy. These compounds have substantially better sublimation fastness than the known, related compounds disclosed in French Pat. No. 2,002,124.

5, 8- dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione belong to a group of compounds that modulate the activity of Janus kinases and are useful in the treatment of diseases related to activity of Janus kinases including, for example, immune-related diseases and cancer (Lamb *et al*., 1998).

1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester is more commonly known as a phthalate. Phthalates are used in a large variety of products ranging from enteric coatings of pharmaceutical pills and nutritional supplements to viscosity control agents, gelling agents, film formers, stabilizers, dispersants, lubricants, binders, emulsifying agents and suspending agents. Phthalates are also frequently used in soft plastic fishing lures, caulk, paint pigments and sex toys made of so-called "jelly rubber."

Phthalates are used in a variety of household applications such as shower curtains, vinyl upholstery, adhesives, floor tiles and cleaning materials. Personal care items containing phthalates include perfume, eye shadow, moisturizer, nail polish, liquid soap, and hair spray (Rudel and Perovich, 2008). The most widely-used phthalates are the di-2-ethyl hexyl phthalate (DEHP), the diisodecyl phthalate (DIDP) and the diisononyl phthalate (DINP). DEHP is the dominant plasticizer used in PVC due to its low cost. Benzylbutylphthalate (BBP) is used in the manufacture of foamed PVC, which is mostly used as a flooring material. Phthalates with small R and R' groups are used as solvents in perfumes and pesticides. They are also known as endocrine disrupters and cause birth defects and metabolic interference.

Octadecanoic acid occurs in many animal and vegetable fats and oils. A colorless, odorless, waxlike fatty acid, $CH_3(CH_2)_{16}COOH$, occurring in natural animal and vegetable fats and used in making soaps, candles, lubricants, and other products (Wootthikanokkhan and Tunjongnawin, 2002).

As there no reports on the biological and safety effects of *C. triloba*, this research in this chapter deals with antimicrobial, antifungal, anti-oxidant, anti-inflammatory and anticancer activities of the hexane crude extracts and purified compounds; 9.10 anthracenedione and 1 hydroxy-4-methyl anthraquinone and determine their safety in *in-vitro* experiments.

3.2. MATERIALS AND METHODS

3.2.1. Determination of Antimicrobial Activity

The antimicrobial activity of methanolic and aqueous plant extracts were carried out on selected bacteria and fungi by evaluating the bactericidal and antifungal effect and the minimum inhibitory concentration on selected bacteria and fungi in a petri dish using the agar disk diffusion method. A standard set of cultures are prescribed in these tests by the National Committee for Clinical Laboratory Standards, USA [NCCLS, 1990].

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that kills all the organisms and is usually determined by dilution methods. It is the antimicrobial susceptibility testing method against which other methods, such as disk diffusion are calibrated. In dilution tests, microorganisms are tested for their ability to produce visible growth on a series of agar plates or in test tubes or microplate wells of broth containing dilutions of the microbial agent.

In determining the MIC values growth indicators are used and not turbidity because plant extracts are frequently turbid or causes precipitates when mixed with microbial growth media. In this project p-iodonitrotetrazolium violet was used.

The p-iodonitrotetrazolium violet (INT) reaction is based on the transfer of electrons from NADH, a product of the threonine dehydrogenase [TDH] catalyzed reaction, to the tetrazolium dye [p-iodonitrotetrazolium violet]. Threonine dehydrogenase [TDH] from bacteria/fungi catalyses the NAD-dependent oxidation of threonine to form 2- amino-3-ketobutyrate and NADH. During the active growth of bacteria/fungi, an electron is transferred from NADH to p-iodonitrotetrazolium violet resulting in a formazan dye, which is purple in colour (Fig. 41). Therefore, the clear zone(s) on the microplate wells indicate areas of inhibition [zones where no active growth of bacteria has taken place].

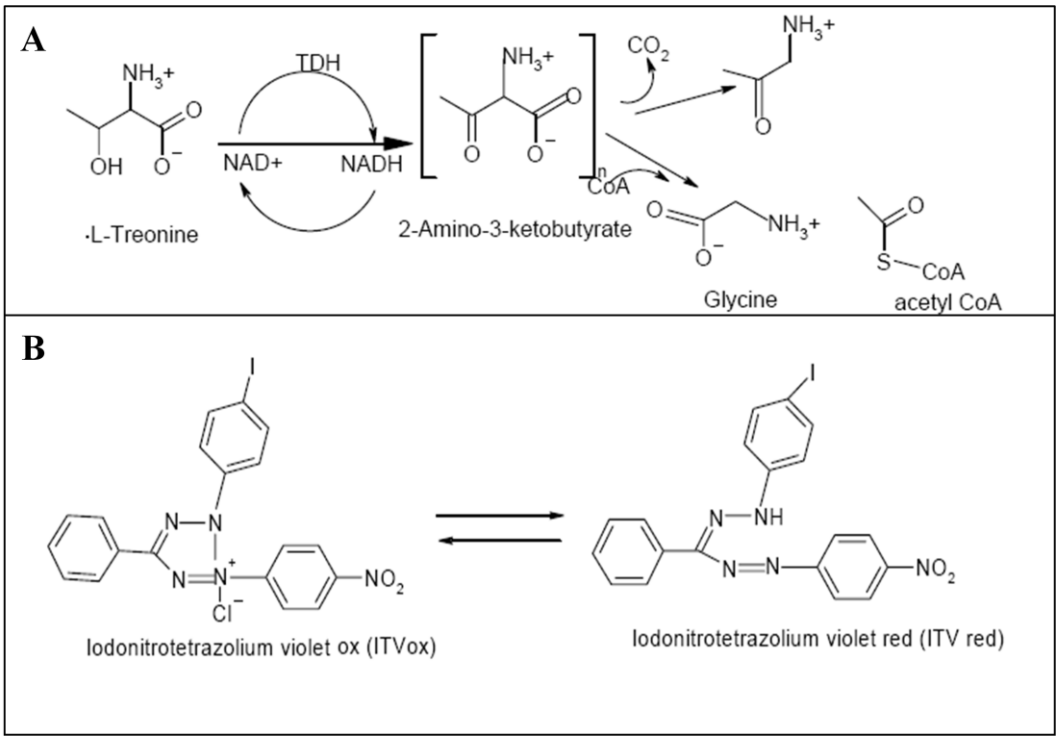


Figure 41: **A- Reaction pathways for the assay of threonine dehydrogenase, B- INT, coupling reagent for the colorimetric assay**.

3.2.2 Antimicrobial Activity Assay

The ten bacteria used as test organisms were as follows: *Bacillus cereus* (DBT*_F), *B. stearothermophilus* (DBT*_Q), *Escherichia coli* (DBT*_L), *Klebsiella oxytoca* (DBT*_AM), *Micrococcus sp.* (DBT*_AR), *Pseudomonas aeruginosa* (DBT*_D), *Proteus mirabilis* (DBT*_O), *Salmonella typhimurium* (DBT*_AF), *Staphylococcus aureus* (DBT*_E) and *S. epidermis* (DBT*_ S).

* DBT is a reference for the Durban University of Technology Culture Collection which is based at the Department of Biotechnology and Food Technology. The cultures were verified by their Gram reactions and antibiotic sensitivity patterns. Stock cultures were prepared from the Culture Collection and stored in micro bank vials using 50% glycerol. When required the cultures were plated out on Tryptone Soya Agar (Biolab) plates and were subsequently grown in Tryptone Soya Broth (Biolab) for 24 h at 37°C. The absorbance of bacterial cells was adjusted to MacFarland Standard of 0.5 which corresponded to 10^8 CFU/ml.

Molten (45°C) sterile tryptone agar (10 ml) in a flask was inoculated with a 0.1 ml of 10^8 cfu/ml of each of the respective bacterial strains. This was poured over the base plates containing 10 ml tryptone agar in sterile 9 cm Petri dishes. 50 µl of plant extracts at different concentrations were pipetted on 5 mm sterile filter paper disks (Whatman No. 1); and air dried in a biological

safety cabinet laminar. Sample containing discs were placed on the surface of the inoculated bacterial plates and incubated at 37°C for 24 h. Dimethyl sulphoxide was used as the negative control and Gentamycin was used a poitive control for the Gram Negative organisms and Ampicillin was used a positive control for the Gram positive organism. All tests were carried out in triplicate.

Minimum inhibitory concentration [MIC] was determined by serial dilution of extracts beyond the level where no inhibition of growth of test organisms was observed (Eloff, 1998). This was performed in microplates by filling all wells, with 50 µL sterile Mueller Hilton broth. In row A, 50 µL [100%] of the extract was placed with a micropipette. From row A, 50 µL was transferred to row B after taking up and releasing three times to ensure adequate mixing. The process was repeated until all the rows were completed and the additional 50 µL from row H was discarded. Two wells were used as a sterility and growth control respectively with the sterility control containing only Oxoid® Mueller Hilton broth (MHB), whilst the growth control containing both MHB as well as test organism.

After adding 50 µL of the bacterial suspension to each row (except for the sterility control), the microplate was sealed and incubated at 37°C at 100% relative humidity overnight. The following morning 50 µL of a 0.2 mg/ml solution of INT (p-iodonitrotetrazolium violet) was added to each row and the plate was returned to the incubator for at least half an hour to ensure adequate colour development. P-INT is a dehydrogenase activity detecting reagent, which is converted into corresponding intensely coloured formazan by metabolically active microorganisms (Navarro, 1998).

Inhibition of growth was indicated by a clear solution or a definite decrease in colour reaction. This value was taken as the minimum inhibitory concentration [MIC] of the extract. Extracts used for MIC determination were either dissolved in acetone or solubilized in DMSO (100 to 200 µl/mg) and made up as a stock solution (200 µg/ml) with distilled water. Well A had typically a final concentration of 100 µg/ml. Positive controls for test organisms were usually made up to a concentration of 1000 µg/ml and are listed in section

3.2.3 Determination of Total activity or Potency

The mathematical model used to determine the total activity is expressed as:

$$\text{Total activity (ml)} = \frac{\text{Amount extracted from 1g of plant material (mg)}}{\text{MIC (mg/ml)}}$$

3.2.4. Determination of Antifungal Activity

For the antifungal activity two strains of fungi *Aspergillus flavus* (DBT*_AR) and *Fusarium verticilloides* (DBT*_FM) was utilized. These were previously isolated from corn samples and stored in the Durban University of Technologies culture collection. These were prepared by growing them on Sabourand Dextrose Agar at 28°C for 4 to 7 days until they sporulated. The spores were collected in 10 ml sterile distilled water, counted in a counting chamber (Neubauer) and the concentration adjusted to 10^6 spores/ml. Sterile distilled water containing the fungal spores (10^6 spores/ml) were poured over the Sabourand Dextrose Agar (SDA) base plates (Biolab). 50 µl of leaf and root extract was transferred onto each of three sterile 5 mm discs (Whatman No. 1). 50 µl of DMSO served as the negative control, and 5 µg/ml Amphotericin B (Fluka, Biochemika), was used as a positive control. Each plant extract and control was tested in triplicate. The plant extracts and ethanol impregnated discs were dried in sterile Petri dishes and incubated at 30°C. Antifungal activities were recorded as the width (mm). The minimum inhibitory concentration (MIC) was taken as the lowest concentration that inhibited growth.

Bioautography (Begue and Kline, 1972) was also used to verify above results. Developed chromatography plates (5 µl of 10 mg/ml = 50 µg) of extracts and fractions were dried over night and sprayed with a suspension of growing cells of the bacteria listed above and incubated at 37°C in a chamber at 100% relative humidity for 18 h. After spraying with tetrazolium violet, clear zones on the chromatogram indicated inhibition of growth after incubating for 1 h at 37°C.

3.2.5. Determination of Antioxidant Activity

The antioxidative properties of the partially purified extracts (CTREH 01, CTREH 02 and CTREh 03) were tested using the DPPH (1.1-diphenyl-2-picrylhydrazyl radical) photometric assay described by (Choi *et al.*, 2002*).*

The freeze dried aqueous leaf and root extract was diluted in methanol (1000 μg/ml). One millilitre of a 0.3 mM DPPH in ethanol was added to 2.5 ml of plant extract and kept at room temperature for 30 minutes. One millilitre ethanol plus plant extract solution (2.5 ml) was used as a blank, while DPPH solution and 2.5 ml ethanol was used as a negative control. The positive control was DPPH solution (1 ml) plus 2.5 ml 1mM Quercitin 3- Rutinoside (Sigma). The radical scavenging activity was measured as the decolourization percentage of the test sample. All tests were carried out in triplicate. The absorbance values were measured in a Varian Cary 1E UV-visible spectrophotometer at 518 nm. The average absorbance values were converted into the percentage antioxidant activity, using the following equation:

$$\% \text{ Decolourization} = \frac{(\text{Av Controls} - (\text{Av sample}_{\text{DPPH}} - \text{Av sample}_{\text{MeOH}})) \text{ X100}}{\text{Av Controls}}$$

Where Av Controls=average absorbance of all DPPH control wells−average absorbance of all methanol control wells; Av sample $_{DPPH}$ average absorbance of sample wells with DPPH and Av sample $_{MEOH}$ = average absorbance of sample wells with methanol.

3.2.6. Determination of the 5-lipoxygenase activity

In normal biological systems 5-lipoxygenase is known to catalyze the oxidation of unsaturated fatty acids containing 1–4 pentadiene structures using arachidonic acid as the biological substrate coverting them into conjugated dienes which results in continuous increase in absorbance at 243 nm. 5-lipoxygenase activity of the plant extracts was determined using the method as published by Evans (1987), Baylac and Racine (2003) and (Alitonou *et al*., 2006) with linoleic acid as the substrate for the 5-lipoxygenase enzyme. Soybean lipoxygenase (EC. 1.13.11.12) was purchased from Fluka and Nordihydroguaiaretic acid (NDGA) [500.38.9] and linoleic acid sodium salt (822-17-3) were obtained from Sigma Chemical Co; potassium phosphate buffer 0.1 M, pH 9 was prepared with analytical grade reagents purchased from standard commercial sources. Deionized water was used for the preparation of all solutions.

Standard linoleic acid was enzymatically converted to conjugated dienes resulting in an increase in absorbance at 234 nm. Absorbance was plotted graphically against the different concentrations used. The slopes of the straight-line portions of the sample and the control curves were used to determine the percentage activity of the enzyme (Lourens *et al*., 2004). Nordihydroguaiaretic acid (NDGA), a known inhibitor of soybean lipoxygenase, was used as a reference drug.

3.2.6.1. 5-Lipoxygenase Assay

The reaction was initiated by the addition of aliquots (50 µl) of a soybean LOX solution (prepared daily in potassium phosphate buffer 0.1M pH 9.0) in a sufficient concentration to give an easily measurable initial rate of reaction to 2.0 ml of sodium linoleate (100 µM) in phosphate buffer; the enzymatic reactions were performed in absence or in presence of inhibitor and their kinetics were compared. The inhibitors were dissolved in DMSO in such a manner that an aliquot of each (30 µl) yielded a final concentration of maximum 100 µg/ml) in each assay. The initial reaction rate was determined from the slope of the straight line portion of the curve and the percentage inhibition of the enzyme activity was calculated by comparing with the control (using 30 µl of DMSO alone instead of 30 µl of the inhibitor solution). Each inhibitor concentration was tested in triplicate and the results averaged; the concentration (µg/ml) of the plant samples that gave 50% inhibition (IC_{50}) was calculated from the outline of the inhibition percentages as a function of the inhibitor concentration. The assay mixture without the plant extract was used as the negative control while one containing nordihydroguaretic acid (NDGA) was used as the positive control.

3.2.7. Determination of Topoisomerase II activity

The topoisomerase inhibition assay was based on the method outlined by Spitzner and Muller, (1988) using the Topo II Assay kit. The TopoGEN Topoisomerase II Drug Screening kit will allow detection of two kinds of topoisomerase inhibitors: those that stimulate formation of cleavable complexes and those that antagonize topoisomerase II action on the DNA. A known topoisomerase II poison was included as a control (etoposide). The DNA substrate (pRYG) included in this assay was ideal for these studies because it contained a single, high affinity topoisomerase II cleavage and recognition site. Topoisomerase II cuts pRYG primarily at a single site at the 54 bp of alternating purine/pyrimidine DNA (Spitzner *et a1*., 1990).

The assay system was based upon evaluating the formation of DNA cleavage products which may be nicked, open circular DNA or linear DNA (Fig. 42). The products were then resolved by ethidium bromide gel electrophoresis.

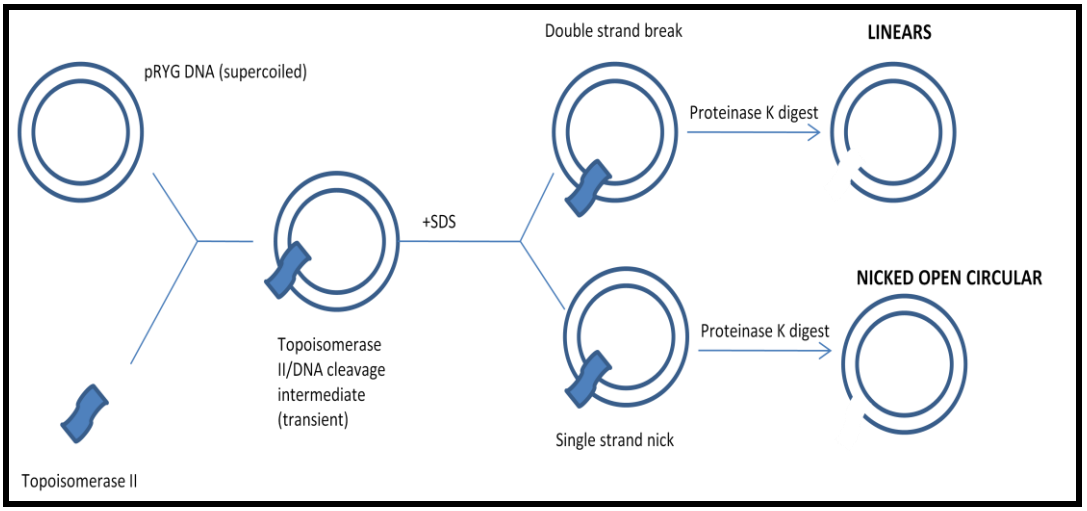


Figure 42: Cleavage pathway of topoisomerase II and pRYG DNA.

3.2.7.1. Topoisomerase II Drug Screening Protocol

3.2.7.1.1. Enzymes and DNAs

Homogeneously purified human topoisomerase II (Cat# 2000H) were used for these studies (Spitzner and Muller, 1988; Trask and Muller, 1983). pRYG (Cat# 2013) and pBR322 DNA was also used in these experiments. A modified protocol as described by Marini *et al*. (1980) was used for this assay.

3.2.7.1.2. Enzyme assays

Eukaryotic topoisomerase II was assayed by decatenation of pRYG DNA. Reactions contained 0.1 µg pRYG DNA (final volume of 20 µl), 50 mM Tris-HCl (pH 8), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM each of dithiothreitol, ATP and 30 µg BSA/ml (topo II reaction buffer Cat# 4040). The reactions were incubated for 15 min at 37°C and terminated with 0.1 vol of stop buffer (5% sarkosyl, 0.025% bromophenol blue, 50% glycerol). One unit of topoisomerase II is defined as the amount of enzyme required to fully decatenate 0.1 µg of pRYG or pBR322 DNA in 15 min at 37°C. Assays designed to detect topoisomerase II inhibitors were carried out in topo II cleavage buffer (30 mM Tris-HCl, pH 7.6, 3 mM ATP, 15 mM 2-mercaptoethanol, 8 mM MgCl2, 60 mM NaCl) in a final volume of 20 µl (Table 13). Reactions were incubated with 4 units of enzyme in the presence or absence of the indicated inhibitor for 30 min at 37°C.

The reactions were terminated with 2 µl of 10% sodium dodecyl sulfate, followed by proteinase K treatment for 15 min at 37°C. After addition of 0.1 vol of loading dye (50% glycerol, 0.025% bromophenol blue) samples were extracted once with an equal volume of chloroform:isoamyl

alcohol (24:1). Following a brief centrifugation in a microfuge, the blue upper layer was loaded directly onto an agarose gel. If the reactions are heated, cooled or treated with high salt prior to SDS, the topoisomerase II breakage and resealing equilibrium may be altered and breaks can reseal. The decatenation products were analyzed on 1% agarose gels run either without or with 0.5 µg ethidium bromide/ml as specified. Electrophoretic analysis of pRYG and pBR322 DNA was performed using standard agarose gel electrophoresis units. Separations designed to resolve supercoiled and relaxed DNA monomers (gyrase assays) were performed at 50 volts. Eukaryotic topo II products were separated at 100 volts which allowed rapid resolution of catenated networks from the minicircles. In gels containing ethidium bromide, appearance of the monomer DNA species was conveniently monitored with a hand-held UV light source; typically, electrophoresis was continued until the bromophenol blue dye front had migrated 50-75% down the gel.

Table 13: A sample reaction (20µl) is shown below.

H_20	Variable, made up to volume (20 µl in this case)
5x Complete Buffer	4 µl (prepared fresh as described above)
pRYG DNA	1 µl (200 to 250 ng is sufficient)
Test Compound (standards and purified extracts)	Variable. (Inhibitor control should be used at a final concentration of 0.1 mM or use 2 µl of a 1 mM stock).
Topoisomerase II	Variable.

The agarose gel (1%) was run to completion until the dye front is at the bottom of the gel. The agarose gel was then stained with 0.5 µg/ml ethidium bromide and results photo-documented. This is a "non-ethidium bromide" gel separation which is optimal for resolving relaxed and supercoiled DNAs.

3.2.8. Cytotoxicity

Cell proliferation inhibition was assessed by the MTT assay (Hanelt *et al*., 1994).

3.2.8.1. Cell Line

Human chronic myelogenous leukaemia (K562) was used in this study. The K562 cell line was purchased from Highveld Biological, Modderfontein, South Africa. The cells were received in an active state and immediately incubated at 37ºC in a humidified incubator (Snjiders Hepa,

United Scientific group, Cape Town South Africa) with 5% CO_2. When the cells were 80% confluent, they were sub-cultured, and stock cultures were stored at -70ºC until required.

3.2.8.2. Cell Maintenance

Cell maintenance was performed according by protocols obtained from Freshney (1987). All cell culture procedures were carried out in a laminar flow cabinet containing a UV light, (Scientific Engineering INC). The unit was swabbed /sterilized with 70% ethanol (Merck, South Africa) before each use. The cells were grown aseptically in 75 cm^2 tissue culture flasks (T 75) (Greiner, Germany) using filter sterilized (0.22 mm) 10% Complete Culture Medium (CCM) which comprised of Dulbecco's modified Eagle's medium (DMEM), containing 10% foetal calf serum and supplemented with antibiotics (penicillin: 10 000 U/ml, streptomycin sulphate: 10 000 U/ml) and 1 mM sodium pyruvate). Cells were incubated in a humidified incubator under 5% CO_2 at 37°C and passaged weekly. All the above media and chemicals were obtained from Highveld Biological, South Africa. The cultures were incubated at 37°C in a humidified incubator. The culture flasks were examined for colour changes and turbidity of the media on a daily basis. This determined the frequency of media changes. The culture was examined under an inverted microscope (Nikon) for cell growth.

The cells were harvested when the culture was 80% confluent and divided into two separate flasks, then more medium was added to each culture flask and incubated at 37°C in a humidified incubator. The cells were enumerated using a haemocytometer. The cell suspension was mixed with equal aliquot of 0.2% Trypan Blue [Biowhittaker, Wakersville (USA)] (v/v 1:1). This mixture was drawn across the grid by capillary action. The volume of cell suspension that occupied one primary square is 0.1mm^3 (1.0mm^2, 0.1mm/ 1.0, 10^4 mL). Only the viable (translucent) cells that lay within or that touched, the left or top boundary were counted.

The number of viable cells per ml in the original sample was calculated as follows:

$$\text{Cells/ml} = \text{Average number of cells per primary square} \times 10^4 \times \text{dilution factor}$$

3.2.8.3. Storage of cells

Storage of cells was performed according by protocols obtained from Freshney (1987). The cells were pelleted and washed twice with pre-warmed Phosphate Buffered Saline, pH 7.2 (PBS). The cells were then resuspended in 0.5 ml FCS and cooled on ice. A 20% dimethylsulphoxide (DMSO) in DMEM (V/V 1:4) was prepared as the cryoprotecting agent and also placed on ice. Equal aliquots (0.5ml) of the cell suspension and the cryoprotective agent were added to a cryotube (Corning, South Africa). The tubes were transferred to the thermos flask and kept overnight at -20°C. The cells were subsequently transferred to a –70°C bio-freezer and stored until required.

3.2.8.4. Regeneration of cells

Regeneration of cells was performed according by protocols obtained from Freshney (1987). Cells were removed from the -70°C biofreezer, swabbed with 70% ethanol and rapidly thawed. The cells were then transferred to 20 ml of pre-warmed 10% CCM in 75 cm^2 tissue culture flasks and incubated at 37°C humidified incubator with a 5% CO_2 atmosphere.

3.2.8.5. MTT assay

The MTT cytotoxity assay was conducted according to Mosmann, *(*1983) with minor modifications. The assay was carried out in 96 well, flat bottomed microtitre plates (Cellstar, Greiner, Germany). Two hundred micro litres of ±1.2 $x10^3$ of cells was added into each well, 20 µl of the plant (1000 µg/ ml, 100 µg/ ml, 10 µg/ ml) extracts were added to the respective wells. In the control wells ,20 µl DMSO and 20 µl media only respectively were added. The plate was incubated in a 37°C humidified incubator with a 5% CO_2 atmosphere for 20 hours. Then 20 µl of MTT reagent (Sigma, St Louis, USA) was added and the plates were then incubated for a further 4 hours at 37ºC in a humidified incubator with 5% CO_2 atmosphere. Subsequently, 100 µl of 100% DMSO was added to each well and the plate was incubated for an additional 1 hour. The absorbance was read at 578 nm on an ELISA plate reader (Digital Analogue Systems, Italy).

The percentage viability was determined using the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

3.2.9. Toxicity Assay

The brine shrimp lethality assay was used with minor modifications (Meyer *et al*., 1982).

3.2.9.1. Sample Preparation

Ten, 100, and 1000 µg/ml of plant extract were dissolved in DMSO and 50 µl was impregnated on filter paper disks. The disks were allowed to dry in an open sterile Petri dish in a biological safety cabinet with a vertical laminar flow for one hour. Control disks were prepared using only DMSO. Three replicates of each dose and the control were tested.

3.2.9.2. Hatching the shrimp

Twenty five milligrams of Class C: *Artemia salina* eggs (Natures Petland, Durban, South Africa) was added to artificial salt water (23 g NaCl, 11g $MgCl_2 \cdot 6H_2O$, 4 g Na_2SO_4, 1.3 g $CaCl_2 \cdot 2H_2O$, 0.7 g KCl in 1000 ml distilled water) and kept at room temperature. The pH was adjusted to 9.0 using Na_2CO_3 to avoid risk of death to the *Artemia* larvae by decrease of pH during incubation. This was incubated in a hatching chamber at room temperature. After 24 hours, 15 ml of yeast solution was added to the chamber for every litre of salt water in order to feed the larvae; 48 hours after the eggs were incubated, the larvae were extracted by picking up the moving larvae and visibly counted.

3.2.9.3. Bioassay

Every vial with 100 µl of plant sample at different concentration (10, 100, 1000 µl) contained 10 larvae of brine shrimp, including the control group, and was filled to 5 ml total volume with artificial salt water. A drop of yeast suspension (3 mg in 5 ml sea water) was added to each vial. The vials were then incubated at 27°C for 24 hours. After 24 hours, dead larvae were counted and percentage death determined.

3.2.10. Ames Mutagenicity Test

The *Salmonella* mutagenicity assay was conducted according to the method described by Maron and Ames, 1983 with minor modifications. The tester strain TA 98 was obtained on disc cultures from MOLTOX^{TM}. The disk cultures described were prepared from master cultures obtained from Dr. B.N. Ames (Berkeley, California, USA)

From the frozen disc culture of the *S. typhimurium* TA 98 and TA 100 tester strain, broth cultures were made. Using a flamed bacteriological needle, one of the culture disks were aseptically removed and inoculated into a sterile 250 ml Erlenmeyer flask containing 25 ml of nutrient broth (Oxoid) and 78 µl of 8 mg/ml Ampicillin (to maintain the stability of the plasmid). The flask was incubated on a shaker (150 rpm) at 37ºC for 16 hours to obtain an optical density at 660 nm of between 1.2 and 1.4.

In a sterile test tube, 100 µl of grown culture was added to 2 ml of 0.05 mM histidine/0.05 mM biotin top agar appendix), vortexed and plated onto a minimal glucose agar plate. The plate was incubated at 37ºC for 48 hours. Well separated colonies were used from this plate for initial broth cultures. Broth cultures of *S. typhimurium* were made by inoculating nutrient broth with master plate colonies. These cultures were incubated on a shaker (150 rpm) at 37 ºC for 24 hours. The crude plant extracts were dissolved in DMSO to obtain concentrations of 100 µg/ml, 1000 µg/ml and 10 000 µg/ml. Sodium azide (NaN_3) is a highly mutagenic compound and was used as a positive control. NaN_3 was dissolved in DMSO to obtain concentrations of 5 µg/ml, 10 µg/ml and 20 µg/ml. Sterile distilled water was used as a negative control.

Three plates were prepared for each concentration of test compound. In a sterile test tube, 100 µl of bacterial culture, 100 µl of test compound and 2.9 ml of soft agar (appendix A) held at 45 ºC, were added. This was briefly mixed with a vortex mixer and poured onto glucose minimal agar plates (Appendix A). Once the agar overlay solidified, the plates were inverted and incubated at 37ºC for 48 hours, after which the number of revertant colonies (i.e. histidine dependant) colonies counted and the mutant frequency determined. The mutant frequency is expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control. This can be expressed in the formula:

$$\textbf{Mutant Frequency} = \frac{\textbf{Revertant number of colonies}}{\textbf{Negative control}}$$

3.3. RESULTS AND DISCUSSION

The antibacterial activity of the root and leaf extracts against *B. cereus*, *S. aureus*, *M. luteus*, *P. aerouginosa*, *S. faecalis* and Gram-negative bacteria: *E. coli*, *S. typhimurium*, *E. aerogenes* and *K. pneumoniae* showed that the root extract showed inhibitory activity against *B. cereus* (Fig. 43) and *M. luteus* (Fig. 45) whereas the leaf extract showed activity against *B. cereus*, *E. aerogenes* (Fig. 44) and *M. luteus*. The activity by the leaf and root extract was lower than that of the control.

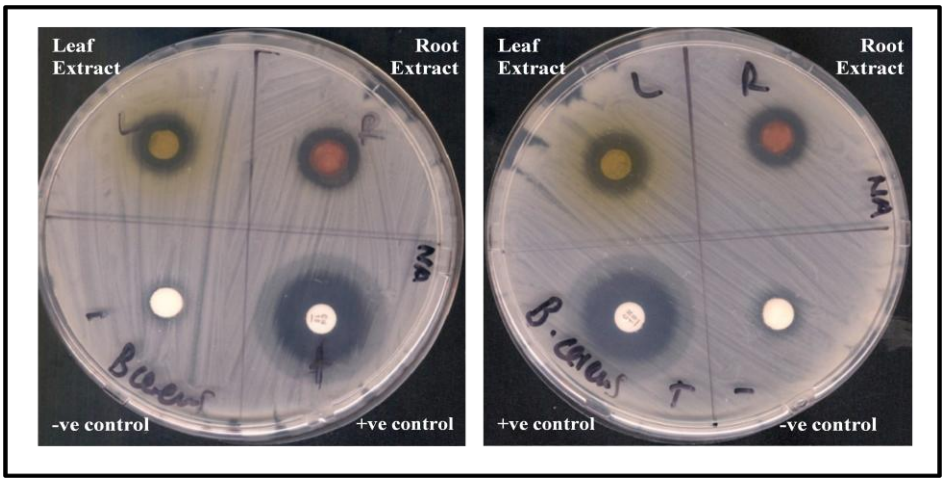


Figure 43: **Antimicrobial screening showing zones of inhibition produced by crude extracts of leaves and roots from *C. triloba* against the gram positive bacteria, *Bacillus cereus.* Ampillicin and dimethylsulfoxide were used as positive control and negative control respectively.**

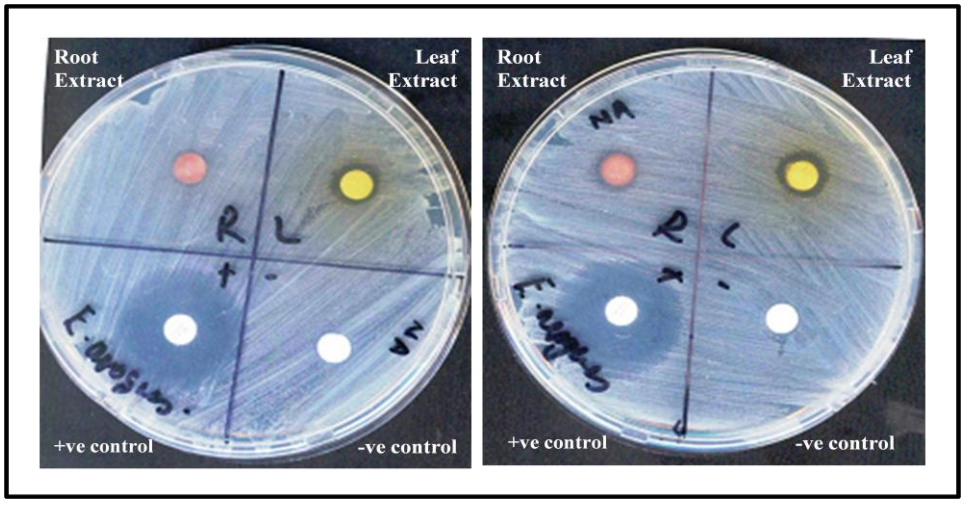


Figure 44: **Antimicrobial screening showing zones of inhibition produced by crude extracts of leaves and roots from *C. triloba* against the gram negative bacteria, *Enterobacter aerogenes.***

Figure 45: **Antimicrobial screening showing zones of inhibition produced by crude extracts of leaves and roots from *C. triloba* against the gram positive bacteria, *Micrococcus luteus*.**

3.3.1. Antibacterial assays – purified extracts (CTREh01, CTREh02 and CTREh03)

Root extracts were further purified by Preparative Thin Layer Chromatography and the extracts CTREh01, CTREh02 and CTREh03 were screened for antibacterial activity. The results of these screens are illustrated in Table 14. The purified extracts exhibited good activity against the Gram positive organisms *Micrococcus luteus, Bacillus cereus* and *Staphylococcus aureus.* Zones of inhibition ranging from 2 – 3 mm were identified in the Gram negative organisms, *Escherichia coli* and *Salmonella typhimurium. Pseudomonas aeroginosa* exhibited no antibacterial activity. DMSO and water did not inhibit growth of organisms. CTREh01, CTREh02 and CTREh03 were characterized and contained a combination of the six isolated compounds namely: 9, 10 anthracenedione, 1-hydroxy-4-methylanthraquinone, 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione, androst-5-ene-3, 17, 19-triol, 1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester and octadecanoic acid. This combination of compounds or the synergy that exists between like molecules produced by the polyketide pathway could have resulted in the increased antibacterial activity.

Test compounds exhibited similar activities against microorganisms, possibly due to similarities between structures and hence structure activity relationships. There appears to be a difference in inhibitory activity between Gram-positive and Gram-negative organisms, with the test

compounds showing significant activity against Gram positive organisms (Fig. 46B, C and E). No clear mechanisms have been identified that specifically indicate how these compounds target microbial invasion but is most probably not due to inhibition of cell wall synthesis. Some compounds appeared to be bactericidal as wells previously showing inhibition had not become infected after prolonged incubation periods.

Table 14: Summary of the antibacterial test using the purified extracts isolated from *C. triloba* roots. Inhibition zones (mm)

Bacterial culture	+ve control	-ve control	CTREh01 (mm)	CTREh02 (mm)	CTREh03 (mm)
Bacillus cereus	14	0	4	3	3
Esherischia coli	20	0	1	2	-
Micrococcus luteus	12	0	4	3	2
Staphylococcus aureus	20	0	4	1	1
Salmonella typhimurium	20	0	3	2	2
Pseudomonas aeroginosa	11	0	-	-	-

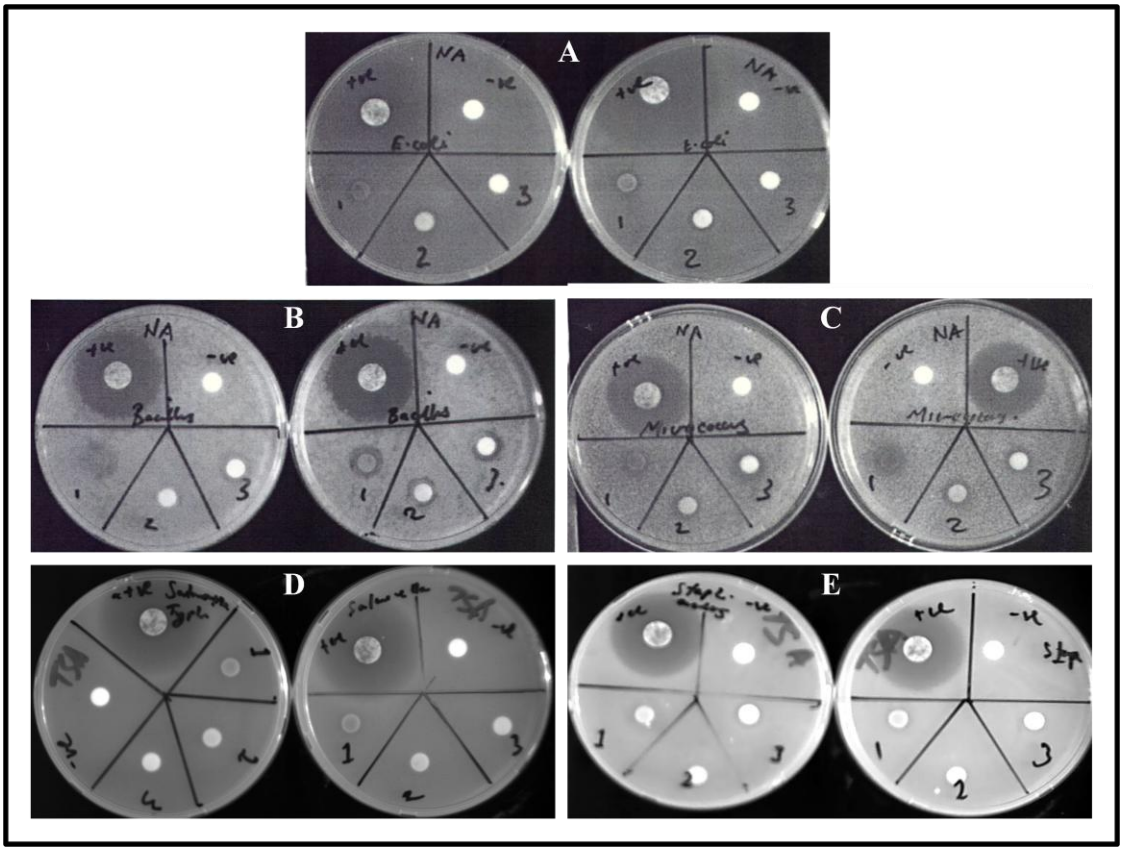


Figure 46: Antimicrobial screening showing zones of inhibition produced by purified extracts of roots from *C. triloba* against the Gram negative and Gram positive bacteria, A- *Escherichia coli, B-Bacillus cereus,* C- *Micrococcus luteus, D- Salmonella typhimurium and E- Staphylococcus aureus.* 1 - CTREh 01, 2 -CTREh 02 and 3 - CTREh 03.

3.3.2. Minimum Inhibitory Concentration (MIC)

The MIC values were calculated using the well dilution method as described in materials and methods. For all organisms, a standard antibiotic, either gentamicin or ampicillin, was included as positive control. Since all compounds were solubilized in acetone and made up to final concentration of 5 mg/ml, the same quantity of acetone to water was included as a negative control (solvent). Gram positive organisms, *S. aureus*, and *M. luteus* (Fig. 47) were inhibited at low concentrations of the crude and purified compounds (40 µg/ml). Gram negative organisms, *S. typhimurium*, and *E. coli* (Fig. 48) were inhibited at higher concentrations of the crude and purified compounds (312 – 620 µg/ml).

The crude extracts showed the highest inhibitory effect against *S. aureus* and *M. luteus* (40 µg/ml) and the lowest activity was exhibited by *E. coli* and *S. typhimurium* (620 µg/ml) [Table 15].

9, 10 anthracenedione inhibited *S. aureus* (>1000 µg/ml), *M. luteus* (40 µg/ml), *S. typhimurium* (620 µg/ml), *E. coli* and *B. cereus* (>1000 µg/ml).

1-hydroxy-4-methyl anthraquinone inhibited *S. aureus* (78 µg/ml), *M. luteus* (40 µg/ml), *S. typhimurium* (125 µg/ml) *E. coli* (150 µg/ml) and *B. cereus* (>1000 µg/ml). No antimicrobial data regarding this compound or 9, 10 anthracenedione could be sourced in the literature.

Structurally related compounds like Zenkequinone B isolated from *Stereospermum zenkeri* (Lenta *et al*., 2007) exhibited potent activity against Gram-negative bacteria, *Pseudomonas aeruginosa* (9,50 µg/ml). Zenkoquinones are complex anthraquinones with a furan ring attached to the main structural backbone of anthraquinones. This complexity in structure determines the antimicrobial potency of the isolated compounds. The antimicrobial activities of quinones may be linked to their properties to complex irreversibly with nucleophilic amino acids in proteins often leading to inactivation of the protein and loss of biological function (Cowan, 1999).

Table 15: MIC results of crude and purified extracts from *C. triloba* (1mg/ml).

Minimum Inhibitory Concentration (MIC) µg/ml						
Bacterial Culture	**Time (min)**	**Crude**	**9, 10 anthracene dione**	**1- hydroxy-4-methyl anthraquinone**	**Ampicillin (AMP)**	**Gentamycin (GEN)**
Salmonella typhimurium	30	0.620	0.620	1.250	0.620	
	60	0.620	0.620	1.250	0.620	
	90	0.620	0.620	0.620	0.620	
Staphylococcus aureus	30	0.040	1.250	0.078	NI*	
	60	0.040	2.500	0.078	NI	
	90	0.040	NI	0.040	NI	
Bacillus cereus	30	1.250	1.250	1.250		0.04
	60	2.500	NI	2.500		0.04
	90	2.500	2.500	2.500		0.04
Escherichia coli	30	0.620	0.312	0.150		0.04
	60	0.156	0.312	0.150		0.04
	90	0.312	0.312	0.312		0.04
Micrococcus luteus	30	1.250	1.250	NI	0.620	
	60	0.040	0.040	0.312	0.620	
	90	0.040	0.040	0.040	0.620	

***NI – no inhibition**

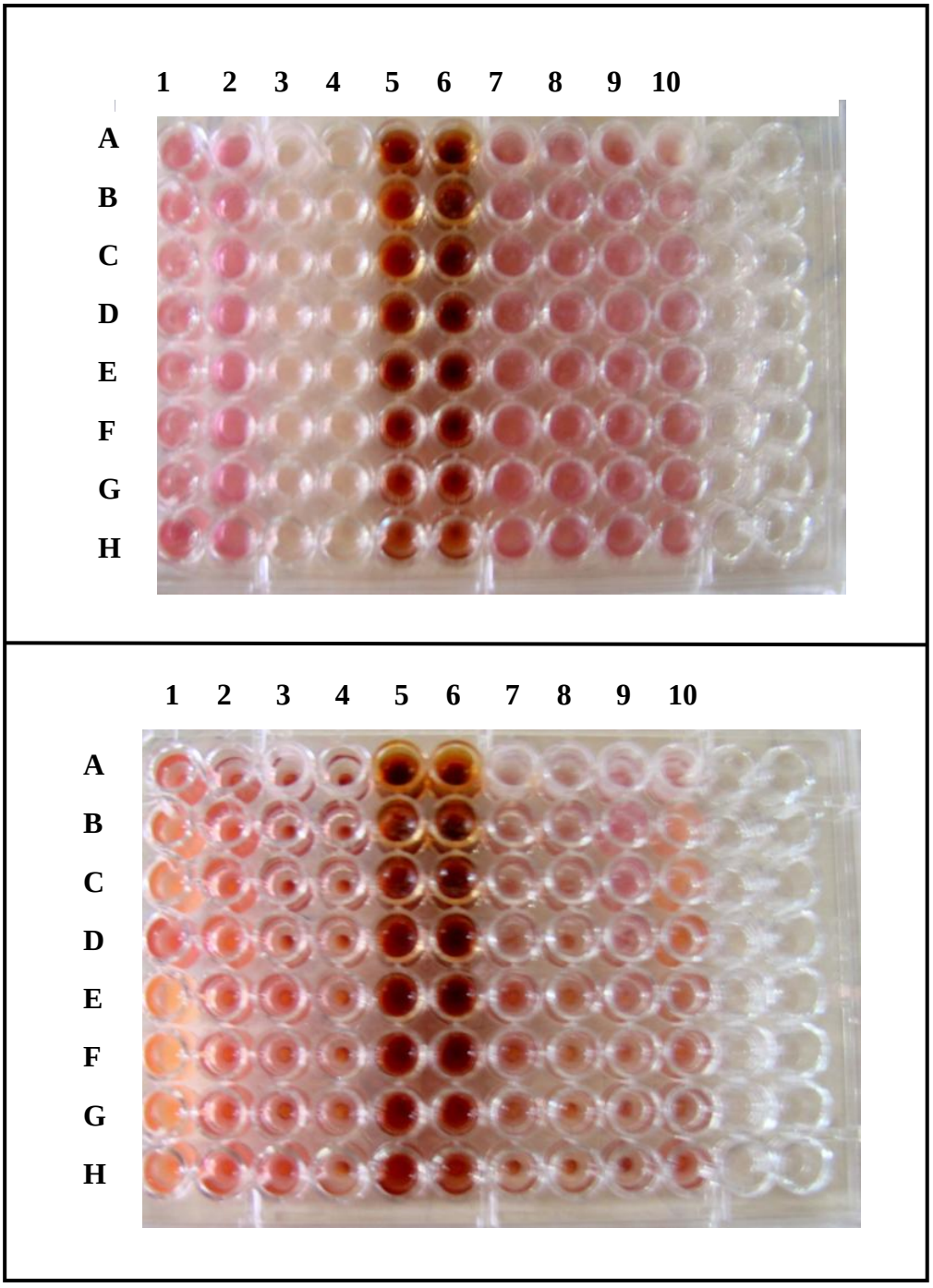


Figure 47: **MIC plate of *B. cereus* (top) and *M. luteus* (bottom) with the concentration in well A = 5 mg/ml, B = 2.5 mg/ml etc. Column 1 A-H represents the sterility control and column 2A-H is the negative control (acetone). Column 3 and 4 A-H is the antibiotic controls, gentamicin and ampicillin (10 mg/ml) respectively. The compounds are represented as follows: Column 5 and 6 A-H is the crude root extract; Column 7/8 A-H is purified 9, 10 anthracenedione and column 9/10 A-H, purified 1-hydroxy-4-methyl anthraquinone.**

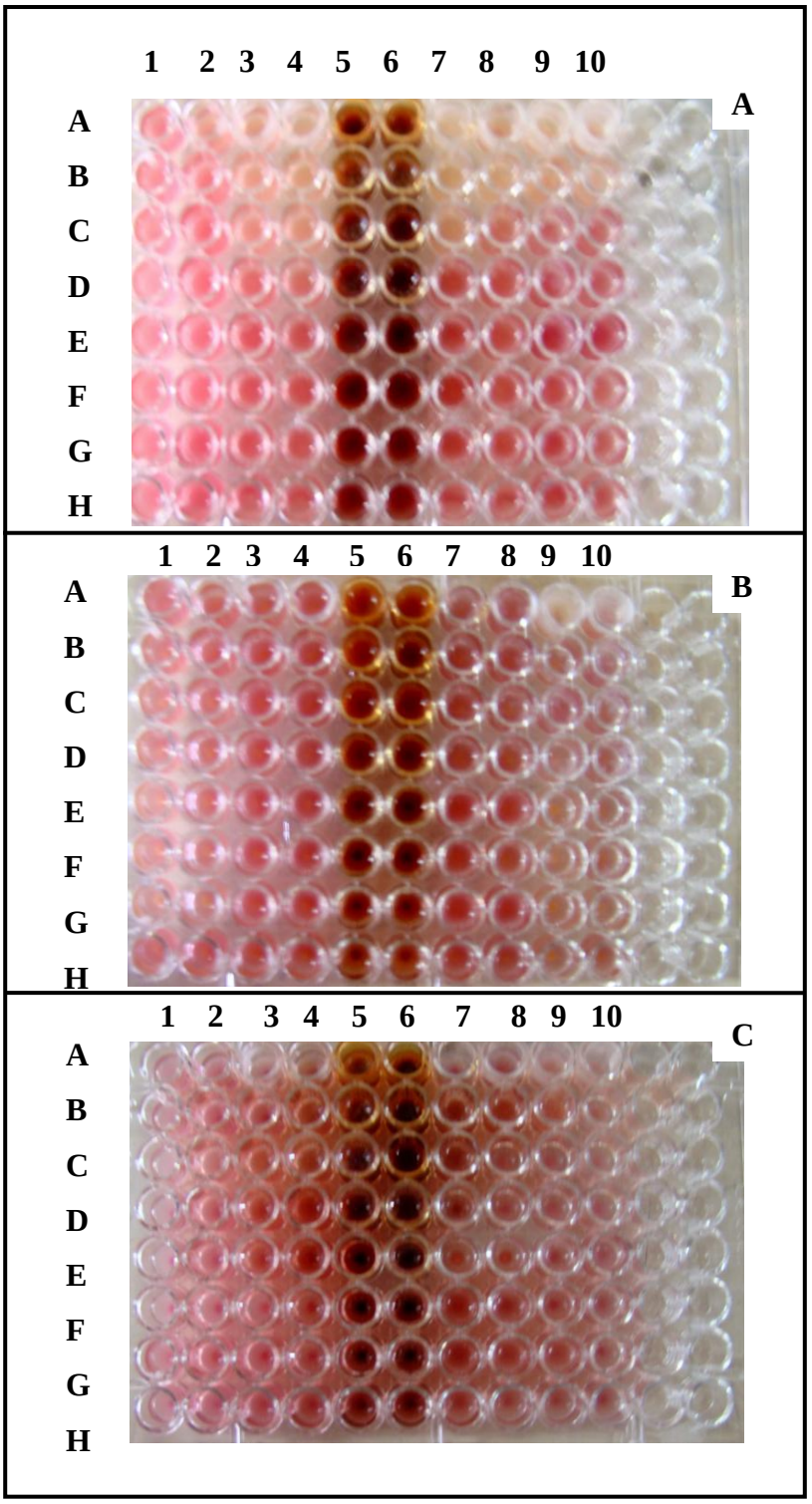


Figure 48: **MIC plate of *S. typhimurium* (A), *S. aureus* (B) and *E. coli* (C) with the concentration in well A = 5 mg/ml, B = 2.5 mg/ml etc. Column 1 A-H represents the sterility control and column 2A-H is the negative control (acetone). Column 3 and 4 A-H is the antibiotic controls, gentamicin and ampicillin (10 mg/ml) respectively. The compounds are represented as follows: Column 5 and 6 A-H is the crude root extract; Column 7/8 A-H is purified 9, 10 anthracenedione and column 9/10 A-H, purified 1-hydroxy-4-methyl anthraquinone.**

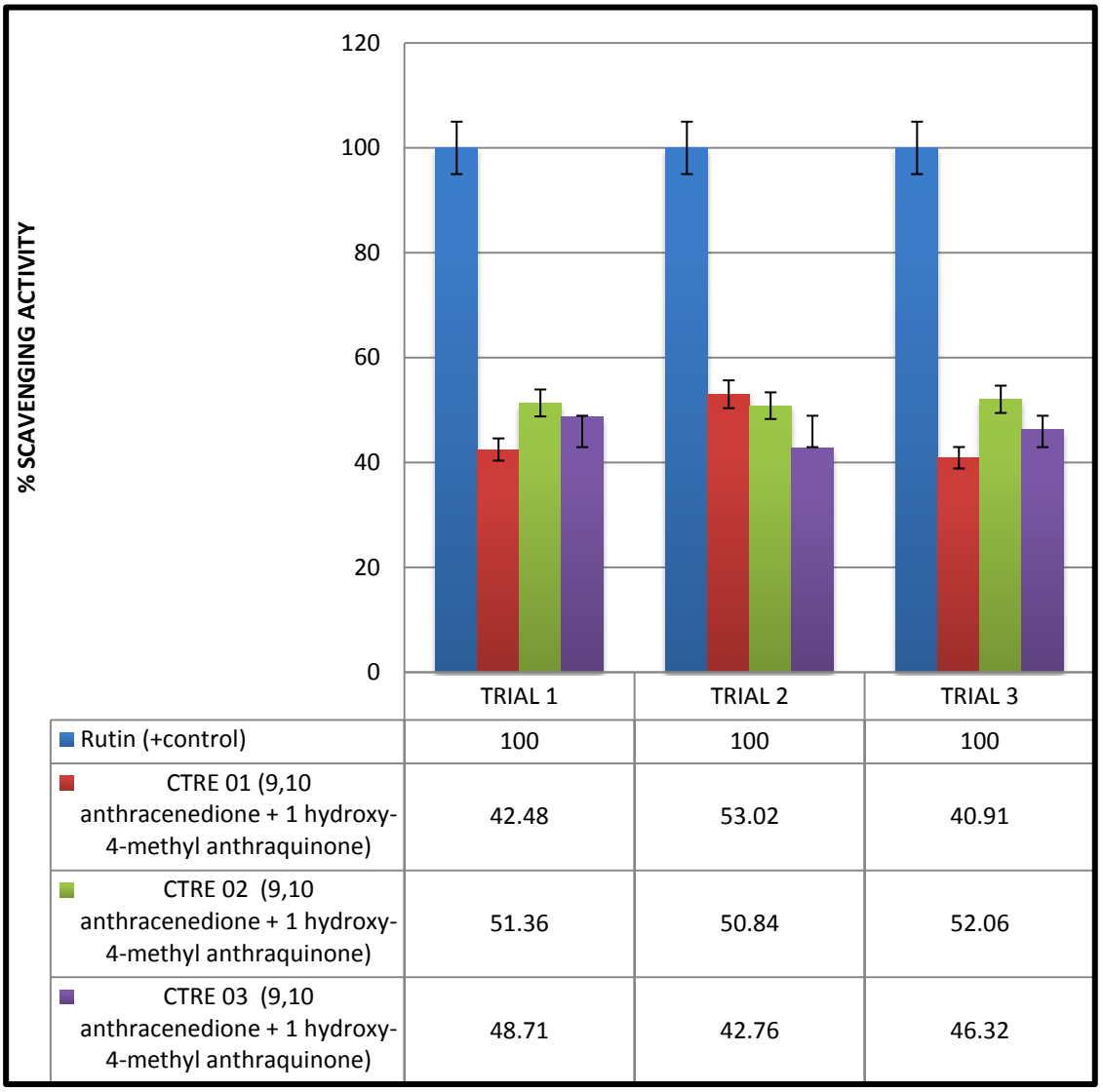
Table 16: Total activity or Potency of purified 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone from *C. triloba*.

Total activity in g/mg/ml	Crude	9.10 anthracenedione	1 hydroxy-4 methyl anthraquinone
Total quantity in mg extracted from 1g	**21.2**	**10**	**8**
Salmonella typhimurium	34	16	12.9
Staphylococcus aureus	530	4	200
Bacillus cereus	8.4	4	3.2
Escherichia coli	67.9	32.05	25.60
Micrococcus luteus	530	250	200

The total activity of crude extracts showed a higher total activity than that of the individual compounds. The Gram positive organisms, *Staphylococcus aureus* and *Micrococcus luteus* had the highest total activity of 530 g/mg/ml. Gram negative *Salmonella typhimurium* and *Escherichia coli* showed moderate activity ranging from 34 to 67.9 g/mg/ml. The purified compounds also exhibited good total activity against Gram positive organisms (200-250 g/mg/ml). The total activity results as well as the minimum inhibitory concentration assays were in agreement with the disc diffusion assays and indicated 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone showed antibacterial activity against Gram positive organisms. No antifungal activity was observed for the two strains of fungi *A. flavus* and *F. verticilloides*.

3.3.3. Antioxidant Activity

Quercetin-3-rutinoside was used as a positive control, which exhibited 100% scavenging activity of free radicals. The root extracts CTREh01, CTREh02 and CTREh03 (1000 µg/ml) showed a ± 50 % reduction of the free radicals. The experiments were carried out in triplicate. Fig. 49 shows the amount of each extract needed for 50% inhibition (IC_{50}). IC_{50} of the standard compound quercetin was 0.01 mg/ml. The highest radical scavenging activity was showed by CTRE 02 with IC50= 1 mg/ml. The radical scavenging activity in the plant extracts decreased in the following order: CTRE 02 > CTRE 03 > CTRE 01. Most of the plants' extracts at different concentrations exhibited more than 50% scavenging activity (Fig. 49).



	TRIAL 1	TRIAL 2	TRIAL 3
Rutin (+control)	100	100	100
CTRE 01 (9,10 anthracenedione + 1 hydroxy-4-methyl anthraquinone)	42.48	53.02	40.91
CTRE 02 (9,10 anthracenedione + 1 hydroxy-4-methyl anthraquinone)	51.36	50.84	52.06
CTRE 03 (9,10 anthracenedione + 1 hydroxy-4-methyl anthraquinone)	48.71	42.76	46.32

Figure 49: Antioxidant activity of the purified extracts (CTREh01, CTREh02 and CTREh03).

3.3.4. Anti-inflammatory assay

In our study we used nordihydroguaiaretic acid **(**NDGA) as a standard for the comparison of anti-inflammatory potential of the three famine plants. Reduced IC_{50} values suggest better inhibitory action on 5 COX. The IC_{50} value of NDGA was 2.5 µg/ml and the extracts showed (Table 17) no anti-inflammatory activity. IC_{50} for the crude leaf and roots extracts of *C. triloba* was 300 µg/ml.

Table 17: Inhibition of 5-lipoxygenase enzyme activity on linoleic acid by various concentrations (ppm) of leaf and root extracts of *C. triloba*

Concentration of plant extract (ppm)	% Inhibition [a] of 5-lipoxygenase activity		
	C. triloba (leaves)	*C. triloba (roots)*	NDGA
100	29.1	17.6	
200	41.6	31.8	
300	54.7	45.0	
400	61.3	52.1	
500	67.8	54.3	
600	67.5	54.1	
1.0			19.5
2.0			41.0
3.0			58.2
4.0			76.9
5.0			89.6
6.0			89.1
IC_{50} (ppm)[b]	**257.3**	**323.8**	**2.5**

a – Mean values obtained from experiments performed in triplicate. b - Mean value determined graphically.

3.3.5. Topoisomerase II Drug Assay – pBR322 and pRYG DNA

Agarose gel electrophoresis of the purified extracts (CTREh01 and CTREh02) on pBR322 DNA by human topoisomerase II is shown in Fig. 50 and on pRYG DNA is shown in Fig. 51. In both instances, lane 1 shows the position of the linear DNA marker which was essential to mark the position of the cleavage product. Lane 2 was a marker of the pBR322 or pRYG DNA, small amounts of nicked open circular DNA was always present in plasmid preparations. Lane 3 shows that incubating plasmid DNA (pBR322 and pRYG) with Human Topoisomerase II gives a relaxed DNA product. The resolution between the relaxed and super-coiled DNA was not ideal. Lane 4 shows the solvent control (DMSO) that clearly demonstrates that the solvent alone was not affecting topoisomerase II activity.

Lanes 4 demonstrates the cleavage of super-coiled DNA to its linearized form. Lane 5 shows cleavage in the presence of a control poison, Etoposide. In the presence of Etoposide, a slight increase in the nicked open circular DNA is seen along with linear DNA cleavage product. Lane 6 shows the inhibitory effect of the purified extract (CTREh01) at 1mM concentration. At this low concentration the enzyme showed only partial inhibition. This is represented by the presence of the nicked open circular DNA along with linear DNA cleavage product. Increasing

the concentration of the purified extracts (CTREh01 & CTREh02) to 5mM resulted in the complete inhibition of the human topoisomerase II enzyme as indicated in Lanes 7 and 8 respectively. In Fig. 52, increasing the concentration of the purified extracts (CTREh01 & CTREh02) to 5mM resulted in the complete inhibition of the human topoisomerase II enzyme as indicated in Lanes 6 and 7 respectively.

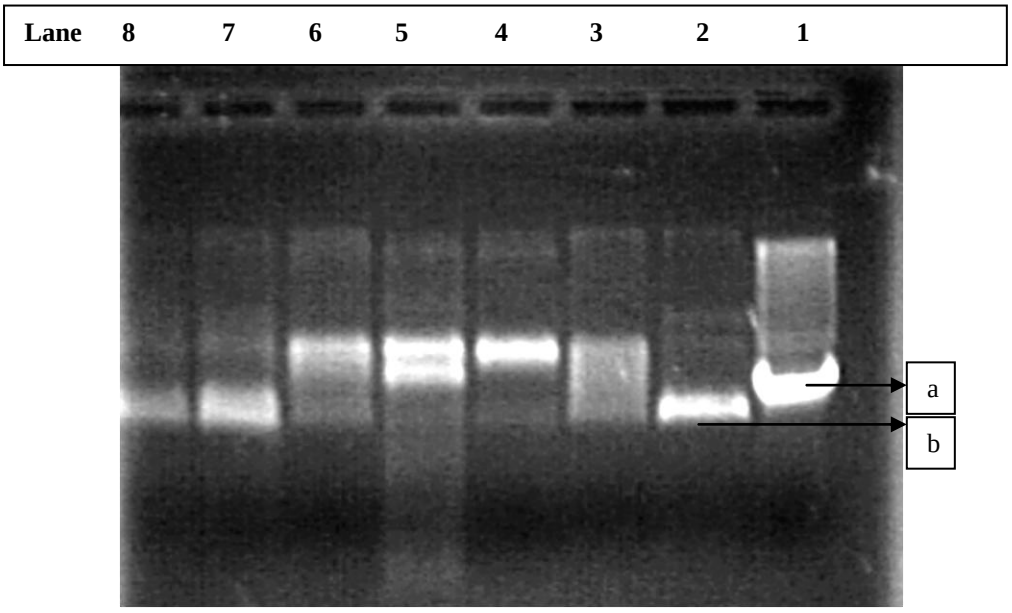


Figure 50: **Agarose gel electrophoresis showing the effect of purified extracts of *C. triloba* on the relaxation of pBR322 by human topoisomerase II. a – Linearized DNA. b – Supercoiled DNA.** Lane 1 - 5µl Linear DNA, 15 µl H_20; Lane 2 – 4µl Buffer [A+B], 1 µl super-coiled DNA, 15 µl H_20; Lane 3 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 13 µl H_20; Lane 4 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 2µl DMSO, 11µl H_20; Lane 5 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 2µl Etoposide, 11µl H_20; Lane 6- 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, [2 µl (1mM isolated CTREh01)], 11µl H_20; Lane 7-8- 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, [2 µl (5mM isolated CTREh01 and CTREh02)], 11µl H_20.

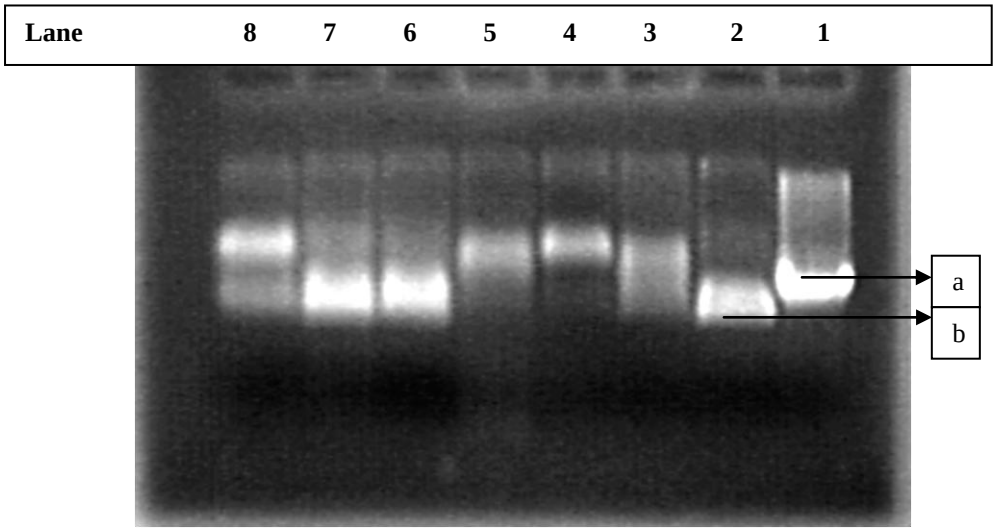


Figure 51: **Agarose gel electrophoresis showing the effect of purified extracts of *C. triloba* on the relaxation of pRYG DNA by human topoisomerase II. a – Linearized DNA. b – Supercoiled DNA**. Lane 1 - 5µl Linear DNA, 15 µl H_20; Lane 2 – 4µl Buffer [A+B], 1 µl super-coiled DNA, 15 µl H_20; Lane 3 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 13 µl H_20; Lane 4 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 2µl DMSO, 11µl H_20; Lane 5 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 2µl Etoposide, 11µl H_20; Lane 6- 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, [2 µl (1mM isolated CTREh01)], 11µl H_20; Lane 7-8- 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, [2 µl (5mM isolated CTREh01 and CTREh02)], 11µl H_20.

3.3.6. Confirmation of Topoisomerase II Activity Using Commercial Standards

Topoisomerase drug assay was carried out against commercial standards from Sigma-Aldrich. The results of the topoisomerase drug assay are illustrated in Fig. 52.

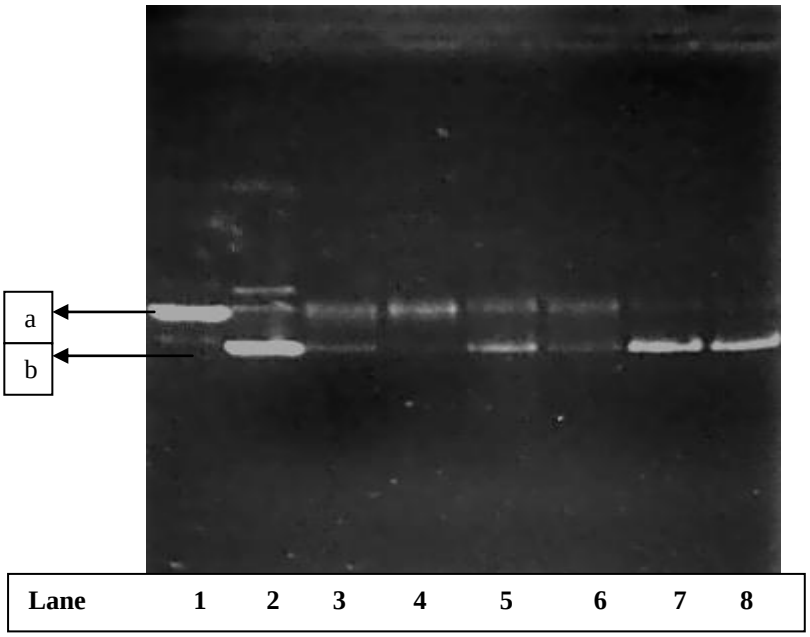


Figure 52: Agarose gel electrophoresis showing the effect of commercial standards (9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone) on the relaxation of pRYG DNA by human topo II. Lane 1 - 5µl Linear DNA, Lane 2 -1 µl super-coiled DNA,; Lane 3- 1µl super-coiled DNA, 2µl Human Topoisomerase II; Lane 4 - 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 2µl DMSO, Lane 5 - 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 2µl Etoposide ; Lane 6- 1 µl super-coiled DNA, 2µl Human Topoisomerase II, [2 µl (1mM 9, 10 anthracenedione)]; Lane 7- 1 µl super coiled DNA, 2µl Human Topoisomerase II, [2 µl (1mM 1 hydroxy -4 methylanthraquinone)]; Lane 8 - 1 µl super-coiled DNA, 2µl Human Topoisomerase II, [2 µl (1mM partially purified CTREh01 containing both 9, 10 anthracenedione and 1-hydroxy -4-methylanthraquinone)]

3.3.7. Topoisomerase II Activity – Purified 9, 10 Anthracenedione, 1 hydroxy-4-methylanthaquinone and 5, 8-dimethoxy-2,3,10,10a tetrahydro-1h,4ah phenanthrene-4,9-dione.

Fig. 53 illustrates the effect of the three purified polyketides on the relaxation of pRYG DNA. 9, 10 anthracenedione showed good potential as a topoisomerase poison by partial inhibition of the topoisomerase II enzyme which was clearly reflected by the partial conversion of supercoiled DNA to linear and nicked open circular DNA. 1-hydroxy -4-methylanthraquinone and 5,8-dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione showed potent activity against the topoisomerase II enzyme by completely inhibiting the formation of the topoisomerase IIα –DNA complex. This type of inhibition is typical of the polyketide group of compounds in which the level of activity of the polyketides is influenced by the presence of specific analogues (amino groups, guanidine and halides).

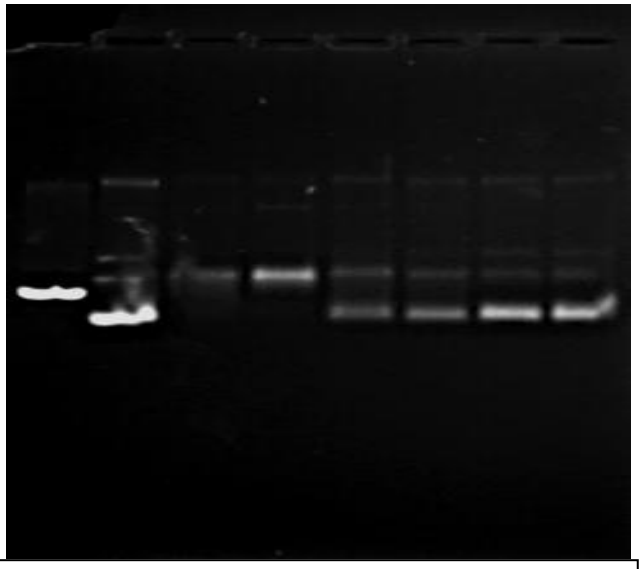


Figure 53: Agarose gel electrophoresis showing the effect of purified secondary metabolites (9, 10 anthracenedione, 1-hydroxy-4-methylanthaquinone and 5,8-dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione) on the relaxation of pRYG DNA by human topo II. Lane 1 - 5µl Linear DNA, 15 µl H_20; Lane 2 – 4µl Buffer [A+B], 1 µl super-coiled DNA, 15 µl H_20; Lane 3 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 13 µl H_20; Lane 4 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 2µl DMSO, 11µl H_20; Lane 5 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, 2µl Etoposide, 11µl H_20; Lane 6- 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, [2 µl (1mM 9.10 anthracenedione)], 11µl H_20; Lane 7- 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, [2 µl (1mM 1 hydroxy -4-methylanthraquinone)], 11µl H_20; Lane 8 - 4µl Buffer [A+B], 1 µl super-coiled DNA, 2µl Human Topoisomerase II, [2 µl (1mM5,8-dimethoxy-2,3,10,10a-tetrahydro-1H,4aH phenanthrene-4,9- dione)], 11µl H_20.

The formation of topoisomerase II-DNA cleavage complexes is required for the enzyme to perform its essential cellular functions (Fig. 54). If the level of cleavage complexes falls too low (left arrow), cells are unable to undergo chromosome segregation and ultimately die of mitotic failure. If the level of cleavage complexes becomes too high (right arrow) the actions of DNA tracking systems can convert these transient complexes to permanent double-stranded breaks in the genetic material. The resulting strand breaks, as well as the inhibition of essential DNA processes, initiate multiple recombination/repair pathways and generate chromosome translocations and other DNA aberrations. If the DNA strand breaks overwhelm the cell, they trigger apoptotic pathways. This is the basis for the actions of several widely prescribed

anticancer drugs. If the concentration of topoisomerase II-mediated DNA strand breaks is too low to overwhelm the cell, chromosomal translocations may be present in surviving populations and trigger the formation of leukemias that involve the MLL (mixed lineage leukemia) gene at chromosome band 1 1q23.

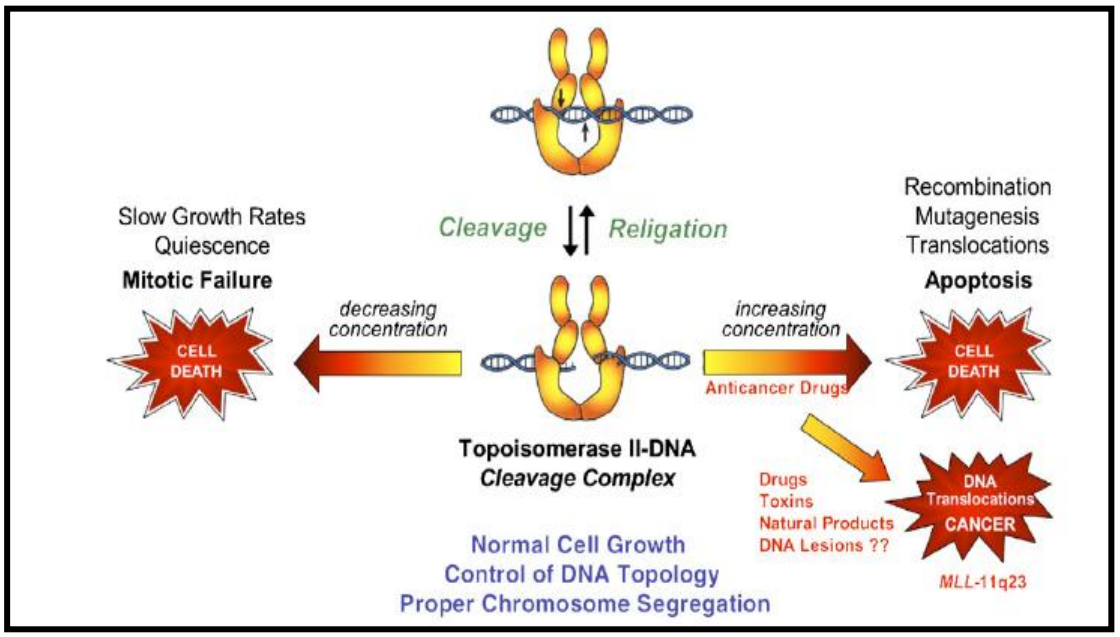


Figure 54: **The effects of increased and decreased topoisomerase II-DNA cleavage complexes in human cellular system.**

3.3.8. Cytotoxicity Assays

3.3.8.1. MTT assay

The MTT assay is a test of metabolic competence based upon assessment of mitochondrial performance. It is a colorimetric assay relying on the conversion of yellow tetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). The results from the MTT assay indicate a distinct pattern showing the decreasing lethality of the purified extracts (CTREh01, CTREh02, and CTREh03) on $HepG_2$ cell lines with decreasing concentration. This shows a direct correlation between the concentration of the purified extracts and the lethality against $HepG_2$ cell lines. At 12.5µg/25µl, all three purified extracts showed significant activity (Fig. 55) against $HepG_2$ cells (41.4 - 73.2% cell death) but when the concentration of the purified extracts were decreased to 1.56µg/25µl, the percentage cell death also decreased (1 -14.4% cell death).

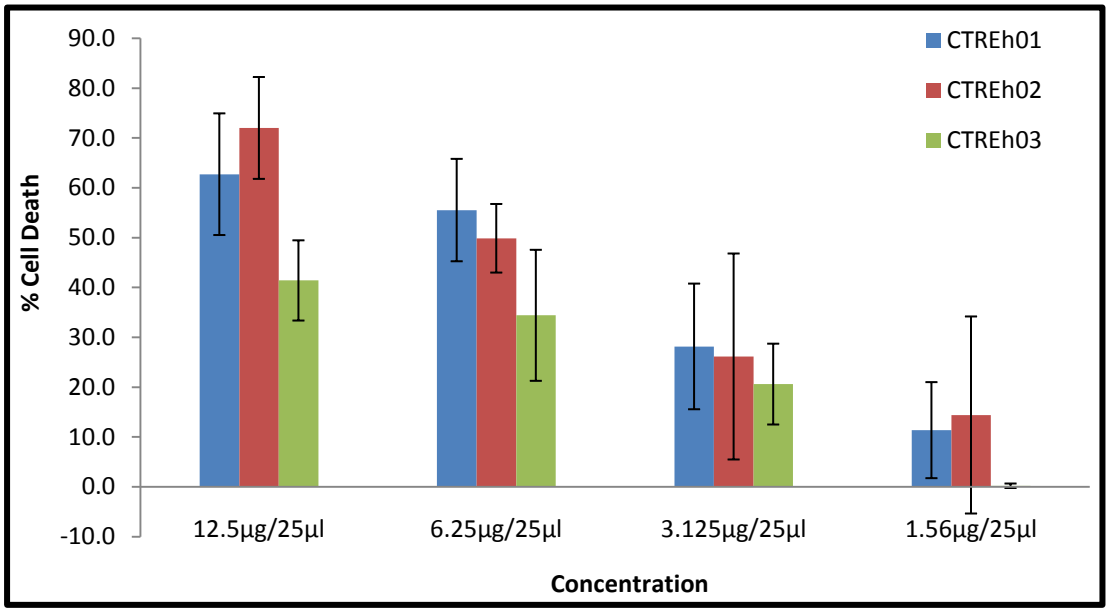


Figure 55: Lethality assay showing effect of purified extracts (CTREh01, CTREh02 and CTREh03) isolated from *C. triloba* on HepG$_2$ cell lines.

3.3.8.2. Brine Shrimp Lethality Assay

A method, utilizing brine shrimp (*Artemia salina*), is a simple bioassay for natural product research. The procedure determines lethal concentrations of active compounds in brine medium. The activities of a broad range of active compounds are manifested as toxicity to the shrimp. A positive correlation exists between brine shrimp lethality and human carcinoma.

The brine shrimp lethality test was conducted on each of the extracts at three concentrations, 10 µg/ml, 100 µg/ml and 1000 µg/ml. Table 18 gives the mean percentage death of shrimp at 24 hours. According to literature in order for a test compound to be considered highly toxic it needs to show shrimp death of 50% or less. For a compound to be considered slightly toxic it needs to show cell death of between 50 – 70%. None of the plants in this assay showed extreme levels of toxicity with all of the plants showing shrimp survival of greater than 90% at the highest concentration tested which was 1000 µg/ml. *Ceratotheca triloba* (CTREh01) displayed some or very little toxicity at concentrations 100 and 1000 µg/ml.

Table 18: Toxicity of purified compounds from *C. triloba* against *Artemia salina*.

	% shrimp death at concentrations (mean %)		
C. triloba	**10 µg/ml**	**100 µg/ml**	**1000 µg/ml**
CTREh01	8	10	10
CTREh02	0	0	0
CTREh03	0	0	0

3.3.9. Mutagenicity assay

The mutant frequency is expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control. Table 19 shows the mutant frequency of the plant extract tested on the *S. typhimurium* TA 98 strain. The greater the number of revertant colonies, the greater the mutant frequency. According to Maron and Ames (1983) a mutagenic potential is assumed if the mutant frequency is greater than 2; a possible mutagenic potential is assumed if the mutant frequency ranges between 1.7 and 1.9; and no mutagenic potential is assumed if the mutant frequency is lower than 1.6. None of the plant extracts up to concentrations of 1000 µg/ml showed any mutagenic potential (Fig. 56). Sodium azide was the chosen mutagen used in this experiment and it showed a mutagenic potential; as the concentration increased so did the number of revertant colonies.

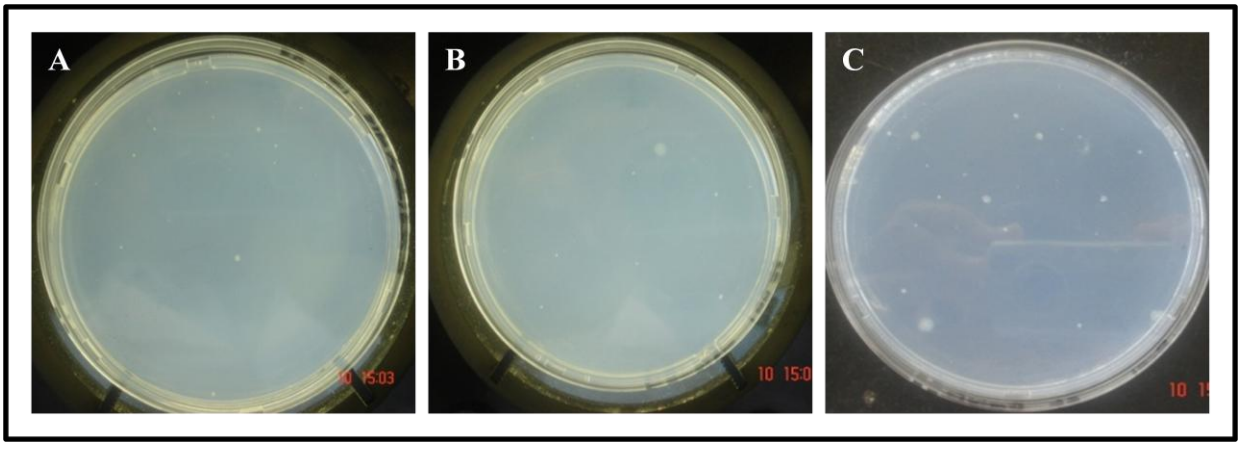


Figure 56: A- Plate with Leaf extract showing revertant colonies B- Plate with root extract showing revertant colonies. C- Plate showing revertant colonies in negative control

Table 19: Number of revertant colonies of *S. typhimurium* in agar plates with leaf and root extracts of *C. triloba* and their mutant frequencies

Test Sample /Agar plate	No of revertant *S. typhimurium* colonies after 48 hrs incubation	Mutant frequency
***C. triloba* leaf extract**	12	0.75
***C. triloba* root extract**	9	0.56
Negative control	16	-

3.3.10. Summary

9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone showed antibacterial activity against *S. aureus, M. luteus, B. cereus* and *E. coli*. Due to the synergistic effect of the individual compounds, the crude extract exhibited good potency (>500) against *S. aureus* and *M. luteus,* medium potency against *E. coli* and *S. typhimurium* (<100) and very low potency against *B. cereus* (<10). Although a similar trend was observed for 9, 10 anthracenedione and 1 hydroxy-4-methylanthraquinone unlike the crude extract. A very low potency against *S. aureus* was observed for 9, 10 anthracenedione and a high potency for 1-hydroxy -4-methyl anthraquinone. Thus 9, 10 anthracenedione is an effective drug against *E. coli* and *S. typhimurium* and 1-hydroxy-4-methyl anthraquinone is effective against *S. aureus* and *M. luteus*.

The root extracts CTREh01, CTREh02 and CTREh03 (1000 µg/ml) showed a ± 50 % reduction of the free radicals. No anti-inflammatory activity was observed. All purified and crude extracts showed potent inhibition of the human topoisomerase II enzyme. The purified extracts showed moderate toxicity against HepG2 cells at high concentrations and no toxicity was observed against brine shrimp larvae. No mutagenicity was observed with the crude extracts using the Ames test.

9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone can be used as antibacterial agents. Their anti-oxidative potential can be exploited for anti-cancer treatment as in many cancers reactive oxygen species are implicated in the aetiology of these cancers. Furthermore, this compound demonstrates potent anti-topoisomerase activity. Thus, the synergistic effect of 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone as anti-topoisomerases and anti-oxidative compounds can contribute to *C. triloba* extracts to fight cancer. However, the use of these compounds as anti-cancer agents needs further testing that can compare the activity of this compound in both normal and cancerous models. Because of the importance of this compound the next chapter explores the production of this compound in suspension cultures.

CHAPTER 4: CALLUS AND CELL SUSPENSION CULTURES OF *C. TRILOBA*

4.1. INTRODUCTION

Currently cancer treatment involves radiotherapy or chemotherapy. These treatment regimens are effective but have many side effects; hence new bioactive compounds such as anthraquinones are being investigated. Anthraquinones are a class of natural compounds that consists of the basic structure of 9, 10-anthracenedione (Bajaj, 1999). Their derivatives currently represent one of the most effective cytostatic and front line therapy for a variety of systematic and solid tumors (Singh *et al*., 2004). Examples of drugs containing the 9, 10 anthracenedione moiety include: daunorubicin and mitoxantrone (McClendon and Osheroff, 2007). From previous studies we have isolated two anthraquinones from the root extracts of *C. triloba*; 9, 10 anthracenedione and 1 hydroxy-4-methyl anthraquinone which are structurally similar to mitoxantrone. The production of anthraquinones from root extracts is however limited as it takes approximately 1-2 years for the *C. triloba* plant to mature to a feasible size, prior to harvesting. This also leads to the destruction of already established plants. Furthermore plant growth is also negatively affected by biological influences (pathogen sensitivity and insects) during the winter months. To overcome these limitations the production of the potential anticancer anthraquinones from *C. triloba* was investigated using plant cell culture technology. Plant cell suspension cultures are the preferred mode of producing phyto-pharmaceutical compounds because they are amendable to good manufacturing practice (GMP) procedures and the compound can be easily scaled up from the shake flask stage to large-scale bioreactors (Schlatmann *et al*., 1995).

Studies (Bulgakov *et al*., 2002, Oliveira *et al*., 2007, Orban *et al*., 2008 J, Han *et al*., 2002) have been conducted on the production of various derivatives of 9, 10- anthracenedione in plant cell cultures; eg. *Rubia cordifolia, Rudgea jasminoides, Rubia tinctorum* L, *Morinda elliptica, Cinchona robusta*. There is no literature that reports the production of 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone from the *C. triloba* cell culture.

In this study, an attempt was made to enhance the yield of anthraquinones produced in *C. triloba* cell suspension cultures using methyl jasmonate. Generally, when plant cells are exposed to chemical and environmental elicitors via specific plant receptors, certain biological responses are triggered which lead to the activation of biosynthesis genes and subsequently the production of plant secondary metabolites (Yukimune *et al*., 1996). The main advantage of using this strategy is that it reduces the time taken to obtain high yields of the secondary metabolites (Barz *et al*., 1988; Eilert, 1987; DiCosmo and Tallevi, 1985). Jasmonates play key role in eliciting biological responses that lead to the accumulation of secondary metabolites (Gundlach *et al*., 1992). Methyl jasmonate was used in this study as it has been proven to increase the production of phyto-pharmaceutically valuable compounds, examples include; paclitaxel and baccatin III from Taxus species (Yukimune *et al*., 1996) and Ajmalicine from *Catharanthus roseus.*

4.2. MATERIALS AND METHODS

4.2.1. Plant material

C. triloba was collected in Durban, Province of Kwazulu Natal, South Africa, and identified by Professor H. Baijnath, a botanist of the University of Kwazulu Natal (Westville), where a voucher specimen was deposited (Durban Botanical Herbarium).

4.2.2. Plant cell culture

The leaves of the *C. triloba* plant were removed and washed with distilled water three times at one minute intervals. Thereafter, they were sterilized with mercuric chloride ($HgCI_2$) (0.1 %) and sodium hypochlorite (NaCIO) (30 % and 40%). The sterilization agents were tested individually and in combinations using the exposure time/s indicated in Table 20. Excess detergent remaining on the leaves was washed off with sterile distilled water at each interval. Leaf disks were submerged in distilled water for 20 minutes, to serve as the control for the experiment. After completion of the surface sterilization process, 0.5 cm square leaf disks were placed on MS medium (Murashige and Skoog, 1962) (six disks per plate) which was prepared using MS basal salt, vitamins, sucrose (130 g/l) and agar (8 g/l) (Sigma-Aldrich, Inc). The medium was supplemented with 1 mg/l each of 2.4 dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (6-BAP) (Sigma-Aldrich, Inc).

All treatments were tested by using two plates of medium and 12 replicates of leaf disks. The prepared explant plates were incubated in the dark at 26˚C for a period of one week. The explants were visually screened on day 7 and the percentage of contamination and the level of tissue damage on each explant were recorded. The best sterilization combination and concentration was determined (Table 20). Callus cultures induced from the explants were transferred onto fresh medium. Maintenance of callus cultures was achieved by sub-culturing callus tissue of a 0.5 cm diameter at 3-4 week intervals on fresh medium.

Table 20: Different sterilization treatments and exposure times for leaf explants

Sterilization agent	Exposure Time (min)
0.1% $HgCl_2$	5
40% NaClO	15
40% NaClO	20
0.1 % $HgCl_2$ + 30% NaClO	5 + 15
0.1 % $HgCl_2$ + 40% NaClO	5 + 15
Water (Control)	20

Approximately 2 g of yellow calli (three weeks old) from the second sub-culture was transferred into 250 ml Erlenmeyer flasks containing 50 ml of MS liquid medium supplemented with1 mg/l of 2,4-D and 6-BAP. The flasks were agitated on a shaker (Infors Ecotron, Polychem supplies cc) at 100 rpm and incubated at 26˚C in the dark phase. The cell suspension cultures were sub-cultured after one week of cultivation by transferring an aliquot of 30 ml of culture into 500 ml Erlenmeyer flasks containing 100 ml of MS medium. Cultures at 100 ml scale provided inocula for conducting shake flask experiments at a 400 ml scale. A growth curve was constructed to obtain sufficient cell mass for elicitation. All flasks were sampled at 7 day intervals to determine the quantity of cell mass in the flask by wet weight analysis. Triplicate samples of the cell suspension culture (2 ml) were vacuum filtered through pre-weighed filters (0.22 µm, 47mm, white grid, Millipore) after which each filter containing wet biomass was measured on an analytical balance (Adventurer ohaus). The wet weight was determined by the following equation: [(Wet weight + filter) – (filter)]* 1000 = wet weight (g/l). Methyl jasmonate (Sigma-Aldrich, Inc) solution was prepared at a concentration of 100 µM in ethanol. An aliquot of 100 ml of the elicitor solution (2.5 µl of ethanol per ml of culture) was filter sterilized (0.22 µm filter) into two flasks on day 21. An equal volume of ethanol was filter sterilized into two flasks to serve as control cultures. The elicitation was conducted for a 9 day period and cell suspension cultures were harvested at day 30 to perform extraction and chromatographic analyses.

4.2.3. Extraction and analysis of anthraquinones

Cell suspension cultures were harvested by centrifugation (Eppendorf centrifuge 5810) at 4000 rpm for 10 minutes at 20˚C. Thereafter cell mass was separated from the supernatant and sonicated (Virsonic, Virtis) at 4 psi for 10 minutes. Anthraquinones were extracted by agitating the cell mass on a shaker (Infors Ecotron, Polychem suppliers. cc) at 180 rpm for 24 hours at room temperature in 100 ml of hexane and the supernatant in 200 ml hexane.

Hexane fractions were separated and concentrated by using a roto-evaporator (Heidolph Laborota 400 efficient) with the water bath set at a temperature of 50°C and the flask rotated at 60 rpm. The residues where dissolved in 10 ml of hexane while the excess residue that was fixed to the flask was dissolved in 5 ml of ethyl acetate. The hexane and ethyl acetate fractions were then pooled and air dried for two days to further concentrate the extract preparation for chromatographic analyses.

4.2.4. Detection of anthraquinones produced in *C. triloba* cells by TLC

Thin layer chromatography was performed to detect anthraquinones in cell and supernatant extracts by using standards; 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone (Sigma-Aldrich, Inc) were prepared at 1 mg/ml concentration using ethyl acetate as a diluent. Approximately 10 µl of each standard solution, 20 µl of the root extract (100 mg/ml of hexane) and 50 µl of the cell extract (dissolved in ethyl acetate) were applied on the TLC silica gel plate (Merck TLC F254 or Silica gel 60 plates). The TLC plates were developed in two mobile phases; petroleum ether: ethyl acetate: formic acid (75:25:1) and ethyl acetate: methanol: water (100:13.5:10). Separated anthraquinones were visualized under visible and ultraviolet light (254 and 360 nm, Camag Universal UV lamp TL-600) after the TLC plates were sprayed with 5 % KOH in ethanol (Wagner *et al*., 1984).

Anthraquinones were also detected by comparison of the root and cell and supernatant extracts. Approximately 20 µl of the root extract (100 mg/ml of hexane) and 50 µl of the cell extract were applied to the TLC silica gel plate (Merck TLC F254 or Silica gel 60 plates). The TLC plate developed in a hexane: ethyl acetate (90:10) mobile phase. Separated anthraquinones were visualized under visible and ultraviolet light (254 and 360 nm, Camag Universal UV lamp TL-600) after the TLC plates were sprayed with 5 % KOH in ethanol (according to Wagner *et al*., 1984, with modifications).

4.2.5. Identification and quantification of anthraquinones by HPLC

Cell extracts were dried at room temperature for 2-3 days and dissolved in 1 ml of ethanol; the filtrates were used for HPLC analysis (according to Fernand *et al*., 2008 with modifications). Separation and quantitative analyses of anthraquinones were performed on a Merck- Hitachi LaChrom system (Darmstadt, Germany) consisting of an D 7000 system controller, four pumps (D7400), a Merck- Hitachi LaChrom (L-7200) auto injector and an Merck- Hitachi LaChrom (L-7200) UV-VIS detector (λ = 260 nm). Separation of the analytes was performed at 40°C on a Licrospher C_{18} (2) column, 100 ˚A pore size, 5µm particle size, 250×4.6mm i.d.column containing a guard column (Merck, Darmstadt, Germany). The analytes were eluted isocratically at a flow rate of 0.4mL/min using acetonitrile/methanol/buffer (25:55:20, v/v), where the buffer is 10mM ammonium acetate (NH_4Ac) at pH 6.8. The injection volume was 10 µL.

4.3. RESULTS AND DISCUSSION

4.3.1. Sterilization of *C. triloba* explant material

A major challenge at the initial stage of the developing C. *triloba* cell culture system was to overcome the contamination in field grown plants, as this was the only available source of explant material when the project started. It was therefore, important to study the effect of two surface sterilization agents on contamination and the leaf tissue. The most prevalent type of contamination in *C. triloba* explants were fungal while bacterial contamination occurred randomly. The percentage explants contaminated with fungi were 100 % with respective treatments due to the spread of the contamination to all explants in the plate. The percentage of bacterial contamination in explants was 8 % and this remained localized to the affected explants (Table 21). Explant plates contaminated with fungi were discarded and the sterile explants that remained unaffected by bacterial contamination were transferred to fresh MS medium. Therefore, in cases where the plant tends to have a high degree of fungal contamination, it should be recommended that one leaf explant be placed in a plate in such cases, an anti-fungal agent can be incorporated into the medium at an appropriate concentration.

Explants treated with $HgCI_2$ (0.1%) for 5 minutes and later in NaClO (30%) for 15 minutes eradicated all contaminants present in the leaf explants, therefore, this treatment was used for initiation of callus. The effectiveness of this treatment could be due to synergistic effect of the two surface sterilization agents as contaminated explants resulted when they were used separately. However, a high level of tissue damage was observed in the explants when this treatment regime was applied (Table 21). A possible reason could be that leaf (light green leaf) explants were exposed to this treatment contained a high level of meristematic tissue. The higher degree of meristematic tissue in an explant the more liable it is to tissue damage caused by the surface sterilization agents. Therefore leaves (green leaves) with a lower level of meristematic tissue were selected and surface sterilized with $HgCI_2$ (0.1%) and NaCIO (30%) to induce callus cultures from the *C. triloba* plant. The level of meristematic tissue is influenced by the formation of prolamellar bodies which become the source of membrane material for the organization of granal and intergranal thylakoids. The process is also accompanied by a two-step increase in the number of plastidial ribosomes which coincides with the extension of the intraplastidial membrane system and by a progressive accumulation of photosynthetic pigments and development of Hill reaction activity (Oliveira, 2006).

Table 21: Percentage contamination and level of tissue damage after sterilization with different treatments

Treatments	Type of contamination	Percentage contamination	Std deviation	Degree of tissue damage
0.1% $HgCI_2$	Fungal	100	0	++++[a]
30% NaCIO	Fungal	100	0	+[c]
40 % NaCIO	Bacterial	16.67	11.78	+++[b]
30 % NaCIO and $HgCI_2$	-	0	0	+++[b]
40 % NaCIO and $HgCI_2$	Bacterial	16.67	11.78	+[c]
Water (control)	Fungal	100	0	++++[a]

n=12, ±: standard deviation, **a)** highest degree of tissue damage, **b)** high degree of tissue damage, **c)** Low degree of tissue damage.

Callus initiation was observed on the surface or cut ends of the explants after 2-3 weeks of inoculation. After five weeks the entire leaf explant was transformed into callus tissue (Fig. 57). Callus cultures induced on MS medium were orange-yellow. Sub-cultured callus tissue produced root hairs and root-like structures after three weeks. The tips of the root-like structures contained a red-orange pigment (Fig. 57). The callus morphological properties were evaluated as previous studies by Banthorpe and White (2000) have shown this parameter can be used to predict whether anthraquinones are being produced in culture. The orange-yellow color of the *C. triloba* callus could be due to the medium used and the production of anthraquinones (Fig. 57). *M. elliptica* leaf explants also produced yellow callus cultures on MS medium and the liquid medium turned yellow when anthraquinones were released from the cells (Abdullah *et al*., 1998). The red- orange pigment observed at the tips of the root like structures of the *C. triloba* callus could be the presence of anthraquinones. Studies conducted by Bais *et al*., (2002) have shown that pigmented regions of the *Hyperium perforatum* (St John's worts) callus contained the polyketide (anthraquinone belongs to this group of compounds) Hypericin. In addition since anthraquinones are natural pigments (Hattori *et al*., 1993), the pigments present in the callus can be used as a marker for selecting high yielding cell lines. According to a study conducted by Mischenko *et al*., (1999), the orange calli accumulated higher anthraquinone content than yellow calli.

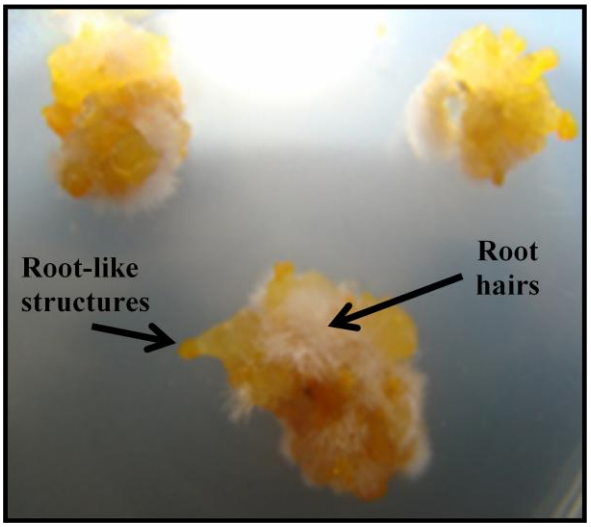


Figure 57: **Orange- yellow callus was induced from *C. triloba* leaf explant and sub-cultured callus developed root hairs and root- like structures.**

4.3.2. Elicitation of cell suspension cultures

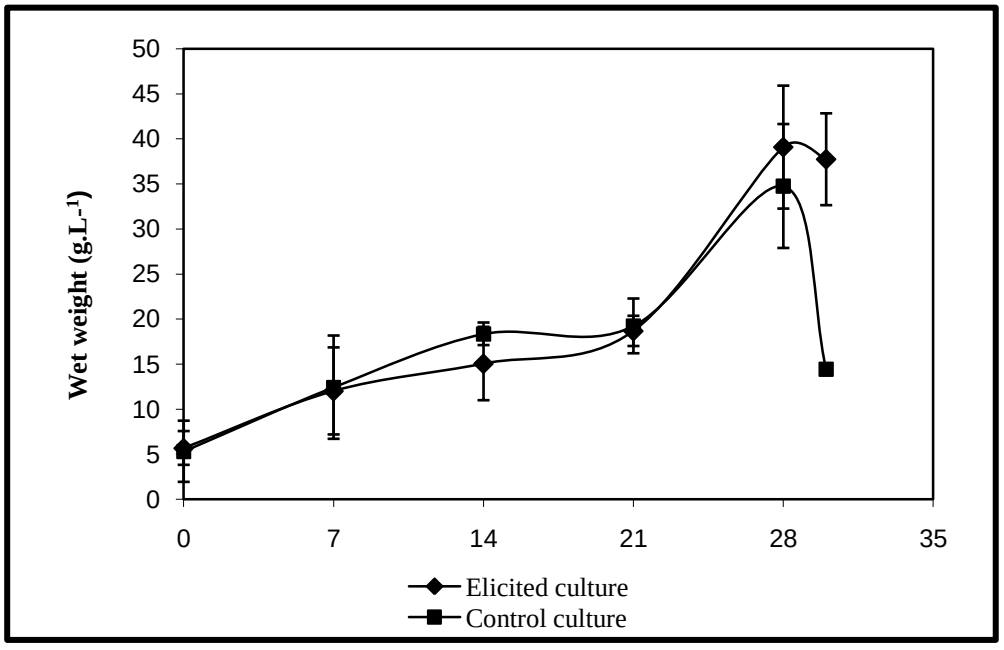


Figure 58: **Production of biomass in *C. triloba* cell suspension cultures.**

The establishment of cell suspension cultures from callus tissue was a key step in developing an efficient cell culture system for producing anthraquinones as liquid cultures have a faster growth rate compared to callus. Callus cultures were transferred into liquid medium, the friable callus tissue dispersed into small aggregates when the flasks were placed on the shaker. A growth curve of *C. triloba* cell suspension cultures (Fig. 58) was generated to assess sufficient biomass to elicit anthraquinones production. The biomass concentration increased from 5.50 g/l to 19 g/l after 20 days of cultivation; however, this increase accounts only for the cell mass that

remained in suspension during sampling as very large aggregates tend to sink to the bottom of the flask (Fig. 58). After 20 days, dense orange-yellow cell suspension cultures with large aggregates were formed. Therefore, cell suspension cultures were treated methyl jasmonate on day 21 to elicit the production of anthraquinones. The production of cell suspension cultures with highly dense cell mass is crucial for obtaining high yields of the plant-derived compound as secondary metabolites are based in intra-cellular parts of the cell (Luckner, 1990). According to Fig. 58, a significant increase in biomass occurred after day 21. This could be due the high level of aggregation that occurred in the control and elicited cultures. A sharp decrease in cell mass occurred in the control on day 30 due to the formation of large aggregates in suspension and nutrient depletion. Figure 59B and 59D shows the aggregates in the elicited culture were smaller than the control culture. A cultivated plant cell suspension culture with a high concentration of cell aggregates is an ideal target for the elicitor as cell aggregation is associated with secondary metabolite production (Bais *et al*., 2002).

Cell suspension cultures turned dark brown 2 days after the addition of methyl jasmonate while control cultures remained orange-yellow (Fig. 59A and 59C). As a result brown aggregates formed in elicited cultures compared to the control culture which produced orange-yellow (Fig. 59B and 59D). Abdullah *et al*., 1998 working with *M. elliptica* also had a similar experience when he performed elicitation studies using methyl jasmonate which resulted in cells turning brown as anthraquinones were produced. The dark brown color of the cell aggregates can be used as an indication of anthraquinones elicitation. Since the accumulation of anthraquinones in *C. triloba* cell suspension cultures is coupled with cell aggregation and cell browning, the cell aggregates should be obtained and assessed for the production of anthraquinones in order to select high yielding cells for future studies.

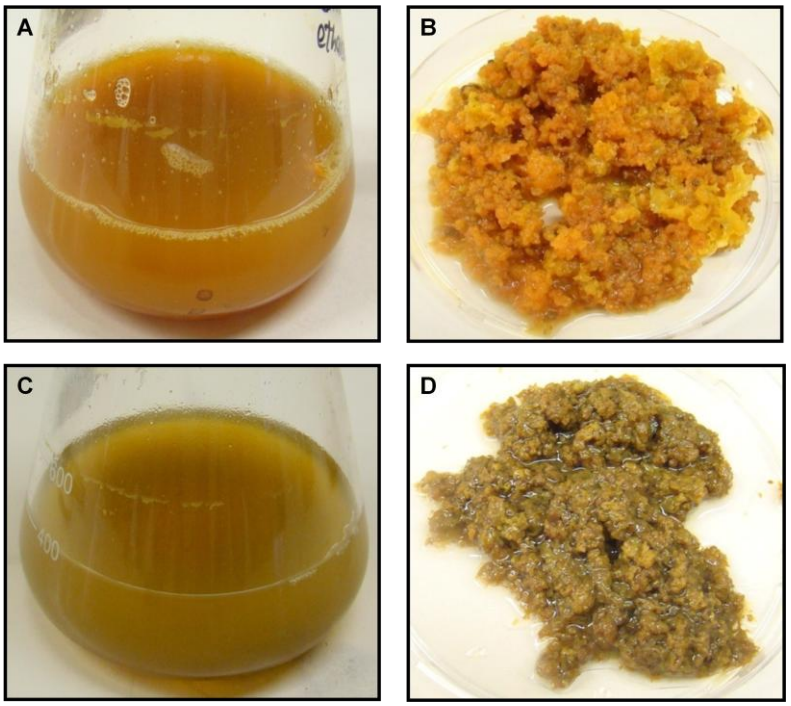


Figure 59: **Effect of methyl jasmonate on cell suspension cultures. The control culture produced orange cell aggregates (A and B) and the elicited culture produced dark brown cell aggregates (C and D)**

4.3.3. Analysis of elicited cell suspension culture extracts

The anthraquinones of interest, 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone could not be detected in control and elicited culture extracts when TLC plate were sprayed with ρ-anisaldehyde (Fig. 60A). Therefore a 5% KOH pre-sprayed plate was viewed with a UV light. No anthraquinones were detected under 254 nm (Fig. 60B). However when the TLC plate was root extract, only 1-hydroxy-4-methyl anthraquinone (yellow fluorescesence) was detected in the intra-cellular extracts of the elicited and control cultures. The anthraquinone standards confirmed the presence of anthraquinones in the intra-cellular extracts of the control and elicited cultures but individual anthraquinones could not be detected as both standards had the same Rf values when the two mobile phases: petroleum ether: ethyl acetate: formic acid (75:25:1) and ethyl acetate: methanol: water (100:13.5:10), were used.

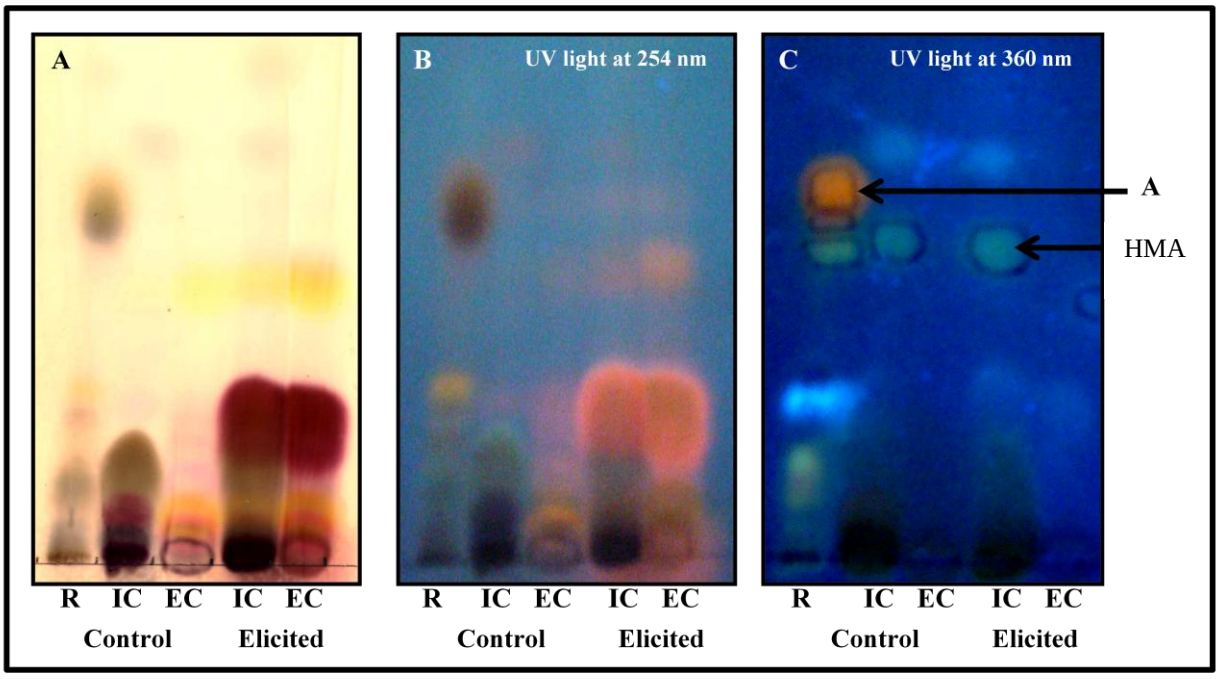


Figure 60: **Detection of anthraquinones in *C. triloba* cell suspension cultures after elicitation. Developed TLC plates were sprayed with and p-anisaldehyde (A) and 5 % KOH (B and C). A- 9, 10 anthracenedione, HMA-1-hydroxy-4-methyl anthraquinone.**

4.3.4. HPLC analysis of anthraquinones in cell cultures

TLC and HPLC analysis showed that anthraquinone accumulation was predominantly intracellularly based as the concentrations of the intracellular extracts were higher than that of the supernatant extracts (Fig. 60C and Table 23). HPLC analysis showed the 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone standards eluted at retention times of 5.90-6.20 minutes and 6.90-7.40 minutes respectively (Fig. 61). The elicited and control culture extract (intra-cellular) profiles showed a peak at 6.91 and 7.05 minutes, respectively. 1-hydroxy-4-methyl anthraquinone was identified in both the extracts (Fig. 62 and 63). In other studies anthraquinones have been detected prior to elicitation as in the case of *Cinchona pubescens* (Wijnsma *et al*., 1984). In contrast cells of *C. robusta* only accumulate anthraquinones after treatment with an elicitor (Schripsema *et al*., 1999). A higher yield of 1-hydroxy-4-methyl anthraquinone (0.75 µg/ml) was found in the elicited culture compared to the control culture extract which was (0.02 µg/ml), (Table 22). Many studies have shown that elicitation increases the production of secondary compounds in cell culture systems (Wang and Zhong, 2002; Yu *et al*., 2002). This strategy was proven to be successful in the *C. triloba* cell culture system as production yield of 1 hydroxy-4-methyl anthraquinone in the elicited culture increased 37.5 - fold after the one month culture period compared to the control culture. The production of anthraquinones in plant cell culture has been enhanced through several other

elicitors. Fungal polysaccharides increased the production of anthraquinones in *Rubia tinctorum L.* Jasmonic acid and salicylic acid was also employed and these elicitors increased the production of pseudopurpurin and alizarin respectively (Orban *et al*., 2008). A yeast elicitor prepared from yeast extract increased the production of naphthoquinones in *R. jasminoides* and also elicited the production of 1, 4 naphthohydroquinone (Oliveira *et al*., 2007). These were not tested in this study but could be investigated to further increase production.

9, 10-anthracenedione was not detected by HPLC in the control and elicited cultures profiles due to co-elution of 9, 10 anthracenedione with other anthraquinones in the sample (Fig. 62 and 63). Co-elution occurs when compounds in a sample do not separate due to the similarity of the structure between the compounds which in turn influences the elution time of the similar compounds.

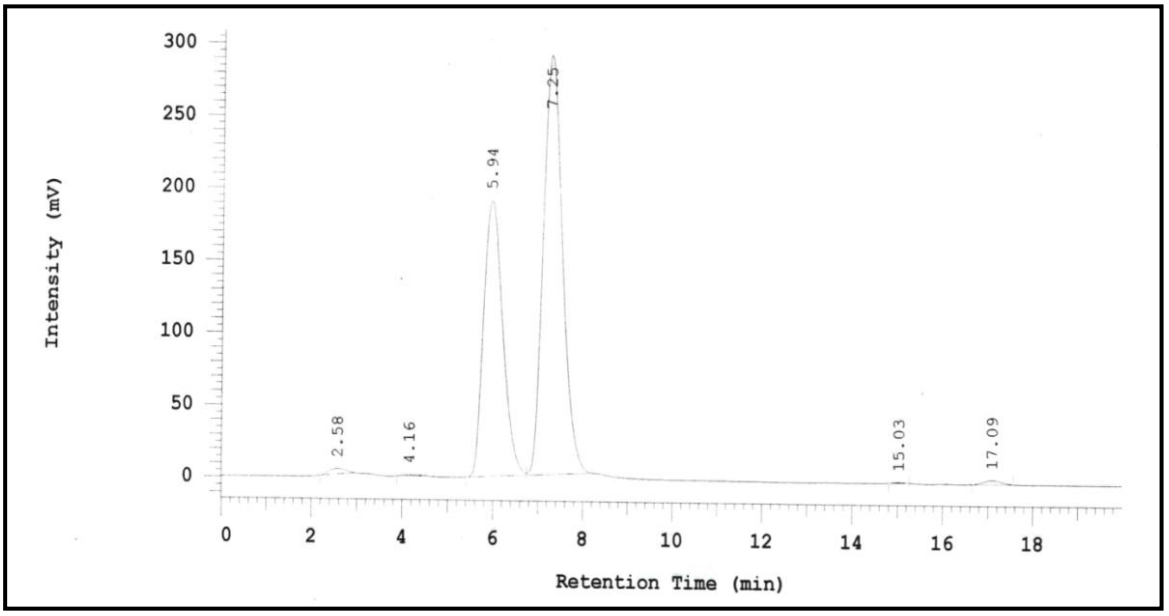


Figure 61: **HPLC chromatogram showing 9, 10 anthracenedione eluted at 5.94 minutes and 1-hydroxy-4-methyl anthraquinone eluted at 7.25 minutes**

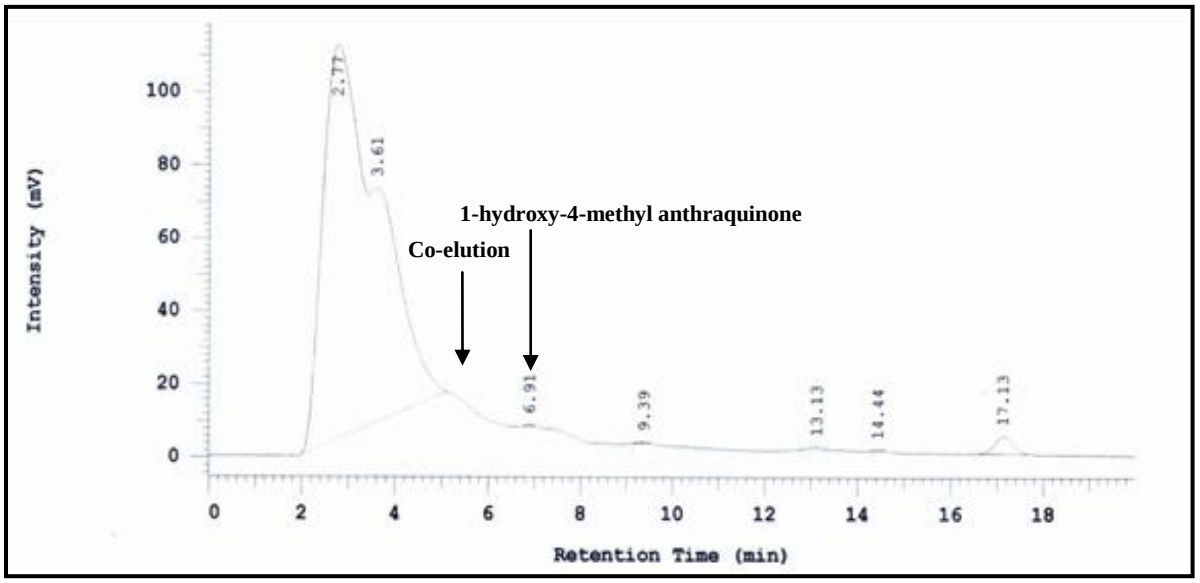


Figure 62: **HPLC profile of the control culture showing the 1-hydroxy-4-methyl anthraquinone peak at 6.91 minutes**

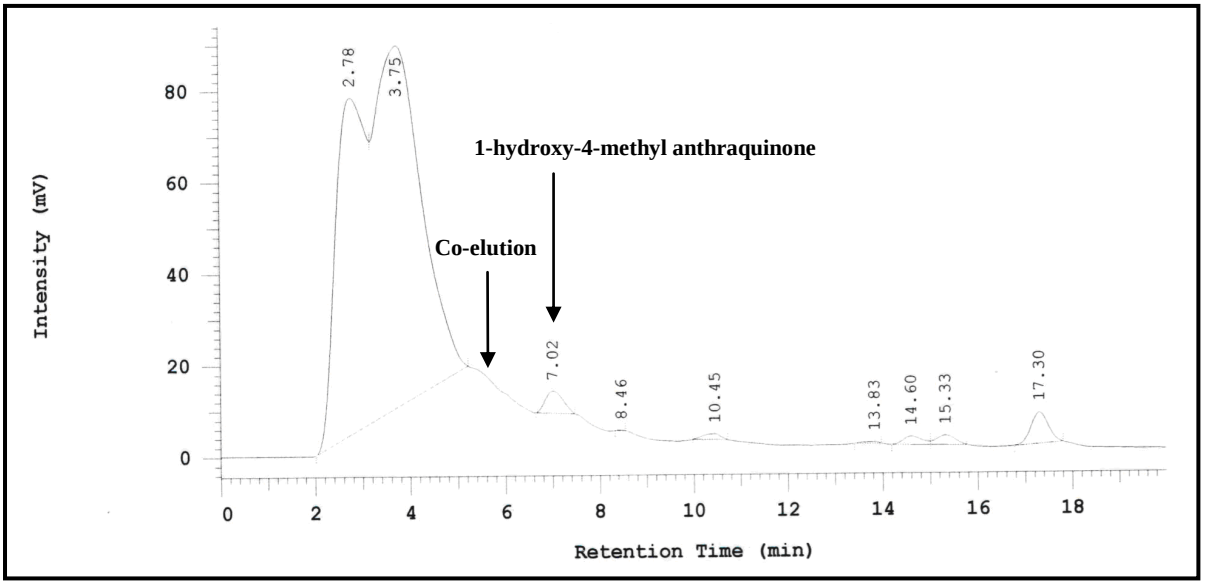


Figure 63: **HPLC profile of the elicited culture showing the 1-hydroxy-4-methyl anthraquinone peak at 7.02 minutes**

Table 22: **Concentration of the identified anthraquinones in elicited and control cultures**

Anthraquinone	Control		Elicited	
	supernatant extract	intracellular extract	supernatant extract	intracellular extract
9,10 anthracenedione	Co-elution	Co-elution	Co-elution	Co-elution
1-hydroxy-4-methyl anthraquinone	-	0.02 µg.ml^{-1}	0.053 µg.ml^{-1}	0.75 µg.ml^{-1}

4.3.5. Summary

This is the first study that shows *C. triloba* cell suspension cultures can be employed by plant cell culture techniques and produce potential bioactive compounds for cancer therapy. Since elicitation with methyl jasmonate does lead to the overproduction of 1-hydroxy-4-methyl anthraquinone in *C. triloba* cell suspension cultures, further studies of the elicitation parameters such as elicitation concentration and duration of elicitation could be evaluated using methyl jasmonate as well as other elicitors to further enhance the production of anthraquinones.

CHAPTER 5: SUMMARY AND GENERAL CONCLUSION

C. triloba is an indigenous widespread annual and is one of only four species of Ceratotheca that is found in Southern Africa (Smithies, 2000). It is used in traditional medicine to treat painful menstruation, stomach cramps, nausea, fever and diarrhea. There is no scientific evidence that describe any biological activity from this plant except for its anti-oxidant activity (Odhav *et al*., 2007) and its nutritional components. This study was undertaken to investigate the phytochemical profile and biological activity of *C. triloba*.

As most bioactives in plants are secondary metabolites we initially determined the major secondary compounds present in *C. triloba.* Major groups of the phytochemical from the crude extracts showed the presence of phlobatannins, saponins, steroids and terpenoids. No flavonoids, tannins and cardiac glycosides were present. Thereafter a TLC protocol was optimized so as to show the maximum number of compounds from the various portals of *C. triloba*. This showed three major compounds (RF values of 0.21, 0.34 and 0.38) which were common in leaves, roots and the stems of *C. triloba*. The three common fractions were pooled by preparative TLC and column chromatography and used for further identification. Despite several different resolving solvent combinations of TLC the three compounds co-eluted and were only partially purified. Subsequently these compounds were separated by LC-MS which showed six different compounds. Using spectral libraries they were found to be anthraquinones, which included 9, 10 anthracenedione (Fig. 64A), 1-hydroxy-4-methylanthraquinone (Fig. 64B) and 5, 8- dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione (Fig. 64C). A steroid was also identified as androst-5-ene-3, 17, 19-triol (Fig. 64D). Two other compounds were also isolated 1, 2 benzenedicarboxylic acid, mono (2-ethylhexyl) ester and octadecanoic acid. The steroid, ester and octodecanoic acid, were not used for further test.

Final confirmation of the isolated compound as 9, 10 anthracenedione was achieved using EI-LCMS as well as a range of NMR experiments. The H^1 NMR, HSQC, HSBC and DEPT spectra of 9, 10 anthracenedione was consistent with the molecular formula $C_{14}H_8O_2$. The HMQC, HMBC and DEPT analysis revealed the 9.10 anthracenedione as a symmetrical structure with the absence of chelated hydroxyl and methyl groups. C^{13} NMR revealed 14 carbon signals which were sorted by HSQC techniques into 12 quartenary carbons and two carbonyl groups detected at δ_C 183.17. The NMR results were in agreement with previously published work on anthraquinones (Marques *et al*., 2000).

The H^1 NMR, HSQC, HSBC and DEPT spectra of 1 hydroxy 4-methyl anthraquinone was consistent with the molecular formula $C_{15}H_{10}O_3$. The HMQC, HMBC and DEPT analysis revealed the 1 hydroxy 4-methyl anthraquinone as a non-symmetrical structure with the presence of chelated hydroxyl and methyl groups. C^{13} NMR revealed 16 carbon signals which were sorted by HSQC techniques into 15 quaternary deshielded carbons and two carbonyl groups detected at δ_C 183.00 and 183.44 respectively.

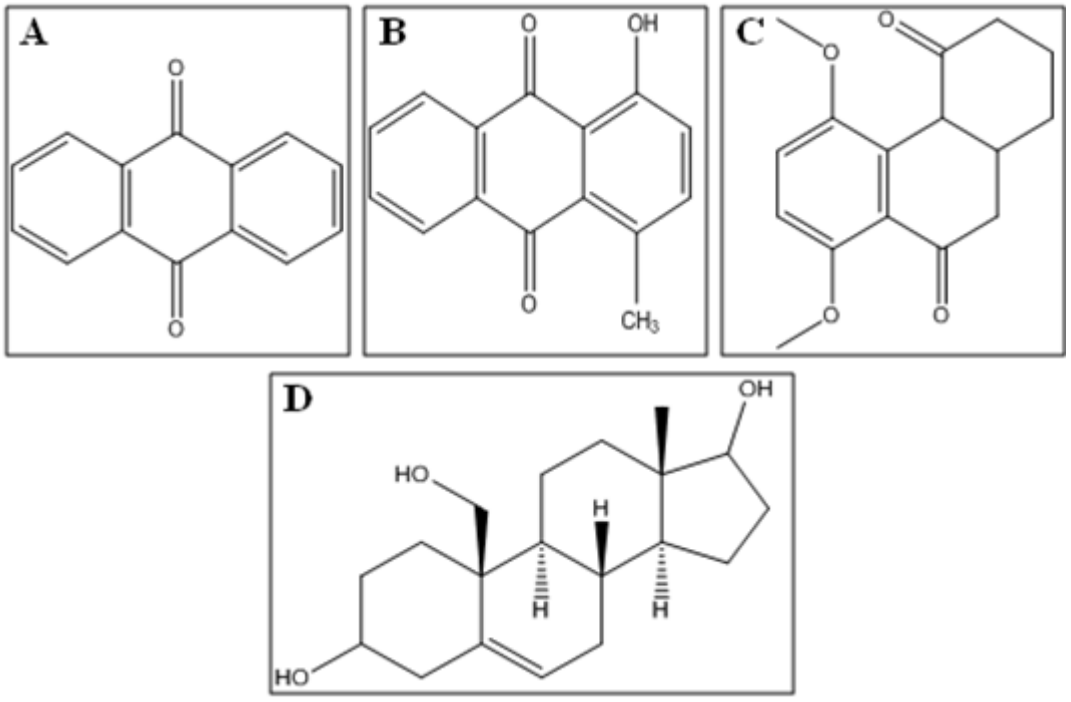


Figure 64: Three novel anthraquinones and a steroid isolated from *C. triloba* roots. A- 9, 10 anthracenedione, B- 1-hydroxy-4-methylanthraquinone, C- 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione, D- androst-5-ene-3, 17, 19-triol.

All the biological activity was tested with crude extracts and with CTREH 01, CTREH 02 and CTREH which were found to be 9, 10 anthracenedione and 1-hydroxy-4-methylanthraquinone and 5, 8- dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione respectively. The root extract showed inhibitory activity against *B. cereus* and *M. luteus and the leaf* extract showed activity against *B. cereus*, *E. aerogenes* and *M. luteus*. Of the purified compounds 9, 10 anthracenedione and 1-hydroxy-4-methylanthraquinone showed inhibitory activity against *Micrococcus luteus, Bacillus cereus* and *Staphylococcus aureus,* and Salmonella typhimurium. The crude extracts showed the highest inhibitory effect against *S. aureus* and *M. luteus* (40 µg/ml) and the lowest activity was exhibited by *E. coli* and *S. typhimurium.* 9.10 anthracenedione inhibited *S. aureus* (>1000 µg/ml), *M. luteus* (40 µg/ml), *S.*

typhimurium (620 µg/ml) *E. coli* and *B. cereus* (>1000 µg/ml). 1 hydroxy-4 methyl anthraquinone inhibited *S. aureus* (78 µg/ml), *M. luteus* (40 µg/ml), *S. typhimurium* (125 µg/ml) *E. coli* (150 µg/ml) and *B. cereus* (>1000 µg/ml). 9, 10 anthracenedione has been previously reported to have potent anticancer, antibacterial and anti-trypanosomal. This study shows that *C. triloba* has anthraquinones with antibacterial properties.

CTREH 01, CTREH 02 and CTREH 03 all had free radical scavenging capacity with 1-hydroxy-4-methylanthraquinone having the highest activity followed by 9, 10 anthracenedione. None of the compounds showed any anti-inflammatory activity. Furthermore, the purified extracts showed moderate toxicity against $HepG_2$ cells at high concentrations and no toxicity was observed against brine shrimp larvae. No mutagenicity was observed with the crude extracts using the Ames test.

The most significant findings of this study was the anti-topoisomerase activity demonstrated by 9, 10 anthraquinone and 1-hydroxy-4-methylanthaquinone. Topoisosmerases are required for the normal functioning of the cell. These enzymes form complexes with DNA. If the levels of these complexes are low than a cell will undergo mitotic failure and die. If these complexes overwhelm the cell than the DNA tracking system convert these to permanent double stranded breaks which trigger the apoptotic pathway. This is the basis of several widely prescribed anti-cancer drugs. The anthraquinones described in this study are anti - Topoisomerase IIα and IIβ which are the targets of a diverse group of natural and synthetic compounds (Fortune and Osheroff, 2000; Wilsterman and Osheroff, 2003: Velez Cruz and Osheroff, 2004) as shown in Table 5. Currently mitoxantrone is one of the commonest drugs to treat prostrate cancer but many cells are showing resistance to the synthetic drug. Thus a biological production of the compound may be a feasible alternative.

As there are several papers highlighting the production of various derivatives of 9, 10-anthracenedione in plant cell cultures (*Rubia cordifolia, Rudgea jasminoides, Rubia tinctorum* L, *Morinda elliptica* and *Cinchona robusta*), we investigated the production of 9, 10 anthracenedione and 1-hydroxy-4-methylanthraquinone from the *C. triloba* cell culture. In this study we show that *C. triloba* produces anthraquinones in callus cultures and also in cell suspension cultures. Of importance is that 9, 10 anthracenedione can be produced in plant cell suspension cultures, which paves the way for large scale production of this anticancer compound under controlled conditions.

In conclusion 9, 10 anthracenedione and 1-hydroxy-4-methyl anthraquinone can be used as antibacterial agents. Their anti-oxidative potential can be exploited for anticancer treatment as many cancers reactive oxygen species are implicated in their aetiology. None of the isolated compounds demonstrate toxicity *in vitro*, and display potent anti-topoisomerase II activity thus indicating novel plant compounds for the treatment of cancer.

CHAPTER 6: REFERENCES

Abdullah, M.A. Ali, A.M. Marziah, M. Lajis, N.H and Ariff, A.B. (1998) Establishment of cell suspension cultures of *Morinda elliptica* for the production of anthraquinones. *Plant Cell, Tissue and Organ Culture*. 54: 173-182.

Adlercreutz, H. (1995) Phytoestrogens: epidemiology and a possible role in cancer protection. *Environmental Health Perspectives.* 103: 103-112.

Aimová, D., Svobodová, L., Kotrbová, V., Mrázová, V., Hodek, P., Hudeček, J., Václavíková, R., Frei, E and Stiborová, M. (2007) The anticancer drug ellipticine is a potent inducer of rat cytochromes P450 1A1 and 1A2, thereby modulating its own metabolism, *Drug Metabolism and Disposition.* 35: 1926–1934.

Alitonou, G.A., Avlessi, F., Sohounhloue, D.K., Agnaniet, H., Bessiere, J.M and Menut, C. (2006) Investigations on the essential oil of *Cymbopogon giganteus* from Benin for its potential use as an anti-inflammatory agent. *International Journal of Aromatherapy*. 16:37-41

Arcamone, F.M. (1998) From the pigments of the actinomycetes to third generation antitumor anthracyclines. *Biochimie*. 80: 201-206.

Ashbolt, J.N. (2004) Microbial contamination of drinking water and disease outcomes in developing regions. *Toxicology*. 198: 229–238.

Au, W.W., Butler, M.A., Matney, T.S. and Loo, T.L. (1981) Comparative structure genotoxicity study of three aminoanthraquinone drugs and doxorubicin. *Cancer Research*. 41: 376-379.

Ayo, R.G., Amupitan, J.O. and Zhao, Y. (2007) Cytotoxicity and antimicrobial studies of 1, 6, 8-trihydroxy-3-methyl-anthraquinone (emodin) isolated from the leaves of *Cassia nigricans* Vahl. *African Journal of Biotechnology*. 6: 1276-1279

Bais, H.P. Walker, T.S. McGrew, J.J. and Vivanco, J.M. (2002) Factors affecting growth of cell suspension cultures of *Hypericum perforatum* L. (St John's wort) and production of hypericin. In Vitro Cell Development and Biology –Plant. 38: 58-65.

Bajaj, Y.P.S. ed., (1999) Biotechnology in Agriculture and Forestry. Berlin: Springer-Verlag.

Barz, W., Daniel, S., Hinderer, W., Jaques, U., Kessman, H., Koster, J., Otto, C and Tiemann, K. (1988) Elicitation and metabolism of phytoalexins in plant cell cultures, Ciba Foundation Symposium. 137:178-198.

Baylac, S. and Racine, B. (2003) Inhibition of 5-Lipoxygenase by essential oils and other natural fragrant extracts. *International Journal of Aromatherapy*. 13: 138-142.

Begue, W.J. and Kline, R.M. (1972) The use of tetrazolium salts in bioautographic procedures. *Journal of Chromatography*. 64: 182-184

British National Formulary (1994) British National Formulary. London: BMA and Royal Pharmaceutical Society of Great Britain.

Bulgakov, V.P., Tchernoded, G.K., Mischenko, N.P., Khodakovskaya, M., Glazunov, V. P., Radchenko, S.V., Zvereva, E.V., Fedoreyev, S.A. and Zhuravlev, Y.N. (2002) Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with the *rolB* and *rolC* genes. *Journal of Biotechnology*. 97: 213-221.

Bulgakov, V.P., Tchernoded, G.K., Mischenko, N.P., Shkryl, Y.N., Glazunov, V.P., Fedoreyev, S.A. & Zhuravlev, Y.N. (2003) Effects of Ca 2b channel blockers and protein kinase/phosphatase inhibitors on growth and anthraquinone production in *Rubia cordifolia* callus cultures transformed by the *rolB* and *rolC* genes. *Planta*. 217: 349-355.

Caron, P.R and Wang, J.C. (1994) Appendix. II: Alignment of primary sequences of DNA topoisomerases. *Advances in Pharmacology*. 29B:271–297

Carr, J.D. and Rogers, C.B. (1986) Chemosystematic studies of the genus Combretum (Combretaceae), I. A convenient method of identifying species of this genus by a comparison of the polar constituents extracted from leaf material. *South African Journal of Botany.* 53: 173-176.

Chattopadhyay, S., Srivastava, A.K. and Bisaria, V.S. (2002) Optimization of culture parameters for production of podophyllotoxin in suspension culture of *Podophyllum hexandrum*. *Applied Biochemistry and Biotechnology.* 102/103: 381-393.

Choi, C.W., Kim, S.C., Hwang, S.S., Choi, B.K., Ahn, H.J., Lee, M.Y., Park, S.H. and Kim, S.K. (2002) Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science.* 163: 1161–1168.

Cowan, M. M. (2000) Plant products as antimicrobial agents. *Clinical Microbiology Reviews.* 12: 564–582.

De Sã Ferrira, I.C.F. and Ferrão Vargas, V.M. (1999) Mutagenicity of medicinal plant extracts in salmonella/microsome assay. *Phytotherapy Research*. 13: 397–400.

DeMarini, D.M., Brock, K.H and Doerr, C.L (1987) Mutagenicity of actinomycin D in mammalian cells due to clastogenic effects. *Mutation Research*. 192: 151-155.

Dicosmo, F. and Tallevi, S.G. (1985) Plant cell cultures and microbial insult: interactions with biotechnological potential. *Trends in Biotechnology* 3: 110-111.

Dixon, R.A. and Gonzales, R.A. (1994) *Plant Cell Culture, A practical approach.* Oxford University Press

Doran, P.M. (2000) Foreign protein production in plant tissue cultures. *Current Opinion in Biotechnology.* 11; 199–204

Drug Information for Malaysia and Singapore (DIMS). (1992) Medical Information for Malaysia and Singapore (MIMS) 21: 204.

Dzierzbicka, K and Kolodziejczyk A.M. (2005) Anthracenedione analogues – synthesis and biological activity. *Polish Journal of Chemistry*. 79: 1-29.

Eilert, U. (1987) Elicitation: Methodology and aspects of application. In: Cell culture and somatic cell genetics of plants. Academic Press. San Deigo. 4: 153-196.

Elgorashi, E.E., Taylor, J.L.S., Verschaeve, L., Maes, A., van Staden, J., De Kimpe, N. (2003) Screening of medicinal plants used in South African traditional medicine for genotoxic effects. *Toxicology Letters* 143: 195–207.

Evans A.T. (1987) Actions of Cannabis constituents on enzymes of arachidonate metabolism: anti-inflammatory potential, *Biochemical Pharmacology*. 36: 2035–2037.

Evenson, D.P., Darzynkiewicz, Z., Staiano-Coico, L., Traganos, F and Melamed, M.R. (1979) Effects of 9,10-Anthracenedione, 1,4-Bis{{2-{(2-hydroxyethyl)amino}-ethyl}amino}-diacetate on Cell Survival and Cell Cycle Progression in Cultured Mammalian Cells. *Cancer Research.* 39: 2574-2581.

Fennell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M and van Staden, J (2004) Assessing African medicinal plants for efficacy and safety: Pharmacological screening and toxicity, *Journal of Ethnopharmacology*. 94: 205–217.

Fernand, V.E. Dinh, D.T. Washington, S.J. Fakayode, S.O. Losso, J.N. Ravenswaay, R.O.V and Warner, I.M. (2008) Determination of pharmacological active compounds in root extracts of *Cassia alata* L. by use of high performance liquid chromatography. *Talanta* 74: 896

Fortune, J.M and Osheroff, N. (2000) Topoisomerase II as a target for anti-cancer drugs: when enzymes stop being nice. *Progress in Nucleic Acid Research & Molecular Biology*. 64: 221-253.

Freshney, R. J. (1987) Culture of Animal Cells. In: A.R. Liss, ed. A Manual of Basic Technique, New York. 245–256.

Frishman, W.H., Sung, H.M., Yee, H.C., Liu, L.L., Keefe, D., Einzig, A.I and Dutcher, J. (1997) Cardiovascular toxicity with cancer chemotherapy. *Current Problems in Cancer*. 21: 301-360.

Giantris, A., Abdurrahman, L., Hinkle, A., Asselin, B and Lipshultz, S.E. (1998) Anthracycline-induced cardiotoxicity in children and young adults. *Critical Reviews in Oncology/Hematology*. 27: 53-68.

Gong, F., Liang, Y.Z., Hui, C., Chau F.T and Chan B.T. (2001a) Determination of volatile components in peptic powder by gas chromatography-mass spectrometry and chemometric resolution. *Journal of Chromatography*. 909: 237-247.

Gong, F., Liang, Y.Z., Xu, Q.S and Chau F.T. (2001b) Gas chromatography-mass spectrometry and chemometric resolution applied to the determination of essential oils in *Cortex cinnamomi*. *Journal of Chromatography*. 905: 193-205

Gopi, C and Vatsala, T.M. (2006) *In vitro* studies on effects of plant growth regulators on callus and suspension culture biomass yield from *Gymnema sylvestre R.Br. African Journal of Biotechnology* 5: 1215-1219.

Grace, O.M., Prendergast, H.D.V., van Staden, J. and Jäger, A.K. (2002) The status of bark in South African traditional healthcare. *South African Journal of Botany*. 68: 21-30.

Guetens G., De Boeck, G. Wood, M. Maes, R.A.A. Eggermont, A.A.M. Highley, M.S. van Oosterom, A.T. de Bruijn E.A. and Tjaden U.R. (2002) Hyphenated techniques in anticancer drug monitoring - I. Capillary gas chromatography-mass spectrometry. *Journal of Chromatography A*. 976: 229-238.

Gundlach, H., Müller, M.J., Kutchan, T.M. and Zenk, M.H (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proceedings of the National Academy of Science*. USA. 89:2389–2393.

Han, Y. S., Van der Heijden, R. & Verpoorte, R. (2001) Biosynthesis of anthraquinones in cell cultures of the Rubiaceae. *Plant Cell Tissue and Organ Culture*. 67:201-220.

Han, Y.S. van der Heijden, R., Lefeber, A.W.M. Erkelens, C. And Verpoorte, R. (2002) Biosynthesis of anthraquinones in cell cultures of *Cinchona* 'Robusta' proceeds via the methylerythritol 4-phosphate pathway. *Phytochemistry* 59: 45-55.

Hanelt, M., Gareis, M and Kollarczik, B. (1994) Cytotoxicity of mycotoxins evaluated by the MTT-cell culture assay, *Mycopathologia*. 128: 167–174.

Harborne, J.B. (1973) Phytochemical methods, London. Chapman and Hall, Ltd. 49-188.

Harborne, J.B. and Harborne, A.J (1998) Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. Kluwer Academic Publishers, London, UK

Harker, W.G., Slade, D.L., Drake, F.H. and Parr, R.L. (1991) Mitoxantrone resistance in HL-60 leukemia cells: reduced nuclear topoisomerase II catalytic activity and drug-induced DNA cleavage in association with reduced expression of the topoisomerase II beta isoform. *Biochemistry* 30: 9953-9961.

Harris, R.K. (1985) Quantitative aspects of high-resolution solid-state nuclear magnetic resonance spectroscopy, *Analyst.* 110: 649–655.

Hattori, M., Akao, T., Kobashi, K and Namba, T. (1993) Cleavages of the O- and C-glucosyl bonds of anthrone and 10, 19′-bianthron derivatives by human intestinal bacteria, *Pharmacology.* 47: 125–133.

Hellwig, S. Drossard, J. Twyman, R.M. Fischer, R. (2004) Plant cell cultures for the production of recombinant proteins. *Nature Biotechnology*. 22: 1415-1422.

Heyman, H. M., Hussein, A.A., Meyer, J.J.M and Lall, N (2009) Antibacterial activity of South African medicinal plants against methicillin resistant *Staphylococcus aureus. Pharmaceutical Biology*. 47:67-71

Higashimoto, M., Purintrapiban, J., Kataoka, K., Kinouchi, T., Vinitketkumnuen, U., Akimoto, S., Matsumoto, H., Ohnishi, Y. (1993) Mutagenicity and antimutagenicity of extracts of three species and a medicinal plant in Thailand. *Mutagenicity Research.* 303: 135–142.

Hortobagyi G.N. (1997) Anthracyclines in the treatment of cancer. An overview. *Drugs*. 54:1-7.

Hsiang, Y.H., Hertzberg, R., Hecht, S. and Liu, L.F. (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase. *Journal of Biological Chemistry*. 260: 14873-14878.

Hutchings, A. (1996) Zulu Medicinal Plants, an Inventory. University of Natal Press, Pietermaritzburg. 104.

Ieven, M., Van den Berghe, D.A., Mertens, F., Vlietinck, A. and Lammens, E. (1979) Screening of higher plants for biological activities 1. Antibacterial activity. *Planta Medica.* 36: 311–321.

Iwalewa, E.O. McGaw, L.J. Naidoo V. and Eloff J.N. (2007) Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. *African Journal of Biotechnology*. 6: 2868-2885.

Jaeger, R.J and Rubin, R.J. (1973) Extraction, localization, and metabolism of di-2-ethylhexyl phthalate from PVC plastic medical devices. *Environmental Health Perspectives*. 3: 95-102

Jäger, A. K., Hutchings, A and van Staden, J. (1996) Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors, *Journal of Ethnopharmacology*. 52: 95–100.

Jelaska, S. Mihaljevic, S. Bauer, N. (1981) Production of biopharmaceuticals, antibiodies and edible vaccines in transgenic plants. *Current Studies of Biotechnology.* 121-127.

Jensen, L.H., Nitiss, K.C., Rose, A., Dong, J., Zhou, J., Hu, T., Osheroff, N., Jensen, P.B., Sehested, M. and Nitiss, J.L. (2000) A novel mechanism of cell killing by anti-topoisomerase II bisdioxopiperazines. *Journal of Biological Chemistry.* 275: 2137-2146

Johnson, R.K., Zee-Cheng, R.K.Y., Lee, W.W., Acton, E.M., Henry, D.W and Cheng, C.C. (1979) Experimental antitumor activity of aminoanthraquinones. *Cancer Treatment Reports*. 63: 425-439

Kassie, F., Parzefall, W., Musk, S., Johnson, I., Lamprecht, G., Sontag, G. and Knasmueller, S. (1996) Genotoxic effects of crude juices from Brassica vegetables and juices and extracts from phytopharmaceutical preparations and spices of cruciferous plants origin in bacterial and mammalian cells. *Chemico-Biological Interactions.* 102: 1–16.

Keefer, S and Zock, J. (2008) In Approaching High Content Screening and Analysis: Practical Advice for Users; Haney, S.A., Eds.; High Content Screening: Science, Techniques and Applications. Wiley- Interscience. 3-24.

Kelmanson, J. E., Jäger, A. K. and van Staden, J. (2000) Zulu medicinal plants with antibacterial activity. *Journal of Ethnopharmacology*. 69: 241-246

Kessman, H., Choudhary, A. and Dixon, R.A (1990) Stress responses in alfalfa (*Medicago sativa* L.). III. Induction of medicarpin and cytochrome P450 activities in elicitor-treated cell suspension cultures and protoplasts. *Plant Cell Reports*. 9: 38-41

Khelifa, T. and Beck, W.T. (1999) Merbarone, a catalytic inhibitor of DNA topoisomerase II, induces apoptosis in CEM cells through activation of ICE/CED-3-like protease. *Molecular Pharmacology*. 55: 548-556.

Koblitz, H. (1988). Anthraquinones. In: F, Constabel and I.K Vasil, eds. 1988. Cell culture and somatic cell genetics of plants. Orlando: Academic Press. 113

Korunaglo, K. Gbeassor, M. Nikabu, O. De Souza, C. and Werner, W. (1992) Effects of three compounds extracted from *Morinda lucida* on *Plasmodium falciparum*. *Planta Medica.* 58: 533-534.

Lachâtre, F., Marquet, P., Ragot, S., Gaulier, J.M., Cardot, P. and Dupuy, J.L. (2000) Simultaneous determination of four anthracyclines and three metabolites in human serum by liquid chromatography-electrospray mass spectrometry. *Journal of Chromatography. B, Biomedical sciences and applications*. 738: 281-291.

Lall, N. and Meyer, J.J.M. (2001) Inhibition of drug-sensitive and resistant strains of Mycobacterium tuberculosis and other bacterial species by diospyrin, isolated from *Euclea natalensis*. *Journal of Ethnopharmacology.* 78: 213-216

Lamb, P., Tapley, P and Rosen J. (1998) Biochemical approaches to discovering modulators of the JAK—STAT pathway. *Drug Discovery Today*. 3: 122-130.

Leistner, E. (1980). Quinonoid pigments. In: F.C. Czygan, ed. Pigments in Plants. Stuttgart: GustavFischer. 352-368.

Leistner, E. (1981) Biosynthesis of plant quinines. In: E. E. Conn, ed. 1981. The Biochemistry of Plants. London: Academic Press. 403-423

Lenta, B.N., Weniger, B., Antheaume, C., Noungoue, D.T., Ngouela, S., Assob, J.C., Vonthron-Sénécheau, C., Fokou, P.A., Devkota, K.P., Tsamo, E and Sewald, N. (2007) Anthraquinones from the stem bark of *Stereospermum zenkeri* with antimicrobial activity. *Phytochemistry*. 68: 1595-1599.

Li X.N., Chi, H., Song, Y.Q. and Liang, Y.Z. (2001) Analysis of the essential oil of *Schisandra chinensis* (Turcz.) Baill. with GC/MS. *Acta Pharmaceutica Sinica.* 36: 215-219.

Lin, J., Opoku, A.R., Geheeb-Keller, M., Hutchings, M.A. Terblanche, S.E., Jager A.K. and van Staden, J. (1999) Preliminary screening of traditional Zulu medicinal plants for anti-inflammatory and anti-microbial activities. *Journal of Ethnopharmacology*. 68**:** 267–274.

Lindsey, K., Jager, A.K., Raidoo, D.M and Staden, J.V. (1999) Screening of plants used by South African traditional healers in the treatment of dysmenorrhoea for prostaglandin-synthesis inhibitors and uterine relaxing activity. *Journal of Ethnopharmacology*. 64: 9–14.

Lindsey, K., Motsei, M.L. and Jäger, A.K. (2002) Screening of South African food plants for antioxidant activity. *Journal of Food Science.* 76:2129–2131.

Lodhi, A. H., Bongaerts, R. J. M., Verpoorte, R., Coomber, S. A and Charlwood, B. V. (1996) Expression of bacterial isochorismate synthase (EC 5.4.99.6) in transgenic root cultures of *Rubia peregrina*. *Plant Cell Reports*. 16: 54-57.

Louie, A.C. and Issel, B.F. (1985) Amsacrine (AMSA): a clinical review. *Journal of Clinical Oncology*. 3: 569-599.

Lourens, A.C.U., Reddy, D., Baser, K. H. C., Viljoen, A.M and Van Vuuren, S.F (2004) In vitro biological activity and essential oil composition of four indigenous South African Helichrysum species. *Journal of Ethnopharmacology*. 95: 253-258

Luckner, M. (1990) Secondary metabolism in microorganisms, plants and animals. Berlin Springer-Verlag.

Luyt, R.P., Jäger, A.K and van Staden J (1999) The rational usage of *Drimia robusta* Bak. in traditional medicine. *South African Journal of Botany* 65: 291–294.

Malonne, H and Atassi, G (1997) DNA topoisomerase targeting drugs: Mechanisms of action and perspectives. *Anticancer Drugs.* 8: 811-822

Mander M. (1997) The Marketing of Indigenous Medicinal Plants in South Africa: A Case Study in KwaZulu-Natal. Investigational Report 164. Institute of Natural Resources, Pietermaritzburg.

Mantri P and Witiak D.T. (1994) Inhibition of cyclooxygenase and 5-lipoxygenase. *Current Medicinal Chemistry.*1; 328–355.

Mantrova, O.V., Dunaeva, M.V., Kuzovkina, I.N., Schneider, B. and Muller-Uri, F. (1999) Effect of methyl jasmonate on anthraquinone biosynthesis in transformed madder roots. *Russian Journal of Plant Physiology*. 46: 248-251.

Marini, J.C., Miller, K.G and Englund, P,T (1980). Decatenation of kinetoplast DNA by topoisomerases. *Journal of Biological Chemistry*. 255:4976-4979

Markovits, Y., Linassier, C., Fosse, P., Couprie, J., Pierre, J., Jacquemin-Sablon, A., Saucier, J.M., Le Pecq, J.B. and Larsen, A.K. (1989) Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. *Cancer Research.* 49: 5111-5117.

Marques, V.L., de Oliveira, F.M., Conserva, L.M., Brito, R.G. and Guilhon, G.M (2000) Dichromenoxanthones from *Tovomita brasiliensis*. *Phytochemistry*. 55: 815-818.

Maron, D.M and Ames B.N. (1983) Revised methods for the Salmonella mutagenicity test. *Mutation Research.* 113: 173-215.

Martin, M.L., Martin, G.L. and Delpuech J.J. (1980) Practical NMR Spectroscopy, Heyden, London, Chapter 9.

McClendon, A. K and Osheroff, N (2007) DNA topoisomerase II, genotoxicity, and cancer, *Mutation Research*. 623: 83–97

McGaw, L, Jäger, A,K. and Staden, J. (1997) Prostaglandin synthesis inhibitory activity in Zulu, Xhosa and Sotho medicinal plants. *Phytotherapy Research*. 11: 113-117

Meyer, B.N., Nerrigni, F.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E and McLaughlin, J.L (1982) Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica.* 45: 31–34

Mischenko, N.P. Fedoreyev, S.A. Glazunov, V.P. Chernoded, G.K. Bulgakov, V.P and Zhuravlev, Y.N. (1999) Anthraquinone production by callus cultures of *Rubia cordifolia*. *Fitoterapia* 70: 552-557.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 65: 55.

Mulabagal, V and Tsay, H-S. (2004) Plant cell cultures - An alternative and efficient source for the production of biologically important secondary metabolites. *International journal of Applied Science and Engineering* 2: 29-48.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum*. 15: 473-497.

Namdeo, A.G. (2007) Plant Cell Elicitation for Production of Secondary Metabolites: A Review. *Pharmacognosy Reviews.* 1: 69-75

National Committee for Clinical Laboratory Standards. (1990) Methods for dilution of antimicrobial susceptibility tests for bacteria that grows aerobically. Second edition, approved standard. NSSLS Doc M7-A2, Villanora, Pa: NCCLS.

Navarro, V., Rojas, G., Delgado, G. and Lozoya, X. (1998) Antimicrobial compounds detected in *Bocconia arborea* extracts by a direct bioautographic method. *Archives of Medical Research.* 29:191–194

Odhav, B., Beekrum, S., Akula, U.S and Baijnath, H. (2007) Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal, South Africa, *Journal of Food Composition and Analysis.* 20: 430–435.

Oliveira, L. (2006) The development of chloroplasts in root meristematic tissue of *Secale cereale* L. seedlings. *New Phytologist.* 91**:** 263 - 275

Oliveira, M.D.C. Negri, G. Salatino, A and Braga, M.R. (2007) Detection of anthraquinones and identification of 1.4-naphthohydroquinone in cell suspension cultures of *Rudgea jasminoides* (Cham.) Mull. Arg. (Rubiaceae). *Revista Brasil. Botanica.* 30: 167-172.

O'Neill, P M. (2004) Medicinal chemistry: A worthy adversary for malaria, Nature, 430: 838-**839.**

Orban, N. Boldizsar, I. Szucs, Z. and Danos, B. (2008) Influence of different elicitors on the synthesis of anthraquinone derivatives in *Rubia tinctorum L.* cell suspension cultures. *Dyes and Pigments* 77: 249-257.

Patterson, L. H. and McKeown, S.R. (2000) AQ4N: a new approach to hypoxia-activated cancer chemotherapy. *British Journal of Cancer*. 83: 1589-1593.

Permana, D., Lajis, N. H., Othman, A. G., Ali, A. M., Aimi, N., Kitajima, M and Takayama, H. (1999) Anthraquinones from *Hedyotis herbacea. Journal of Natural Products.* 62: 1430-1431

Perry, P.J., Gowan, S.M., Reszka, A.P., Polucci, P., Jenkins, T.C., Kelland, L.R and Neidle, S. (1998) 1,4- and 2,6-disubstituted amidoanthracene-9,10-dione derivatives as inhibitors of human telomerase. *Journal of Medicinal Chemistry*. 41: 3253-3260.

Perry, P. J., Read, M. A., Davies, R. T., Gowan, S. M., Reszka, A. P., Wood, A. A., Kelland, L. R and Neidle, S. (1999) 2,7-Disubstituted amidofluorenone derivatives as inhibitors of human telomerase. *Journal of Medicinal Chemistry*. 42: 2679-2684

Pillay, C.C.N., Jäger, A.K., Mulholland D.A. and Van Staden, J. (2001) Cyclooxygenase inhibiting and anti-bacterial activities of South African *Erythrina* species, *Journal of Ethnopharmacology*, 74, 231–237.

Plant Tissue Culture Protocols, Plant Biotechnology. (2008) Surface sterilization of plant explants and orchid seed. [Online]
Available from:
http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Plant_Biotechnology/Tissue Culture_Protocols/Explant_ Sterilization.html [Accessed March 2007]

Poirer, T. I. (1986) Mitoxanthrone. *Drug Intelligence & Clinical Pharmacy*. 20: 97-105

Rabe, T and Van Staden, J. (1997) Antibacterial activity of South African plants used for medicinal purposes. *Journal of Ethnopharmacology*. 56: 81-87.

Rabe, T and Van Staden, J. (2000) Isolation of an antibacterial sesquiterpenoid from *Warburgia salutaris*. *Journal of Ethnopharmacology.* 73: 171–174.

Rabe, T., **Mullholland, D and Van Staden, J**. (2002) Isolation and identification of antibacterial compounds from *Vernonia colorata* leaves. *Journal of Ethnopharmacology.* 80: 91-94.

Raleigh, S.M., Wanogho, E., Burke, M.D., McKeown, S.R. and Patterson, L. H. (1998) Involvement of human cytochromes P450 (CYP) in the reductive metabolism of AQ4N, a hypoxia activated anthraquinone di-N-oxide prodrug. *International Journal of Radiation Oncology-Biology-Physics.* 42: 763-767**.**

Ramachandra Rao. S. and Ravishankar, G.A. (1998) Biotransformation of phenylpropanoid compounds to vanilla flavour metabolites in immbolized cell cultures of *Capsicum frutescens.* 4th International Food Convention, Mysore, 44 (Abstract volume)

Ramachandra Rao. S. (2000) Biotechnological production of phyto- pharmaceuticals. *Journal of Biochemistry, Molecular Biology and Biophysics* 4: 73-102

Ramachandra Rao. S. and Ravishankar, G.A. (2002) Plant cell cultures: chemical factories of secondary metabolites. *Biotechnological Advances.* 20: 101-153

Ramesar, S., Baijnath, H., Govender, T and Mackraj, I. (2008) Angiotensin I-Converting Enzyme Inhibitor Activity of Nutritive Plants in KwaZulu-Natal. *Journal of Medicinal Food*. 11: 331–336

Ramos-Valdivia, A.C., van der Heijden, R. & Verpoorte, R. (1997a) Isopentenyl diphosphate isomerase: a core enzyme in isoprenoid biosynthesis. A review of its biochemistry and function. *Natural Product Reports*. 14: 591-603.

Ramos-Valdivia, A.C., van der Heijden, R. & Verpoorte, R. (1997b) Elicitor-mediated induction of anthraquinone biosynthesis and regulation of isopentenyl diphosphate isomerase and farnesyl diphosphate synthase activities in cell suspension cultures of *Cinchona robusta* How. *Planta.* 203: 155-161.

Ramos-Valdivia, A. C., van der Heijden, R. & Verpoorte, R. (1998) Isopentenyl diphosphate isomerase and prenyltransferase activities in rubiaceous and apocynaceous cultures. *Phytochemistry*, 48: 961-969.

Rasoanaivo, P and Ratsimamanga-Urverg, S. (1993) Biological evaluation of plants with reference to the Malagasy flora. NAPRECA, Madagascar.72–83.

Reid, K.A., Jäger, A.K., Light, M.E., Mulholland, D.A and Van Staden, J. (2005) Phytochemical and pharmacological screening of Sterculiaceae species and isolation of antibacterial compounds, *Journal of Ethnopharmacology* 95: 285–291

Rosenthal, G.A., and Berenbaum M.R (1991) Herbivores, Their Interactions with Secondary Plant Metabolites. San Diego, CA: Academic Press.

Sawai, J., Doi, R., Maekawa, Y. and Kojima, H. (2002) Indirect conductimetric assay of antibacterial activities. *Journal of Industrial Microbiology and Biotechnology.* 29: 296–298

Schlatmann, J.E., Koolhaas, C.M.A., Vinke, J.L., ten Hoopen, H.J.G and Heijnen, J.J. (1995) The role of glucose in ajmalicine production by *Catharanthus roseus* cell cultures. *Biotechnology and Bioengineering*. 47: 525–534

Schripsema, J., Ramos-Valdivia, A. and Verpoorte, R. (1999) Robustaquinones, novel anthraquinones from an elicited *Cinchona robusta* suspension culture. *Phytochemistry*. 5: 55–60.

Schimmer, O., Kruger, A., Paulini, H. and Haefele, F. (1994) An evaluation of 55 commercial plant extracts in the Ames mutagenicity test. *Pharmazie*. 49: 448–451.

Schimmer, O., Haefele, F and Kruger, A (1988) The mutagenic potencies of plant extracts containing quercetin in Salmonella typhimurium TA98 and TA100. *Mutation Research.* 206: 201–208.

Simantiras, M. and Leistner, E. (1992) O-succinylbenzoate: coenzyme A ligase from anthraquinone producing cell suspension cultures of *Galium mollugo. Phytochemistry*. 31: 2329-2335.

Shale, T. L., Stirk, W. A. and van Staden, J. (1999) Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity. *Journal of Ethnopharmacology*. 67: 347-354

Singh, R., Geetanjali, S and Chauchan, S.M.S. (2004) 9.10 anthraquinones and their biologically active compounds from the genus *Rubia*. *Chemical Biodiversity*. 1: 1241-1264.

Smith, I.E (1983) Mitoxantrone (Novantrone): a review of experimental and early clinical studies.*Cancer Treatment Reviews*. 10: 103–115

Smithies, S.J. (2000) Pedaliaceae. In: O. A. Leistner, ed. 2000. Seed plants of southern Africa: families and genera. Pretoria: National Botanical Institute. 436-437.

Sofowara, A. (1993). Medicinal plants and Traditional medicine in Africa. Spectrum Books Ltd, Ibadan, Nigeria. 289.

Spitzner, J.R. and Muller, M.T (1988) A consensus sequence for cleavage by vertebrate DNA topoisomerase II. *Nucleic Acids Research*. 16: 5533-5556.

Spitzner, J.R., Chung, I.K and Muller, M.T (1990) Eukaryotic topoisomerase II preferentially cleaves alternating purine–pyrimidine repeats, *Nucleic Acids Research.* 18: 1–11.

Stalman, M., Koskamp, A. M., Luderer, R., Vernooy, J. H. J., Wind, J. C., Wullems, G. J. & Croes, A. F. (2003) Regulation of anthraquinone biosynthesis in cell cultures of *Morinda citrifolia. Journal of Plant Physiology.* 160: 607-614.

Sugino, A., Higgins, N.P., Brown, P.O., Peebles, C.L. and Cozzarelli, N.R. (1978) Energy coupling in DNA gyrase and the mechanism of action of novobiocin. *Proceeding of the National Academy of Sciences.* 75: 4838-4842.

Swaine, D.J., Loadman, P.M., Bibby, M.C., Graham, M.A. and Patterson, L,H. (2000) High-performance liquid chromatographic analysis of AQ4N, an alkylaminoanthraquinone N-oxide. *Journal of Chromatography. B, Biomedical sciences and applications*. 742: 239-245

Taniguchi, M. and Kubo, I. (1993) Ethnobotanical drug discovery based on medicine men's trials in the African savanna: screening of East African plants for antimicrobial activity II. *Journal of Natural Products*. 56: 1539-1546.

Taticek, R.A. Moo-Young, M. Legge, R.L. (1991) The scale-up of plant cell culture: engineering considerations. *Plant Cell and Tissue Organ Culture.* 24: 139 – 159.

Taylor, J.L.S. and van Staden, J. (2002) "COX-1 and COX-2 inhibitory activity in extracts prepared from *Eucomis* species, with further reference to extracts from *E. autumnalis*" *South African Journal of Botany*. 68; 80-85

Taylor, J.L.S., Elgorashi, E.E., Maes, A., Van Gorp, U., De Kimpe, N., van Staden, J and Verschaeve, L. (2003) Investigating the safety of plants used in South African traditional medicine: testing for genotoxicity in the micronucleus and alkaline comet assays. *Environmental and Molecular Mutagenesis.* 42: 144–154.

Tetyana, P., Prozesky, E. A., Jäger, A. K., Meyer, J. J. M and Van Staden, J. (2002) Some medicinal properties of *Cussonia* and *Schefflera* species used in traditional medicine. *South African Journal of Botany*. 68; 51–54.

Thatte, U., Bagadey, S and Dahanukar, S. (2000) Modulation of programmed cell death by medicinal plants, *Cellullar and Molecular Biology* 46; 199–214.

Thomas, X and Archimbaud, E (1997) Mitoxantrone in the treatment of acute myelogenous leukemia: A review. *Hematology and Cell Therapy.* 39: 63-74.

Thomson, R. H. (1976) Quinones: nature, distribution and biosynthesis. In: T. W. Goodwin, ed 1976. Chemistry and Biochemistry of Plant Pigments. London: Academic Press.527-549.

Trask, O.K and Muller, M.T (1983) Biochemical characterization of topoisomerase I purified from avian erythrocytes. *Nucleic Acids Research* II, 2779-2800

Trease, G.E and Evans, W.C. (1989) Pharmacognosy. 11th edn. Brailliar Tiridel Can. Macmillian publishers.

Trejo-Tapia, G. Jimenez-Aparicio, A. Villarreal, M. Rodriguez- Monroy, M. (2001) Broth rheology and morphological analysis of *Solanum chrysotrichum* cultivated in a stirred tank. *Biotechnology Letters.* 23; 1943-1946.

Tripathi, L. and Tripathi, J.N. (2003) Role of biotechnology in medicinal plants. *Tropical Journal of Pharmaceutical Research* 2: 243-253

Van Belle, S.J.P., Shoemaker, T.J., Verwey, S.L., Paalman, A.C.A. and McVie, J.G. (1985) Ion-paired high-performance liquid chromato- graphic determination of mitoxantrone in physiological fluids. *Journal of Chromatography*. 337: 73.

Van der Berg, A. J. J and Labadie, R. P. (1989) Quinones. In: M, Dey., J. B. Harborne, eds. 1989. Methods in Plant Biochemistry. London:Academic Press, 413-491.

Van der Walt, L. (2001) "*Ceratotheca triloba* (Bernh.) Hook.f." [Online] Available at http://www.plantzafrica.com/plantcd/ceratothectrilob.htm. [Accessed: 20 February 2005]

Vane, J. R. (2004) Towards a better aspirin, *Nature.* 367: 215–216

Velez-Cruz, R and Osheroff, N. (2004) DNA topoisomerases: type II, in: Encyclopedia of Biological Chemistry, Elsevier Inc. 806-811.

Vershaeve, L., Kestens, V., Taylor, J.L.S., Elgorashi, E.E., Maes, A., Van, L., De kimpe, N. and Van Staden, J. (2004) Investigation of antimutagenic effects of selected South African medicinal plant extracts. *Toxicology In Vitro.* 18**:** 29–35.

Wagner, H., Bladt, S and Zgainski, E, M (1984) Plant Drug Analysis, Springer-Verlag, Berlin ISBN 0-387-13195-7.

Wang, Z. Y. and Zhong, J. J. (2002) Repeated elicitation enhances taxane production in suspension cultures of *Taxus chinensis* in bioreactors. *Biotechnology Letters.* 24: 445-488

White, H.L. and Glassman A.T. (1974) A simple radiochemical assay for prostaglandin synthetase. *Prostaglandins*. 7: 123-129.

Wijnsma, R. Go, J.T.K.A. Harkes, P.A.A. Verpoorte, R. Baerheim-Svendsen, A. (1984) In: Han, Y.S. van der Heijden, Lefeber, A.W.M. Erkelens, C. Verpoorte, R. 2002. Biosynthesis of anthraquinones in cell cultures of *Cinchona* 'Robusta' proceeds via the methylerythritol 4-phosphate pathway. *Phytochemistry* 59: 45-55.

Wilstermann, A. M and Osheroff, N. (2003) Stabilization of eukaryotic topoisomerase II-DNA cleavage complexes, *Current Topics in Medicinal Chemistry*. 3: 1349-1364

Wink, M. (2000) Biochemistry of Plant Secondary Metabolism: Annual Plant Review, vol 2. Academic, Sheffield, UK, 15

Wiseman, L.R and Spencer, C.M (1997) Mitoxantrone. A review of its pharmacology and clinical efficacy in the management of hormone-resistant advanced prostate cancer. *Drugs and Aging*. 10: 473-485.

Wootthikanokkhan, J. and Tunjongnawin, P (2002) Investigation of the effect of mixing schemes on cross-link distribution and tensile properties of natural–acrylic rubber blends. *Polymer Testing.* 22: 305–312.

Young, C.W. and Raymond, V. (1986) Clinical assessment of the structure- activity relationship of anthracyclines and related synthetic compounds. *Cancer Treatment Reports.* 70: 51-63

Yu, L.J. Lan, W.Z. Qin, W.M. Jin, W.W. and Xu, H.B. (2002) Oxidative stress and taxol production induced by fungal elicitor in cell suspension cultures of *Taxus chinensis*. *Plant Biology.* 45: 459-461.

Yuan, Y.J., Ma, Z.Y., Wu, J.C and Zeng, A.P (2002) Fungal elicitor- induced cell apoptosis in suspension cultures of *Taxus chinensis* var. *mairei* for taxol production. *Process Biochemistry.* 38: 193-198.

Yukimune, Y., Tabata, H., Higashi, Y and Hara, Y. (1996) Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in taxus cell suspension cultures. *Nature Biotechnology*. 14: 1129-1132.

Zhong, J.J. (2002) Plant Cell Culture for Production of Paclitaxel and Other Taxanes. *Journal of Bioscience and Bioegineering*. 94: 591- 599.

Zschocke, S, Drewes, S.E, Paulus, K, Bauer, R. and van Staden, J. (2000) "Analytical and pharmacological investigation of *Ocotea bullata* (black stinkwood) bark and leaves" *Journal of Ethnopharmacology*. 71: 219-230

APPENDICES

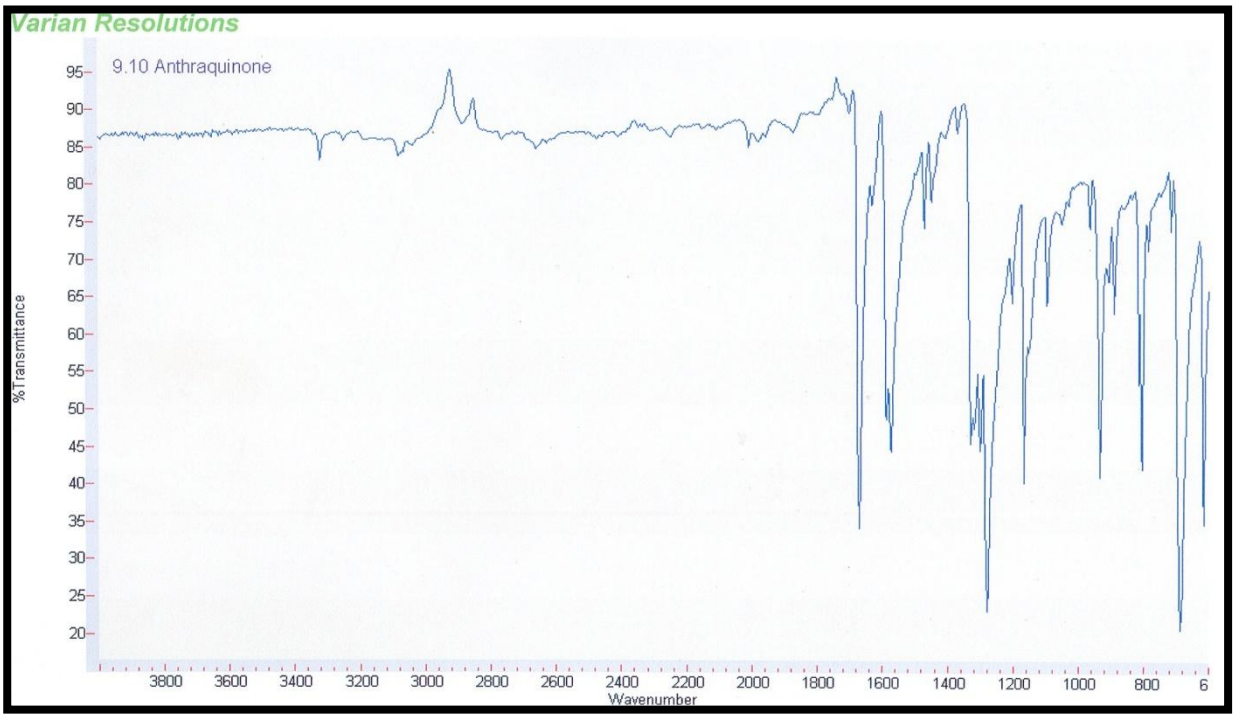


Figure A1: **The IR spectra (Fig. A1 - appendices) showed absorption bands for the conjugated carbonyls (υ 1590 cm^{-1}) and the aromatic rings (υ 1590 and 1480 cm^{-1})**

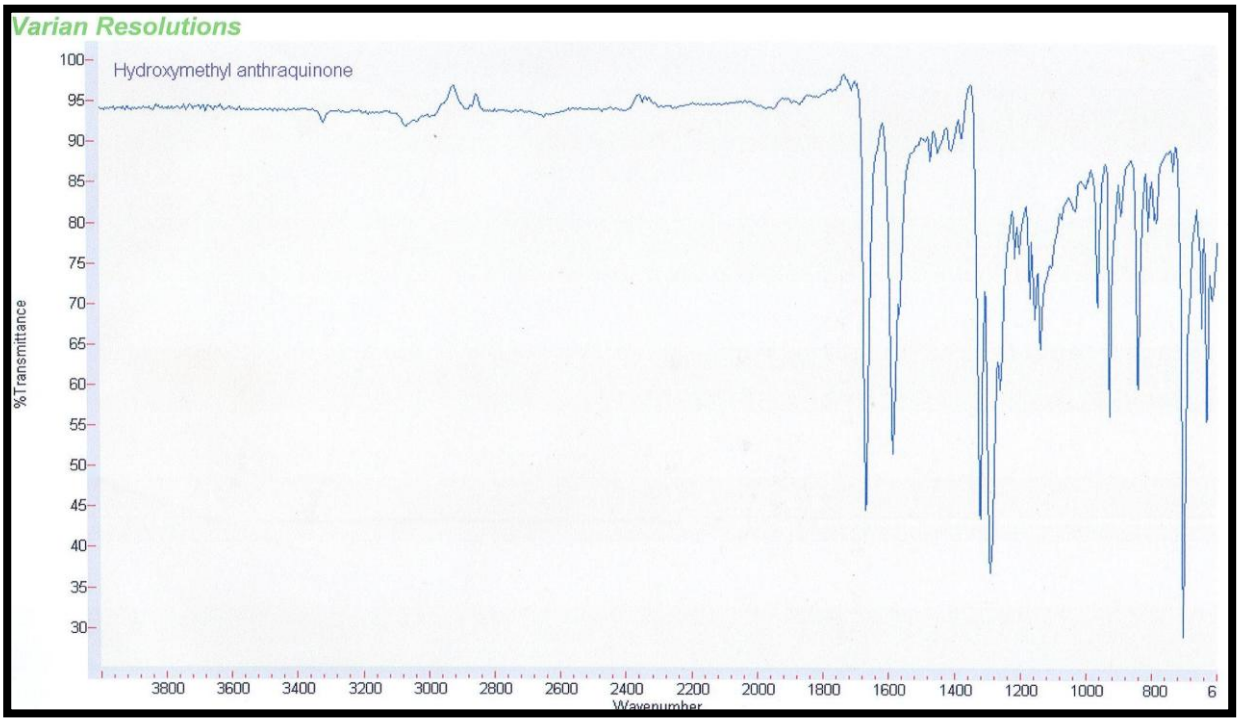


Figure A2: **IR spectra of 1-hydroxy-4-methyl anthraquinone showed absorption bands for a hydroxyl group, the conjugated carbonyls and the aromatic rings.**