



GENETIC TRANSFORMATION OF *Ceratotheca triloba* FOR THE PRODUCTION OF ANTHRAQUINONES FROM HAIRY ROOT CULTURES

Leeann Naicker

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

***SUBMISSION APPROVED FOR EXAMINATION**

SUPERVISOR

Professor B. Odhav (PhD)

DATE

CO-SUPERVISOR

Dr V. Mohanlall (D Tech)

DATE

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Students signature

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
6-BAP	6-Benzylaminopurine
AN	9,10-anthracenedione
CIA	Chloroform: isoamyl alcohol
CS	Cell suspension culture
CSIR	Council of Scientific and Industrial Research
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DU-145	Prostate adenocarcinoma cells
EC	Extra-cellular extract
EDTA	Ethylenediaminetetraacetic acid
EI-LC-MS	Electron Ionization- Liquid Chromatography-Mass Spectroscopy
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric
IC	Intra-cellular extract
MA	1-hydroxy-4-methylanthraquinone
MS	Murashige-Skoog medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAA	1-Naphthaleneacetic acid
NaClO	Sodium hypochlorite
OD	Optical Density
PBS	Phosphate saline buffer
PDA	Photodiode array
P-TLC	Preparative Thin Layer Chromatography
R _f	Resolution factor
R _t	Retention time
RT	Root extract
SDS	Sodium dodecyl sulphate
ST	Shoot culture
UV-Vis	Ultraviolet-Visible light spectroscopy
TLC	Thin layer chromatography

ACCEPTED PUBLICATION

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ABSTRACT

Many secondary metabolites that have been extracted from medicinal plants have been used as source of clinical drugs. However, the concentration of the active metabolites in plants is generally low. An attractive alternative for producing these important secondary metabolites is via plant tissue culture technology. More particularly, the genetic transformation of a plant tissue by *Agrobacterium rhizogenes* has been employed for producing high yields of secondary metabolites. In a previous study, three structurally similar anthraquinones: 9,10-Anthracenedione, 1-Hydroxy-4-methylanthraquinone and 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, and one steroid; Androst-5-ene-3, 17, 19-triol were isolated from the root extracts of *C. triloba*. The anthraquinones have shown to exhibit the anticancer mechanism which involves the inhibition of the activity of the human topoisomerase II enzyme that transforms supercoiled DNA to linear DNA. However, these anthraquinones were found in very low concentrations. Therefore, in this study we used plant cell and tissue culture systems (cell suspension, shoot and hairy root cultures) of *C. triloba* to increase the production of anthraquinones.

Since the establishment of *C. triloba in vitro* plant systems required a source sterile explants, a protocol that involved the use of NaClO was optimized for the sterilization and subsequent germination of *C. triloba* seeds which were micro-propagated into shoot cultures. These cultures provided a source explants for the induction of callus and hairy root cultures. The biomass of these plant cell and tissue cultures were subsequently bulked up for the extraction for anthraquinones and the yields were compared followed by fractionation and identification of the major compounds. The bioactivity of the fractions was evaluated by testing their cytotoxicity on cancer cells and anti-topoisomerase activity.

The sterilization protocol that provided sterile seeds was found to be a solution of 30% NaClO at an exposure time of 10 minutes. From the sterilized seeds shoot cultures were established on MS medium. The leaf explants of the shoot cultures were then used to induce callus cultures which subsequently were transferred to liquid medium whereby the total biomass of suspension cultures increased from 4 g to 134.18 g (wet weight). Also hairy roots cultures were established from stem explants with a low cell density inoculum of *A. rhizogenes* at a transformation efficiency of 73%. The growth of these hairy roots was slow in hormone free medium. This was overcome with the use NAA and IAA which increased the

biomass from 1.03 g in the control culture (without hormone) to 23.91 g and 46.13 g respectively.

An evaluation of the anthraquinones in the field root and hairy root, cell suspension and shoot culture extracts was carried out by using their Thin Layer Chromatography profiles and the High Performance Liquid Chromatography profiles as well as the standards, 9,10-Anthracenedione and 1-Hydroxy-4-methylanthraquinone. TLC analysis showed that the RF values of the fractions CT01 and CT02 matched the RF values of anthraquinones standards while HPLC analysis revealed that hairy root cultures supplemented with IAA (125.03 $\mu\text{g}.\text{mg}^{-1}$) or NAA (98.25 $\mu\text{g}.\text{mg}^{-1}$) produced a higher concentration of anthraquinones than the control culture (without hormone) (13.33 $\mu\text{g}.\text{mg}^{-1}$), the field roots (33.51 $\mu\text{g}.\text{mg}^{-1}$) and the shoot (3.23 $\mu\text{g}.\text{mg}^{-1}$) and cell suspension cultures (13.17 $\mu\text{g}.\text{mg}^{-1}$). Due to co-elution of the compounds in HPLC analysis, six fractions were isolated by Preparative Thin Layer Chromatography from the hairy root extract (obtained from the culture supplemented with NAA) and were coded as CT01, CT02, CT03, CT04, CT05 and CT06. The compounds in these fractions were identified by Electron Ionization-Liquid chromatography-Mass Spectroscopy and it was found that the hairy roots produced one acridone derivative; 5-Methoxy-2-nitro-10H-acridin-9-one, one naphthoquinone derivative; 2H-Naphto[2,3-b]pyran-5,10-dione, 3,4-dihydro-2,2-dimethyl- and seven anthracenedione derivatives. These were: i) 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, ii) 9,10-Anthracenedione, 2-methyl-, iii) 1-Hydroxy-4-methylanthraquinone, iv) 9,10-Anthracenedione, 2-ethyl-, v) 1,5-Diaminoanthraquinone, vi) Phenanthrene, 3,6-dimethoxy-9-methyl-, vii) 9,10-Anthracenedione, 1,4-dimethyl-.

Fractions CT01 (5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, 9,10-Anthracenedione, 2-methyl- and 1-Hydroxy-4-methylanthraquinone) and CT02 (9,10-Anthracenedione, 2-ethyl-) were cytotoxic to the DU-145 cancer cell line at concentrations of 125 $\mu\text{g}.\text{mg}^{-1}$ to 1000 $\mu\text{g}.\text{mg}^{-1}$. These fractions also showed anti-topoisomerase activity as they inhibited the conversion of supercoiled DNA into linear DNA.

In conclusion this is the first study that describes the transformation of *C. triloba* by *A. rhizogenes* mediated transformation and compares the production of anthraquinones in *C. triloba* hairy roots to the field roots, shoot and cell suspension cultures. This study has

indicated that hairy root cultures is a high-yielding production system for anthraquinones (5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, 1-Hydroxy-4-methylantraquinone, 9,10-Anthracenedione, 2-methyl- and 9,10- Anthracenedione, 2-ethyl-) which could have the potential to be used in cancer therapy. In addition the discovery of *C. triloba* hairy roots having the biosynthetic capacity to synthesize five valuable anthraquinone derivatives that are not found the field roots has also been revealed.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Ceratotheca triloba (Bernh.) E. Mey. ex. Hook.f. is one of four species found in Southern Africa. In a previous study, three structurally similar anthraquinones; 9,10-Anthracenedione, 1-Hydroxy-4-methylanthraquinone and 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, and one steroid; Androst-5-ene-3, 17, 19-triol were isolated from the root extracts of *C. triloba* (Mohanlall *et al.*, 2011). Anthraquinones are an important group of natural products that are found in plants (Korunaglo *et al.*, 1992; Bajaj, 1999). Derivatives of the anthraquinone molecule have shown to exhibit various pharmacological and biological activities which include: anticancer, antibacterial, antitrypanosomal and antineoplastic activities (Baguley, 1991; Monneret, 2001; Dzierzbicka and Kolodziejczyk, 2005; Preobrazhenskaya *et al.*, 2006). These compounds have also been used in the production of dyes (French Pat. No. 2,002,124; U.S. Pat. No. 2,533,178). The anthraquinones from *C. triloba* natural roots have shown to inhibit the activity of the human topoisomerase II enzyme which transforms supercoiled DNA to linear DNA. This mechanism is currently the basis of many anticancer compounds such as the current and commonly used drug, mitoxantrone (Caron and Wang, 1994; Bassi and Palitti, 2000). Mitoxantrone (1, 4-dihydroxy-5, 8-bis {[2-(2-hydroxyethyl) amino] amino}-9, 10-anthracenedione) is used in combating a variety of cancerous diseases but it has low activity and is cardiotoxic (Morreal *et al.*, 1990; Seiter, 2005; Bidwell *et al.*, 2007). Many derivatives of this drug have been designed and synthesized and have shown better anti-tumour activity (Hsiao *et al.*, 2008). However, these derivatives have very complex structures and/or exhibit chirality, thus in many cases organic synthesis is not cost effective (Oksman-Caldentey and Inze, 2004). Therefore extractions of field grown plants have been used for the production of these important secondary metabolites.

Depending on the plant species, the concentration of the active metabolites in plants is generally low. Plant cell suspension cultures have been considered as an alternative to agricultural processes for producing valuable secondary metabolites; however these cultures produce secondary metabolites at very low concentrations (Zhong, 2002). In addition some secondary metabolites are not synthesized in cell suspension cultures as undifferentiated plant cells often lose partial of their biosynthetic potential. Hence specialized cells or differentiated

plant tissues are required to produce these secondary metabolites (Suzuki *et al.*, 1999; Palazon *et al.*, 2006). Most of the research efforts that have used differentiated cultures have focused on hairy (transformed) root cultures. These cultures have made a significant contribution to the production of secondary metabolites (Zhou and Wu, 2006). For example, taxol is an important anticancer compound that has been produced by hairy roots of *Taxus brevifolia* (Zhou and Wu, 2006). *Agrobacterium rhizogenes* is the bacterium that infects plants which subsequently form hairy roots that are characterized by their high growth rate, genetic stability and the ability to grow in hormone free media (Flores *et al.*, 1987; Aird *et al.*, 1988; Schmülling *et al.*, 1988; Petersen *et al.*, 1989; Estruch *et al.*, 1991; Escobar and Dandekar, 2003). These genetically transformed roots can produce valuable secondary metabolites (used as pharmaceuticals, cosmetics and food additives) at a comparable or higher level to that of intact plants (Giri and Narasu, 2000a).

The aim of this study was to compare the production of anthraquinones from hairy root cultures induced by *A. rhizogenes*-mediated transformation to cell suspension and shoot cultures of *C. triloba*.

This was achieved by the following objectives which were to:

- i) Germinate the seeds of *C. triloba*
- ii) Induce callus cultures on MS medium from leaves of shoot cultures
- iii) Establish cell suspension cultures using callus cultures
- iv) Induce hairy roots on *C. triloba* leaf explants by *A. rhizogenes*-mediated transformation
- v) Compare the production of anthraquinones in field roots and in shoot, cell suspension and hairy root cultures by using TLC and HPLC
- vi) Determine the chemical structures of the compounds produced by *C. triloba* hairy root cultures using EI-LC-MS
- vii) Determine the cytotoxicity of the extracts from field roots and hairy roots on the DU-145 cancer cell line using the MTT assay
- viii) Determine the anti-topoisomerase activity of the fractions isolated from the hairy root culture extract by using the TopoGEN Topoisomerase II Drug Screening kit

The diagram below shows an overview of this thesis which is divided in five chapters namely; introduction and literature review, materials and methods, results, discussion and, summary and conclusion. The results are not shown the diagram.

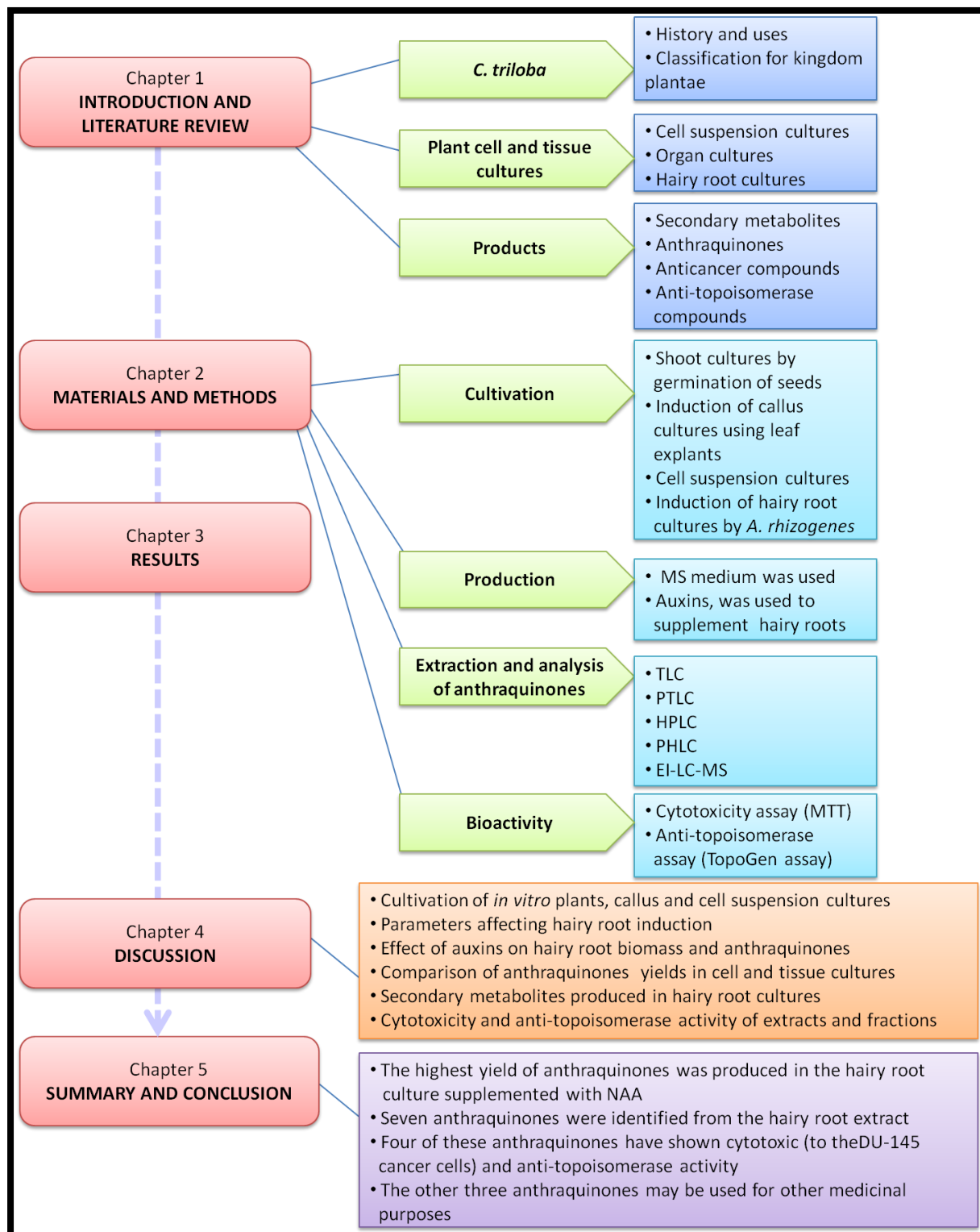


Figure 1: An overview of the thesis.

1.2 *Ceratotheca triloba*

C. triloba is a South African annual plant that is found in the summer rainfall areas of South Africa, mainly the grasslands. The plant is commonly called South African Foxglove, Wild foxglove, Vingerhoedblom, Ludvonca (Swazi), Udonqa (Swazi, Zulu), Undoncalwabathwa or Udonqabathwa (Zulu). There are only four known species of *Ceratotheca* that are found in Southern Africa (Smithies, 2000). The genus name “*Ceratotheca*” means a horned capsule which is derived from the Greek words *kerato* (horned) and *theke* (a case). The species name *triloba* is derived from Latin, meaning three-lobed, alluding to leaves (Hutchings, 1996). The plant was first named *Sporledera triloba* Bernh. in 1842 and it has undergone several name changes since then (Table 1). According to the current classification system (Table 2) it belongs to the family Pedaliaceae (Wunderlin and Hansen, 2002). This family of plants is characterized by having mucilaginous hairs which give the stems and leaves a slimy or clammy feel. The fruits have hooks or horns. *C. triloba* germinates optimally in disturbed areas like roadsides, where they grow, flower and seed before winter. The height of the plant depends on the water uptake during summer. The leaves are soft, green and about 50 mm long; they are divided into three lobes with a bluntly serrated margin. The plant has pink flowers with red stems or white flowers with yellow-green stems (Figure 2A). The flowers are 50 mm long with 5 lobes. The bottom lobe is longer than the others and has streaks of delicate lines running down the throat of the flower. The small seeds are black and are located in 30 mm long fruits which have very sharp horns at the tips (Figure 2B). The green fruits turn brown and dry and split open to release the flat pear shaped seeds. The stems and leaves of the plant are covered with fine white hairs. *C. triloba* is slightly sticky and when crushed it produces a strong unpleasant smell (van der Walt, 2001).

C. triloba is used in many traditional cultures. Some people soak the whole plant in water and use it as a substitute for soap or shampoo. The plant is also used in traditional medicine to treat painful menstruation, stomach cramps, nausea, fever and diarrhea (Tredgold, 1986). Other traditional uses include the preparation of infusions of the leaves which are administrated as an abortifacient and its use for the treatment of diarrhea and gastro-intestinal cramps (Watt and Breyer-Brandwijk, 1962; Roberts, 1990; Pooley, 1998; Van Wyk and Gericke, 2000).

Studies show that *C. triloba* serves as a good source of energy and magnesium (Odhav *et al.*, 2007). Literature on the biological activities of *C. triloba* show that the plant has no angiotensin 1-converting enzymes (Ramesar *et al.*, 2008). Extracts of the plant can be used to control diabetes as it inhibits α -amylases and also has anti-oxidant activity (Odhav *et al.*, 2010). In addition the plant has been shown to inhibit lipo-oxygenase, thus it can be used as an anti-inflammatory agent. Of significance to this work is the work carried out recently in our laboratory which has shown the root extracts of *C. triloba* contains three anthraquinones, namely: 9,10-Anthracenedione, 1-Hydroxy-4-methylanthraquinone and 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, and one steroid, Androst-5-ene-3, 17, 19-triol (Mohanlall *et al.*, 2011). The structures of these compounds are shown in Figure 3. The anthraquinones have shown to inhibit the human topoisomerase II enzyme which transforms supercoiled DNA to linear DNA. This mechanism is currently the basis of many anticancer compounds such as mitoxantrone.

Due to the low production of anthraquinones in the roots, a recent study was conducted whereby cell suspension cultures of *C. triloba* were established for the production of anthraquinones by methyl jasmonate elicitation. However low yields of anthraquinones were produced (Naicker *et al.*, 2010) (Appendix 1). Therefore this study is based on the induction of hairy root cultures from *C. triloba* by *A. rhizogenes* mediated transformation with the intention of producing higher yields of anthraquinones.

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

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

Table 2: Classification for Kingdom Plantae

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 <i>Ceratotheca</i> Endl. - ceratotheca P
Species - <i>Ceratotheca triloba</i> (Bernh.) E. Mey. ex Hook. f.

(Wunderlin and Hansen, 2002)

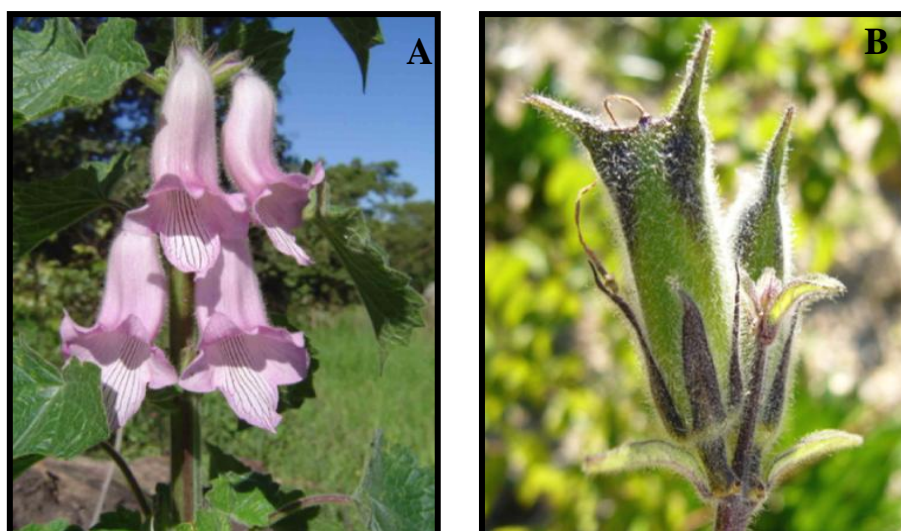


Figure 2: Wild Foxglove - *C. triloba* plant (A) and seed pods (B) (van der Walt, 2001).

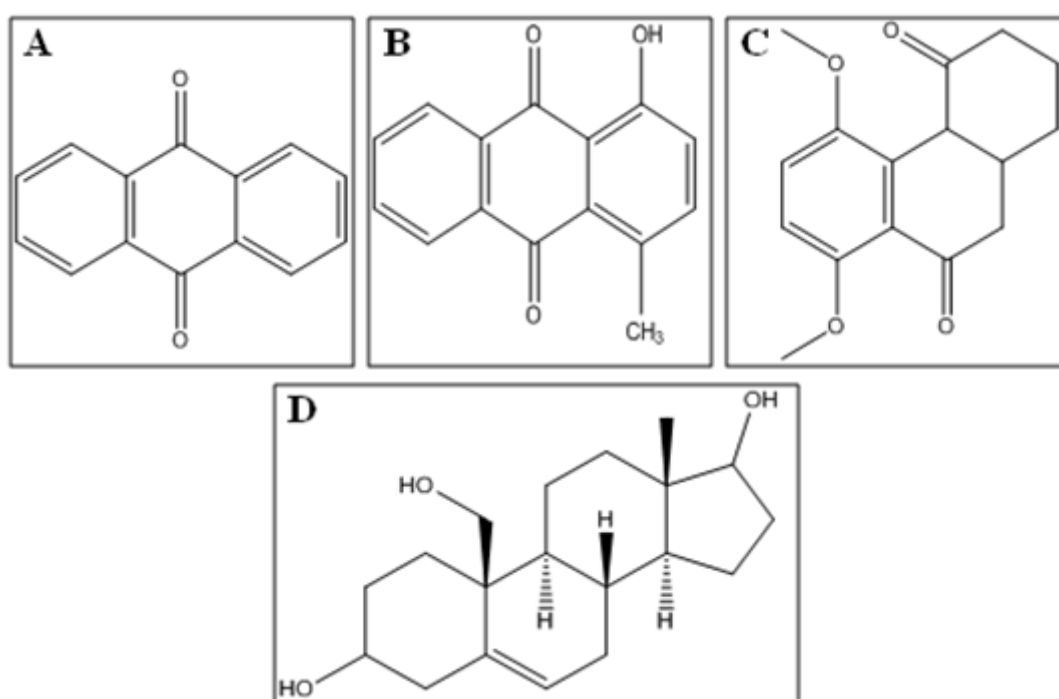


Figure 3: Three novel anthraquinones and a steroid isolated from *C. triloba* roots. 9, 10-Anthracenedione (A), 1-Hydroxy-4-methylantraquinone (B), 5, 8-Dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione (C), Androst-5-ene-3, 17, 19-triol (D) (Mohanlall *et al.*, 2011).

1.3 Plant cell and tissue culture systems

Plant cell and tissue culture technology has many advantages over field cultivation of many plants, as well as drawbacks. Some plants do not withstand field cultivation due to the impact of biological influences (pathogen sensitivity and insects) in nature (Mulabagal and Tsay, 2004), there is risk of extinction for over-harvested plants and geopolitical barriers limit their accessibility. Plant cell and tissue culture has the potential to overcome many of these barriers and they also offer an attractive alternative for the production of high-value secondary metabolites.

- Plant cells are biosynthetically totipotent which means plant cells in culture retain their complete genetic information and therefore have the ability to produce a range of secondary metabolites that are found in the parent plant (Ramachandra and Ravishankar, 2002).
- Product profiles of the *in vitro* plant culture and parent plant can differ, thus novel metabolites can be produced (Ramachandra and Ravishankar, 2002).
- Plant cell culture biotransformation systems can allow for the conversion of inexpensive precursors to novel or valuable compounds (Ramachandra and Ravishankar, 2002).
- Plant cultures can accumulate higher levels of the plant derived product through optimization of cultural conditions (Ramachandra and Ravishankar, 1998).
- The plant derived metabolite can be produced all year round as *in vitro* plant cultures are independent of climate or season (Ramachandra and Ravishankar, 2002).
- 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. It also eliminates contamination with fertilizers and pesticides. Good Manufacturing Practice (GMP) is easily implemented at all stages of the metabolite production, *in vitro* (Hellwig *et al.*, 2004).

There are many different types of cell and tissue culture systems which include undifferentiated (callus and suspension cultures) and differentiated cultures (root, shoot and transformed hairy root cultures). The type of culture chosen depends on whether the same metabolites as the parent plant can be produced by the plant cell or tissue culture, the product profile, the genetic stability and the growth profile of the plant cell or tissue culture. The key characteristics of the different plant cell and tissue culture systems are described in Table 3. The major advantage of hairy root cultures is that it produces compounds that are similar to roots of the parent plant, they are genetically stable and they grow faster than untransformed roots and shoots.

Table 3: Characteristics of different types plant cell and tissue cultures

Characteristics	Undifferentiated cell mass (Callus & cell suspension cultures)	Organ cultures (Roots & shoots)	Transformed tissues (eg; hairy roots)
Metabolite production	Produces same metabolites as parent plant (Ramachandra and Ravishankar, 2002)	Produces metabolites that require cell differentiation (Fu, 1998)	Produces root-derived metabolites (Giri and Narasu, 2000a)
Product profiles	Differ from parent plant (Fu, 1998)	Similar to parent plant (Endo and Yamada, 1985)	Similar to parent plant (Spencer <i>et al.</i> , 1990)
Genetic stability	Low (Charlwood and Moustou, 1988; Miura <i>et al.</i> , 1988)	High (Charlwood and Moustou, 1988; Miura <i>et al.</i> , 1988)	High (Aird <i>et al.</i> , 1988)
Growth profile	Rapid growth cycles (Fu, 1998)	Slow growth cycles	Growth faster than untransformed roots/shoots (Flores <i>et al.</i> , 1987)

1.3.1 Callus culture

A callus culture is an undifferentiated tissue that develops around an injured or cut part of a plant (leaves, stems or roots) in nature or *in vitro*. 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 (growth medium) and physical of the plant cells have to be met. One of the most commonly used plant growth medium, Murashige and Skoog (MS) was developed for tobacco tissue culture (Murashige and Skoog, 1962). The significant feature of 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, cytokinins 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 the 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. Figure 4 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).

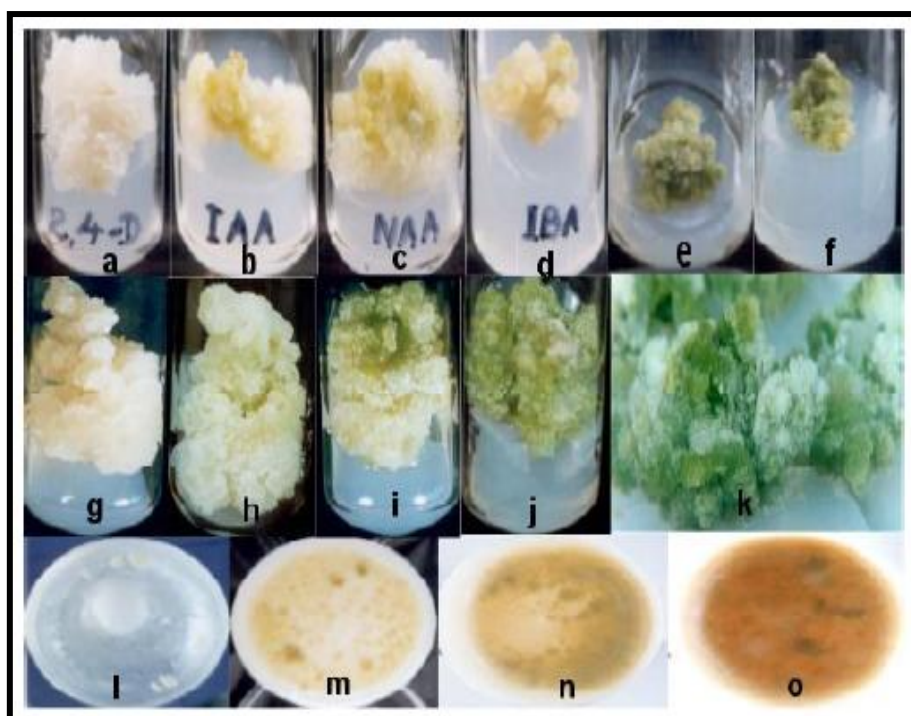


Figure 4: Callus (a-k) and suspension culture (l-o) developments from *G. sylvestre* Gopi and Vatsala, 2006).

1.3.2 Plant cell suspension culture

Callus cultures are used to prepare cell suspension cultures by agitating friable callus tissue in liquid medium in shake flasks (Hellwig *et al.*, 2004). 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 as a result of the secretion of extra-cellular polysaccharides which causes the cells to attach to each other after cell division (Taticek *et al.*, 1991; Chattopadhyay *et al.*, 2002). Cell suspension cultures have been researched for the production of secondary metabolites due to their rapid growth cycles as nutrient uptake is enhanced in submerged conditions (Tripathi and Tripathi, 2003). A further advantage of suspension cultures is that secondary metabolites are intracellular based, therefore, enhanced nutrient uptake in liquid cultures allows for the cultivation of plant cell masses at high concentrations before biosynthesis of secondary metabolites is induced. (Tripathi and Tripathi, 2003). Plant cell suspension culture systems can be adapted to fermentation technology and thus bioreactors can be used for large scale production of secondary metabolites (Fu, 1998; Eibl and Eibl, 2008). Furthermore, the environmental factors (temperature, light, pH, gas composition and osmotic pressure) chemical factors (carbon/nitrogen sources), phytohormones, precursors and elicitors (Zhong, 1995; Jaziri *et*

al., 1996; Zhong, 2002) can also be manipulated to influence secondary metabolite production. However, the major disadvantage is that a low concentration of the cell culture derived secondary metabolite limits the industrial exploitation of plant cell suspension cultures (Zhong, 2002). Despite this, plant cell technology has shown to be a promising approach in terms of the production of paclitaxel from *Taxus* species. ESCAgenetic (San Carlos, CA, USA) and Phyton (Ithaca, NY, USA) cultivated *Taxus* cell suspension cultures in 2500 litre and 75,000 litre bioreactors respectively (Smith, 1995). Also, Samyang Genex (Taejon, Korea) achieved the commercial production of paclitaxel in plant cell suspension cultures in 2001 (Choi *et al.*, 2002). Other secondary metabolites that have been produced in cell suspension cultures are summarised in Table 4.

Table 4: Groups of secondary metabolites produced in cell suspension and tissue cultures of higher plants

Phenylpropanoids	Alkaloids	Terpenoids	Quinones	Steroids
Anthocyanins	Acridines	Carotenes	Anthraquinones	Cardiac glycosides
Coumarins	Betalaines	Monoterpenes	Benzoquinones	Pregnenolone derivatives
Flavonoids	Quinolizidines	Sesquiterpenes	Naphthoquinones	
Hydroxycinnamoyl derivatives	Furonoqui nones	Diterpenes		
Isoflavonoids	Harringtonines	Triterpenes		
Lignans	Isoquinolines			
Phenolenones	Indoles			
Proanthocyanidins	Purines			
Stilbenes	Pyridines			
Tanins	Tropane alkaloids			

Adapted from Stockigt *et al.*, (1995)

1.3.3 Organ culture

An organ culture is an isolated organ cultivated *in vitro*. There are two types. Determinate organs which develop to a defined size and shape (example: leaves, flowers and fruits) and indeterminate organs which have the potential to grow without limit (example: shoot and root cultures). The apices (tips) of stems are cultured to cultivate shoot cultures and the apices of lateral roots are cultured to establish root cultures (George *et al.*, 2008). The cultivation of indeterminate organs *in vitro* has been developed for the production of secondary metabolites that require the use of certain biosynthetic enzymes which are only specific to the plant organ of interest (Suzuki *et al.*, 1999; Palazon *et al.*, 2006). Examples of secondary metabolites that have been produced in organ cultures are shown in Table 5.

Table 5: Secondary metabolites produced in organ cultures

Plant	Type of culture	Secondary metabolite	Reference
<i>Silybum marianum</i>	Root	Flavonolignan	Alikaridis <i>et al.</i> , 2000
<i>Withaina somnifera</i>	Shoot	Withaferin	Ray and Jha, 2001
<i>Mentha arvensis</i>	Shoot	Terpenoid	Phatak and Heble, 2002
<i>Hypericum perforatum</i>	Organ	hypericin	Wilken <i>et al.</i> , 2005
<i>Panax ginseng</i>	Adventitious root	Ginsenoside	Jeong and Park, 2006
<i>Lavandula officinalis</i>	organ	Rosmarinic acid	Wilken <i>et al.</i> , 2005
<i>Scopolia parviflora</i>	Adventitious root	Scopolamine, hyoscyamine and tropane alkaloids	Min <i>et al.</i> , 2007

1.4 *Agrobacterium rhizogenes*-mediated transformation for the induction of hairy root cultures

Transformed organ cultures are achieved by *Agrobacterium*-mediated transformation which is derived from the gram negative soil bacteria, *Agrobacterium tumefaciens* and *A. rhizogenes*. These bacteria attack dicotyledonous plant tissues in nature at a wounded site; possibly caused by insect or mechanical damage. The wounded site produces phenolic compounds that attract the bacteria by chemotaxis which subsequently leads to infection of the plant cells by the bacteria (Balandrin *et al.*, 1985). This activity causes crown gall disease if the plant is infected by *A. tumefaciens*, or hairy root disease if it is infected by *A. rhizogenes* (Escobar and Dandekar, 2003). Hairy root disease is characterized by the formation of a number of small roots with fine hairs that proliferate rapidly at the site of infection (Balandrin *et al.*, 1985).

Hairy root cultures offer a reliable source of secondary metabolites for large scale feasibility and long term stability for the following reasons:

- Hairy root cultures have a growth rate as high as or higher than normal roots (untransformed) due to their extensive branching which results in many meristems (Charlwood and Charlwood, 1991; Flores *et al.*, 1999). The average doubling time after inoculation is 24-90 hours (Payne *et al.*, 1991).
- Hairy roots are capable of synthesizing metabolites specific to the parent plant as well as novel metabolites that cannot be detected in the mother plant or other types of *in vitro* plant tissue cultures (Nader *et al.*, 2006).
- Stable integration of the Ri T-DNA (root inducing transfer DNA) into the plant genome allows for the genetic stability of the transformed root cultures. In addition these root cultures produce secondary metabolites over successive generations without losing their biosynthetic capability (Giri and Narasu, 2000a). Therefore hairy root lines are a promising source for the constant and standardized production of secondary metabolites.
- Hairy roots also offer a valuable source of root-derived phytochemicals that can be used as pharmaceuticals, cosmetics and food additives (Giri and Narasu, 2000a).

The infection process and the induction of hairy roots are directed by several regions contained in the Ri (Root inducing) plasmid carried by the bacterium. The *vir* region contains 6 to 8 genes which encode for proteins that are involved in the transformation of the plant cells by transferring the right and left T-DNA (Transfer-DNA) regions (delimited by border sequences) of the plasmid into the plant genome (Bensaddek *et al.*, 2008). The right T-DNA region is involved in biosynthesis of auxin and opines (amino acid sugar derivatives) (Gartland, 1995). The left T-DNA-region contains four *rol* genes A, B, C and D which enhance the auxin and cytokine synthesis in the plant cells. These genes are responsible for the hairy root phenotype. The T-DNA regions thus confer the plant cells ability to grow in the absence of exogenous plant hormones (Schmülling *et al.*, 1988; Petersen *et al.*, 1989; Estruch *et al.*, 1991). The infection and transformation process is illustrated in Figure 5 (Tzfira *et al.*, 2004). The process comprises of ten steps (Tzfira and Citovsky, 2006): **1)** recognition and attachment of the bacterium to the host plant cells; **2)** and the sensing of specific plant signals by *Agrobacterium*'s VirA/VirG two component signal-transduction system; **3)** this leads to the activation of the *vir* gene region and a mobile copy of the T-DNA is then generated by the VirD1/VirD2 protein complex; **4)** the single stranded T-DNA forms a complex with VirD2 protein; this is called the immature T-complex; **5)** this complex then associates with VirE2 to form the mature complex which travels through the pilus and; **6)** host-cell cytoplasm; **7)** and is actively imported into the host-cell nucleus; **8)** once inside the nucleus, the complex is recruited to a point of integration; **9)** stripped of the *vir* proteins and **10)** integrated into the plant host genome. The process described above is used by *A. tumefaciens* to induce crown gall tumors in dicotyledonous plants however Giri and Narasu, (2000a) describes a similar process for *A. rhizogenes*-mediated transformation which involves the Ri plasmid instead of the Ti (Tumor inducing) plasmid.

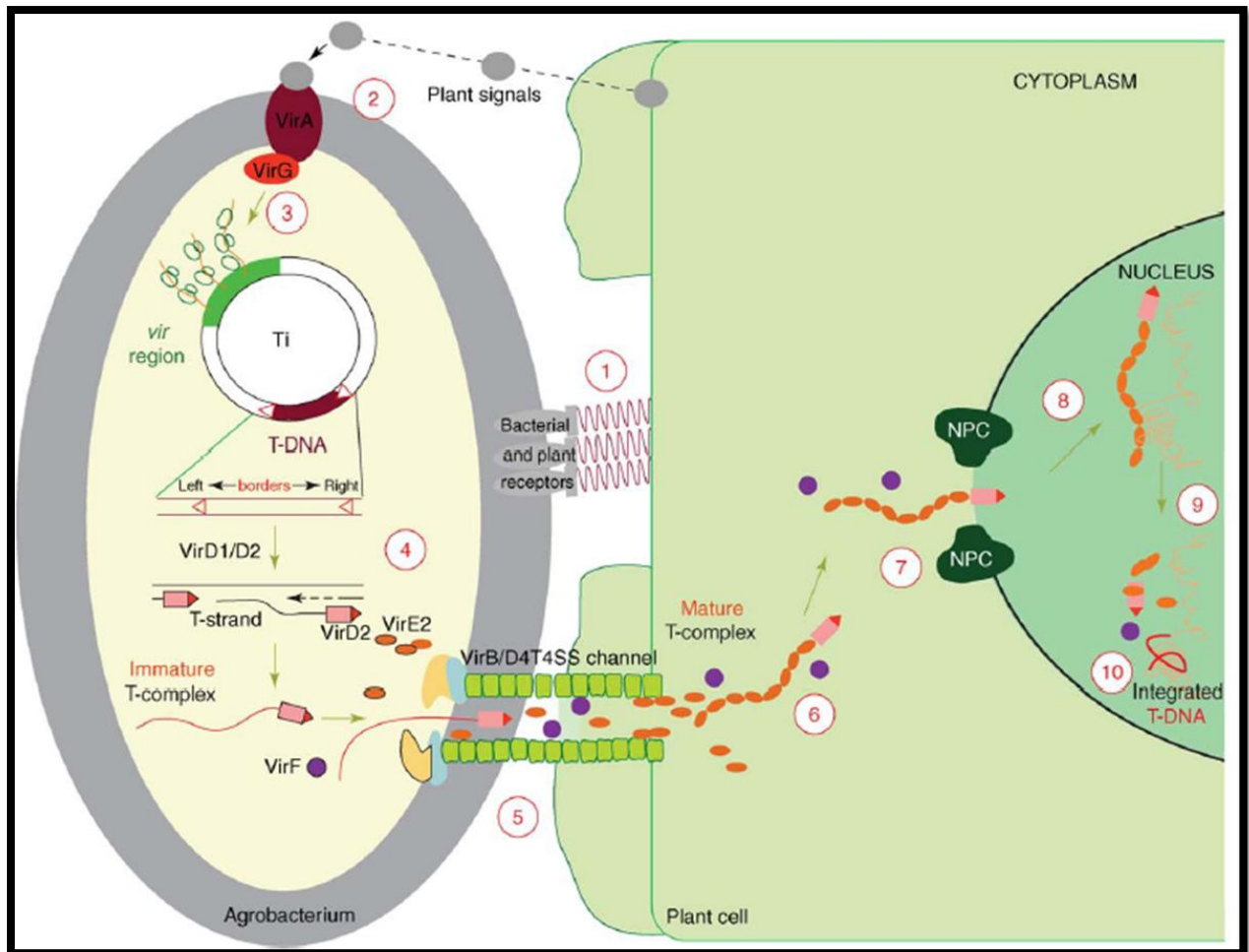


Figure 5: A model of the *Agrobacterium*-mediated genetic transformation process (Tzfira *et al.*, 2004).

The natural transformation process by *A. rhizogenes* can be performed under *in vitro* conditions by using protocols designed for the infection of various plant species. In order for *A. rhizogenes* to mediate the transformation of a plant tissue, contact between bacteria and plants cells must be induced. This can be achieved by direct injection of bacterial suspension into the plant tissue or immersion of the plant tissue into the bacterial suspension. The later procedure can be enhanced with vacuum filtration (Tomilov *et al.*, 2007). This procedure involves wounding of the explant tissue before inoculation with bacteria. In addition the use of excised tissues and leaf disks increases the contact surface between the plant tissue and the bacteria (Wang *et al.*, 2002). Alternative procedures such as micro-wounding through electroporation or sonication can be implemented for hard to transform plants (Matsuki *et al.*, 1989; Trick and Finer, 1997; Le Flem *et al.*, 2004). Transformation of the explant with bacteria occurs during the co-cultivation on solid medium for approximately two to three

days. The explant is then transferred to solid medium containing an antibiotic to eliminate the bacteria. Cefotaxime (250-500 mg.l⁻¹) or timentin (200-300 mg.l⁻¹) is usually used. The explants are then transferred onto hormone-free solid medium and incubated in the dark phase at 20-25°C. Hairy roots appear after 1-4 weeks, these are transferred to Erlenmeyer flasks containing hormone-free liquid medium. The typical phenotype of the transformed root is highly branched and covered with a mass of tiny root hairs. Hairy root cultures have an average doubling time of approximately 2-3 days (Bensaddek *et al.*, 2008). The success of the *Agrobacterium* transformation method can be highlighted by the constantly increasing number of plant species that have been transformed for the establishment of hairy root cultures: 29 species in 1987, 116 species in 1990 and 185 species from 41 families in 2004 (Kuzovkina and Schneider, 2006).

However, the success of the transformation procedure depends on various factors which include: the plant species and age of the plant tissue (younger plant tissues are generally more sensitive to bacterial infection); the explants used for infection (young tissues of sterile plantlets such as hypocotyl segments, cotyledons, petioles and leaves commonly used) and the bacterial concentration used. Sub-optimal concentrations can lower availability of the level of bacteria for transforming the plant cells while high concentrations decrease the availability of the level of bacteria due to competitive inhibition (Kumar *et al.*, 1991; Sevon and Oksman-Caldentey, 2002; Bensaddek *et al.*, 2008). The growth medium also significantly influences hairy root induction. Media containing a high concentration of salt favour hairy root formation of some plants (example, LS medium). Low salt media (example, B5) favour excessive bacterial growth and therefore the explant needs to be treated by transferring it several times onto fresh medium containing antibiotic (Linsmaier and Skoog, 1965; Gamborg *et al.*, 1976).

The choice of the bacterial strain is also an important factor since some plants are very resistant to infection by *Agrobacterium*. Monocotyledonous plants are more difficult to transform than dicotyledonous plants (Bensaddek *et al.*, 2008). There are several types of *A. rhizogenes* strains, examples include: opine, mannopine, agropine and cucumopine. Opine strains are wild types and have been used to produce hairy roots from medicinal plants (Bensaddek *et al.*, 2008). Mannopine strains transfer only the left T-DNA region (Schmülling *et al.*, 1988; Petersen *et al.*, 1989). Agropine strains transfer both the left T-DNA region and right T-DNA region to the plant host genome (Schmülling *et al.*, 1988; Petersen *et al.*, 1989).

The restriction map of a Ri plasmid (pRiA4b) of an agropine strain is shown in Figure 6 (Huffman *et al.*, 1984). Hairy roots cell lines obtained by infection with different *A. rhizogenes* strains exhibit different morphologies due to the different Ri plasmids harbored by the strains (Nguyen *et al.*, 1992). The degree of virulence of different strains is also based on the different Ri plasmids harbored by the strains (Bensaddek *et al.*, 2008). For example; LBA 9402 strain is a hypervirulent strain that has been used to transform hard to transform plants such as *Centaurea erythraea*, *Hyoscyamus muticus*, *Saponaria vaccaria* and *Gentiana macrophylla* (Vanhala *et al.*, 1995; Piatczak *et al.*, 2006; Schmidt *et al.*, 2007; Tiwari *et al.*, 2007)

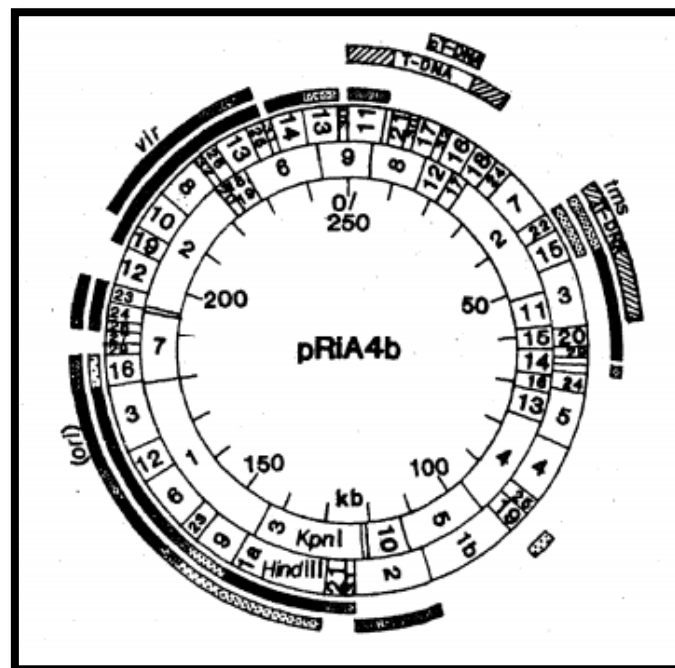


Figure 6: Restriction map of a Ri plasmid (pRiA4b) of an agropine strain. *Vir* represents the virulence region; *ori* is the origin of replication and *tms* represents the auxin genes (Huffman *et al.*, 1984).

1.5 Secondary metabolites production in hairy root cultures

Although the biosynthesis of secondary metabolites in hairy roots is genetically controlled, chemical (sucrose concentration, exogenous growth hormone and the nature of the nitrogen source) and environmental factors (light, temperature and pH) can affect growth, total biomass yield and secondary metabolite production (Rhodes *et al.*, 1994; Giri *et al.*, 1997; Nussbbaumer *et al.*, 1998). In order to maximize biomass and metabolite production, modification of the culture medium is required; this can involve change in the sugar, nitrogen and/or phosphorus source (Bensaddek *et al.*, 2008). Sucrose is the best carbon source; it is hydrolyzed into glucose and fructose during assimilation by the plant cells in culture (Srinivasan *et al.*, 1995). Studies have also been conducted on many plant species whereby the product that leaches into the medium can be recovered by adsorbents. The culture medium is then rejuvenated to maintain the supply of nutrients. Leaching is accomplished by the addition a permeabilization agent (example: Tween 20) into the medium which results in the transient release of secondary metabolites (Boitel *et al.*, 1996). Pietrosiuk *et al.* (2007) describes the different factors involved in culturing and producing secondary metabolites from *Catharanthus roseus* hairy root cultures. Figure 7 shows *C. roseus* hairy roots cultured from shoot and artificial seed explants. Other examples of secondary metabolites produced in hairy root cultures are shown in Table 6 (Zhou and Wu, 2006).

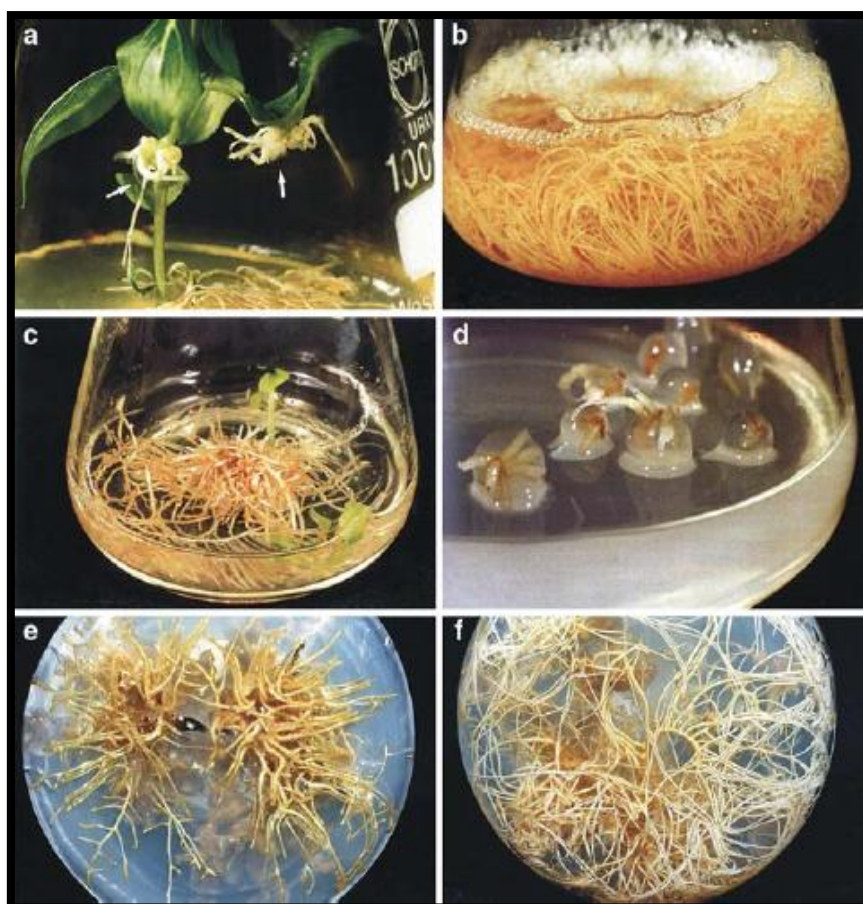


Figure 7: Hairy root cultures of *C. roseus*. Hairy roots emerged at the sites of infection after 7-10 days by using *A. rhizogenes* (ATCC 15843) (a). Hairy roots cultured in B5 medium liquid medium without hormones (b). Transgenic plants regenerated from *C. roseus* hairy roots in half strength B5 medium without hormones (c). Artificial seeds obtained by encapsulating *C. roseus* hairy roots in sodium alginate (d). Hairy roots were cultured from artificial seeds of *C. roseus* on NN medium containing 0.5 mg.l⁻¹ IBA and 0.1 mg.l⁻¹ kinetin (e). Hairy roots were cultured from artificial seeds of *C. roseus* on NN medium containing 0.5 mg.l⁻¹ IBA and 0.1 mg.l⁻¹ BAP (f). The difference in morphology of the hairy roots can be seen when different hormones were used (Pietrosiuk *et al.*, 2007).

Table 6: Hairy root cultures of medicinal plants and their secondary metabolites

Plant	Product/s
<i>Anisodus tanguticus</i>	Scopolamine
<i>Artemisia annua</i>	Artemisinin
<i>Cassia obtusifolia</i>	Steroids
<i>Cassia obtusifolia</i>	Betulinic acid, chrysophanol, physcion, 8-O-methylchrysophanol, 1-hydroxy-7-methoxy-3-methylanthraquinone, 1-O-methylchrysophanol, aloe-emodin
<i>Astragalus membranaceus</i>	Astragaloside IV, polysaccharides
<i>Cyanotis arachnoidea</i>	20-Hydroxyecdysone
<i>Dioscorea zigerensis</i>	Diosgenin
<i>Glycyrrhiza uralensis</i>	Flavonoids
<i>Gynostemma pentaphyllum</i>	Triterpenoid saponins
<i>Panax ginseng</i>	Saponins
<i>Panax japonicus var. major</i>	Saponins
<i>Panax quinquefolium</i>	Saponins
<i>Phytolacca esculenta</i>	Saponins
<i>Polygonium multiflorum</i>	Rhein, emodin
<i>Pueraria lobata</i>	Isoflavonoids
<i>Rheum palmatum</i>	Anthraquinones
<i>Rheum wittrochii</i>	Aloe-emodin, rhein, chrysophanol, emodin, physcion, 8-O-methylchrysophanol
<i>Salvia miltiorrhiza</i>	Tanshinone I, tanshinone IIA, cryptotanshinone
<i>Saussurea involucrata</i>	Sytingin, rutin, hispidulin
<i>Solanum photeinocarpum</i>	Glycoalkaloids, saponins
<i>Stephania delavayi</i>	Isoquinoline alkaloids
<i>Taxus brevifolia</i>	Taxol

(Zhou and Wu, 2006)

1.6 The production of anticancer compounds in hairy root cultures

“Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths in 2008” (Globocan, 2008). Several chemo-preventive agents have been developed and are used for the treatment of cancer but the toxicity of these agents limits their use (Kathiresan *et al.*, 2006). Thus there is a great interest to research new and better treatments. The plant kingdom is one of the most attractive sources of novel anticancer compounds. Various potent anticancer compounds from higher plants have been identified by the National Cancer Institute in the United States of America through conducting an intensive screening program (Suffness and Douros, 1982). However, there are certain limitations towards using plants; the concentration of the active compounds present in the plants is generally low, the growth rate of plants is slow and geographical or environmental conditions affects the accumulation of the active compounds (Yesil-Celiktas *et al.*, 2010). Thus, the economical production of the active compounds by extraction of the intact plant is a difficult task. Furthermore, many anticancer compounds isolated from higher plants are secondary metabolites and have complex structures which are difficult to chemically synthesize (Oksman-Caldentey and Inze, 2004). Although plant tissue culture technology is not a very cost effective option it is undoubtedly one of the most appropriate approaches to solve the above problems if the active compounds could not be manufactured by extraction or chemical processes. Therefore, over the last decade research studies have focused on applying plant tissue culture technology for the possible commercial production of anticancer drugs such as taxol, vinblastine, vincristine, camptothecin derivatives and podophyllotoxin (Table 7).

Table 7: Anticancer compounds produced by hairy root cultures

Plant	Compound/s	Treatment	Reference/s
<i>Taxus cuspidata</i>	Taxol	Ovarian, breast, non-small cell lung	Rowinsky and Donehower, 1995; Kim <i>et al.</i> , 2009
<i>Catharanthus roseus</i> L. (G.) Don	Vinblastine [Velban®], vincristine [Oncovin®]	Acute leukaemia, small-cell lung cancer, cervical, breast, head and neck cancer	Ataei-Azimi <i>et al.</i> , 2008; Schmelzer and Gurib-Fakim, 2008
<i>Ophiorrhiza pumila</i>	Camptothecin derivatives (irinotecan [Campto®], topotecan [Hycamtin®])	Colorectal and ovarian cancers	Bookman, 1999; Mathijssen <i>et al.</i> , 2001; Vanhoefer <i>et al.</i> , 2001; Mathijssen <i>et al.</i> , 2002; Sudo <i>et al.</i> , 2002
<i>Podophyllum hexandrum</i>	Podophyllotoxin (used as a precursor for production of etoposide [VP-16-213] and teniposide [VM-26])	Lung cancer, testicular cancer, a variety of leukemias and other solid tumours	Holthius, 1988; Stahelin and von Wartburg, 1991; Imbert, 1998; Giri and Narasu, 2000b

1.7 Analysis of the compounds in plant cell and tissue culture extracts

Chromatographic techniques are used in plant cell and tissue culture studies to separate, detect, identify and isolate the compounds of interest in the plant extracts.

Thin Layer Chromatography (TLC) is used to separate the compounds from the extract and detect the compounds of interest by using commercial standards. This method involves the use of glass or aluminum plates that are pre-coated with a sorbent (example, silica gel) which varies in thinness depending the amount of sample to be loaded. The extract is loaded at approximately 2 cm from the bottom of the TLC plate for both preparative and analytical plates. The plate is lowered into a tank containing the solvent which migrates up the plate and separates the compounds in the extract based on their polarity. There are several reagents that are available for the visualization of the separated compounds. The advantage of using TLC is that it is a highly cost-effective qualitative technique as a large number of samples can be analyzed or separated simultaneously.

HPLC is widely used to identify and quantify compounds in the extract. It involves the use of different types of stationary phases and a pump which moves the mobile phase and the mixture of compounds through the column. A detector then provides the retention times of the compounds in the extract which is presented in a computer-generated chromatogram (Holme and Peck, 1998). The retention time of a standard can be compared to that of the compound in the extract to determine its identity. The analytical sensitivity of this method can be enhanced depending on the detector used. Detectors can be UV based such as the photodiode array (PDA) detector which allows acquisition of the UV spectra of eluting peaks between wavelengths of 190 nm to 800 nm. The PDA detector can also be used to detect compounds with poor UV characteristics such as terpenoids and polyketides.

1.8 Structural elucidation of compounds

Structural elucidation of a compound is crucial as it provides the molecular structure of the compound which is important since the 3-D arrangement of the functional groups on the molecule influences its biological activity. Structure elucidation is achieved by using classical spectroscopic techniques such as: Nuclear Magnetic Resonance (NMR) 1-D, 2-D Proton NMR, C^{13} NMR, Infra Red (IR), X-Ray and Mass Spectrometry (MS) analysis. MS will be discussed further as this method of structural elucidation was used in the study.

MS is one of the most sensitive and selective methods as it is used to provide information on the mass and structure of a compound. MS is combined with liquid chromatography (LC) to allow for the separation of the compounds in the extract and subsequent partial structural identification. MS involves the use of a range of methods to ionize compounds and then separate the ions (Gong *et al.*, 2001). The common methods of ionization are electron impact (EI) and electron capture ionization (ECI). EI is primarily configured for the selection of positive ions and ECI is configured for selection negative ions. EI is used for routine analysis and provides a reproducible mass spectrum that allows for the partial structure of a compound to be obtained by library searching (Gong *et al.*, 2001).

Once the sample is introduced into the system, a molecular ion is produced by the removal of an electron or the addition of proton/s. This is followed by fragmentation to produce ionized fragments (fragment ions). The fragmentation pattern is characteristic to a compound. The mass spectrometer separates and measures the mass of the ions by using a mass-to-charge

ratio (m/z). The ions that are formed have a single, positive charge ($z = 1$), thus $m/z =$ the mass of the ion. In a mass spectrum plot, the relative amounts of ions are shown as their relative abundance on the y axis. The m/z (mass) value is displayed on the x-axis (Figure 8). The height of each peak on the chromatogram is shown as a percentage of the biggest peak (the base peak) (Holme and Peck, 1998).

The partial structural identification of a compound is obtained by using software that performs the computerized identification by comparing the unknown mass spectrum against reference libraries of mass spectra.

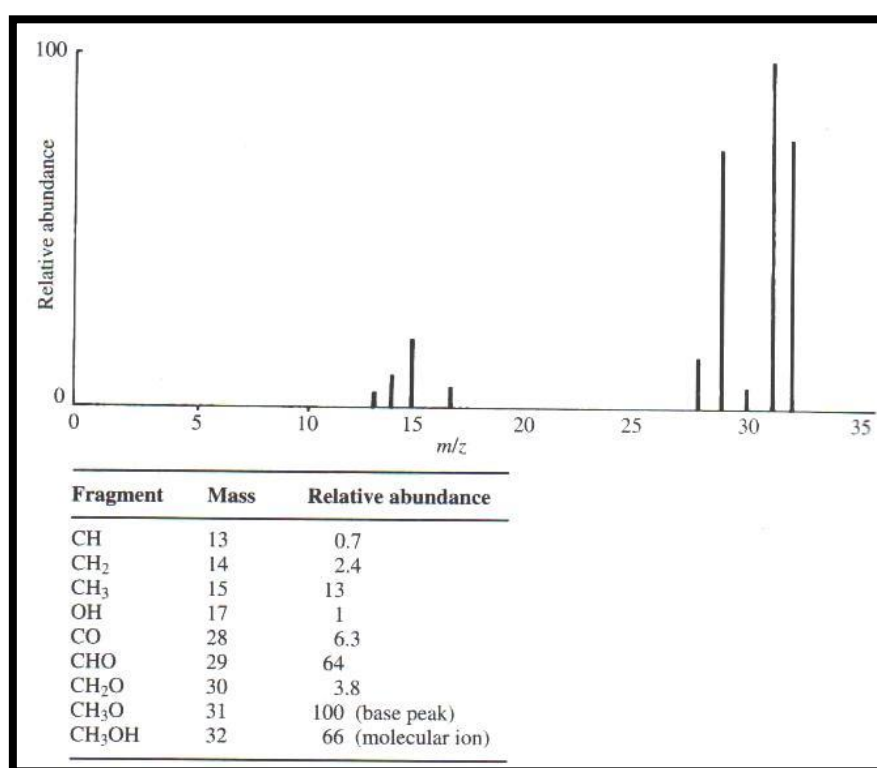


Figure 8: Mass spectrum of methanol (Holme and Peck, 1998).

1.9 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 contain derivatives that consist of the basic structure of 9,10-anthracenedione (Bajaj, 1999). Anthraquinones can be divided into alizarin and emodin types based on two main biosynthetic pathways which occur in higher plants. The alizarin types are formed via chorismate/o-succinylbenzoic acid pathway and only have one of the rings unsubstituted (Kobliz, 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 to a large extent in the plant families: Fabaceae (*Cassia*, *Araroba*), Rhamnaceae (*Rhamnus*, *Frangula*) and Polygonaceae (*Rheum*, *Rumex*, *Fagopyrum*) (Bajaj, 1999).

Anthraquinones are widely applied in the pharmaceutical, food and dye industry. In the pharmaceutical industry, natural and synthetic derivatives of 9,10-anthracenedione are beneficial to mammals and humans as they can display antibacterial, antitrypanosomal and antineoplastic activities (Dzierzbicka and Kolodziejczyk, 2005). In addition several commercial pharmaceutical products that have been developed contain anthraquinone derivatives. Anthraquinone glycosides are used to produce Pyralvex which is used 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 the 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). Anthracycline antibiotics are also anthraquinone derivatives and are key substances that have been known to be used for therapy of several cancers (Dzierzbicka and Kolodziejczyk, 2005).

Table 8: Anticancer anthracenedione derivatives

Source	Anthraquinones	Cancer line	Reference
Daylies (<i>Hemerocallis spp.</i>)	Kwanzoquinone C, kwanzoquinone E, 2- hydroxychrysophanol, rhein	MCF-7 (breast cancer), SF-268 (CNS cancer), HCT (colon cancer), NCI (lung cancer)	Cichewicz <i>et al.</i> , 2004
<i>Hedyotis diffusa</i>	2-hydroxy-3- methylantraquinone, 1- methoxy-2- hydroxyanthraquinone	SPC-1-A, Bcap37, HepG2	Shi <i>et al.</i> , 2008
Traditional Chinese herbs	6-nitro-1-methyl-1-hydroxy-3,8- dimethoxy-9,10- anthraquinone	MDA-MB-453 (breast cancer), Calu-3 (lung cancer adenocarcinoma)	Yan <i>et al.</i> , 2008
Chemically synthesized	Alchemix (1-{2-[<i>N,N</i> -bis(2- chloroethyl)amino]ethylamino}- 4-{2- [<i>N,N</i> - (dimethyl)amino]ethylamino}- 5,8-dihydroxy-9,10- anthracenedione)	2780AD, 2780/cp70 (ovarian carcinoma cell lines)	Pors <i>et al.</i> , 2003
Chemically synthesized (Sigma Chemical Co., USA)	Emodin (1,3,8-trihydroxy-6- methyl-antraquinone)	HepG2/C3A, PLC/PRF/5, and SK-HEP-1 (hepatoma cell lines)	Shieh <i>et al.</i> , 2004
<i>Rheum palmatum</i> L.	Emodin (1,3,8-trihydroxy-6- methyl-antraquinone)	HL-60 (leukemia cell line)	Chen <i>et al.</i> , 2002
Chemically synthesized	(<i>S,S</i>)-1,4-bis[2-(4- methylsulfanyl-butylamino) ethylamino]-5,8- dihydroxyanthracene-9,10- dione	A549 (non-small cell lung cancer), DU145 (androgen- independent prostate cancer), HT-29 (colorectal cancer), MCF- 7 (breast cancer), Hep3B and HepG2 (hepatocellular carcinoma).	Hsiao <i>et al.</i> , 2008

1.10 Mechanism of action of anticancer compounds

1.10.1 The cell cycle

In order to understand the mechanism of action of anticancer compounds, it is important to be familiar with the human cell cycle. The cell cycle can be divided into five phases (Figure 10). The S-phase is when the chromosomal DNA replicates. This phase is followed by the M (mitosis) phase where the segregation of the chromosomes into two daughter cells occurs (Giordano and Kasten, 1998). In between the S and M phase are the two gap phases, G1 and G2. The G1 phase proceeds after the M phase, during this time the cell becomes responsive to negative and positive growth signals. The G2 phase proceeds after the S phase where the cell prepares for entry into the M phase. The final phase is the G0 phase where the cell exits from the G1 into the G0 phase if it is deprived of the appropriate growth promoting signals (Garrett, 2001).

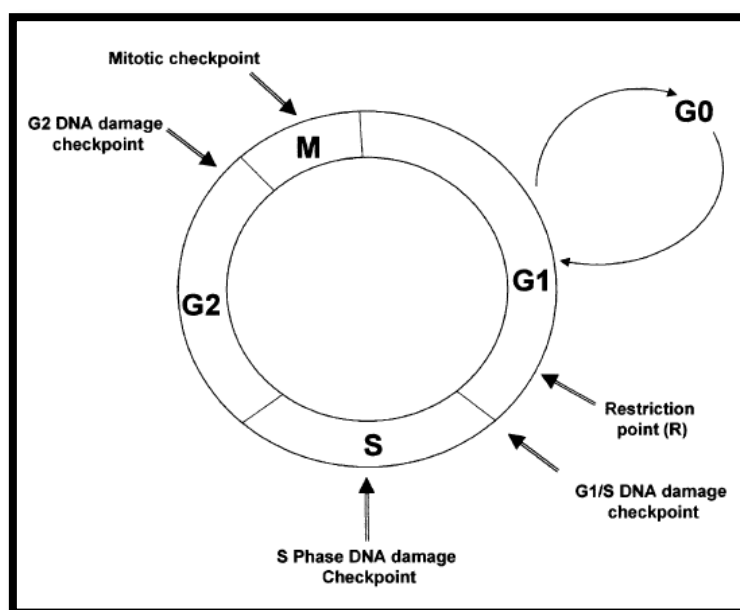


Figure 10: The cell cycle and the check points involved (Garrett, 2001).

The progression of the normal cell cycle is controlled by a family of kinases called cyclin dependent kinases (cdks) and regulated by positions within the cell cycle called the check points (MacLachlan *et al*, 1995) (Figure 10). Hartwell and Weinert, (1989) first defined the cell cycle check points as a ‘mechanism that maintains the observed order of events of each cell cycle’. This means that there are a number of key genes that participate in the check

points which function to see if the integrity of the genome is retained throughout the normal cell cycle (Garrett, 2001). Normal cells protect themselves against the exposure to growth-limiting conditions or toxic agents by using the checkpoint control mechanism (Kufe *et al.*, 2003). For example, if normal cells undergo DNA damage by UV radiation, they arrest in the G1 phase in order to repair the DNA prior to replication. However if any of the genes involved in the check points are mutated, the integrity of the cell's genome will be at stake and the cancer cell cycle may proceed. Cancer cells exhibit poor check points and therefore they are more susceptible to the cytotoxic effects of drugs such as anti-topoisomerases (Kufe *et al.*, 2003).

1.10.2 Anti-topoisomerase drugs

Anti-topoisomerase drugs cause DNA damage to cancer cells by inhibiting enzymes known as DNA topoisomerases (McClendon and Osheroff, 2007). These enzymes function to open transient, protein-bridged, single- or double-stranded DNA breaks. DNA is passed through these breaks so that topological problems can be solved and accumulated torsion stress can be relieved during cellular transactions of the DNA molecule. Topoisomerases have been classified as type I and II according to their ability to cleave single- or double- stranded DNA molecules, respectively (Caron and Wang, 1994). In cancer therapy anti-topoisomerase drugs inhibit DNA topoisomerase II (Figure 11) and prevent the disentangling of the cell's DNA and subsequently causes cell death via apoptosis (McClendon and Osheroff, 2007).

There are a diverse group of natural and synthetic compounds that target topoisomerase II α and II β (Fortune and Osheroff, 2000; Wilsterman and Osheroff, 2003; Velez Cruz and Osheroff, 2004), some of which are described in Table 9. These compounds are potent *in vitro* and in human cells. They are widely used as some of the most successful chemotherapeutic drugs for the treatment of human cancer and malignancies. Studies have shown that the cytotoxic effect of anthraquinones on cancer cells is due to their ability to inhibit the DNA topoisomerase II enzyme which in turn leads to the activation of apoptotic pathways. For example, Hsiao *et al.* (2008) showed that (S,S)1,4-bis[2-(4-methylsulfanyl-butylamino)ethylamino]-5,8-dihydroxyanthracene-9,10-dione inhibited the topoisomerase II enzyme and induced chromosomal DNA breaks which led to S and G2 phase arrest of the cell cycle and activation of apoptotic pathways in prostate cancer cells.

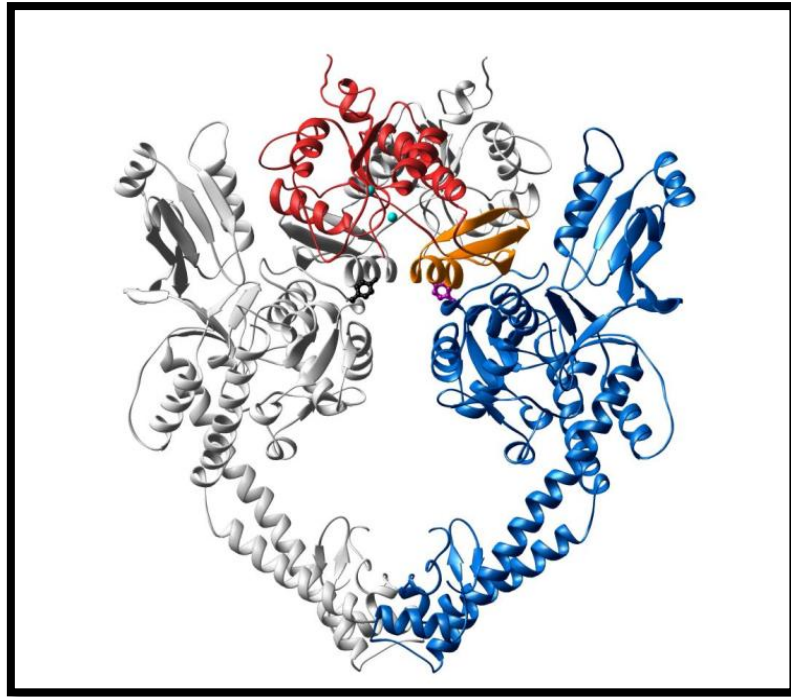


Figure 11: High resolution 3-D crystallography image of the binding and cleavage core of type II topoisomerase from *Saccharomyces cerevisiae*. DNA topoisomerase II is a highly conserved protein dimer with a heart-shaped form and large holes (Caron and Wang, 1994).

Table 9: 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 <i>et al.</i> , 1987
Anthracenediones	Mitoxantrone	II	stabilize cleavable complex	Harker <i>et al.</i> , 1991
Anthracyclines	Doxorubicin	II	stabilize cleavable complex	Au <i>et al.</i> , 1981
Ellipticines	2-methyl-9-OH-ellipticinium acetate	II	stabilize cleavable complex	Aimova <i>et al.</i> , 2007
Coumarine	Novobiocin	bacterial gyrase (sub B)	interferes with ATPase activity of Topo II	Sugino <i>et al.</i> , 1978
Isoflavonoids	Genistein	II	PTK inhibitor and cleavable-complex blocker	Markovits <i>et al.</i> , 1989 Adlerkreutz, 1995
Alkaloid	Camptothecin and its derivatives	I	stabilize cleavable complex	Hsiang <i>et al.</i> , 1985
bis-piperazinediones	ICRF-159, 193	II	inhibits DNA relaxation and cleavable complex formation	Jensen <i>et al.</i> , 2000
Anthracenyl peptides	Merbarone	II	inhibits cleavable complex formation	Khelifa and Beck, 1999

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant material

Seed pods and roots of *C. triloba* were collected from wild plants in Durban, Kwa-Zulu Natal, South Africa. The plant was identified by using available floral keys and verified by botanist, Professor H. Baijnath. A voucher specimen was deposited in the Ward Herbarium at the University of Kwa-Zulu Natal (Westville campus). Seed pods were collected in March and April when they were dried. The pods were broken to remove the mature seeds which were stored in a closed bottle at room temperature. The roots were harvested and damaged or infected roots were discarded. Healthy roots were dried in the laboratory at room temperature for 5-8 days or until they broke easily by hand. These were then ground to a fine powder using a Wareing blender and stored in a closed bottle at room temperature until required.

2.2 Seed germination and shoot culture

All plant tissue culture procedures were conducted in a laminar flow cabinet (Scientific Engineering INC). The laminar flow cabinet was sterilized by UV light and disinfected by swabbing with 70% ethanol (Merck, South Africa) before use. All tissue culture instruments (forceps and scalpel) were sterilized by dipping in 70 % ethanol and then passing through a flame.

Mature seeds were selected by placing the seeds in water in a Petri dish. The heavy, swollen seeds (mature seeds) sank to the bottom of the Petri dish while immature seeds floated on the top. The mature seeds were removed with forceps and surface sterilized for germination. In order to determine the optimum time for effective surface sterilization the seeds were exposed to 30% NaClO for 0 (control), 5, 10, 15 and 20 minutes. The seeds were then washed with sterile distilled water. Five seeds were placed in each Petri dish containing solid MS medium (Murashige and Skoog, 1962). A replicate of 5 Petri dishes were prepared for each exposure time. The Petri dishes were incubated under visible light at room temperature. The seeds were then examined for contamination after two weeks and the treatment regime that produced the highest percentage of sterile seeds was used for germination seeds to produce shoot cultures for the *in vitro* transformation experiment.

The germinated seedlings (3 weeks old) were cultivated into shoot cultures in 12 cm culture vessels containing MS medium which was supplemented with 1 mg.l⁻¹ of 6-BAP (Sigma-Aldrich, Inc) and 0.5 mg.l⁻¹ of IAA (Sigma-Aldrich, Inc). The seedlings were incubated for 45 days in a growth chamber at 26°C under standard cool white fluorescent light with a flux rate of 35 µmols⁻¹m⁻² and a 16 h photoperiod.

2.3 Cultivation of callus cultures

2.3.1 Induction of callus cultures

The leaves of *in vitro* shoot cultures were removed and cut into 1 cm square disks using a scalpel. They (5 leaf disks per plate) were placed on MS medium supplemented with 1 mg.l⁻¹ of 2,4-D and 1 mg.l⁻¹ of 6-BAP (Sigma-Aldrich, Inc). These plates were then placed in a cardboard box and incubated in the dark phase at 26°C for 4 weeks.

2.3.2 Microscopic analysis of callus

Initiated callus cultures from the explants in Petri dishes were placed under the dissecting microscope (Nikon SM2800) to view callusing of the explant and morphological parameters (colour and root-like structures) of induced callus at 100 x magnification.

2.3.3 Maintenance of callus cultures

Callus cultures induced from the explants were transferred onto Petri dishes containing MS medium using a sterile forceps (5 calli per plate). The Petri dishes were placed in a box and incubated in a cupboard in the dark phase at 26°C. The callus cultures were sub-cultured at 3 week intervals by transferring callus tissue of 0.5 cm diameter on to fresh medium.

2.4 Cultivation of cell suspension cultures

Inoculum for the cell suspension cultures were initiated by transferring approximately 1 g of callus (three weeks old) from the second sub-culture into four 1000 ml Erlenmeyer flasks containing 50 ml of MS liquid medium which was supplemented with 1 mg.l⁻¹ of 2,4-D and 1 mg.l⁻¹ of 6-BAP. The flasks were agitated on a shaker (Infors Ecotron, Polychem supplies

cc) at 100 rpm at 26°C in the dark phase for one week. Thereafter, 150 ml of MS medium was added to the inocula and the flasks were incubated for a further 2 months. At the end of cultivation period the cell mass of suspension cultures was determined by centrifuging 50 ml volumes at 4000 rpm for 10 minutes at 20°C. The total wet weight of the cell suspension cultures was determined by the following equation: (mass of beaker + cell mass) – (mass of beaker) = total wet weight of cell suspension cultures from four flasks.

2.5 Production of hairy root cultures

2.5.1 Cultivation of *A. rhizogenes*

A. Rhizogenes strain 15834 was provided on Yeast extract peptone (YEP) agar by the Council of Scientific and Industrial Research (CSIR), South Africa. Stock cultures of *A. rhizogenes* were prepared by streaking out a loopful of the culture onto YEP agar plates (An *et al.*, 1988). The strain was cultivated for 48 hours at 30°C and stored at 4°C in a refrigerator. The stock culture was used to prepare a cell suspension culture by streaking out a loopful of *A. rhizogenes* onto a YEP agar plate which was incubated for 24 hours at 30°C. A loopful of this culture was then used to inoculate a 250 ml Erlenmeyer flask containing 50 ml of YEP liquid medium. The flasks were placed on a shaker and incubated for 24 hours at 30°C. The optical density (OD) of the culture was determined spectrophotometrically at 600 nm and the culture was diluted with YEP medium to an OD of 1, 0.5 and 0.2 for the transformation experiment.

2.5.2 Culture, storage and regeneration of *A. rhizogenes*

A. rhizogenes was cyro-preserved by using the following protocol. A 24 hour liquid culture of *A. rhizogenes* was prepared as described in section 2.5.1. An aliquot of 1.5 ml of glycerol was added to 8.5 ml of the liquid culture. Aliquots of 1 ml of the prepared cells were then transferred to cryovials which were stored in an ultra-freezer at -80°C. When required the cryovial was removed from the ultra-freezer, thawed and swabbed with 70% ethanol. A loopful of the cells was used to inoculate a 250 ml Erlenmeyer flask containing 50 ml YEP liquid medium. The flask was placed on a shaker and incubated for 24 hours at 30°C (Van Asma, 1995).

2.5.3 Hairy root induction in *C. triloba* using *A. rhizogenes*

Shoots of the *in vitro* grown plants were cut at the stems and the leaves and stems were used as explants. These were inoculated with a sterile scalpel dipped in three concentrations (OD of 1, 0.5 and 0.2) of the *A. rhizogenes* culture. Leaf explants were inoculated by pricking and stem explants were cut. Explants inoculated with a scalpel dipped in YEP medium served as the control. The infected and control explants were placed on MS medium and incubated for 3-4 weeks at 26°C under standard cool white fluorescent light with a flux rate of 35 $\mu\text{mol s}^{-1}\text{m}^{-2}$ and a 16 h photoperiod. The response of the explants to Agrobacterium in terms of hairy root emergence was measured by determining the transformation efficiency: (number of hairy roots/number of explants) X 100. Induced hairy roots were excised and then decontaminated by treating them with 500 mg.l⁻¹ of cefotaxime for five days, and thereafter with 200 mg.l⁻¹ of cefotaxime for eight days. The sterility of the hairy roots was confirmed by placing 1 cm of the root onto a YEP plate which was incubated at 30°C for three days. Once the roots were confirmed to be sterile the entire hairy root culture was placed on MS medium and maintained by sub-culturing onto fresh medium at three week intervals.

2.6 Effect of auxins on hairy root biomass production

In order to improve the growth of hairy roots two auxins were used, NAA at 1 mg.l⁻¹ and IAA at 1 mg.l⁻¹. These hormones were filter sterilized into MS medium. Inoculum cultures were prepared by transferring approximately 200 mg hairy roots into 1 L flasks containing 30 ml of MS medium. Three flasks containing medium with no hormone served as the control cultures. All cultures were incubated at 26°C in a shaker under standard cool white fluorescent light with a flux rate of 35 $\mu\text{mol s}^{-1}\text{m}^{-2}$ and a 16 h photoperiod. An aliquot of 70 ml of medium was added to the inoculum cultures after 3 weeks and a final volume of 100 ml of medium was added to the flasks after a further 3 weeks. Cultures were then incubated for 4 weeks. Thereafter the biomass was removed from the medium using a forceps and the increase in wet weight was determined by the following equation: (final wet weight – initial weight = increase in wet weight).

2.7 Examination of the morphological characteristics of adventitious roots and hairy roots

Shoot cultures were sub-cultured on Petri plates containing MS medium supplemented with 1 mg.l⁻¹ of IBA to induce adventitious roots. Induced hairy roots were transferred onto plain MS medium and MS medium supplemented with 1 mg.l⁻¹ of NAA. The plates were incubated at 26°C under standard cool white fluorescent light with a flux rate of 35 $\mu\text{mol s}^{-1}\text{m}^{-2}$ and a 16 h photoperiod. The morphology of adventitious roots and hairy roots were examined according to colour and branching of the lateral roots.

2.8 Extraction and analysis of anthraquinones

2.8.1 Extraction of anthraquinones from field roots and shoot and cell suspension cultures

The preparation of the field roots, shoot and cell suspension cultures for extraction varied but the preparation of the different extracts was the same. This is outlined below:

Field roots: the ground material of the field roots was extracted with hexane by agitation on a shaker at 180 rpm for 24 hours at room temperature.

Shoot cultures: the shoot cultures were dried at 30°C in an oven and crushed to a fine powder in a Waring blender. The powder was extracted in 100 ml of hexane at room temperature by agitation on a shaker at 180 rpm for 24 hours.

Cell suspension cultures: The cell mass was harvested by centrifuging 50 ml volumes of suspension culture at 4000 rpm for 10 minutes at 4°C. The supernatant was transferred to a 1 L flask and a pellet was obtained which represented the cell mass that was disrupted by sonication (Virsonic, Virtis) at 4 psi for 10 minutes. The cell mass (used to obtain the intra-cellular extract) and the supernatant (used to obtain the extra-cellular extract) were agitated in 100 ml of hexane in 1 L flasks on a shaker at 180 rpm for 24 hours at room temperature.

Preparation of the intra-cellular extracts involved using the following protocol. Filtration was used to separate the powder or the cells from the hexane extract which was then 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 residue was dissolved in 10 ml of hexane and transferred to a glass bottle which was covered with foil to prevent light from degrading the compounds. The extract was dried under an air current for 3 days and used for chromatographic analyses. For the extra-cellular extract the hexane fraction was slowly poured out into a flask and concentrated as described above.

2.8.2 Extraction of anthraquinones from hairy root cultures

The hairy root biomass from the experiment described in section 2.6 was macerated using a scalpel. Anthraquinones were extracted by agitating the macerated root mass (used to obtain the intra-cellular extract) and the supernatant (used to obtain the extra-cellular extract) in 200 ml of hexane on a shaker at 180 rpm for three days at room temperature. The hexane extracts were removed and stored in a glass bottle and this procedure was carried out three times. All hexane extracts were 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 were dissolved in 5 ml of hexane and transferred to a glass bottle which was covered with foil. The extracts were dried for 2-3 days under an air current and used for chromatographic analyses.

2.8.3 Profile of compounds and detection of anthraquinones in the field root extract and in plant cell and tissue culture extracts by TLC

Thin layer chromatography (TLC) was performed to compare the compounds and detect anthraquinones in *C. triloba* field root extract and plant cell and tissue culture extracts by using commercial standards; 9,10-Anthracenedione and 1-Hydroxy-4-methylanthraquinone (Sigma-Aldrich, Inc) (1 mg.ml⁻¹ dissolved ethyl acetate). Approximately 10 µl of each standard solution, 50 µl of the field root extract and plant cell and tissue culture extracts were applied to the TLC silica gel plate (Merck TLC F₂₅₄). Hexane: ethyl acetate (90:10) was used as the mobile phase. The developed TLC plate was visualized under ultraviolet light at 312 nm and 264 nm (Camag Universal UV lamp TL-600). The TLC plate was also sprayed with

p-anisaldehyde solution (13.31 ml of anisaldehyde in 250 ml of ethanol and 2.5 ml of H₂SO₄) and heated at 120°C in a oven for 15 minutes as described by Wagner *et al.* (1984)

2.8.4 Quantification of anthraquinones by HPLC

HPLC analysis was carried out according to the method of Fernand *et al.* (2008) using commercial standards. A standard curve was generated with 10, 20, 50, 5, and 2 µg.ml⁻¹ of 9,10- anthracenedione and 1-hydroxy-4-methylanthraquinone (Sigma-Aldrich, Inc) which were dissolved in ethyl acetate. The field root extract, hairy root, shoot and cell suspension culture extracts were dissolved in ethyl acetate. The filtrates of the extracts were used for HPLC analysis. Separation and quantitative analyses of anthraquinones were performed on a Merck- Hitachi LaChrom system (Darmstadt, Germany) consisting of a D7000 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 C18 (2) column, 100 °Å pore size, 5µm particle size, 250×4.6 mm i.d.column containing a guard column (Merck, Darmstadt, Germany). The analytes were eluted isocratically at a flow rate of 0.4 ml.min⁻¹ using an acetonitrile/methanol/buffer (25:55:20, v/v). The buffer used as 10 mM ammonium acetate at pH 6.8. The injection volume was 10 µL.

2.8.5 Preparative TLC

Column chromatography was tried but due the structural similarity of the compounds this method was not used and the compounds were isolated by Preparative TLC (PTLC). The hairy root (cultured in NAA) extract was used for bulking of compounds. In order to isolate the fractions by PTLC, a reference TLC plate was prepared by using the hairy root extract and field root extract according to the method described in section 2.8.3. This plate was not sprayed with p-anisaldehyde solution. The location of the bands (A to I) and fractions (CT01 to CT06) were marked off on the unsprayed plate using a pencil under UV light at 312 nm and 264 nm.

PTLC was performed using the following method. Approximately 100 µl of the hairy root (cultured in NAA) extract was applied in a band across the TLC plate (Merck TLC F₂₅₄) with at least 1 cm on either side. Hexane: ethyl acetate (90:10) was used as the mobile phase. The

developed TLC plate was visualized under ultraviolet light at 312 nm and 264 nm (Camag Universal UV lamp TL-600) and the fractions were marked off with pencil. This plate was compared to the reference plate to ensure that each fraction contained the correct band/s. A scalpel blade was used to scrape off the fractions from the TLC plate. The silica of each fraction was suspended in 20 ml of ethyl acetate in 50 ml centrifuge tubes. The tubes were shaken to dissolve the isolated compounds and then left to stand for 15 minutes for the silica to settle to the bottom. A pipette was used to draw out the compounds which were filtered into glass bottles covered in foil. The bottles were placed under an air current to evaporate the ethyl acetate and the powdered form the fractions was stored at room temperature until required to be used for biological activity assays and EI-LC-MS analysis.

2.8.6 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 C₁₈ column (150 x 2.1 mm, 3.5 µm) maintained at 40⁰C. 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 10. The 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 90⁰C, the expansion region temperature at 80⁰C and the source temperature at 225⁰C. The total volume of post-column eluent was sent to the TMD detector and helium was used as the nebulisation gas at 30 L/h. The TMD detector was tuned every day prior to starting an analysis run and caffeine was injected as the test compound to ensure functionality of the total system. The injection volume was 5 µl for all fractions.

Table 10: 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

2.9 Biological activity of the field root and hairy root extracts and isolated fractions of *C. triloba*

2.9.1 Cytotoxicity of the extracts and isolated fractions on cancer cells

2.9.1.1 Cell Line

Prostate adenocarcinoma cells (DU-145) was used in this study. The DU-145 cell line was provided by Dr Strini Naidoo at Nelson R Mandela School of Medicine, Department of Therapeutics and Medicines Management, University of Kwa-Zulu Natal. The cells were received in a 25 cm² tissue culture flask and were incubated at 37°C in a humidified incubator (Snjiders Hepa, United Scientific group, Cape Town, South Africa) with 5% CO₂. After three days the cells were 80% confluent and they were transferred to a 75 cm² flask (Greiner, Germany).

2.9.1.2 Cell Maintenance

All cell culture procedures were conducted in a laminar flow cabinet (Scientific Engineering INC) which was sterilized by UV-light and swabbing with 70% ethanol (Merck, South Africa) before each use. DU-145 cells were grown in a monolayer with Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Inc) containing glucose (4,5 g.l⁻¹), 1 mM L-glutamine and 1 mM sodium pyruvate. This medium was supplemented with 10% heat inactivated foetal calf serum (FCS) and 1 % antibiotic-antimycotic solution (Sigma-Aldrich, Inc). The cells were maintained in an exponential growth phase by sub-culturing every 2-3

days after the flasks became 80 % confluent. The following protocol was used to sub-culture the cells: the medium was removed from the flasks and the monolayer of cells was washed with 5 ml of phosphate saline buffer (PBS). An aliquot of 1 ml of trypsin was then added to the flasks which were incubated at 37°C in a humidified incubator with 5% CO₂ for three minutes. The flasks were then tapped on the side for 30 seconds to de-attach the monolayer (Figure 12A and 12B). Figure 12C and 12D shows the de-attached DU-145 cells. Thereafter 10 ml of medium was added to each flask and 5 ml of the cell culture was transferred to a new flask. A final aliquot of 20 ml of medium was added the flasks and they were incubated at 37°C in a humidified incubator with 5% CO₂. The cells were monitored for contamination by examining colour changes and turbidity of the media on a daily basis. The cells were also examined under an inverted microscope (Nikon) for cell growth.

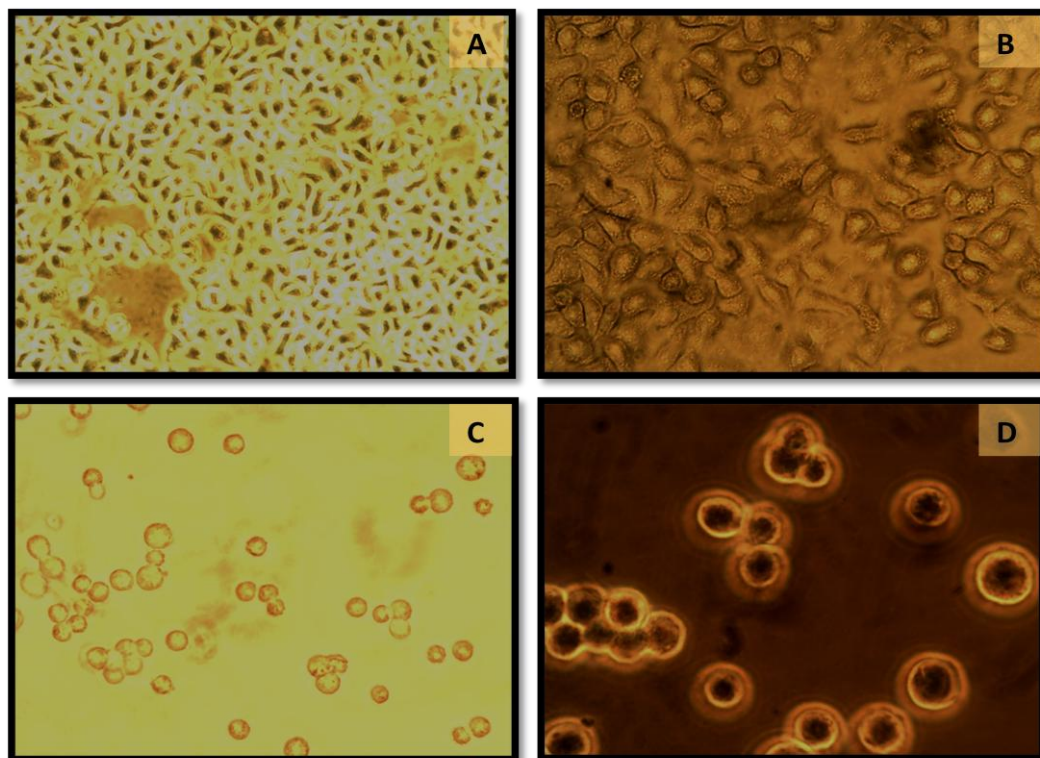


Figure 12: Microscopic observation of the morphology of DU-145 cells. The monolayer showed spindle shaped cells at 100x (A) and 200x (B) magnification. De-attached cells appeared rounded at 100x (C) and 400x (D) magnification.

2.9.1.3 Storage of cells

Cell culture flasks that were 80 % confluent were washed with 5 ml of PBS. The cells were trypsinized as described in section 2.9.1.2. An aliquot of 10 ml of DMEM medium was added to the flasks and the cells were centrifuged at 1500 rpm for 10 minutes to form a pellet. The cells were then re-suspended in 2 ml of cryo-protective medium (10 % DMSO, 10% FBS and 80% DMEM) and aliquots of 1 ml of the cells were added to cryotubes (Corning, South Africa) which was placed on ice to allow for slow cooling. The tubes were transferred to thermos flask and kept overnight at -20°C. The cells were subsequently transferred to a -85°C ultra-freezer and stored until required.

2.9.1.4 Regeneration of cells

Cells were removed from the -85°C ultra-freezer, swabbed with 70% ethanol and rapidly thawed. The cells were then transferred to 20 ml of pre-warmed DMEM (supplemented with 10% FCS and 1 % antibiotic-antimycotic solution) in 75 cm² tissue culture flasks and incubated at 37°C in a humidified incubator with a 5% CO₂ atmosphere.

2.9.1.5 Enumeration of cells

Trypan blue stain is an exclusion dye that is used for counting viable cells. This method is based on the principle that intact viable cells do not take up the dye while non-viable cells with altered membrane do. Therefore, viable cells remain translucent and non-viable cells appear blue. Staining with trypan blue also facilitates the visualization of cell morphology. (Strober, 2001). An aliquot of 100 µl of trypan blue [Biowhittaker, Wakersville (USA)] was mixed with 100 µl of cell suspension culture in a centrifuge tube which was incubated at room temperature for 1 minute. An aliquot 10 µl of the mixture was loaded into the two chambers of the Neubauer haemocytometer. The cells in the centre square and the four 1 mm corner squares of the two chambers were counted. The volume of cell suspension that is available in one primary square is 0.1 mm³ (1.0 mm², 0.1 mm/1.0, 10⁴ ml). Therefore, the following equation was used to determine the number of cells in suspension:

$$\text{Cells.ml}^{-1} = \text{Average number cells in the two chambers} \times 10^2 (\text{dilution factor}) \times 10^4$$

2.9.1.6 Drug treatment

The field root and hairy root extracts and fractions CT01 and CT02 (isolated by PTLC from the extract of hairy root cultures supplemented with NAA) were prepared by dissolving 5 mg of the powdered extract or fraction in 1 ml of DMSO. These stock solutions were further diluted with DMEM to four concentrations ($1000\ \mu\text{g}.\text{ml}^{-1}$, $500\ \mu\text{g}.\text{ml}^{-1}$, $250\ \mu\text{g}.\text{ml}^{-1}$ and $125\ \mu\text{g}.\text{ml}^{-1}$) which were used to treat the cells. An aliquot of 200 μl of DMSO was added to 800 μl DMEM medium and this was used as a solvent control.

2.9.1.7 Cytotoxicity assay

The effect of the field root and hairy root extracts and isolated fractions (CT01 and CT02) on the DU-145 cells were determined by using the MTT assay method. This method is simple, accurate and yields reproducible results. This assay is based on the ability of mitochondrial dehydrogenases such as succinate dehydrogenase present in metabolically active cells to cleave the tetrazolium ring; thus reducing the yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan crystals which can be dissolved by acidified isopropanol or 100 % DMSO. The resulting purple solution is measured spectrophotometrically. The number and the metabolic activity of surviving cells are determined by the quantity of formazan generated by the cells. Therefore the increase or decrease in the number of cells is responsible for the concomitant change in the amount of formazan formed which indicates the degree of cytotoxicity caused by the test compound (Mossman, 1983).

The MTT assay was performed according to the procedure described by Mossman, (1983) with modifications. An aliquot of 200 μl (2×10^5 cells) of the DU-145 cell suspension was pipetted into 96 well microtitre plates. PBS was added to the outer wells to prevent evaporation of the medium. The plates were incubated for 24 hours to allow the cells to attach to the wells. The medium was then removed and the monolayer was washed with PBS. Medium containing the test extract or fraction was added to the wells at four concentrations ($1000\ \mu\text{g}.\text{ml}^{-1}$, $500\ \mu\text{g}.\text{ml}^{-1}$, $250\ \mu\text{g}.\text{ml}^{-1}$ and $125\ \mu\text{g}.\text{ml}^{-1}$). DMSO at a concentration of 0.2 %, in DMEM was added to the wells as the solvent control. DMEM (negative control) was also added to the wells to determine if DMSO had any effect on the cells. The test extract or fraction, solvent control and negative control were tested in replicates of five. The microtitre

plates were incubated for 3 days at 37°C in a humidified incubator with 5% CO₂. At end of the incubation period the medium was replaced with 50 µl of MTT (5 mg.ml⁻¹) and the plates were incubated for four hours at 37°C. Thereafter an aliquot of 100 µl of DMSO was then added to the wells to dissolve the formazan crystals that formed in metabolically active cells. The plates were shaken at room temperature for 2 minutes to ensure complete dissolution of the formazan crystals. The absorbance of the formazan solutions were read at 590 nm using an ELISA plate reader (Digital Analogue Systems, Italy) and the percentage cytotoxicity of the cells was determined by using the following equations:

$$\text{A) \% Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100 \quad \text{B) \% Cytotoxicity} = [100 - \% \text{ Cell viability}]$$

2.9.2 Anti- topoisomerase activity of the isolated fractions

2.9.2.1 Topoisomerase II Drug Screening Kit

The TopoGEN topoisomerase II Drug Screening kit (TopoGEN, Inc, USA) allows for detection of two types of topoisomerase inhibitors. The first type involves those that stimulate the formation of cleavable complexes and the second type involves those that antagonize topoisomerase II action on the DNA. Thus the kit can be used to conduct two types of assays (cleavage complex and catalytic reactions). A known topoisomerase II poison, etoposide which is included in the kit was used a positive control. A DNA substrate (pRYG) that contains a single, high affinity topoisomerase II cleavage and recognition site which is ideal for these studies is also included. Topoisomerase II cuts pRYG DNA primarily at a single site at the 54 base pair of alternating purine/pyrimidine DNA (Spitzner *et al.*, 1990). This is an advantage as it is possible to conserve enzyme, when a DNA substrate which has a high affinity site is used, given that cleavage analysis requires a higher amount of enzyme than catalytic assays. This assay system is based on the evaluating the formation of DNA cleavage products which may be nicked, open circular DNA or linear DNA. The products are resolved by ethidium bromide gel electrophoresis (Figure 13).

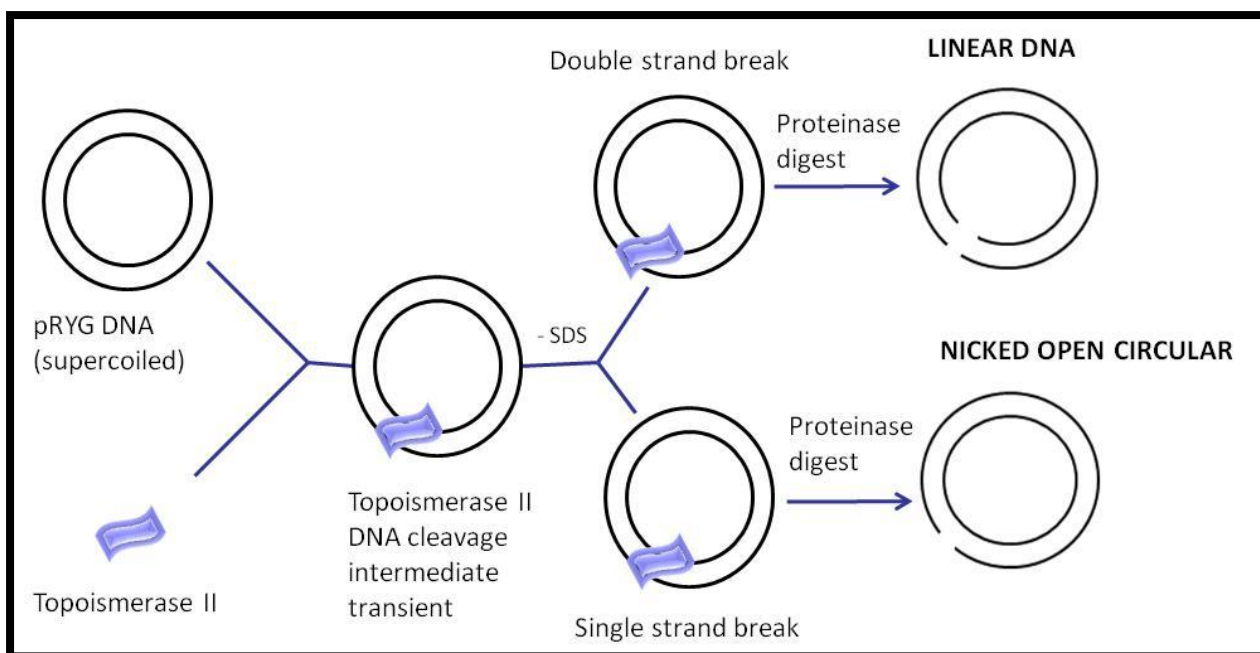


Figure 13: Cleavage pathway of topoisomerase II and pRYG DNA (Topogen manual, cat # 1009-2)

2.9.2.2 Preparation of extracts

Fractions CT01 and CT02 (isolated by PTLC from the extract of hairy root cultures supplemented with NAA) were dissolved in DMSO at four concentrations ($1000 \mu\text{g}.\text{ml}^{-1}$, $500 \mu\text{g}.\text{ml}^{-1}$, $250 \mu\text{g}.\text{ml}^{-1}$ and $125 \mu\text{g}.\text{ml}^{-1}$).

2.9.2.3 Enzyme and DNAs

Homogeneously purified human topoisomerase II (Cat# 2000H; TopoGEN, Inc, USA) and pRYG DNA (substrate) was used in the study. The topoisomerase drug screening kit (Cat # 1009-2; TopoGEN, Inc, USA) was used to conduct the enzyme assays by using a modified protocol described by Marini *et al.* (1980).

2.9.2.4 Enzyme assay

The kit contains a catalytic buffer that is used to test for catalytic activity and a cleavage buffer that is used to test for cleavage complex formation. The latter is more difficult to detect as higher levels of the enzyme is required for cleavage complex formation (typically 2-6 units per reaction). In our studies cleavage complex reactions were conducted. The reactions were assembled on ice in micro-centrifuge tubes (water, buffer, DNA, test fraction/compound and enzyme). A sample reaction is shown in Table 11. The reaction volumes (20 μ l) were limited to the volume that could be loaded into the wells of the agarose gel. After the enzyme was added, the tubes were transferred to a heating block to initiate the reaction. Reactions were incubated for 40 minutes at 37°C and terminated at 37°C by rapid addition of 1/10 volume (2 μ l) of 10 % SDS to facilitate trapping of the enzyme in the cleavage complex. The reactions were not cooled, heated or treated with high salt prior to the addition of SDS as these actions can alter the topoisomerase II breakage and resealing equilibrium and the breaks can reseal. Proteinase K solution was then added at 50 μ g. ml⁻¹ (1.1 μ l of a 1 mg.ml⁻¹ solution) to digest the enzyme at 37°C for 20 minutes. An aliquot of 0.1 vol. (1.2 μ l) of 10 x gel loading buffer (0.25 % bromophenol blue, 50 % glycerol) was added to the samples and they were cleaned by extraction with the addition of 20 μ l of chloroform: isoamyl alcohol (CIA) (24:1). The samples were spun for 5 seconds in a microfuge and the upper blue coloured aqueous phase was withdrawn and loaded onto a 0.8 % agarose gel. The agarose gel was run at 1.5-2 V/cm (measured between electrodes) (70 V) until the dye reached the bottom of the gel. (The running buffer was 1x TAE buffer, prepared from a 50x stock solution [242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA made up to 1 L solution with milli Q water and the pH was adjusted to 7 with HCL]). The gel was stained with 0.5 μ g.ml⁻¹ ethidium bromide solution (5 μ l of a 10 mg.ml⁻¹ stock solution per 100 ml of distilled water) for 30 minutes and results were photo-documented. This method involved the use of a non-ethidium bromide gel which is optimal for resolving relaxed and supercoiled DNA.

Table 11: A sample reaction (20µl) is shown below

H₂O	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 fraction/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	4 µl (4 Units/µl)

CHAPTER 3: RESULTS

3.1 Seed germination and shoot culture

The optimum exposure time to NaClO for maximum seed germination and minimum contamination was determined by exposing the *C. triloba* seeds to 30% NaClO for 0, 5, 10, 15 and 20 minutes. It was found that the percentage of seed germination decreased as the exposure time of the seeds to NaClO increased. Seeds treated for 5 minutes had 33% germination but 47% of seeds were contaminated. At a 10 minute exposure time 26% of the seeds germinated and 6% were contaminated. Seeds treated for 15 minutes showed only 6% germination and 6% of seeds were contaminated. Seeds treated for 20 minutes had 0% germination. These results are shown in Figure 14. Hence, an exposure time of 10 minutes to 30% NaClO was chosen to sterilize *C. triloba* seeds. The sterilized seeds were germinated on MS medium for two to three weeks (Figure 15A). Thereafter the seedlings were grown on MS medium containing 1 mg.l⁻¹ 6-BAP and 0.5 mg.l⁻¹ IAA for shoot development (Figure 15B).

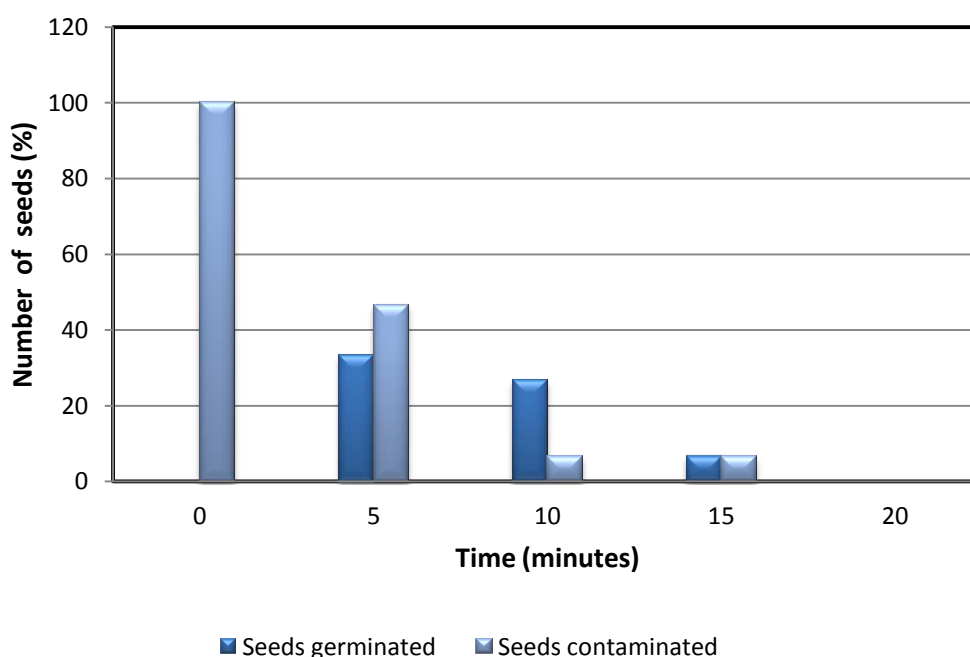


Figure 14: Percentage of seeds that germinated and the percentage of seeds that were contaminated after treatment with 30% NaClO.

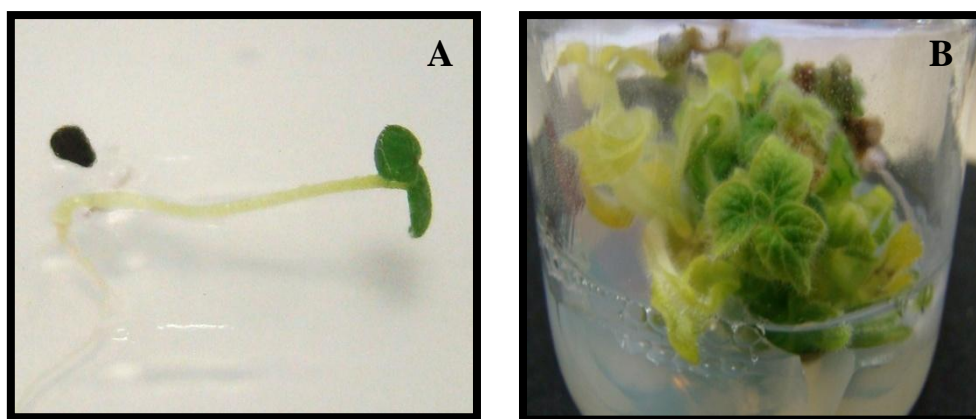


Figure 15: Germinated seedling (3 weeks old) of *C. triloba* on MS medium containing no hormone (A). Shoot cultures (45 day old) of *C. triloba* on MS medium containing 1 mg.l^{-1} of 6-BAP and 0.5 mg.l^{-1} IAA (B).

3.2 Callus culture

Callus initiation was observed on the surface or cut ends of the leaf explants after 2-3 weeks of inoculation. White crystal type callus cells were viewed under the inverted microscope during the early stages of the culturing period (results not shown). Callus cultures induced from *C. triloba* leaf explants on MS medium were orange-yellow in colour (Figure 16A and 16B). Sub-cultured callus tissue produced root hairs and root-like structures after three weeks (Figure 16C and 16D). The tips of the root-like structures appeared orange when viewed under the inverted microscope (results not shown). Thus callus tissue was successfully maintained on MS medium.

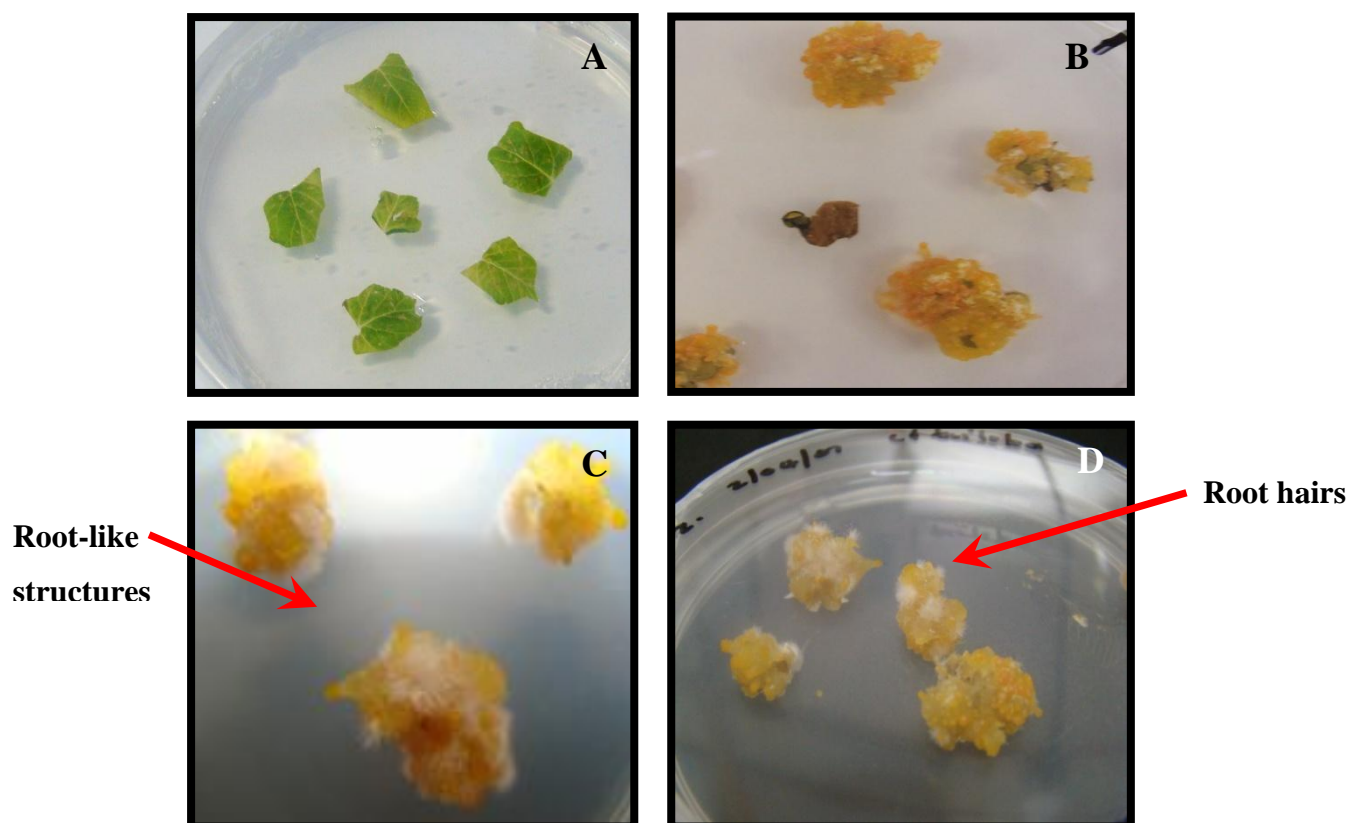


Figure 16: *C. triloba* leaf explants from shoot cultures were placed on MS medium (A) and orange-yellow calli were induced (B). These were sub-cultured and root hairs and root-like structures developed on the calli (C and D).

3.3 Cell suspension culture

When callus cultures were transferred into flasks containing MS liquid medium, the friable callus tissue dispersed into small aggregates after the flasks were agitated on the shaker. Actively growing cell aggregates were observed under the inverted microscope at 400x magnification in the inoculum at a 50 ml scale (results not shown). This allowed cell suspension cultures to be scaled up to a 200 ml scale. After one month of cultivation the cell suspension cultures turned from orange to brown in colour (Figure 17A and 17B) and after two months the total biomass of cell suspension cultures increased from 4 g to 134.18 g (wet weight).

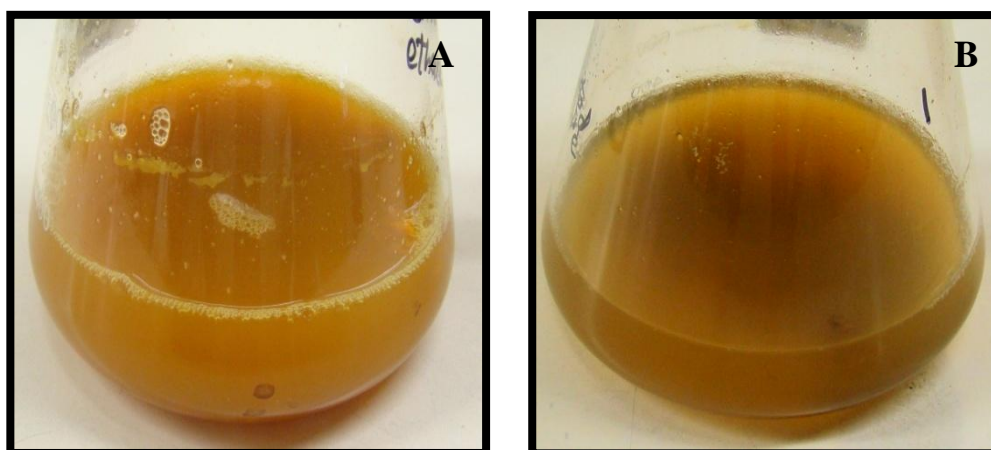


Figure 17: Cell suspension culture was orange in colour after one month (A) and thereafter turned brown after two months (B).

3.4 Hairy root culture

The transformation ability of *A. rhizogenes* 15834 was tested against *C. triloba* leaf and stem explants which were obtained from shoot cultures. Leaf explants were not susceptible to *A. rhizogenes* infection as no hairy roots emerged from wounded regions when a concentration of 1, 0.2 and 0.5 OD of bacteria was used. Stem explants were susceptible to *A. rhizogenes* infection at an OD of 0.2. Hairy roots emerged from the infected stem explants after two weeks of inoculation. More than 73% of stem explants were transformed after three weeks (Table 12). Higher concentrations of agrobacterium killed the stem explant tissue as it turned brown and no hairy roots were induced. After four weeks the hairy roots became highly branched (Figure 18A and 18B) and were sub-cultured to MS medium containing 500 mg.l⁻¹ cefotaxime. Hairy roots kept on this medium for more than five days resulted in browning and they stopped growing. Therefore, they were transferred onto MS medium containing a lower concentration of cefotaxime (200 mg.l⁻¹). After eight days on this medium, the sterility test showed that the hairy roots were sterile as no bacterial colonies grew on YEP solid medium.

Table 12: Concentration of *A. rhizogenes* used to transform *C. triloba* and the transformation efficiency of leaf and stem explants

Explant	Concentration of bacteria (OD)	No. of explants transformed	Transformation efficiency (%)
Leaf	Control	None	0
	1	None	0
	0.5	None	0
	0.2	None	0
	Control	None	0
Stem	1	None	0
	0.5	None	0
	0.2	11	73.33
	Control	None	0



Figure 18: *C. triloba* hairy roots were induced on MS medium and became highly branched after 4 weeks (A). The hairy roots emerged from stem explants (B).

3.5 Effect of auxins on hairy root biomass production

Hairy roots were cultured in MS liquid medium containing no hormone (control culture) and 1 mg.l^{-1} of IAA or 1 mg.l^{-1} of NAA. Hairy root cultures containing no hormone did not grow well as the entire hairy root biomass turned brown after four weeks (Figure 19A, 19B and 20). In contrast, cultures that contained IAA or NAA turned brown while their lateral roots were yellow in colour and continued growing (Figures 19C, 19D, 19E, 19F). Hairy root cultures containing no hormone and 1 mg.l^{-1} of IAA or 1 mg.l^{-1} of NAA produced a mean biomass of 1.03 g, 23.91 g and 46.13 g, respectively (Figure 20). This was a 23.21 and a 44.79 fold increase in the mean biomass of cultures containing hormones in comparison to the control culture. Thus MS medium containing NAA produced the highest mean biomass (46.13 g) (Figure 20 and 19F).

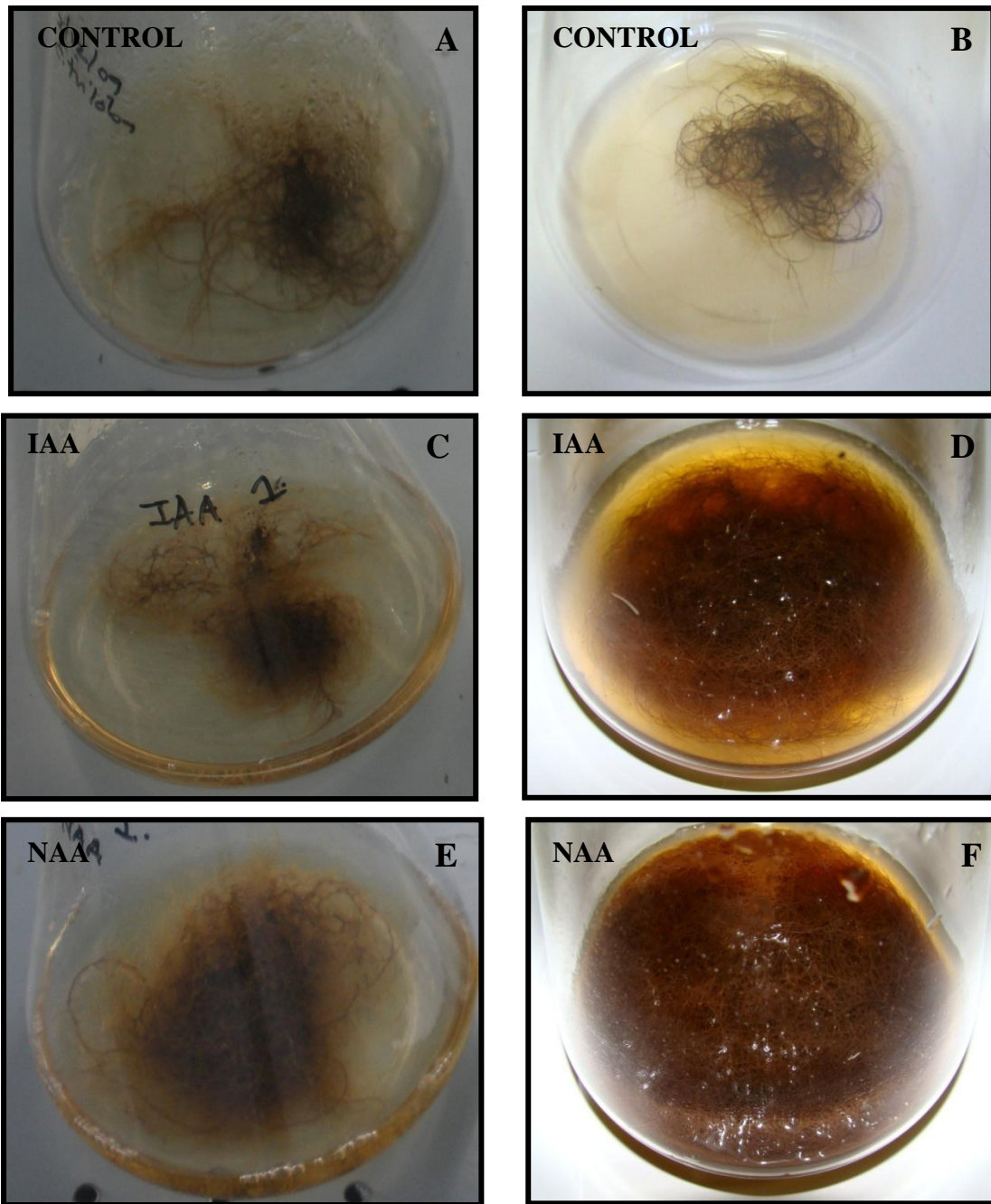


Figure 19: Hairy root cultures cultivated MS medium containing no hormone (A and B) and 1 mg.l⁻¹ of IAA (C and D) or 1 mg.l⁻¹ of NAA (E and F). One month old cultures (A, C, E); two month old cultures (B, D, F).

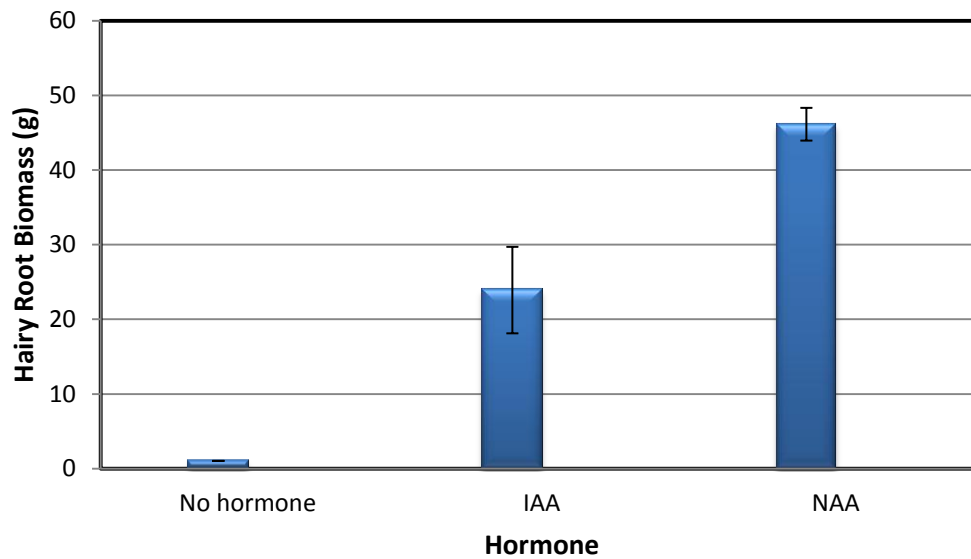


Figure 20: Mean biomass of hairy root cultures (n=2) cultivated in MS medium containing no hormone (control) and 1 mg.l⁻¹ of IAA or 1 mg.l⁻¹ of NAA.

3.6 Morphological characteristics of adventitious and hairy roots

Hairy roots cultured on MS medium containing no hormone were slow growing. Therefore, the morphology of adventitious roots (non-transformed roots) and hairy roots were examined to ensure that the hairy roots induced were as result of *Agrobacterium* transformation. Adventitious roots were induced in *C. triloba* shoot cultures using MS medium containing 1 mg.l⁻¹ of IBA (Figure 21A). Morphologically, these roots were highly branched and were white in colour. In contrast, the hairy roots cultured with no hormone or with NAA were less branched and turned brown as the biomass increased in the plate (Figure 21B and 21C). Hence, based on the morphological features it can be assumed that in this study the induced hairy roots resulted from *Agrobacterium* transformation.

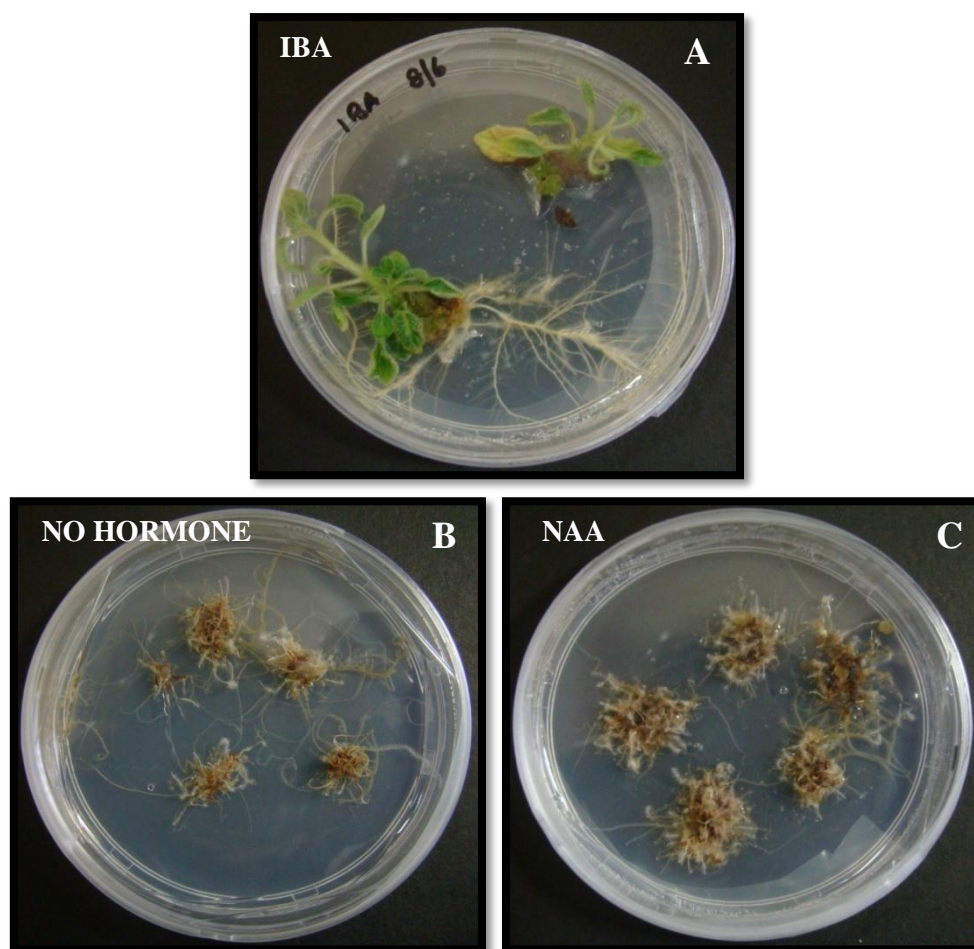


Figure 21: Adventitious roots induced from *C. triloba* shoot culture on MS medium supplemented with 1 mg.l⁻¹ of IBA (A). Hairy root cultures cultivated on MS medium containing no hormone (B). Hairy root cultures cultivated on MS medium supplemented with 1 mg.ml⁻¹ of NAA (C).

3.7 Profile of compounds and detection of anthraquinones in the field root extract and plant cell and tissue culture extracts by TLC

The *R_f* value profiles of the extracts from *C. triloba* field roots and hairy root, shoot and cell suspension cultures were compared by visualization of the developed TLC plate under UV light at 312 nm (Figure 22A) and 264 nm (Figure: 22B); and by using p-anisaldehyde spray reagent (shows bands A to I in Figure 22C). These results tabulated in Table 13.

The TLC plate visualized under UV light at 312 nm showed that the field root extract profile contained 6 bands, the hairy root extract profiles contained 7-11 bands and the shoot and cell suspension culture extract profiles contained 3-7 bands (Figure 22A).

The TLC plate visualized under UV light at 264 nm showed the positions of the commercial standards; 9,10-Anthracenedione (AN) and 1-Hydroxy-4-methylanthraquinone (MA) which had an R_f value of 0.55 and 0.60, respectively (Figure 22B). The R_f value of band D (0.55) in the hairy root and cell suspension culture extracts correlated to R_f values of these standards (Figure 22B). Individual anthraquinones could not be identified due the similarity of the R_f values of the standards.

There were three other bands that had a R_f value similar to the anthraquinone standards these were: band A (0.71) (Figure 22A) which was present in field root and hairy root extracts, band B (0.70) (Figure 22B) which was present only in hairy root culture extracts and band C (0.64) (Figure 22B) which was present in the field root and hairy root, shoot and cell suspension culture extracts.

The four bands (band A, B, C and D) of interest were predominant in the extract of hairy roots cultivated in the presence of NAA. Therefore Preparative TLC (PTLC) was performed using this extract and the all four bands were scraped off the plate. Because of co-migration bands A, B and C were isolated together and were coded as fraction CT01 and band D was isolated separately and was coded as fraction CT02. These two fractions were used to conduct the MTT and anti-topoisomerase assay as they correlated to the anthraquinone standards.

Four other fractions; CT03 (band E), CT04 (band F and G), CT05 (band H) and CT06 (band I) (Figure 23) were also isolated from the hairy root (supplemented with NAA) extract by PTLC, their R_f values are shown in Table 13. Figure 23 shows the bands and fractions marked on the unsprayed TLC plate which was used as a reference to isolate each fraction by PTLC.

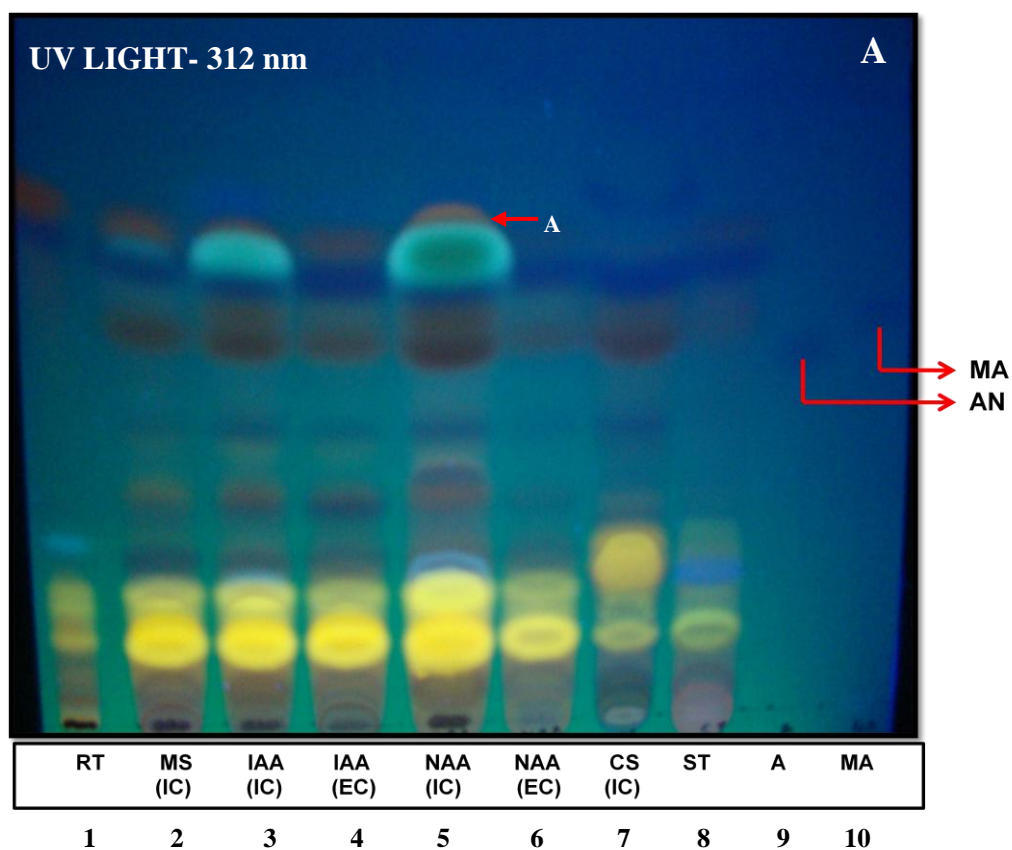


Figure 22: (continued)

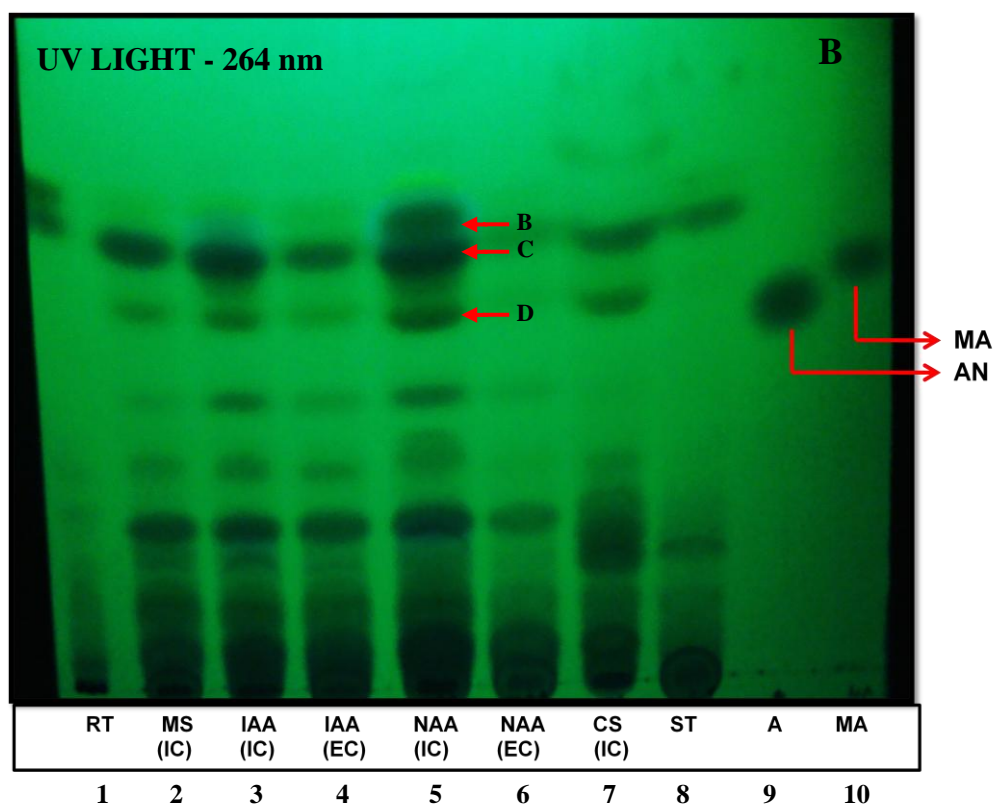


Figure 22: (continued)

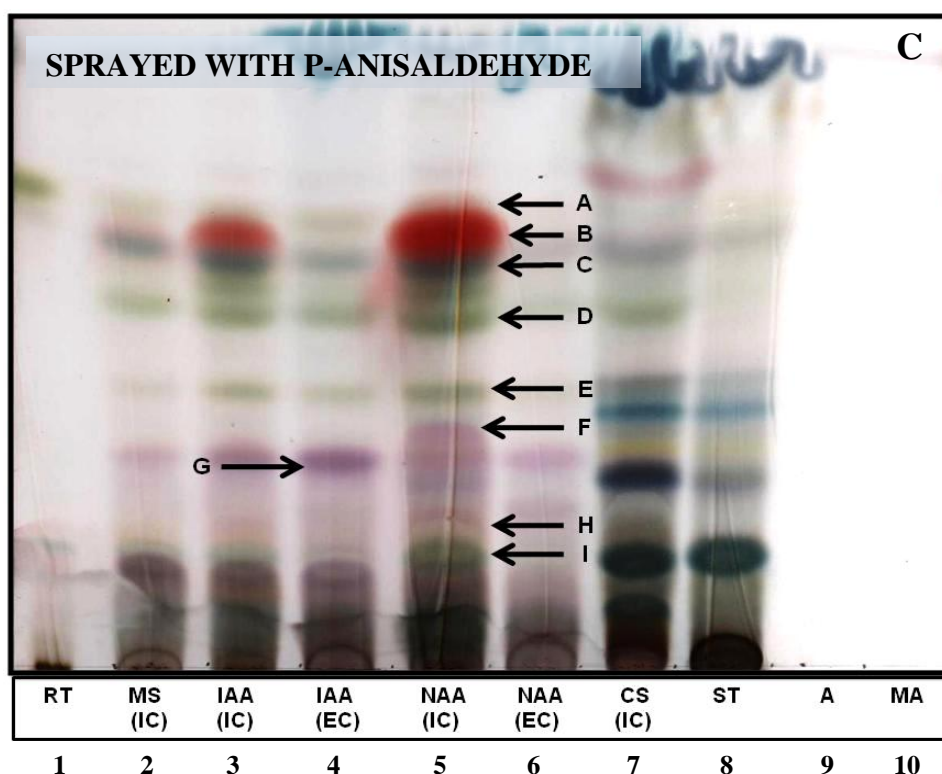


Figure 22: Separation of compounds and detection of anthraquinones from the field root extract and plant cell and tissue culture extracts of *C. triloba*. The TLC profiles of the field root extract and hairy root, shoot and cell suspension culture extracts of *C. triloba* were compared under UV light at 312 nm (A) and 264 nm (B) and by using p-anisaldehyde spray reagent (C). Field root extract (RT) (lane 1). Hairy root extract from the cultures cultivated with no hormone (lane 2), hairy root extracts from the cultures supplemented with IAA or NAA (lane 3-6). Cell suspension culture extract (CS) (lane 7). Shoot culture extract (ST) (lane 8). 9,10-Anthracenedione (AN) (lane 9), 1-Hydroxy-4-methylanthraquinone (MA) (lane 10). Intra-cellular extract (IC), Extra-cellular extract (EC).

Table 13: R_f values of the TLC bands from *C. triloba* field root and plant cell and tissue culture extracts

Plant Material	Hormones added to medium	Fraction CT01 (Band A)	Fraction CT01 (Band B)	Fraction CT01 (Band C)	Fraction CT02 (Band D)	Fraction CT03 (Band E)	Fraction CT04 (Band F)	Fraction CT04 (Band G)	Fraction CT05 (Band H)	Fraction CT06 (Band I)
Field roots		0.74	-	0.67	-	-	-	-	-	0.17
Hairy roots	No hormones (control) (IC)	0.71	0.65	0.64	0.56	0.44	-	0.34	0.24	0.17
	IAA (IC)	0.70	0.70	0.62	0.55	0.44	-	0.34	0.24	0.18
	IAA (EC)	0.68	-	0.63	0.55	0.44	-	0.33	0.24	0.17
	NAA (IC)	0.72	0.70	0.63	0.54	0.44	0.38	0.34	0.25	0.17
	NAA (EC)	-	-	0.64	0.55	-	-	0.33	0.24	0.17
Cell suspension	2,4-D and 6-BAP (IC)	-	-	0.64	0.55	-	-	0.34	-	0.19
Shoot culture	6-BAP and IAA	-	-	0.64	-	-	-	-	-	0.20

IC (Intra-cellular extract), EC (Extra-cellular extract). The bands are representative of the TLC plate shown in **Figure 22A and 22C**.

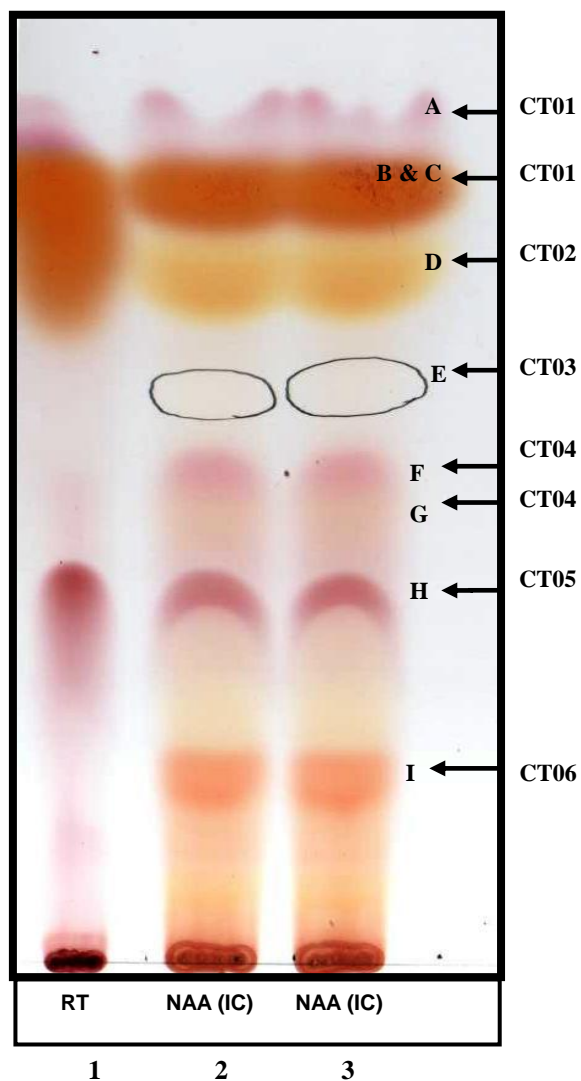


Figure 23: Unsprayed TLC plate showing the bands and fractions from *C. triloba* field root and hairy root (supplemented with NAA) extracts. Fraction CT03 was marked under UV light at 264 nm. Field root extract (RT) (lane 1), hairy root extract from the cultures supplemented with NAA (lane 2 and 3). Intra-cellular extract (IC).

3.8 Quantification of anthraquinones by HPLC

HPLC analysis showed that the 9,10-Anthracenedione and 1-Hydroxy-4-methylanthraquinone standards eluted at retention times (Rt) of 3.72-3.75 minutes and 4.07-4.12 minutes, respectively (Figure 24 and 25). The field root extract showed peak at 3.57 minutes (Figure 26). The hairy root culture extracts showed a peak between 3.60-3.70 minutes (Figures 27, 28, 29). The shoot and cell suspension cultures extracts showed a peak at 3.55 and 3.68 minutes, respectively (Figure 30 and 31). Thus anthraquinones were present in the plant cell and tissue culture extracts. However, individual anthraquinones were not identified due to co-elution and therefore, the total amount of anthraquinones was calculated.

The concentration of anthraquinones in *C. triloba* field root extract and plant cell and tissue culture extracts were calculated by using the standard curve of 9,10-Anthracenedione (Table 14). Hairy root cultures supplemented with IAA ($125.03 \mu\text{g}.\text{mg}^{-1}$ [intra-cellular extract]) or NAA ($98.25 \mu\text{g}.\text{mg}^{-1}$ [intra-cellular extract]) produced a higher concentration of anthraquinones compared to the control culture ($13.33 \mu\text{g}.\text{mg}^{-1}$ [intra-cellular extract]). This was a 9.38 and a 7.37 fold increase, respectively. Also hairy root cultures supplemented with IAA or NAA produced a higher concentration of anthraquinones compared to the field roots of *C. triloba* ($33.51 \mu\text{g}.\text{mg}^{-1}$). This was a 3.73 and a 2.93 fold increase, respectively. Shoot ($3.23 \mu\text{g}.\text{mg}^{-1}$) and cell suspension cultures ($13.17 \mu\text{g}.\text{mg}^{-1}$) produced a lower concentration of the anthraquinones in comparison to the field roots and hairy roots (supplemented with hormones). Therefore, the hairy root culture extracts were used to test the anthraquinones by using bioactivity assays.

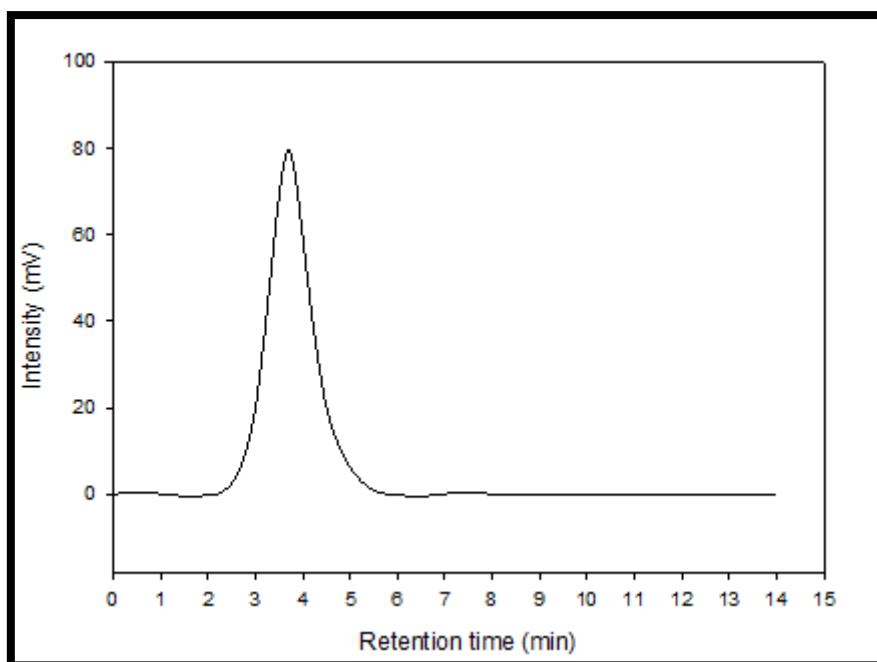


Figure 24: HPLC chromatogram showing 9.10-Anthracedione eluted at 3.72 minutes.

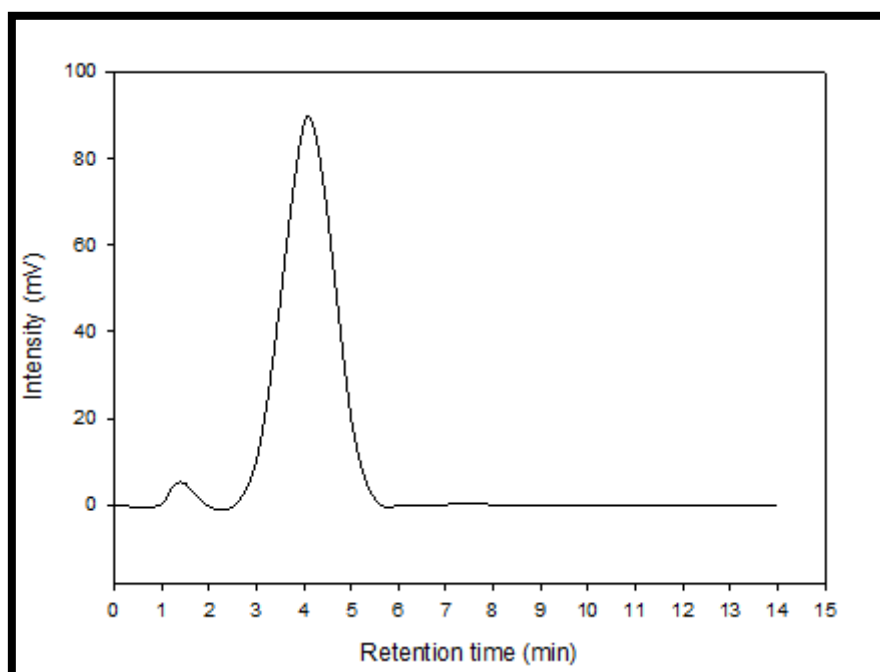


Figure 25: HPLC chromatogram showing 1-Hydroxy-4-methylanthraquinone eluted at 4.07 minutes.

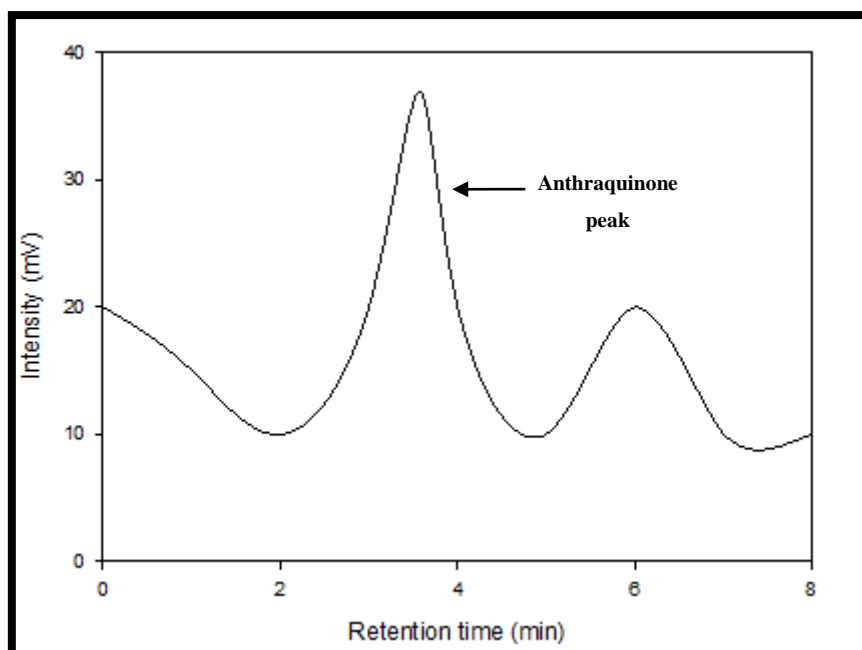


Figure 26: HPLC chromatogram of *C. triloba* field root extract showing the anthraquinone peak eluted at 3.57 minutes.

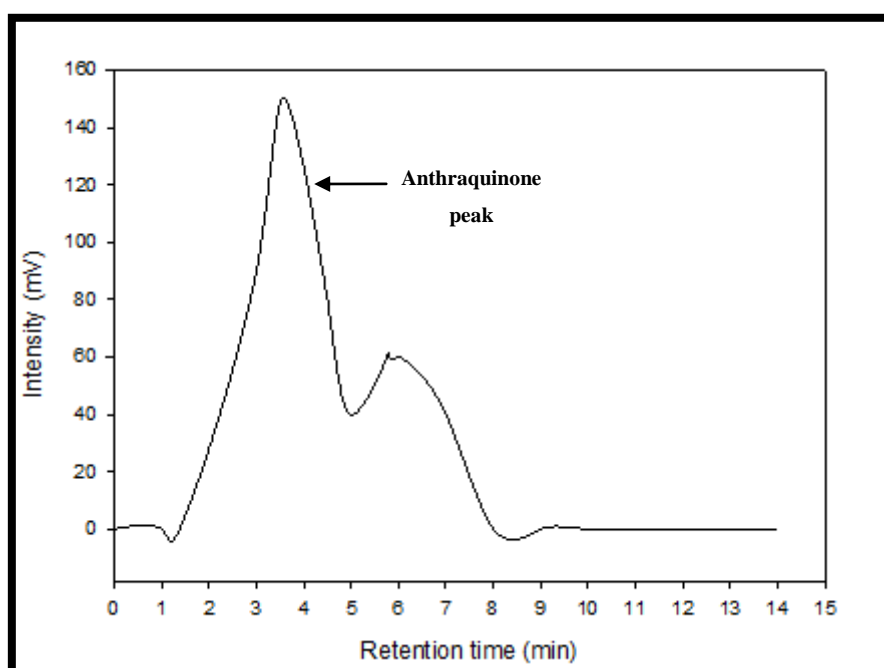


Figure 27: HPLC chromatogram of the hairy root (cultivated in MS medium without hormone) intra-cellular extract showing the anthraquinone peak eluted at 3.60 minutes.

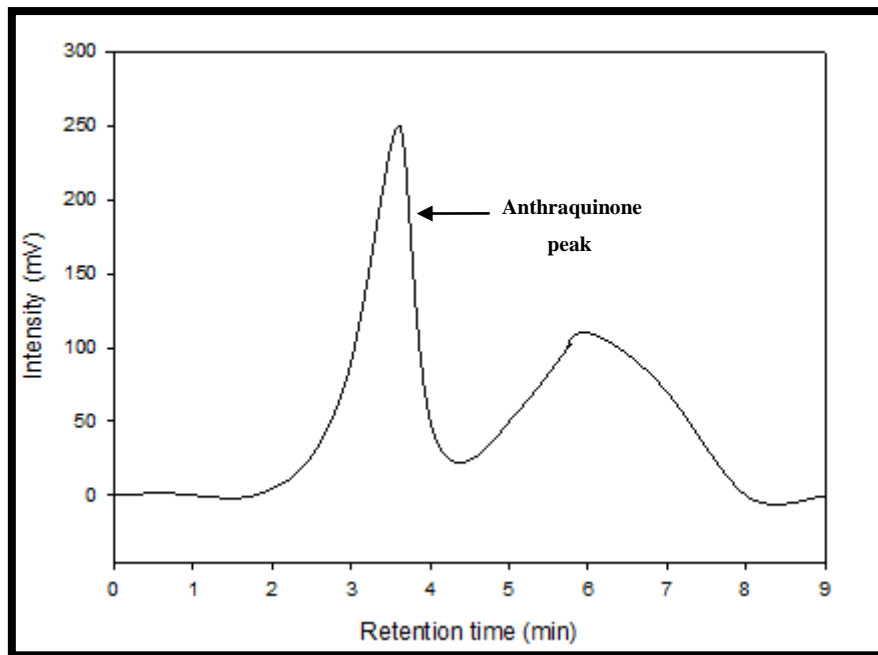


Figure 28: HPLC chromatogram of the hairy root (cultivated in MS medium supplemented with IAA) intra-cellular extract showing the anthraquinone peak eluted at 3.62 minutes.

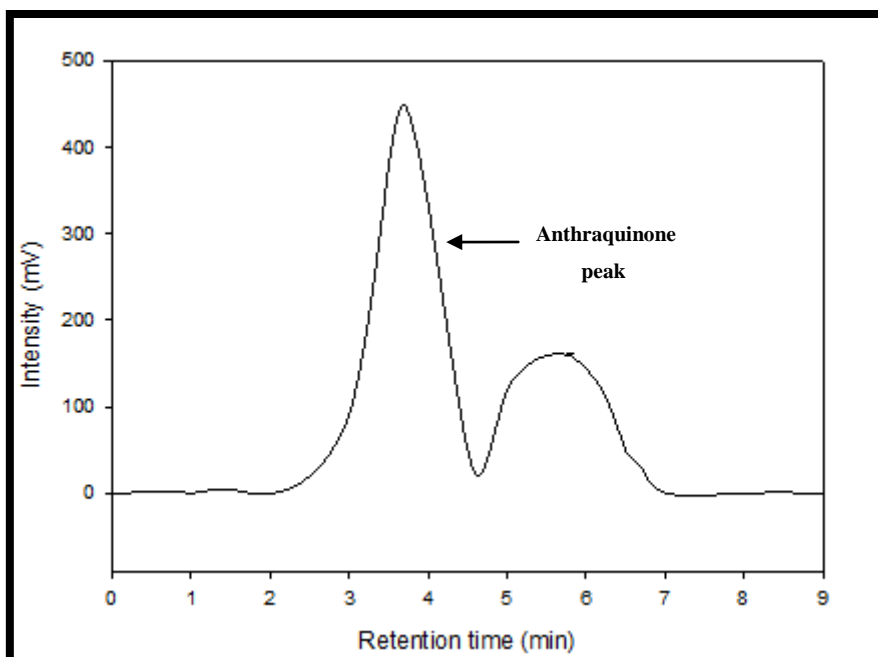


Figure 29: HPLC chromatogram of the hairy root (cultivated in MS medium supplemented with NAA) intra-cellular extract showing the anthraquinone peak eluted at 3.70 minutes.

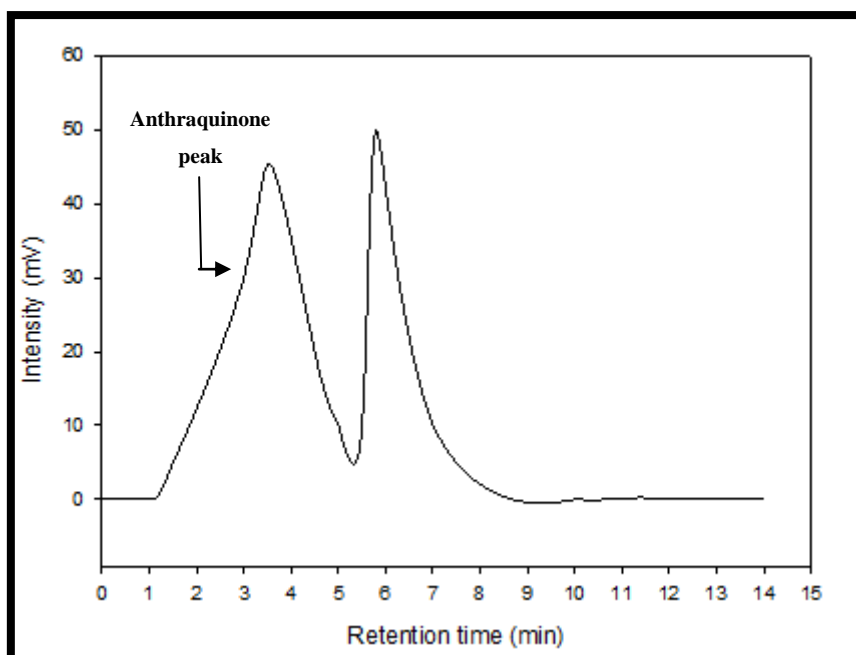


Figure 30: HPLC chromatogram of the shoot culture extract showing the anthraquinone peak eluted at 3.55 minutes.

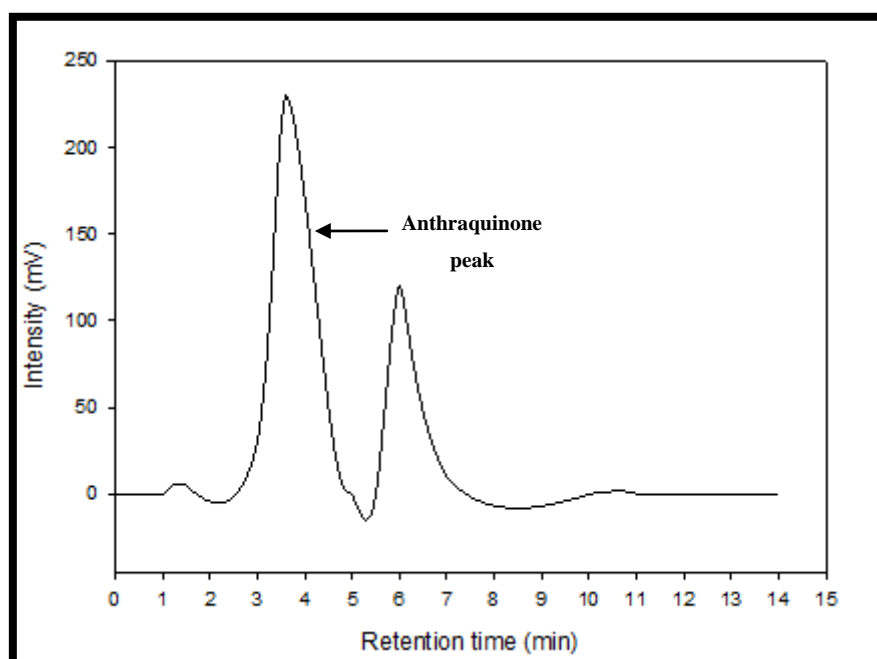


Figure 31: HPLC chromatogram of the cell suspension culture intra-cellular extract showing the anthraquinone peak eluted at 3.68 minutes.

Table 14: Concentration and yield of anthraquinones from *C. triloba* field root and plant cell and tissue culture extracts

Plant material	Hormones	Extract (mg)	Anthraquinones ($\mu\text{g}\cdot\text{mg}^{-1}$)	Yield of anthraquinones in the extract (mg)	Fold increase (comparison to field roots)
Natural roots		14	33.51	0.47	-
Hairy roots	No hormones (control) (IC)	37.7	13.33	0.50	-
	IAA (IC)	38.8	125.03	4.85	3.73
	IAA (EC)	13.3	0.0037	0.00005	-
	NAA (IC)	81.4	98.25	8.00	2.93
	NAA (EC)	5.3	117.23	0.62	3.50
Cell suspension	2,4-D and 6-BAP (IC)	53.1	13.17	0.70	-
Shoot culture	6-BAP and IAA	39.6	3.23	0.13	-

IC (Intra-cellular extract), EC (Extra-cellular extract). Fold increase is representative of the increase in the concentration of anthraquinones found in hairy roots cultures compared to *C. triloba* field roots.

3.9 Preparative High Performance Liquid Chromatography and UV-Vis spectroscopy of compounds isolated from hairy roots

The fractions isolated by Preparative Thin Layer Chromatography (PTLC) were coded as CT01, CT02, CT03, CT04, CT05 and CT06. These fractions did not contain pure compounds. Therefore, Preparative High Performance Liquid Chromatography (PHPLC) was used to separate the compounds in fractions CT01 to CT06. Figures 32 to 36 shows the PHPLC chromatograms of the fractions of interest (CT01 to CT05) and Table 15 shows that these five fractions contained similar compounds which eluted retention times between 15.753 to 27.6919 minutes. The compounds were further analyzed by scanning for the maximum absorption wavelength in the UV light range between 200-600 nm. Figures 37 to 41 illustrates the UV scans and the results are summarized in Table 15. All retention times were measured in minutes.

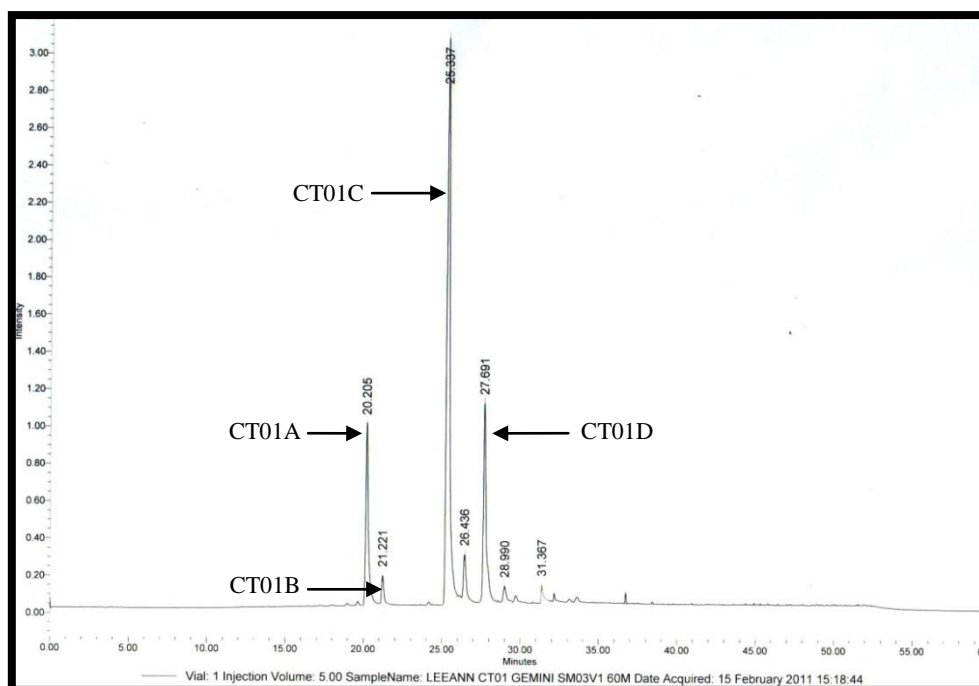


Figure 32: Preparative High Performance Liquid Chromatography of fraction CT01 showing the presence of four distinct compounds of interest at varying retention times. CT01A- Retention time (Rt) = 20.2082, CT01B- Rt = 21.2212, CT01C- Rt = 25.3353 and CT01D- Rt = 27.6919.

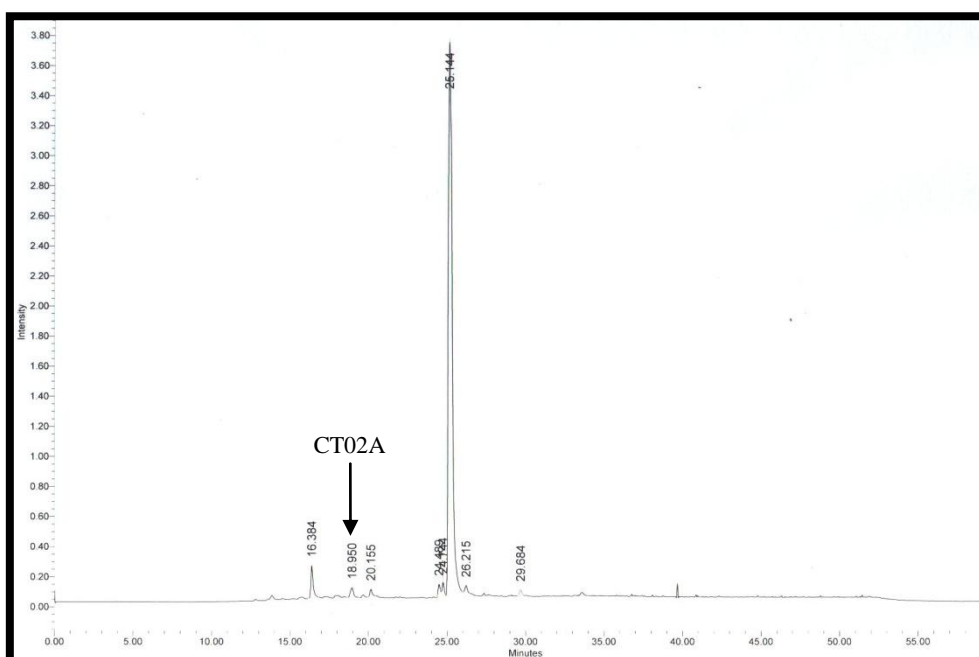


Figure 33: Preparative High Performance Liquid Chromatography of fraction CT02 showing the presence of a compound of interest. CT02A-Retention time (Rt) = 18.9471.

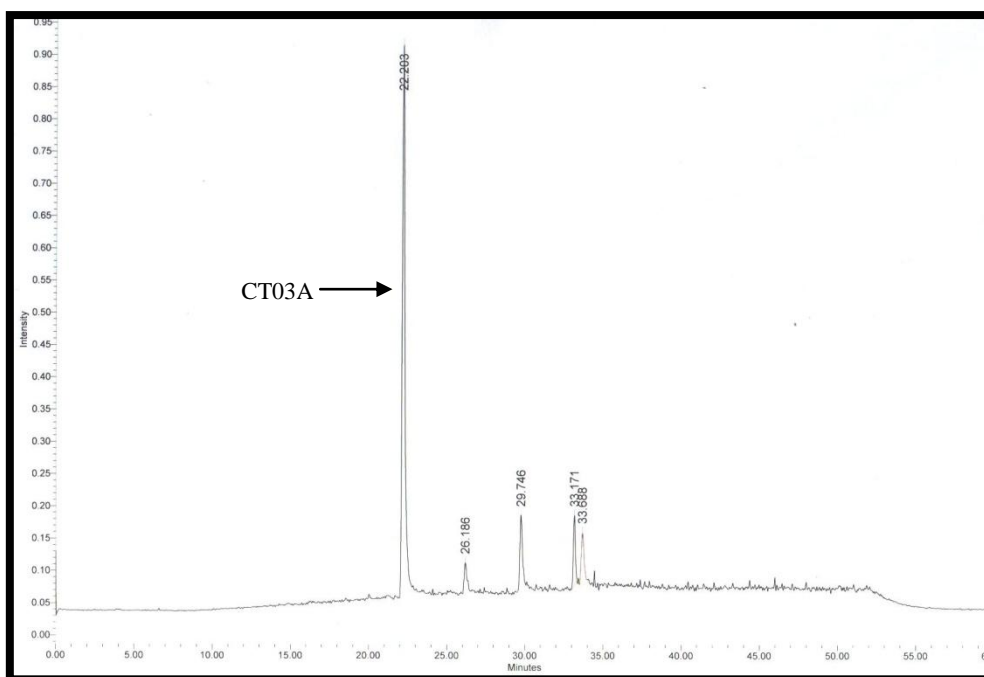


Figure 34: Preparative High Performance Liquid Chromatography of fraction CT03 showing the presence of one distinct compound of interest. CT03A- Retention time (Rt) = 22.2032.

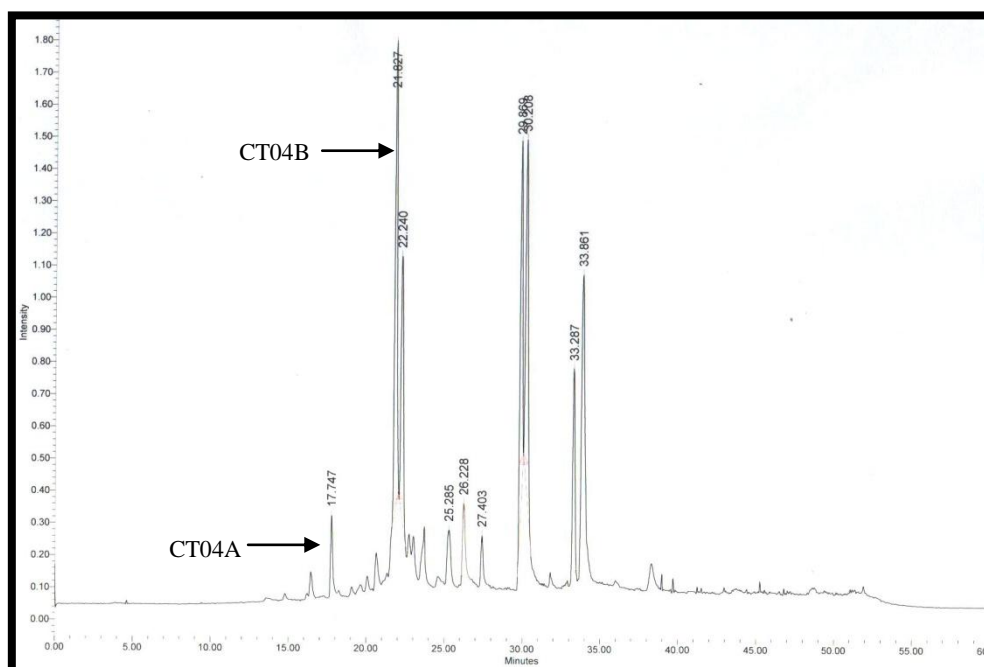


Figure 35: Preparative High Performance Liquid Chromatography of fraction CT04 showing the presence of two distinct compounds of interest at varying retention times. CT04A-Retention time (Rt) =17.7481 and CT04B- Rt = 21.831.

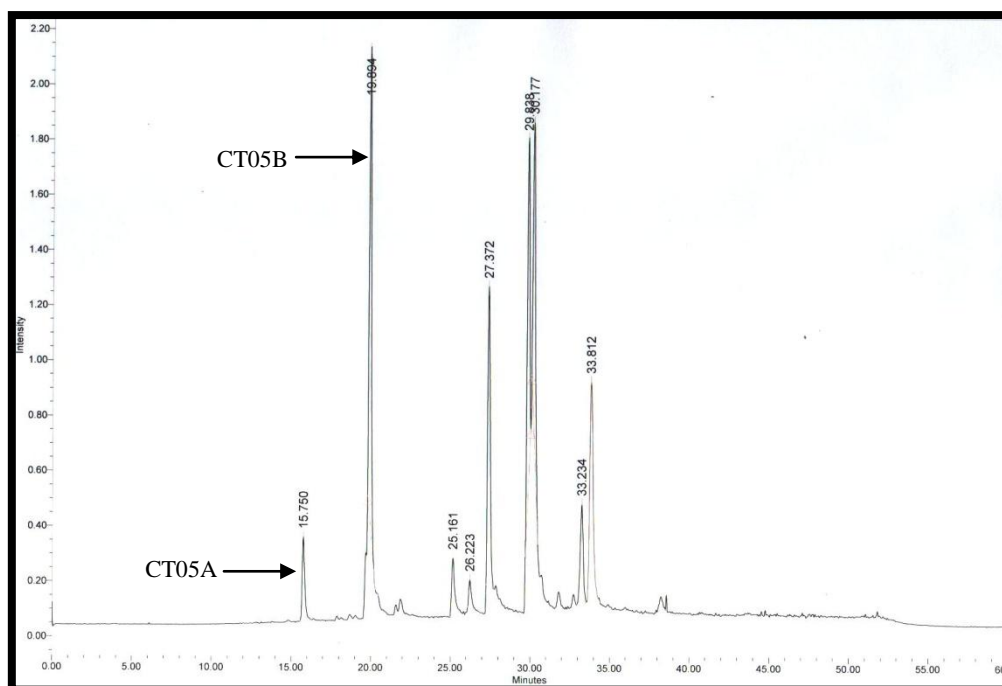


Figure 36: Preparative High Performance Liquid Chromatography of fraction CT05 showing the presence of two distinct compounds of interest. CT05A Retention time (Rt) = 15.750 and CT05B- Rt =19.8981.

Table 15: Summary of the PHPLC peaks of interest of fractions CT01 to CT05 from *C. triloba* hairy roots

Fractions and compounds	Retention time (Rt) in P-HPLC	Wavelengths (nm) of the peaks of interest in UV-Vis spectral analysis
CT01		
CT01A	20.2082	267.5, 413.9
CT01B	21.2212	259.3, 300.7, 355.4
CT01C	25.3353	205.2, 256.9, 330.4, 513.1
CT01D	27.6919	256.9, 329.2, 411.5
CT02		
CT02A	18.9471	206.4, 256.9, 328.0, 504.6, 522.8
CT03		
CT03A	22.2032	256.9, 360.9
CT04		
CT04A	17.7481	205.2, 268.7, 331.6, 365.9, 517.9, 553.2
CT04B	21.831	256.9, 330.4, 410.3
CT05		
CT05A	15.753	251.0, 278.2, 332.8
CT05B	19.8981	205.2, 256.9, 392.2

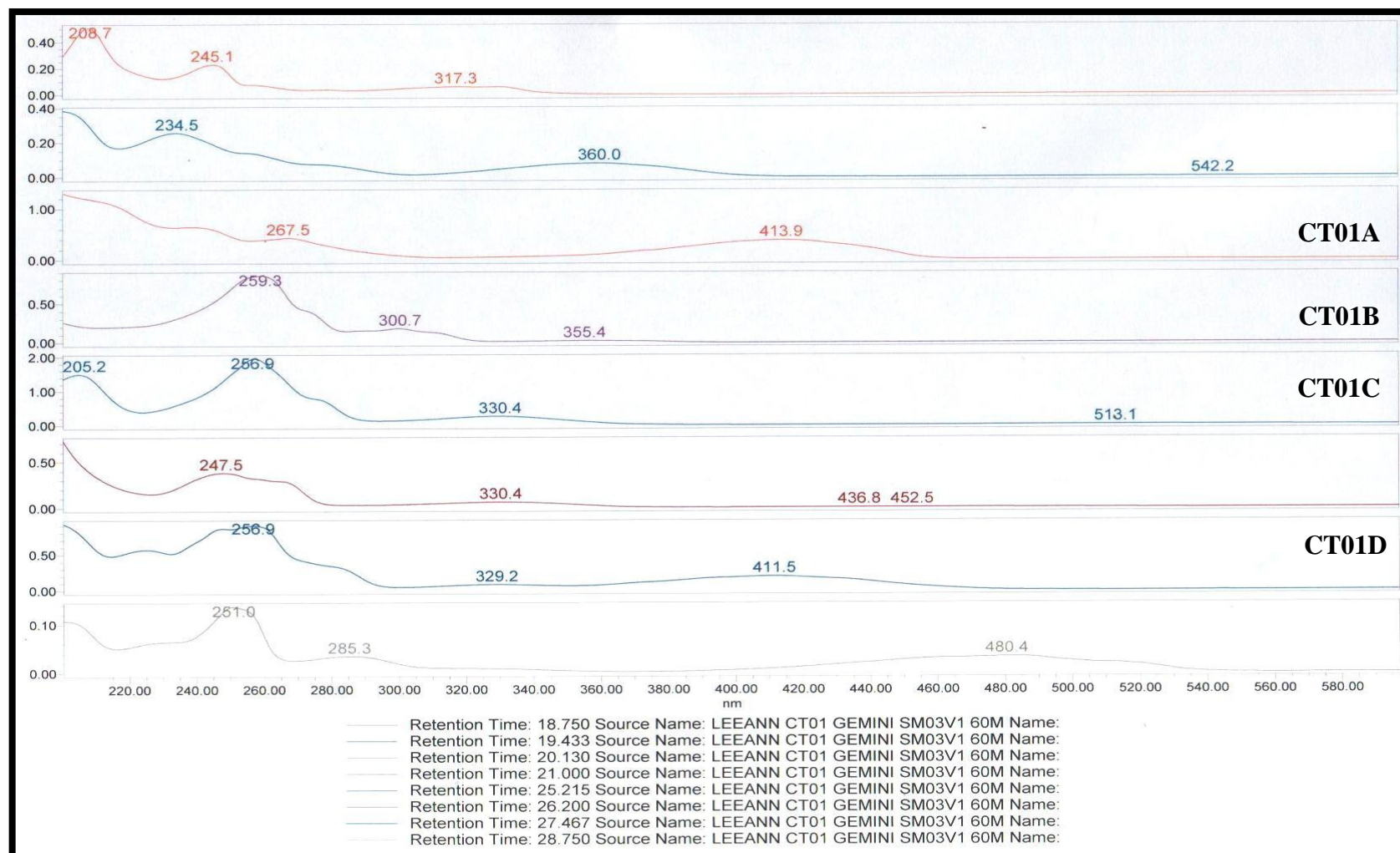


Figure 37: UV scan of fraction CT01 showing the peaks of interest, CT01A- Retention time (Rt) =20.130, CT01B-Rt = 21.000, CT01C-Rt=25.215 and CT01D-Rt = 27.467 isolated from P-HPLC and their maximum absorption wavelengths.

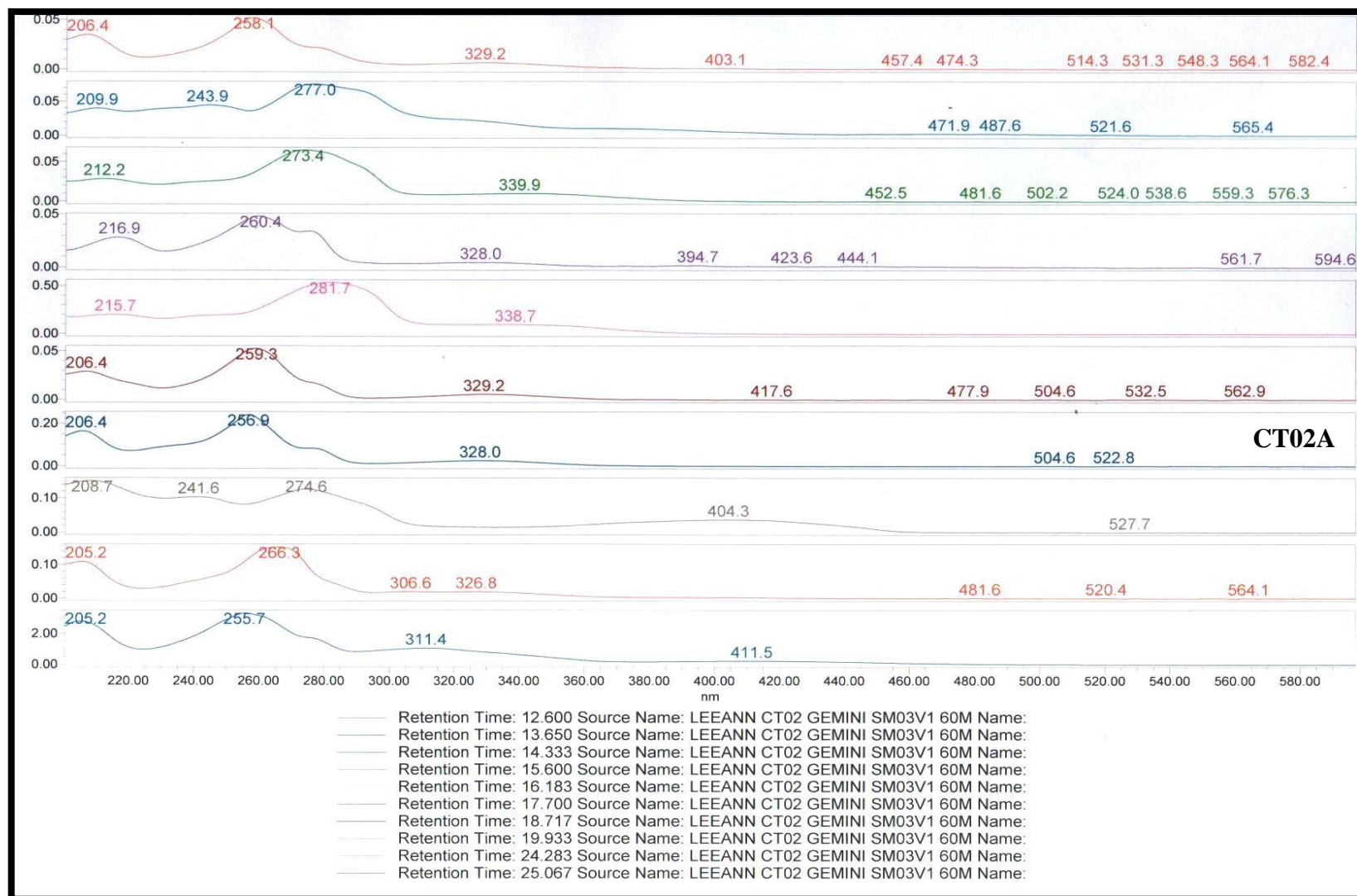


Figure 38: UV scan of fraction CT02 showing the peak of interest, CT02A- Retention time = 18.717 isolated from P-HPLC and its maximum absorption wavelength.

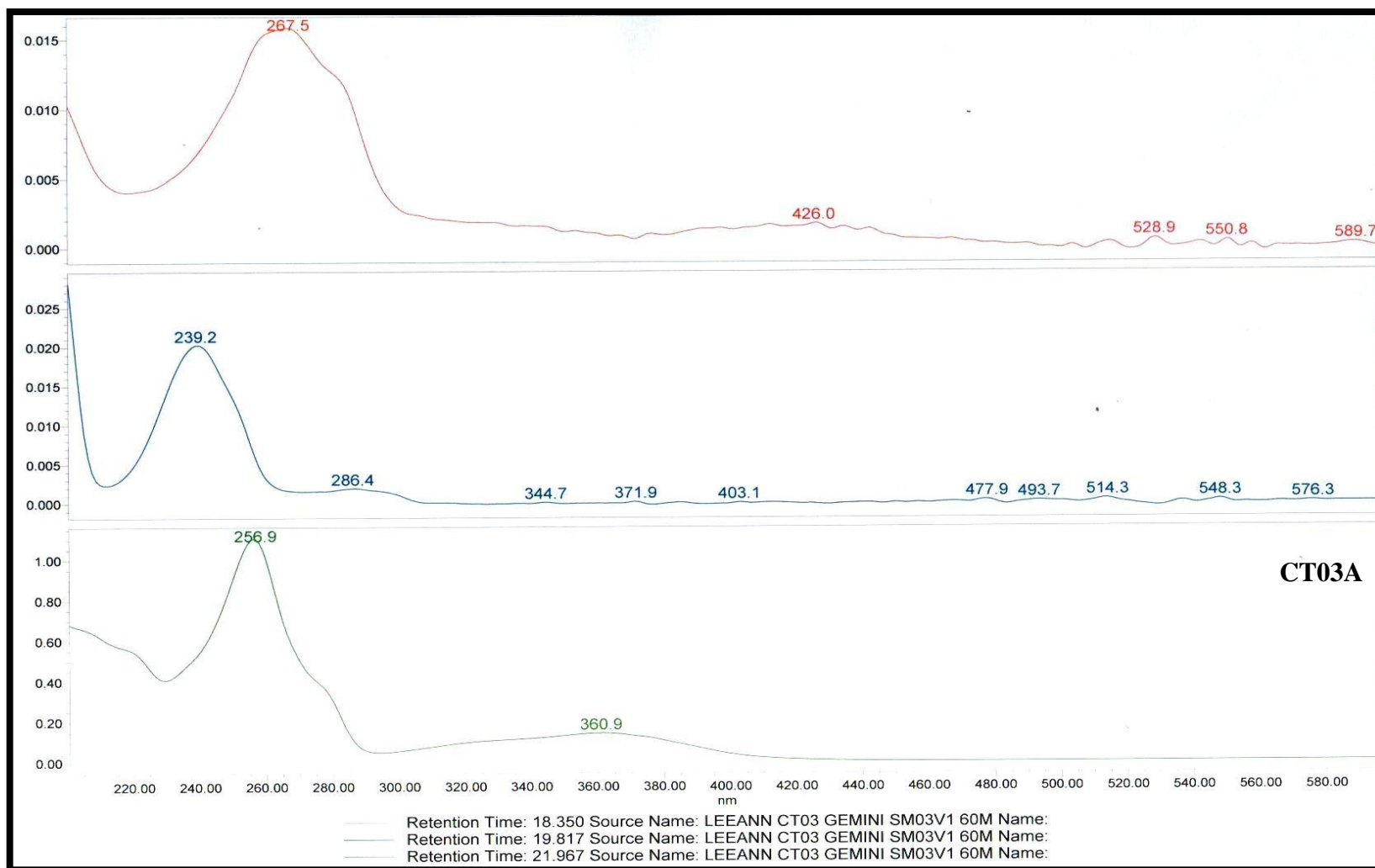


Figure 39: UV scan of fraction CT03 showing the peak of interest, CT03A- Retention time = 21.967 isolated from P-HPLC and its maximum absorption wavelength.

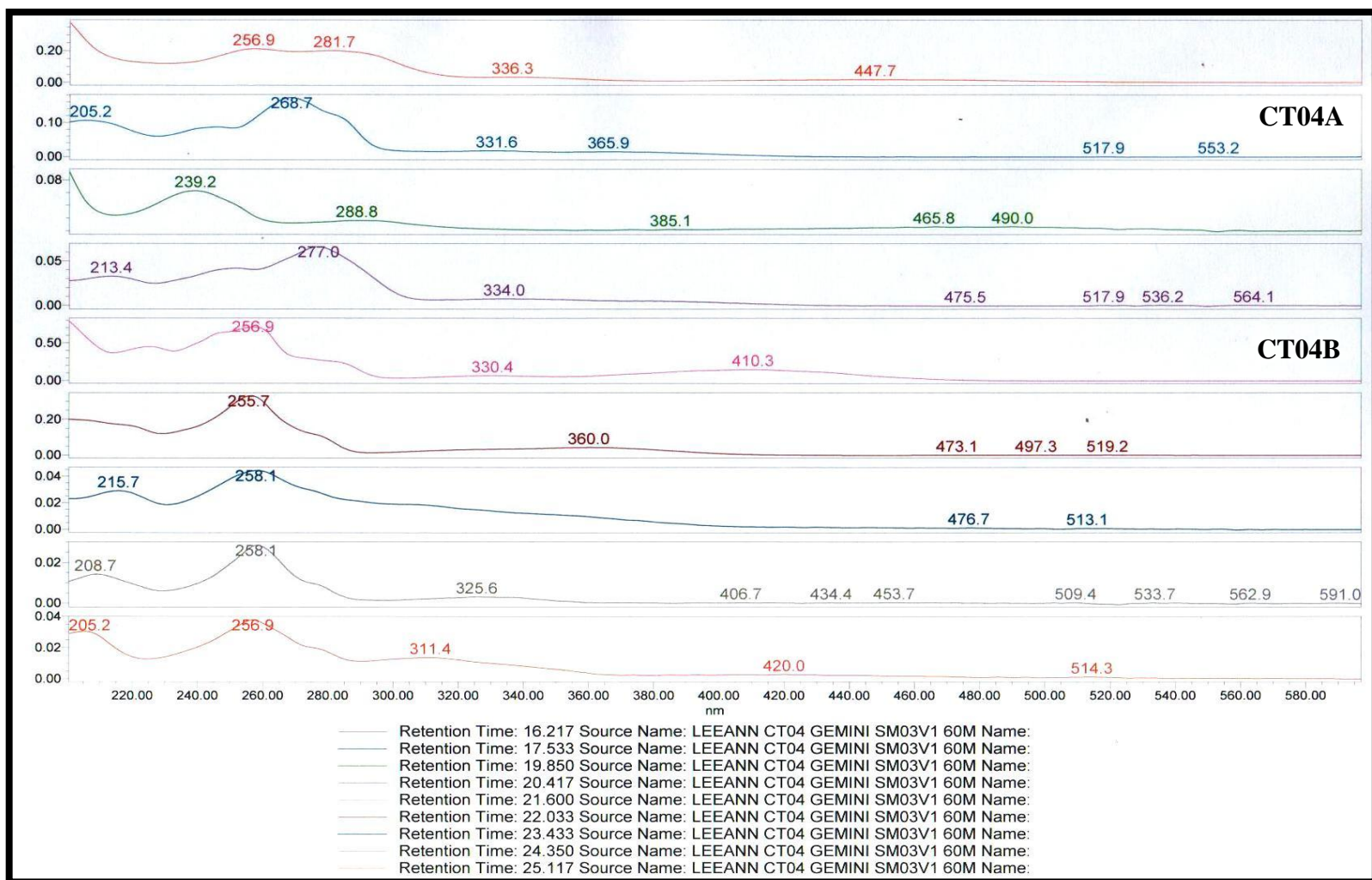


Figure 40: UV scan of fraction CT04 showing the peaks of interest, CT04A- Retention time = 17.533 and CT04B- Rt = 21.600 isolated from P-HPLC and their maximum absorption wavelengths.

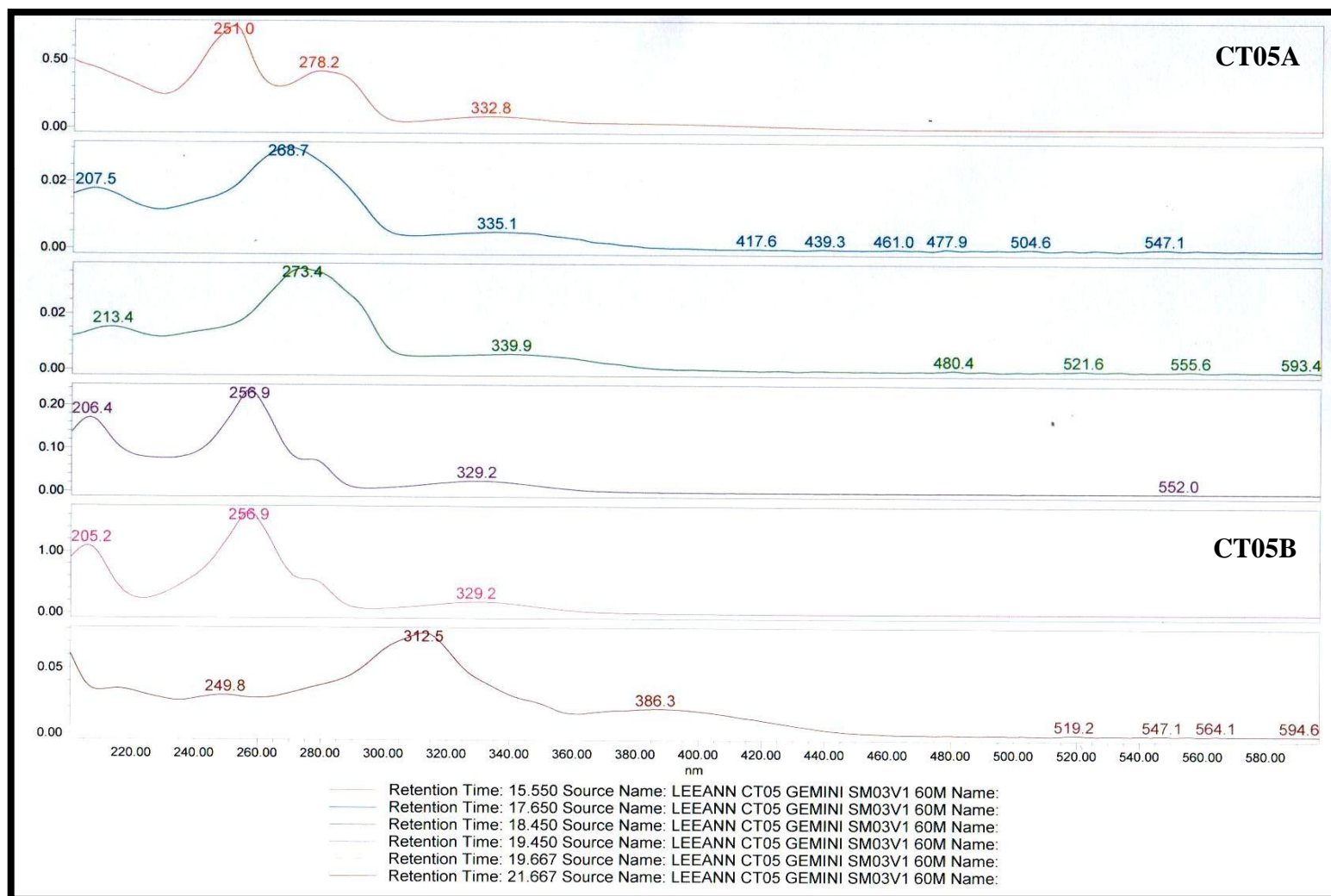


Figure 41: UV scan of fraction CT05 showing the peaks of interest, CT05A- Retention time = 15.550 and CT05B-Rt = 19.667 isolated from P-HPLC and their maximum absorption wavelengths.

3.10 EI-LC-MS analysis of purified compounds of *C. triloba* hairy roots

Elucidation of compound structure was achieved EI-LC-MS analysis. The mass spectra of the compounds are shown in Figures 42 to 47. The isolated compound's (from PHPLC) mass spectrum was compared with a library match to obtain its structure. An example is shown in Figure 48 whereby compound CT01A mass spectrum was matched to the mass spectrum of 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione. The library matches of the other compounds are shown in Appendix 2.

According EI-LC-MS analysis one acridone derivative; 5-Methoxy-2-nitro-10H-acridin-9-one (Figure 49), one naphthoquinone derivative; 2H-Naphto[2,3-b]pyran-5,10-dione,3,4-dihydro-2,2-dimethyl- (Figure 53) and seven anthracenedione derivatives were identified from *C. triloba* hairy root cultures. These were 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione (Figure 49), 9,10-Anthracenedione, 2-methyl- (Figure 49), 1-Hydroxy-4-methylanthraquinone (Figure 49 and 51), 9,10- Anthracenedione, 2-ethyl- (Figure 50), 1,5-Diaminoanthraquinone (Figure 52), Phenanthrene, 3,6-dimethoxy-9-methyl- (Figure 52) and 9,10-Anthracenedione, 1,4-dimethyl- (Figure 53). Six other compounds were also identified, these were 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl (Figure 52), n-Hexadecanoic acid (Figures 50 to 54), 1,2-Benzenedicarboxylic acid, diisooctyl ester (Figure 51 and 52), Octadecanoic acid (Figures 51 to 54), 9,12-Octadecadienoic acid (Z,Z)- (Figures 52 to 54) and 6-Octadecenoic acid, (Z)- (Figure 52 and 54).

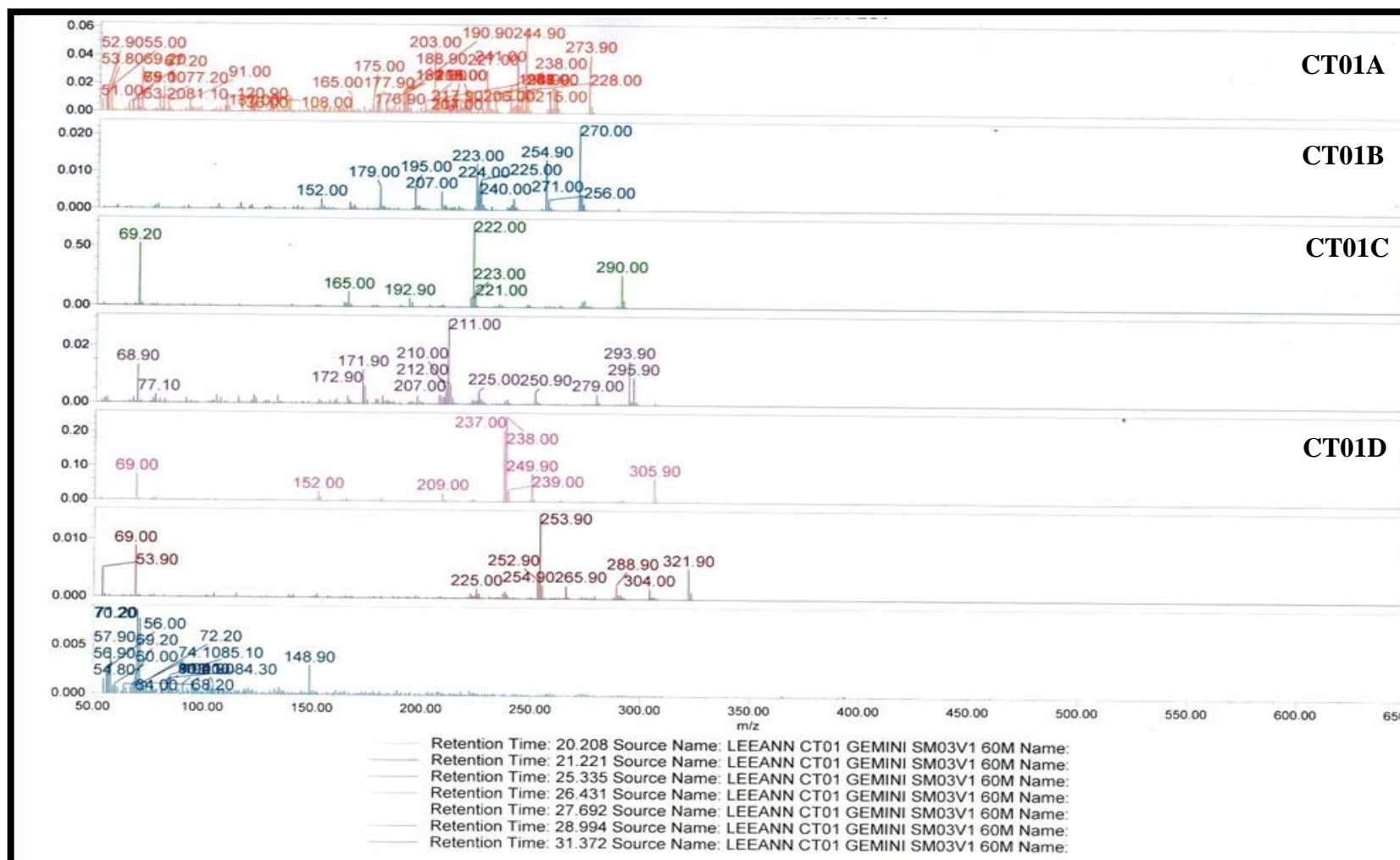


Figure 42: EI-LC-MS scan of the separated compounds from P-HPLC of fraction CT01. CT01A- Rt = 20.208, CT01B-Rt = 21.221, CT01C-Rt = 25.335 and CT01D-Rt = 27.692.

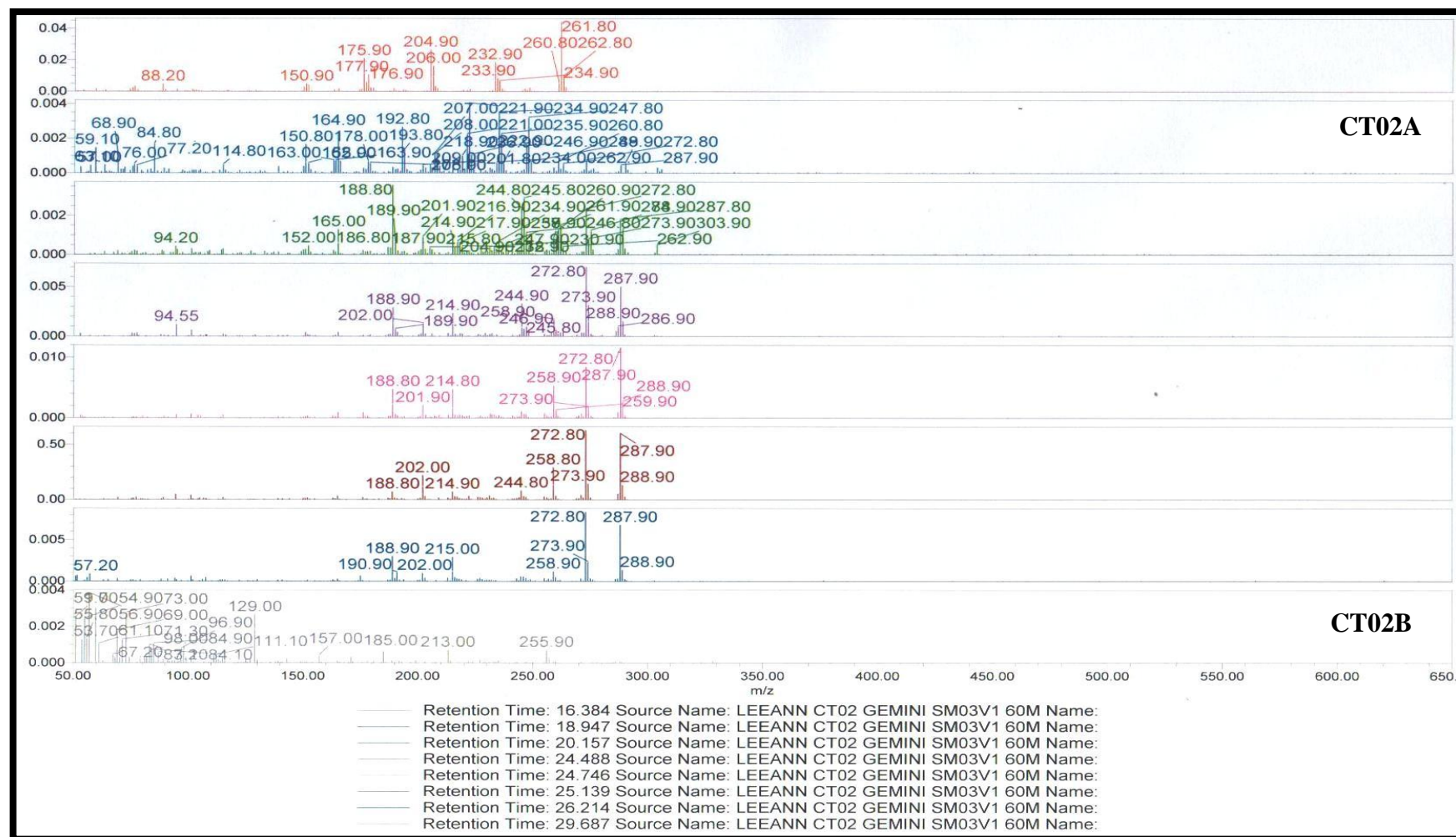


Figure 43: EI-LC-MS scan of the separated compounds from P-HPLC of fraction CT02. CT02A-Rt = 18.947 and CT02B-Rt = 29.387.

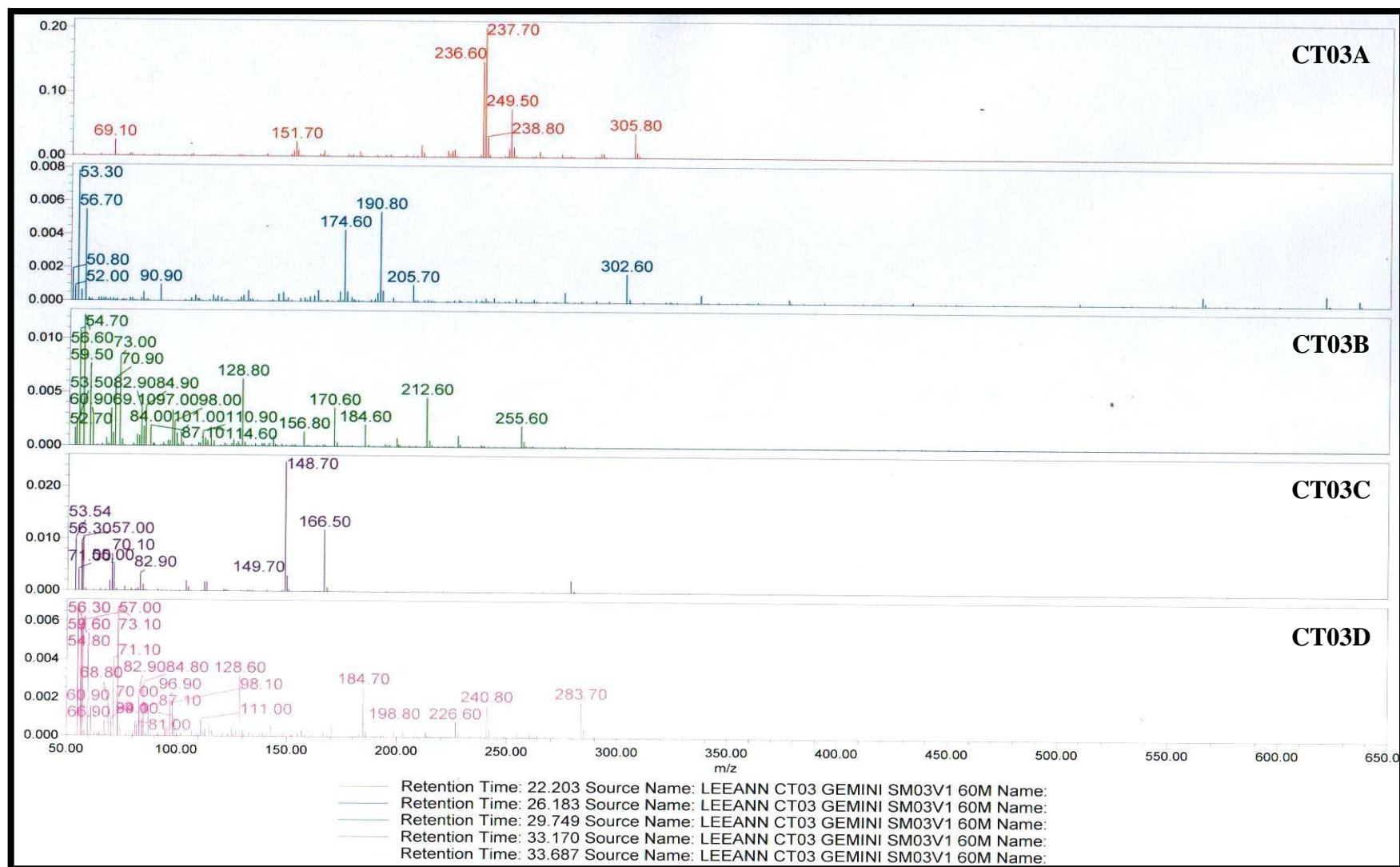


Figure 44: EI-LC-MS scan of the separated compounds from P-HPLC of fraction CT03. CT03A- Rt = 22.203, CT03B-Rt = 29.749, CT03C-Rt = 33.170 and CT03D-Rt = 33.683

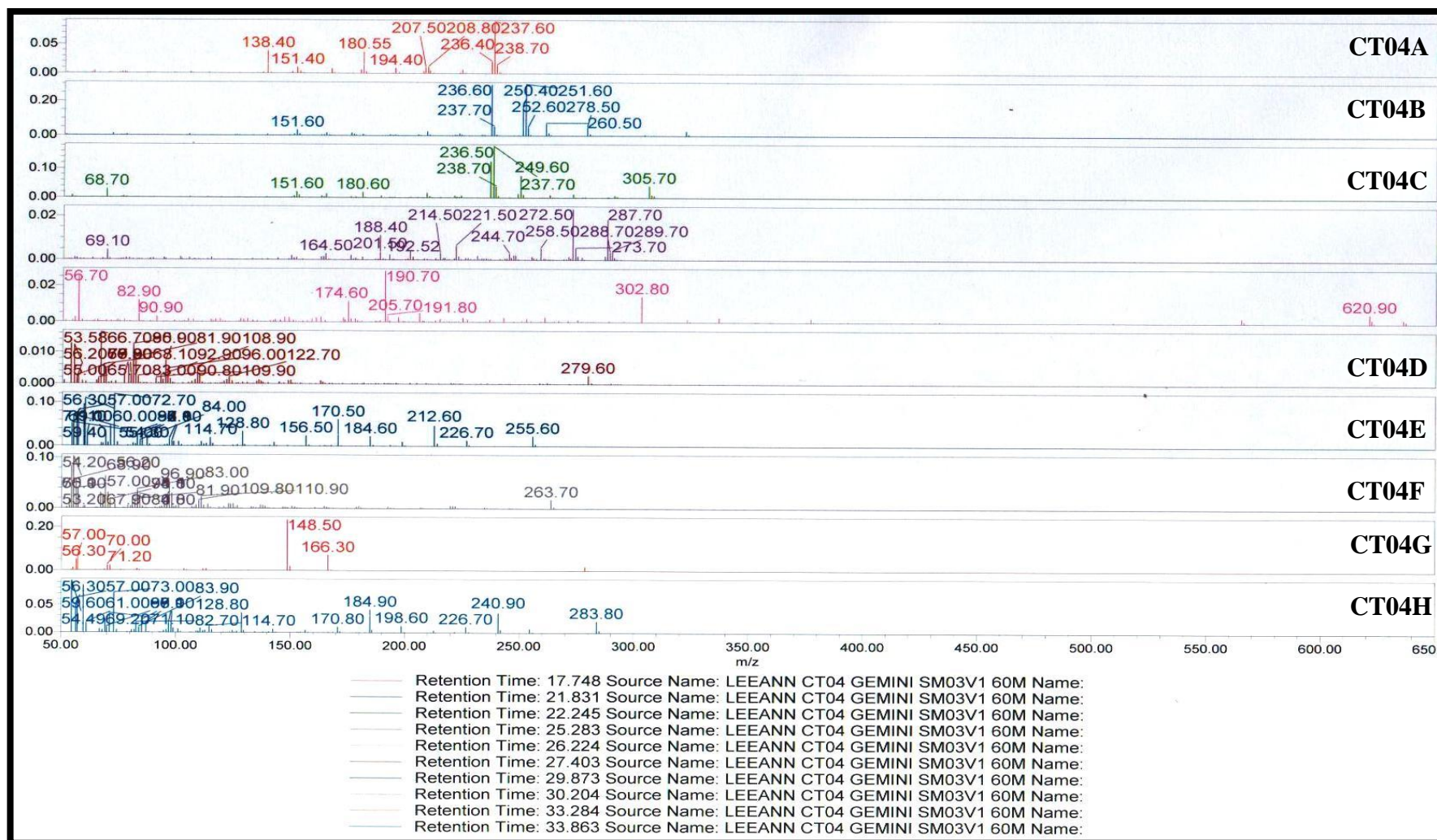


Figure 45: EI-LC-MS scan of the separated compounds from P-HPLC of fraction CT04. CT04A- Rt = 17.748, CT04B-Rt = 21.831, CT04C-Rt = 22.245, CT04D-Rt = 27.403, CT04E-Rt = 29.873, CT04F-Rt = 30.204, CT04G-Rt = 33.284 and CT04H-Rt- 33.863.

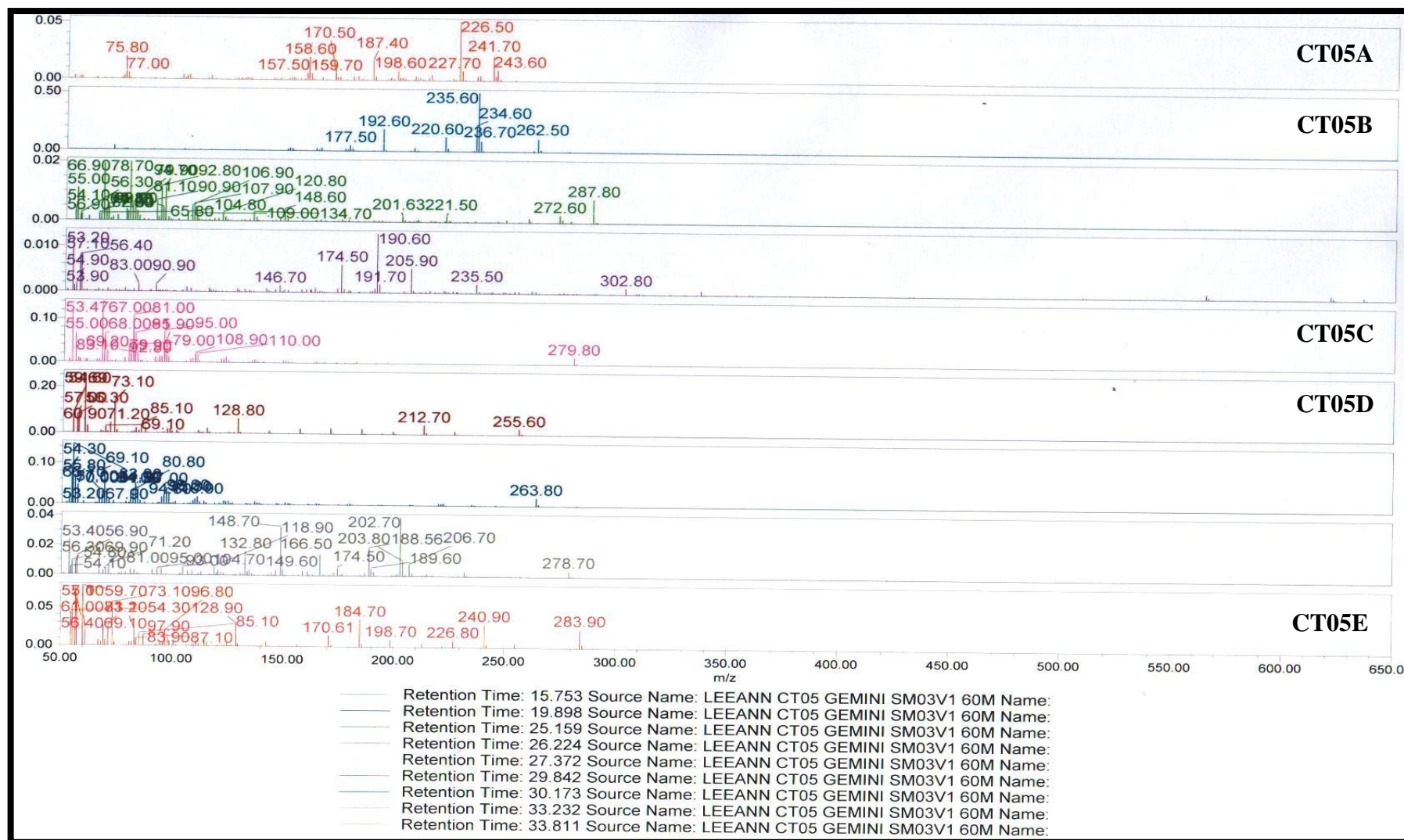


Figure 46: EI-LC-MS scan of the separated compounds from P-HPLC of fraction CT05. CT05A-Rt = 15.753, CT05B-Rt = 19.898, CT05C-Rt = 27.372, CT05D-Rt = 29.842 and CT05E-Rt = 33.811.

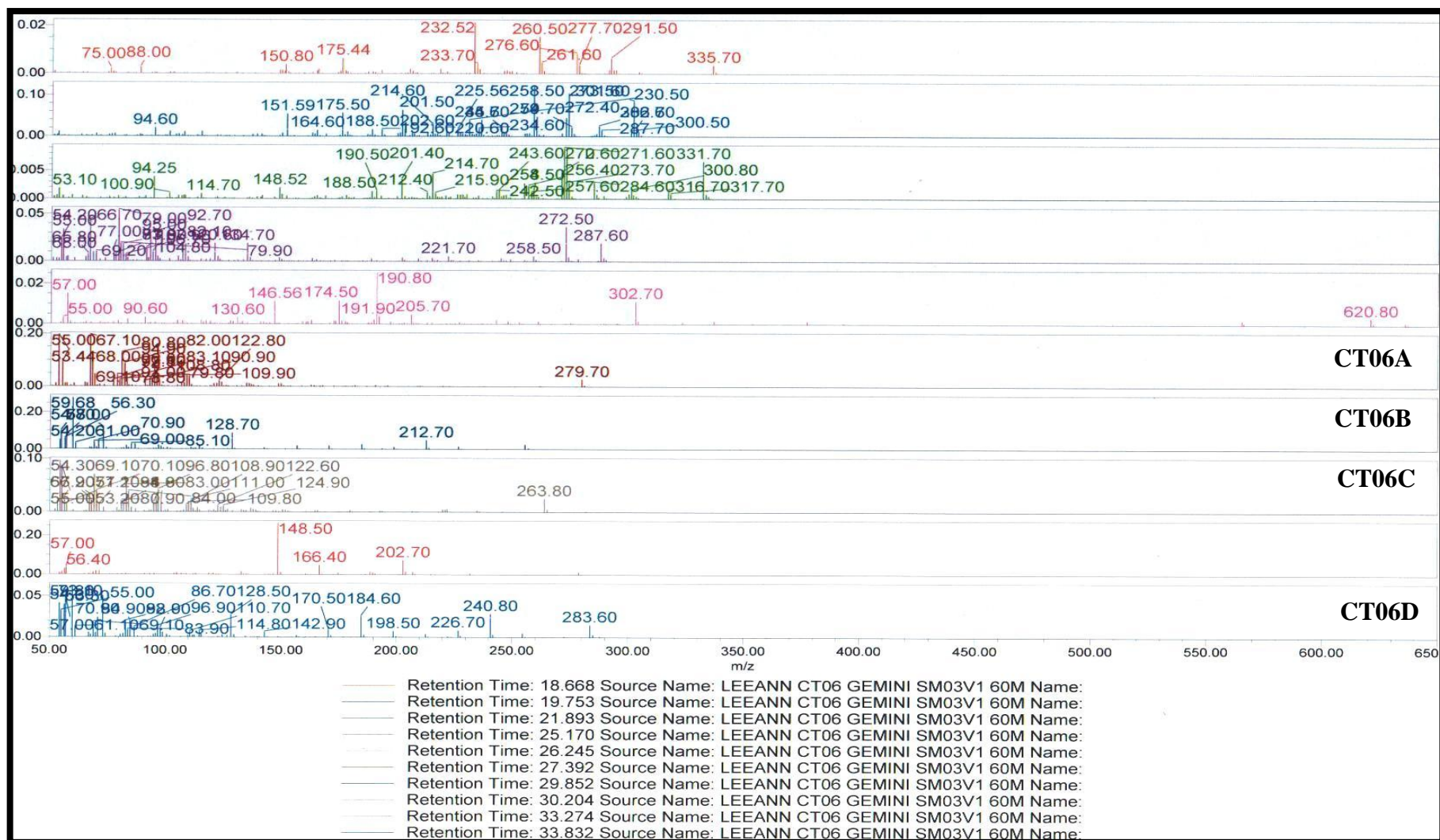


Figure 47: EI-LC-MS scan of the separated compounds from P-HPLC of fraction CT06. CT06A-Rt = 27.392, CT06B-Rt = 29.852, CT06C-Rt = 30.204 and CT06D-Rt = 33.832.

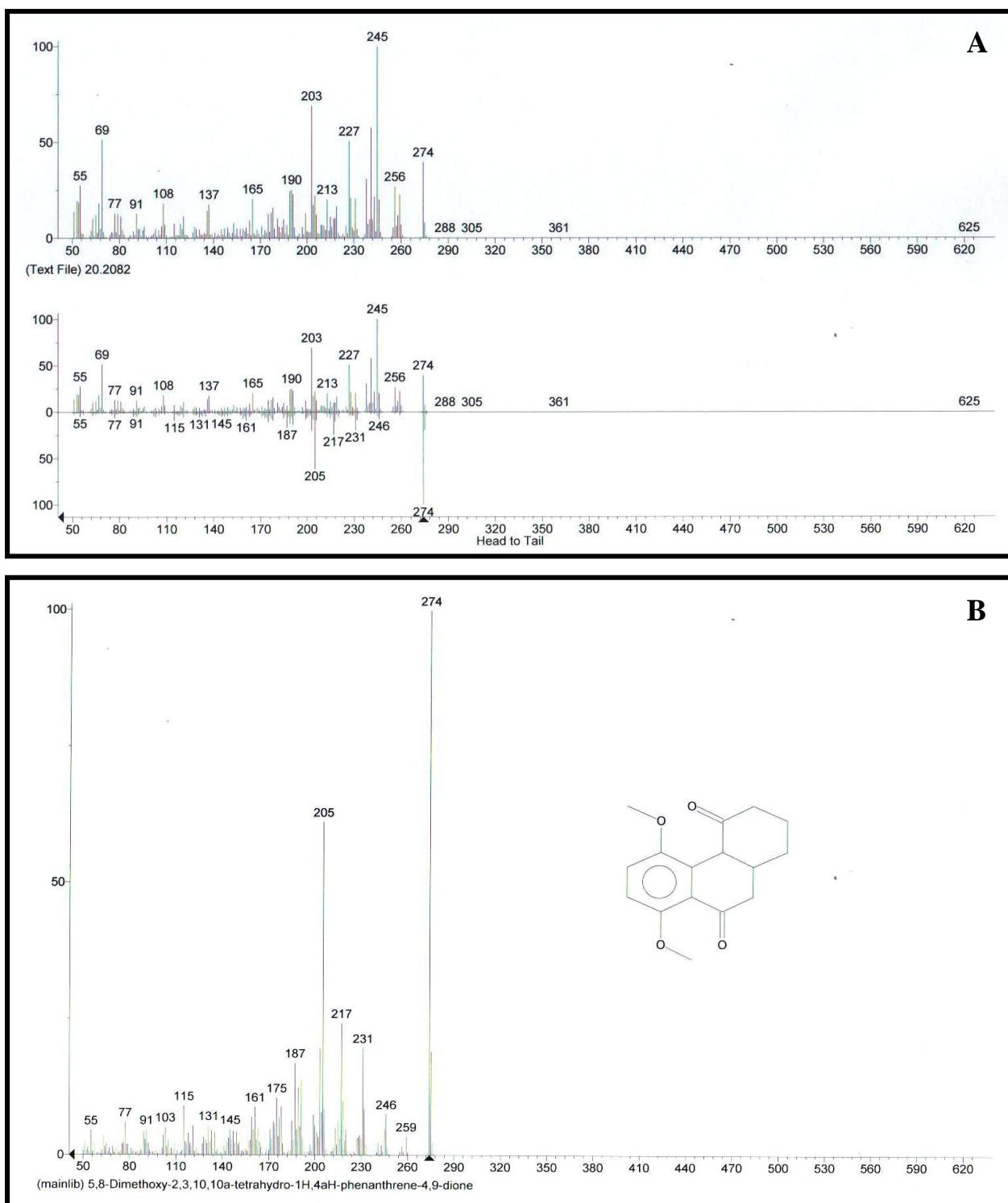


Figure 48: Library match of 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione (compound CT01A) isolated from *C. triloba* hairy roots. A-library template of 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione and B-isolated 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione from *C. triloba* hairy roots.

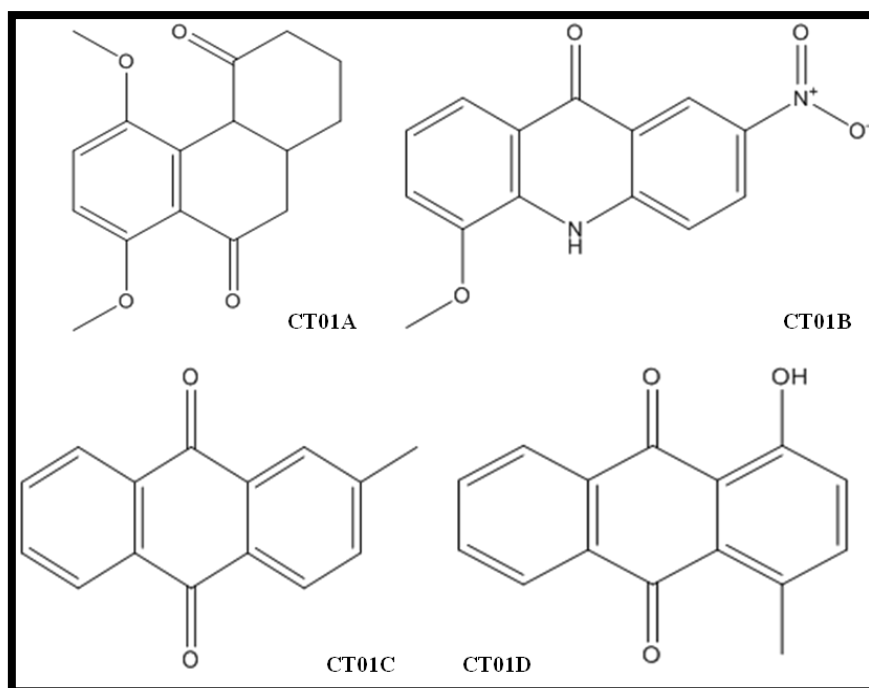


Figure 49: Compounds isolated from fraction CT01. CT01A-5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, CT01B-5-Methoxy-2-nitro-10H-acridin-9-one, CT01C-9,10-Anthracenedione, 2-methyl-, CT01D-1-Hydroxy-4-methylantraquinone.

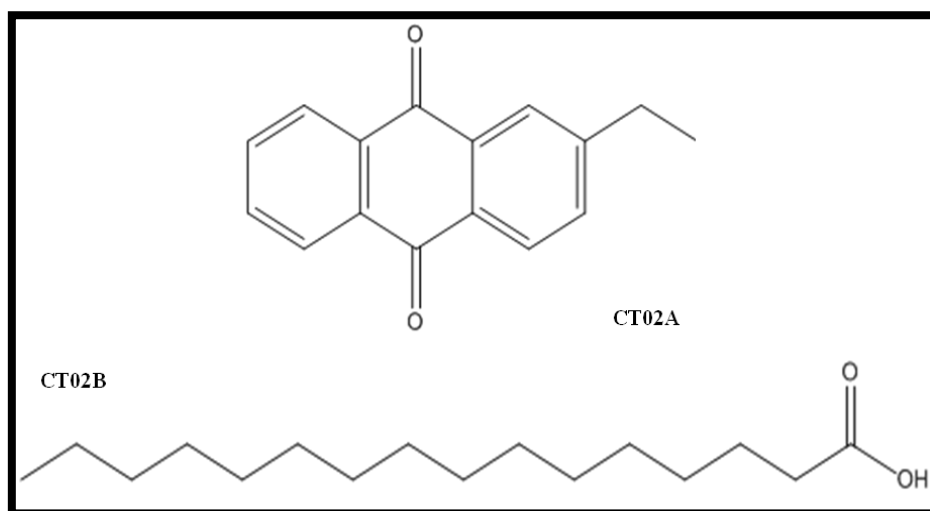


Figure 50: Compounds isolated from fraction CT02. CT02A-9,10- Anthracenedione, 2-ethyl-, CT02B- n-Hexadecanoic acid.

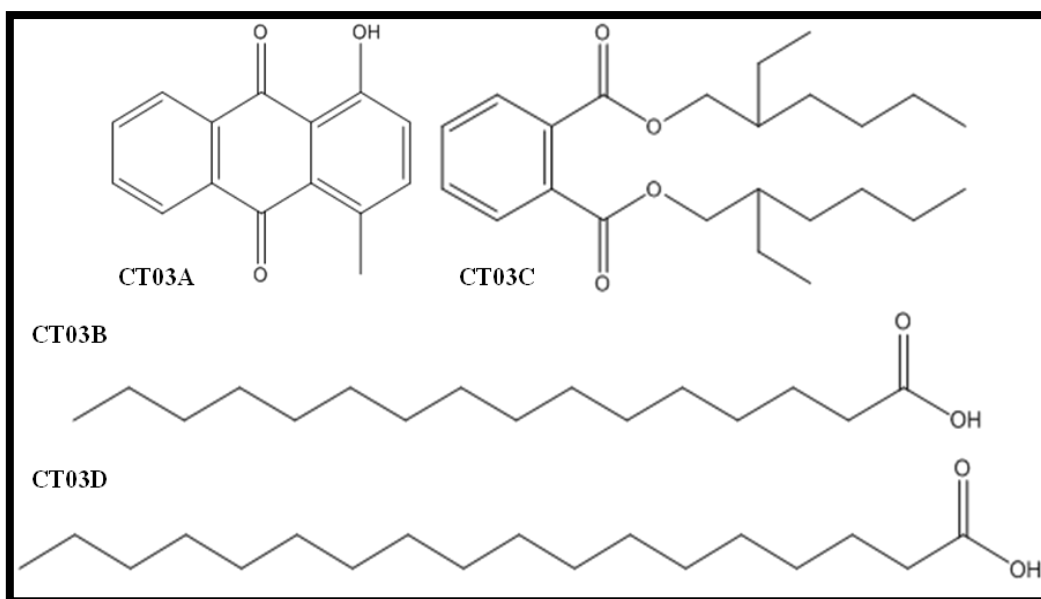


Figure 51: Compounds isolated from fraction CT03. CT03A-1-Hydroxy-4-methylantraquinone, CT03B- n-Hexadecanoic acid, CT03C-1,2-Benzenedicarboxylic acid, diisooctyl ester, CT03D-Octadecanoic acid.

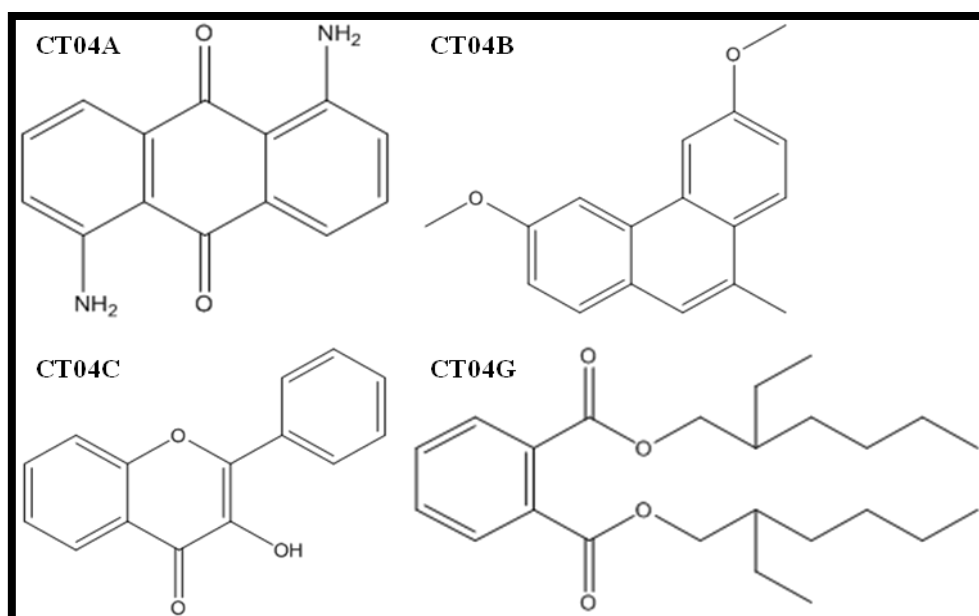


Figure 52: Compounds isolated from fraction CT04. CT04A-1,5-Diaminoanthraquinone, CT04B-Phenanthrene, 3,6-dimethoxy-9-methyl-, CT04C-4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl, CT04G-1,2-Benzenedicarboxylic acid, diisooctyl ester.

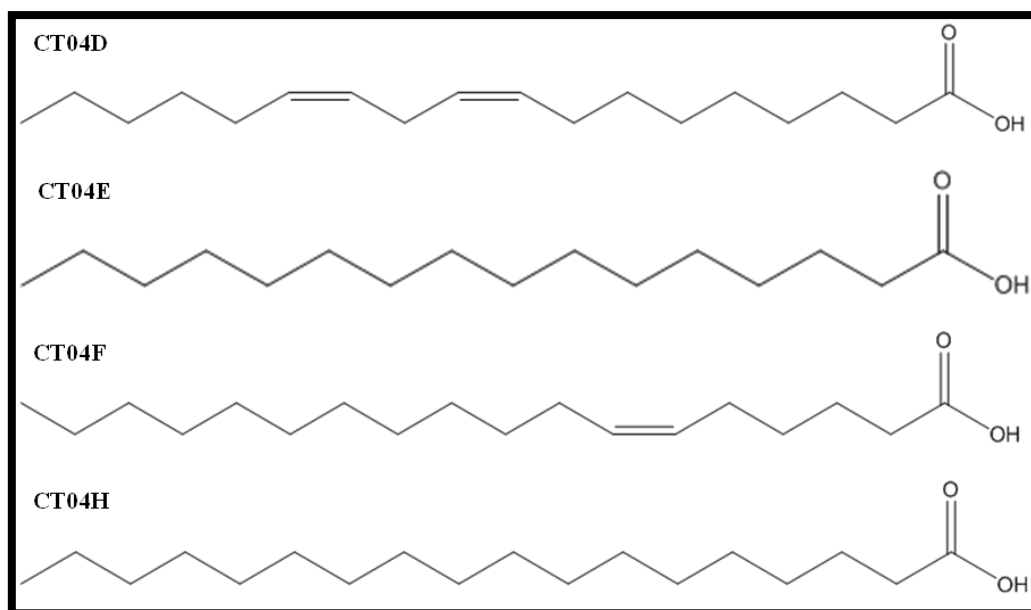


Figure 52: (continued) Compounds isolated from fraction CT04. CT04D-9,12-Octadecadienoic acid (Z,Z)-, CT04E-n-Hexadecanoic acid, CT04F-6-Octadecenoic acid, (Z), CT04H- Octadecanoic acid.

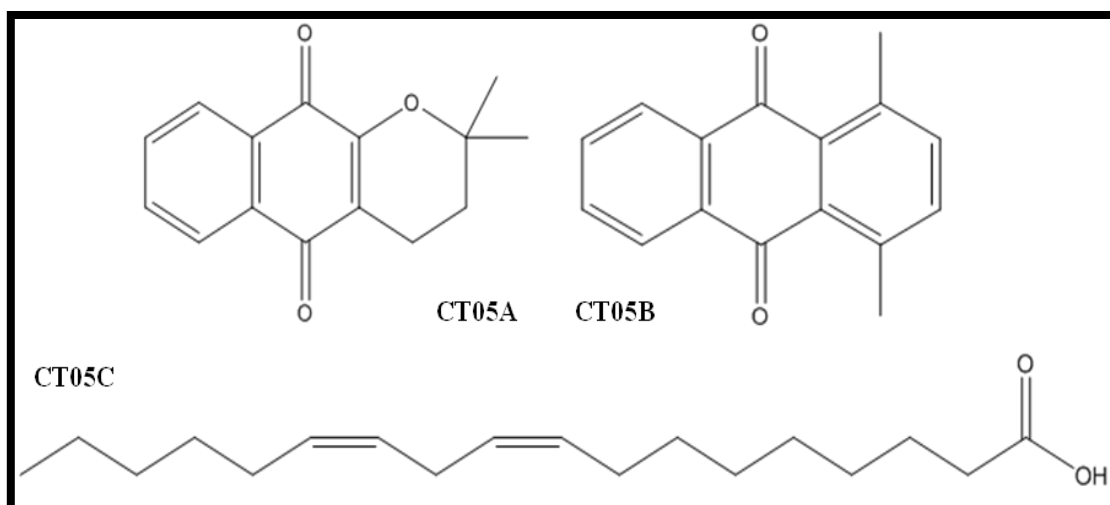


Figure 53: Compounds isolated from fraction CT05. CT05A-2H-Naphto[2,3-b]pyran-5,10-dione, 3,4-dihydro-2,2-dimethyl-, CT05B-9,10-Anthracenedione, 1,4-dimethyl-, CT05C-9,12-Octadecadienoic acid (Z,Z)-.

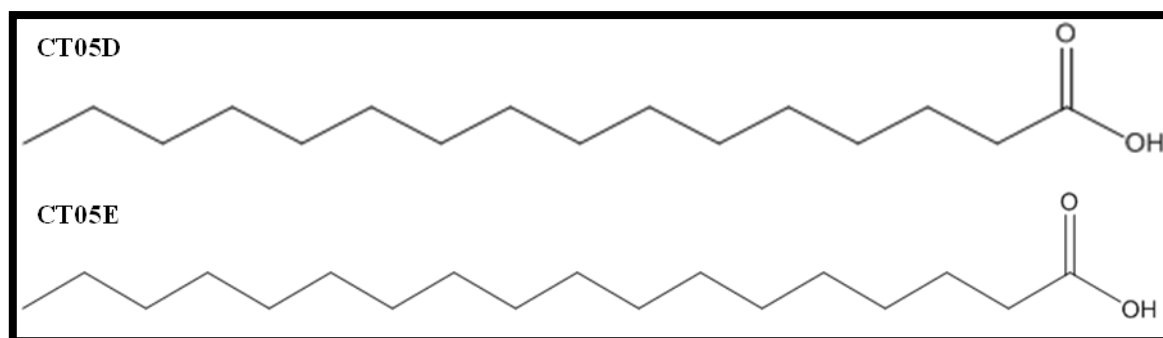


Figure 53: (continued) Compounds isolated from fraction CT05. CT05D-n-Hexadecanoic acid, CT05E-Octadecenoic acid.

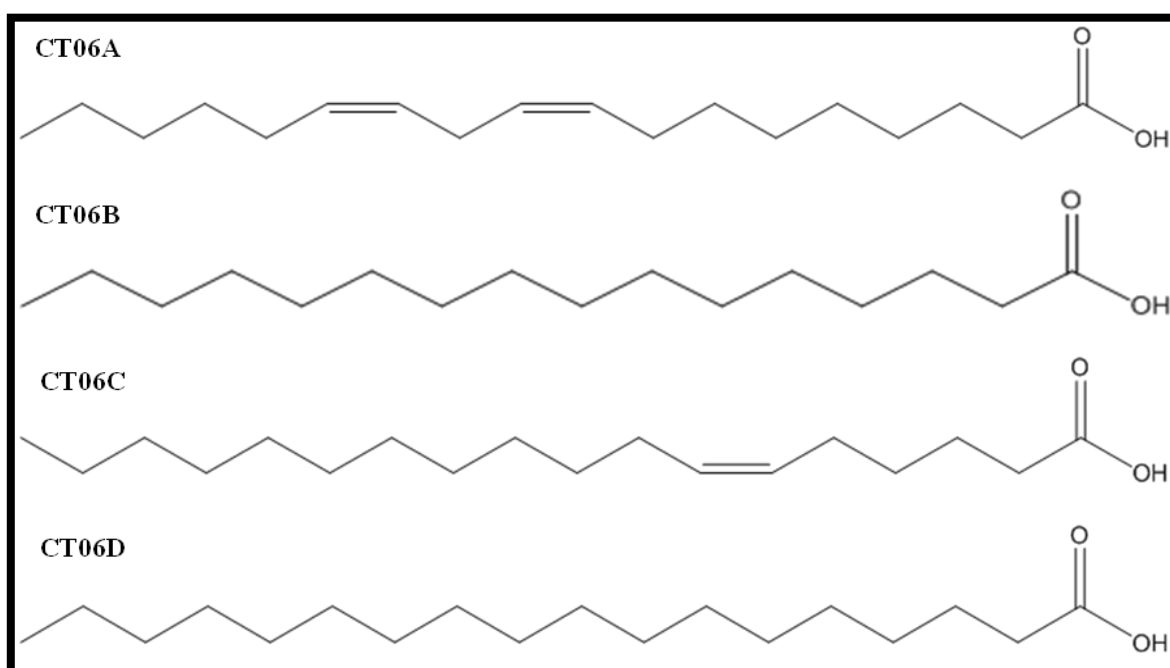


Figure 54: Compounds isolated from fraction CT06. CT06A-9,12-Octadecadienoic acid (Z,Z)-, CT06B-n-Hexadecanoic acid, CT06C-6-Octadecenoic acid, (Z)-, CT06D- Octadecenoic acid.

3.11 Biological activity of *C. triloba* extracts and isolated fractions

3.11.1 Determination of the cytotoxicity of *C. triloba* extracts and isolated fractions

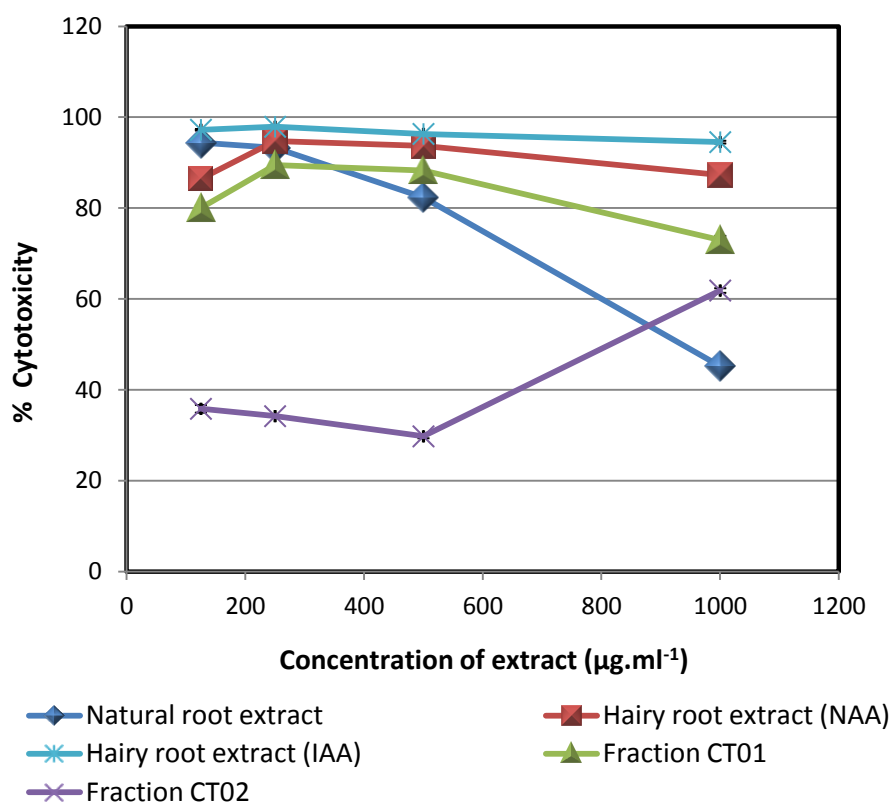


Figure 55: Percentage cytotoxicity of the field root and hairy root (from the cultures supplemented with IAA or NAA) extracts and fractions CT01 and CT02 on the DU-145 cancer cell line.

The extracts from the field roots and hairy roots cultivated in MS medium supplemented with IAA or NAA had a high cytotoxic effect on the DU-145 cancer cell line. These results are shown in Figure 55. The cytotoxicity of the field root and hairy root extracts and fraction CT01 (from PTLC) was reduced in a concentration dependent manner from a concentration of 250 $\mu\text{g.ml}^{-1}$ to 1000 $\mu\text{g.ml}^{-1}$. Whereas the cytotoxicity of fraction CT02 (from PTLC) decreased slightly from a concentration of 125 $\mu\text{g.ml}^{-1}$ to 500 $\mu\text{g.ml}^{-1}$ and thereafter it increased from 500 $\mu\text{g.ml}^{-1}$. Thus the order of cytotoxicity for the extracts and fractions on the DU-145 cell line at a 1000 $\mu\text{g.ml}^{-1}$ concentration was: hairy root extract (from the culture supplemented with IAA) > hairy root extract (from the culture supplemented with NAA) > fraction CT01 > fraction CT02 > field root extract, respectively.

3.11.2 Determination of the anti-topoisomerase II activity of fractions CT01 and CT02 from the hairy root culture extract

The effect of fractions CT01 and CT02 (from the extract of hairy roots supplemented with NAA) on the relaxation of pRYG DNA by human topoisomerase II is shown in Figure 56 and 57. In both instances, lane 1 shows the position of the linear pRYG DNA marker as it was important to mark the position of the linear cleavage product. Lane 2 shows the position of the supercoiled pRYG DNA marker and small amounts of nicked open circular DNA which is always present in plasmid preparations. Lane 3 shows the control which was the pRYG DNA incubated with human topoisomerase II; this reaction gave a relaxed DNA product. Lane 4 shows the solvent control which was the pRYG DNA incubated with human topoisomerase II and DMSO; this reaction also gave a relaxed DNA product but more linear DNA was formed. Lane 5 shows the formation of a linear DNA cleavage product in the presence of a control poison, Etoposide. Lane 6-9 shows the inhibitory effect of fractions CT01 and CT02 on topoisomerase II at 1000 $\mu\text{g}.\text{ml}^{-1}$, 500 $\mu\text{g}.\text{ml}^{-1}$, 250 $\mu\text{g}.\text{ml}^{-1}$ and 125 $\mu\text{g}.\text{ml}^{-1}$. A higher inhibition effect of the topoisomerase II enzyme was detected when a high concentration (1000 $\mu\text{g}.\text{ml}^{-1}$) of the fractions was tested (lane 6). This was represented by the presence of a low concentration of cleavage products which were nicked open circular DNA along with some linear DNA. The inhibition effect of the fractions decreased as the concentration of the fractions decreased. This was revealed by the presence of a higher concentration of cleavage products which were nicked open circular DNA, linear DNA cleavage product and relaxed DNA. Thus the fractions CT01 and CT02 did inhibit the human topoisomerase II enzyme and the degree of inhibition of the enzyme was dependant on the concentration of the fraction.

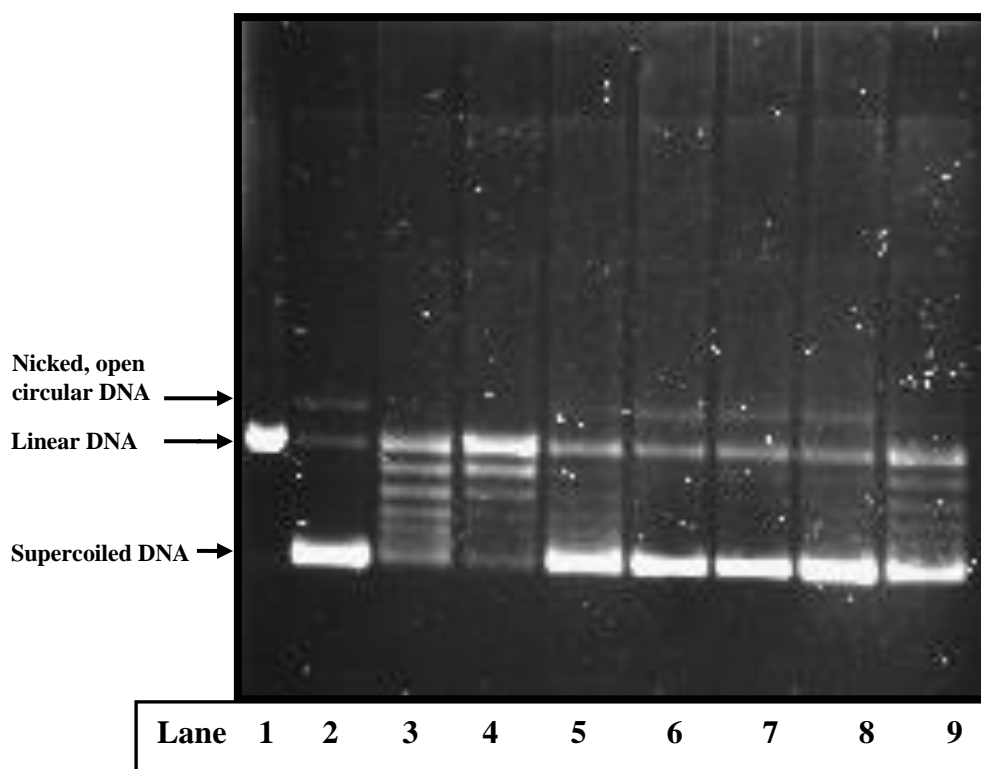


Figure 56: Agarose gel electrophoresis showing the effect of fraction CT01 from *C. triloba* hairy root culture extract on the relaxation of pRYG DNA by human topoisomerase II. Lane 1 - 5µl linear DNA, 15 µl H₂O; Lane 2 – 4 µl buffer [A+B], 1 µl super-coiled DNA, 15 µl H₂O; Lane 3 – 4 µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 11 µl H₂O; Lane 4 – 4 µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl DMSO, 9 µl H₂O; Lane 5 – 4 µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2µl etoposide, 9 µl H₂O; Lane 6 - 4µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl fraction CT01 (1000 µg.ml⁻¹), 9 µl H₂O; Lane 7 - 4µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl fraction CT01 (500 µg.ml⁻¹), 9 µl H₂O; Lane 8 - 4µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl fraction CT01 (250 µg.ml⁻¹), 9 µl H₂O; Lane 9 - 4µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl fraction CT01 (125 µg.ml⁻¹), 9 µl H₂O.

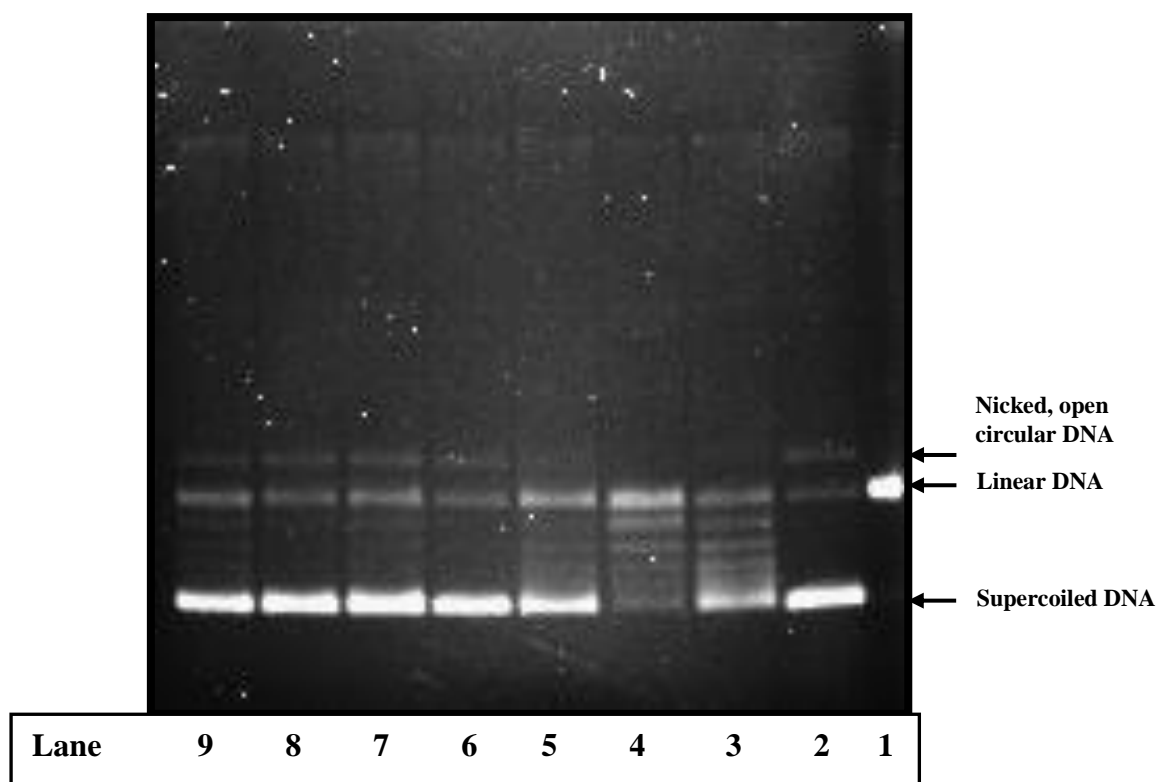


Figure 57: Agarose gel electrophoresis showing the effect of fraction CT02 from *C. triloba* hairy root culture extract on the relaxation of pRYG DNA by human topoisomerase II. Lane 1 - 5µl linear DNA, 15 µl H₂O; Lane 2 - 4 µl buffer [A+B], 1 µl super-coiled DNA, 15 µl H₂O; Lane 3 - 4 µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 11 µl H₂O; Lane 4 - 4 µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl DMSO, 9 µl H₂O; Lane 5 - 4 µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2µl etoposide, 9 µl H₂O; Lane 6 - 4µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl fraction CT02 (1000 µg.ml⁻¹), 9 µl H₂O; Lane 7 - 4µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl fraction CT02 (500 µg.ml⁻¹), 9 µl H₂O; Lane 8 - 4µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl fraction CT02 (250 µg.ml⁻¹), 9 µl H₂O; Lane 9 - 4µl buffer [A+B], 1 µl super-coiled DNA, 4 µl human topoisomerase II, 2 µl fraction CT02 (125 µg.ml⁻¹), 9 µl H₂O.

CHAPTER 4: DISCUSSION

Cancers are characterized by the dysregulation of cell signaling pathways at multiple steps. These could occur at an epigenetic level which involves DNA methylation or chromatin acetylation, inhibition of microtubule dynamics or inhibition of winding and unwinding of DNA during cell division which is mediated by topoisomerase I and II enzymes. Currently, there are drugs that are marketed based on inhibiting topoisomerases that are effective against different forms of cancer. Some of these are topoisomerase I inhibitors (examples include: irinotecan and topotecan) whilst others are topoisomerase II inhibitors (examples include: etoposide and teniposide) (Amin *et al.*, 2009). Topoisomerase II inhibitors mainly belong to a group of compounds known as anthracyclines. Recent bench and clinical scale research indicates that alternative forms of this group of compounds will continue to be highly valuable in improving the overall efficacy of anti-topoisomerases (Amin *et al.*, 2009).

In a previous study three anthracycline compounds which are anthraquinones; 9,10-Anthracenedione, 1-Hydroxy-4-methylanthraquinone and 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione were isolated from the root extracts of *C. triloba* (Mohanlall *et al.*, 2011). These compounds have shown to exhibit anti-bacterial and anti-topoisomerase activity, but they were found in very low concentrations. Therefore, in this study we used plant cell and tissue culture systems (cell suspension, shoot and hairy root cultures) of *C. triloba* to increase the production of anthraquinones. Although cell suspension and shoot cultures were studied, the emphasis was on hairy root cultures since they have been successful in producing increased amounts of valuable secondary metabolites. Examples include: taxol from *Taxus brevifolia*, diosgenin from *Dioscorea zingiberensis*, alkaloids from *C. roseus*, artemisinin from *Artemisia annua*, anthraquinones from *Rheum palmatum*, scopolamine from *Anisodus tanguticus* and emodin from *Polygonium multiflorum* (Zhou and Wu, 2006; Pietrosiuk *et al.*, 2007).

4.1 Cultivation of *in vitro* *C. triloba* plants

In order to transform *C. triloba* by using *A. rhizogenes*, sterile explants were required. In our previous study a sterilization treatment protocol was set up to sterilize leaf explants which were obtained from field plants (Naicker *et al.*, 2010) (Appendix 1). However, when plant collections were done for this study fungal contaminants still grew on the treated explants. Therefore, the seeds of the plant were collected and sterilized with 30% NaClO for 10 minutes and subsequently the sterilized seeds were germinated to supply seedlings that were cultivated into shoot cultures which provided explants for induction of callus cultures and for transformation of *C. triloba* by *A. rhizogenes*.

4.2 Callus culture

C. triloba leaf explants produced orange-yellow calli and orange pigmented regions were observed in the root-like structures of the calli (Figure 16B and 16C). These observations could be due to the production of anthraquinones as indicated in several studies. For example, Abdullah *et al.* (1998) showed that *Morinda elliptica* leaf explants produced yellow calli and the liquid medium of cell suspension cultures turned yellow when anthraquinones were released from the cells. Also Bais *et al.* (2002) showed that the red pigmented regions of *Hypericum perforatum* (St John's worts) calli were due the production of hypericin (a compound that belongs to the anthracycline group). In addition a study conducted by Mischenko *et al.* (1999) showed that orange calli accumulated higher anthraquinone content than yellow calli. Thus, these studies and our results indicate that the pigmentation of calli can be used for selecting high yielding cell lines.

4.3 Cell suspension culture

C. triloba cell suspension cultures had a high cell density (134.18 g - wet weight). The cultivation of high cell density cultures is important for extracting high yields of plant-derived compounds as secondary metabolites are based in intra-cellular parts of the cells (Luckner, 1990). The cell suspension cultures of *C. triloba* turned from orange to brown after two months of cultivation. This phenomenon was observed in *M. elliptica* cells which turned brown when anthraquinones were produced (Abdullah *et al.*, 1998). Furthermore, in a previous study by Naicker *et al.* (2010) (Appendix 1), 1-Hydroxy-4-methylantraquinone

was extracted from *C. triloba* cell suspension cultures which were also brown in color. Thus the browning of these plant cell cultures can indicate the time to harvest the cells from suspension cultures for extraction of anthraquinones.

4.4 Factors affecting the induction of hairy roots

The transformation of *C. triloba* to produce hairy roots was dependent on the type of explant used (young tissues of sterile plantlets, hypocotyl segments, cotyledons, petioles and young stems and leaves are normally used). In this study stem and leaf explants of shoot cultures were used and it was found that stem explants gave the highest transformation efficiency while leaf explants were not responsive to transformation by *A. rhizogenes* (Table 12). The concentration of *A. rhizogenes* used was also significant as it influenced the survival of the explants. A low concentration of *A. rhizogenes* transformed the stem explants whereas high concentrations killed the explants (Table 12). The concentration of cefotaxime used to decontaminate the induced hairy root cultures was also significant. Incomplete destruction of *A. rhizogenes* led to browning, slow growth rates and deterioration of the induced culture and eventually culture loss. Thus, the transformation of *C. triloba* was successfully accomplished by using stem explants (from shoot cultures) which were inoculated with *A. rhizogenes* strain 15834 at an optical density of 0.2 (A_{600}) for incubation at 26°C for 3-4 weeks and subsequently the induced hairy roots were decontaminated with cefotaxime at concentrations of 500 mg.ml⁻¹ for five days and 200 mg.ml⁻¹ for eight days.

4.5 The effect of auxins on *C. triloba* hairy root biomass and anthraquinone production

C. triloba stem explants infected with *A. rhizogenes* formed hairy roots four weeks after inoculation. The growth rate of these hairy roots was slow in MS liquid medium without hormone supplementation (Figure 58). This result contradicted the findings of Estruch *et al.* (1991) who indicated that hairy roots can grow rapidly in hormone free medium. The mean doubling time of hairy roots after inoculation varies from 24 to 90 hours, but in some cultures this time is even longer, for example the doubling time of *Galphimia glauca* hairy roots was 6 days (Nader *et al.*, 2006) and as long as 15 days has been reported for *Cinchona* hairy roots (Geerlings *et al.*, 1999). Thus great variations exist from one species to another.

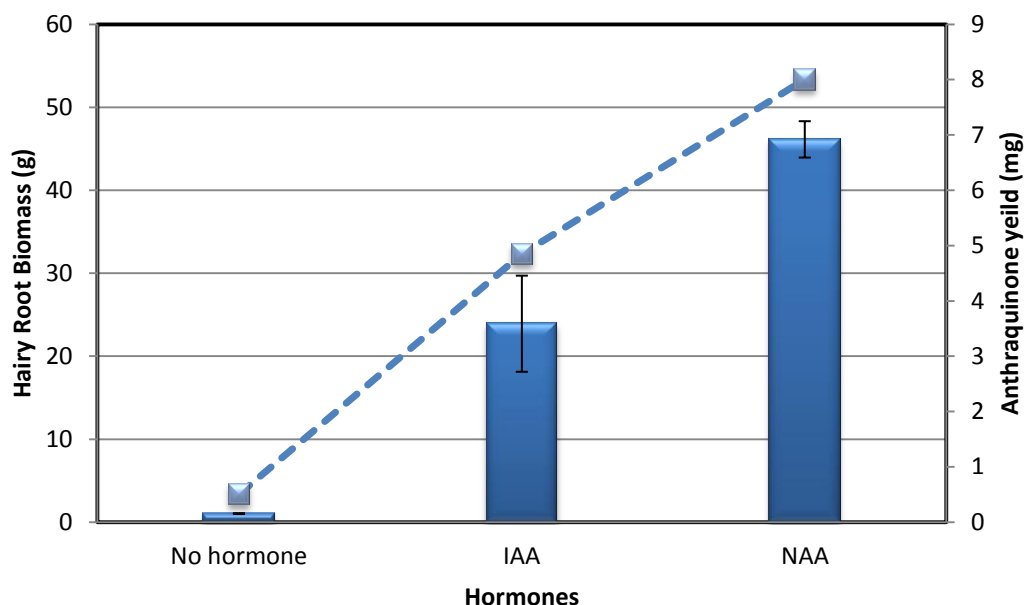


Figure 58: Correlation of the biomass production to anthraquinone yields from *C. triloba* hairy root cultures.

It has been reported that the improvement of the culture medium can increase the growth rate of slow growing hairy roots as well as the yield of secondary metabolites. In our study we found that the addition of auxins; IAA or NAA into the medium significantly increased the mean biomass by 23.21 and 44.79 times, respectively in comparison to the control culture which was cultivated in hormone free medium (Figure 58). A study by Balvanyos *et al.* (2001) also showed that the addition of NAA into the medium increased the biomass production of *Lobelia inflata* L. hairy root cultures.

Figure 58 shows that the anthraquinone production in *C. triloba* hairy root cultures increased with the amount of biomass produced which in turn was influenced by the auxin that was added into the medium. This was evident as higher yields of anthraquinones were produced in hairy root cultures that were supplemented with IAA or NAA (4.85 mg and 8.00 mg of anthraquinones, respectively) as compared to the control culture (0.50 mg of anthraquinones). A recent study by Park and Lee, (2009) also showed this effect when *Rubia akane* Nakai hairy roots were supplemented with an auxin. They used three auxins; IAA, IBA and NAA at concentrations of 0.1, 0.5 and 1 mg.l⁻¹ each and found that the anthraquinone (alizarin and purpurin) production also increased with the yield of biomass produced. In addition, NAA performed the best in terms of both biomass and anthraquinone production. *R. akane* hairy

roots were then cultivated in SH medium (Schenk and Hildebrandt, 1972) supplemented with 5 mg.l⁻¹ of NAA which increased the yield of the biomass by 1.16 times and the yields of alizarin and purpurin by 1.31 and 1.30 times, respectively in comparison to the control culture (without hormone). The enhancement of the biomass of hairy root cultures and secondary metabolite production by the addition of an auxin into the medium has also been demonstrated in *Lippia dulcis*, *L. inflata* and *Panax hybrid* hairy root cultures (Sauerwein *et al.*, 1991; Balvanyos *et al.*, 2001; Washida *et al.*, 2004).

4.6 Comparison of the yields of anthraquinones from the hairy root extracts to the field root, shoot and cell suspension culture extracts

HPLC analysis showed that the concentration of anthraquinones extracted from the hairy root cultures of *C. triloba* exceeded the concentration extracted from the roots of the parent plant (Table 14). This is possible as there is evidence that the secondary metabolites produced by hairy roots in culture are same as those usually synthesized in the intact parent roots and have similar or higher yields. For example, the content of indole alkaloids (ajmalicine, serpentine, vindoline and catharanthine) in the hairy root cultures of *C. roseus* was similar or higher than that of the amounts measured in the field roots (Pietrosiuk *et al.*, 2007). Studies show that the biosynthesis of secondary metabolites in hairy root cultures is directed by the effect of the oncogenes (the four *rol* genes A, B, C and D) (Schmülling *et al.*, 1988; Petersen *et al.*, 1989; Estruch *et al.*, 1991). For example, it has been reported that a correlation exists between the expression of the *rolC* gene and the production of *C. roseus* alkaloids (Palazon *et al.*, 1998). Furthermore a study reported by Bulgakov *et al.* (2002) showed that an association exists between the *rol* genes and anthraquinone production. In this study callus cultures of *Rubia cordifolia* were transformed with *rol* genes and the level of anthraquinones (purpurin and munjistin) was as high as 4.8% of the dry weight extract. This was much higher than the yields reported by Mischenko *et al.* (1999) for *R. cordifolia* field roots (0.2% of the dry weight extract) and non-transformed callus cultures (0.62-1.22% of the dry weight extract). The *rol* genes have also been considered to be potential activators of secondary metabolism in transformed plants as shown in Solanaceae, Araliaceae, Rubiaceae, Vitaceae and Rosaceae families (Bulgakov, 2008). Thus the effect of the *rol* genes on secondary metabolism may probably be the reason for the higher anthraquinone yields in hairy root cultures of *C. triloba*. The concentration of anthraquinones in the shoot and cell suspension culture extracts of *C.*

triloba was also evaluated. HPLC analysis showed that the shoot culture extract contained a lower concentration of anthraquinones than the hairy root culture and field root extracts (Table 14). According to literature shoot cultures can produce high yields of secondary metabolites for example; *Frangula alnus* and *Frangula rupestris* shoot cultures (Kovačević and Grubišić, 2005). However, some shoot cultures produce lower quantities of secondary metabolites than the parent plant for example; *Gentianella austriaca* shoot culture (Vinterhalter *et al.*, 2008).

The cell suspension culture extract also contained a lower concentration of anthraquinones than the hairy root and field root extracts (Table 14). A major limitation of cell suspension cultures is their lack of ability to synthesize secondary metabolites at the same level as the intact parent plant. This could be due to the lower degree of differentiation and organization of cell suspension cultures compared to organ cultures, and the location of key enzymes involved in the biosynthetic pathways (Palazon *et al.*, 2006). There are certain genes and proteins that are required to produce the secondary metabolites of interest. For example, the expression *pmt* gene is essential for the synthesis of scopolamine and the expression of this gene is specific to the pericycle of the roots (Suzuki *et al.*, 1999). In the case of *C. triloba* the anthraquinones of interest are predominantly synthesized in the roots and thus root differentiation of the plant tissue is required to express certain genes in order for high yields of anthraquinones to be synthesized. In addition, in a previous study *C. triloba* cell suspension cultures were treated with methyl jasmonate as an elicitor for increasing the production of anthraquinones. However, the results showed that low levels of 1-Hydroxy-4-methylanthraquinone were produced (Naicker *et al.*, 2010). Therefore in terms of the levels anthraquinone synthesized, *C. triloba* hairy roots are the preferable mode of production over shoot and cell suspension cultures.

This is the first study that shows the induction of *C. triloba* hairy root cultures and compares concentration of anthraquinones from *C. triloba* hairy root extracts to the field root, shoot and cell suspension culture extracts. Further optimization of culture parameters (the selection of *A. rhizogenes* strain, medium composition, influence of pH and temperature and the effect of elicitors) can allow for the scale-up of the cultivation of *C. triloba* hairy roots for the mass production of anthraquinones in bioreactors. For example, *Catharanthus trichophylleus* hairy roots were cultivated at a 20 L scale and 17 monomeric indole alkaloids were isolated including vindoline, ajmalicine, lochnericine and tabersonine (Pietrosiuk *et al.*, 2007).

Advances in bio-techniques and plant tissue culture technology have provided new means for commercial processing of medicinal plants and their valuable phytochemicals. For example, the German company ROOTec has specialized in the large-scale cultivation of hairy root cultures for the production of valuable secondary metabolites such as camptothecin and podophyllotoxin (anticancer drugs) (Shuler and Kargi, 1992).

4.7 Secondary metabolites produced by *C. triloba* hairy roots

The secondary metabolites produced by *C. triloba* hairy root cultures (supplemented with NAA) were characterized by TLC, HPLC and definitive identification by EI-LC-MS analysis. The identified metabolites were: one acridone derivative; 5-Methoxy-2-nitro-10H-acridin-9-one, one naphthoquinone derivative; 2H-Naphto[2,3-b]pyran-5,10-dione, 3,4-dihydro-2,2-dimethyl- and seven anthracenedione derivatives; i) 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, ii) 9,10-Anthracenedione, 2-methyl-, iii) 1-Hydroxy-4-methylanthraquinone, iv) 9,10-Anthracenedione, 2-ethyl-, v) 1,5-Diaminoanthraquinone, vi) Phenanthrene, 3,6-dimethoxy-9-methyl- vii) 9,10-Anthracenedione, 1,4-dimethyl-. With the exception of two compounds, 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione and 1-Hydroxy-4-methylanthraquinone none of these compounds were found in the field root extracts of *C. triloba* in earlier studies by Mohanlall *et al.* (2011). It has been reported that hairy root cultures can produce derivatives of the compounds found in the parent plant. For example, Furumoto *et al.* (2007) found 2-geranyl-1,4-naphthoquinone in the hairy root culture extract of *Sesamum indicum* L. This compound is not produced the field roots of *S. indicum* but is a derivative of the anthrasesamone biosynthetic pathway which occurs in the field roots. Thus the five anthracenedione derivatives which are not produced in *C. triloba* natural roots formed in the hairy root cultures as a result of them being precursors or derivatives of the anthraquinone biosynthetic pathway. The production of different derivatives of a group of compounds in culture could also be due to the influence of the medium composition as indicated in a study by Parr *et al.* (1988) who reported that the use of standard B5 medium affected the number of alkaloids that was produced in *C. roseus* hairy roots.

According to literature it is evident that the compounds isolated from *C. triloba* hairy root extract could contribute to the extract having potentially versatile biological activity. For instance, 5-Methoxy-2-nitro-10H-acridin-9-one (from *C. triloba* hairy root extract) belongs to a group of compounds known as acridones. Triazoloacridone is an example of a compound from this group and it has been shown to exhibit antitumor activity. Other compounds that belong to this group include: imidazoacridone (exhibits antitumor activity), acridone-10-yl acetic acid [Neovir] (exhibits antiviral activity), 3-chloro-6-(2-diethylamino-ethoxy)-10-(2-diethylaminoethyl) acridone (exhibits antimalarial activity) and 1, 3, 4 oxadiazole derivatives of acridone (exhibits antimicrobial activity). In addition a variety of other acridone derivatives have shown to exhibit biological activities which have been summarized in a review by Kumar *et al.* (2011).

2H-Naphto[2,3-b]pyran-5,10-dione,3,4-dihydro-2,2-dimethyl- is a naphthoquinone known as gunacin. Naphthoquinones have been reported to possess antibacterial, antifungal and anticancer activity. Gunacin is an antibiotic that exhibits good inhibition activity against mycoplasmas and Gram-positive bacteria including multi-resistant strains. It exhibits weak inhibition activity against Gram-negative bacteria with exception of *Proteus vulgaris* which is more susceptible. Gunacin has shown to have antifungal activity against *Trichophyton mentagrophytes*. This compound has also shown to inhibit DNA synthesis *in vivo* and has acute toxicity in mice (Werner *et al.*, 1979).

The hairy root culture extract was rich in anthraquinones. Derivatives of the anthraquinone molecule have been researched in earlier studies. Reports have shown that 9,10-Anthracenedione, 1,4-bis [2-[(2-hydroxyethyl)amino] -ethyl]amino] -diacetate at concentrations ranging from 0.05 $\mu\text{g}.\text{ml}^{-1}$ to 10.0 $\mu\text{g}.\text{ml}^{-1}$ caused alteration of the kinetics of chinese hamster, friend murine leukemia and SK-L7 cells which were cultivated *in vitro*. Exposure to the drug blocked cell cycle progression at the G₂-M phases after 2 to 18 hours. In addition the drug was tested on the cell cycle progression of normal phytohemagglutinin-stimulated lymphocytes and it had little effect (Evenson *et al.*, 1979). Anthracenedione-based drugs are known to exhibit prominent anticancer properties and are widely applied in clinical practice (Hortobagyi, 1997; Thomas and Archimbaud, 1997; Wiseman and Spencer, 1997; Arcamone, 1998). The mechanism of action of these drugs has been attributed to their ability to inhibit the topoisomerase II enzyme (Malonne and Atassi, 1997).

A disubstituted aminoanthraquinone derivative, 1,5-Diaminoanthraquinone was also isolated from the hairy root extract of *C. triloba*. Disubstituted aminoanthraquinone derivatives represent one of the most important classes of anticancer agents as the biological activity of the anthraquinone derivative is influenced by the disubstituted side chains (Johnson *et al.*, 1979). These can be a protonable nitrogen which can be represented by the presence of a $(CH_2)_2$ spacer between the ring and side chain nitrogens and a 1, 4 location for the two side chains. Huang *et al.* (2005) assessed the biological activity of anthraquinone derivatives which were amino-substituted in the side chains located at positions 1 and 8 of the anthraquinone ring system. The study was proposed to give an indication whether the anthraquinone family could be used as adjuncts to enhance the inhibition of the growth of cancer cells. The study showed disubstituted aminoanthraquinone derivatives inhibited the growth of rat glioma C6 cells and human hepatoma G2 cells. In addition the presence of hydroxyl groups in aminoanthraquinone derivatives also gives these compounds higher biological potency and efficacy. For example, the hydroxyl groups at positions 5 and 8 in mitoxantrone allows it to have potent activity against breast cancer, acute leukaemias and non-Hodgkin's lymphoma and marginal activity against non-small cell lung cancer, Hodgkin's lymphoma, myeloma and cancer of the liver, prostate, bladder, head and neck (Smith, 1983; Poirier, 1986; Young and Raymond, 1986). Mitoxantrone is now licensed and clinically used in many countries for the treatment of breast cancer and acute leukemias (Zagotto *et al.*, 2000).

5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione belongs to a group of compounds known as phenanthrenequinones. This compound is used for modulation of the activity of Janus kinases as well as in the treatment of diseases that are related to the activity of Janus kinases which include immune-related diseases and cancer (Lamb *et al.*, 1998). Recently eleven phenanthrenequinones were isolated from the ethyl acetate extract of *Calanthe arisanensis*. These compounds were tested for cytotoxic activity against seven human cancer cell lines (lung [A549], prostate [PC-3 and DU-145], colon [HCT-8], breast [MCF-7], nasopharyngeal [KB] and vincristine-resistant nasopharyngeal [KBVIN]). It was found that the phenanthrenequinones were cytotoxic to the breast cancer cell line, MCF-7 and the prostate cancer cell lines, PC-3 and DU-145 (Lee *et al.*, 2009).

1-Hydroxy-4-methylantraquinone is used by the textile industry as an excellent source of synthetic dyes, however in recent years derivatives of this compound have been utilized as dyes for synthetic fibers (French Pat. No. 2,002,124; U.S. Pat. No. 2,533,178).

The bioactivity of three anthracenedione derivatives was assessed by a scientist in our laboratory. 9,10 Anthracenedione and 1-Hydroxy-4-methylantraquinone exhibited antibacterial activity against Gram-positive organisms. In addition purified anthraquinones isolated from *C. triloba* roots exhibited anti-topoisomerase activity. 9,10- Anthracenedione was shown to partially inhibit the topoisomerase II enzyme which was represented by the partial conversion of supercoiled DNA to linear and nicked open circular DNA. 1-Hydroxy -4-methylantraquinone and 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione exhibited potent activity against the topoisomerase II as these compounds inhibited the complete formation of the topoisomerase II–DNA complex. Thus the fractions (CT01 and CT02) isolated from *C. triloba* hairy roots were analyzed using the TopoGEN Topoisomerase II Drug Screening kit to determine if they also display anti-topoisomerase activity.

Six other compounds were also identified from the *C. triloba* hairy root extract. These were: 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl; n-Hexadecanoic acid; 1,2-Benzenedicarboxylic acid, diisooctyl ester; Octadecanoic acid; 9,12-Octadecadienoic acid (Z,Z)- and 6-Octadecenoic acid, (Z)-.

4.8 The cytotoxicity and anti-topoisomerase II activity of *C. triloba* extracts and fractions, CT01 and CT02

The bioactivity of fractions, CT01 and CT02 was evaluated as these fractions correlated to the anthraquinone standards when the hairy root extract (from the culture supplemented with NAA) was analyzed by TLC. Fractions CT03 to CT05 were not tested in the biological activity assays as they did not correlate to the anthraquinone standards. However, the EI-LC-MS results showed that anthracenedione derivatives were present in them and thus these derivatives may also have the potential to exhibit biological activity.

Currently synthetic anthracenedione derivatives such as mitoxantrone and doxorubicin are being used in the treatment of cancer. However a number of cell lines have developed resistance to these drugs (Dietel *et al.*, 1990; Taylor *et al.*, 1991; Nakagawa *et al.*, 1992; Futscher *et al.*, 1994; Hazlehurst *et al.*, 1999; Doyle and Ross, 2003; Ma and Wink, 2010). This reason and the presence of three potentially anticancer anthracenedione derivatives in *C. triloba* roots had encouraged us to determine the biological activity of *C. triloba* extracts and isolated fractions.

According to the MTT assay results *C. triloba* field root and hairy root extracts (from the cultures supplemented IAA or NAA) and the isolated fractions (CT01 and CT02) had a high cytotoxic effect on the DU-145 cancer cell line. This effect was dependent on the concentration used as high concentrations of the extracts and fraction CT01 decreased the cytotoxic effect on the cells. This may be due to the presence of pro-proliferative compounds in the extracts and fraction CT01.

The results of this experiment directed us to perform the anti-topoisomerase assay which showed that both fractions CT01 and CT02 inhibited the topoisomerase II enzyme. Since there is evidence that the cytotoxic action of anthraquinones such as doxorubicin and mitoxantrone is attributed to these drugs being potent topoisomerase inhibitors *in vitro* and in human cells (McClendon and Osheroff, 2007), we can conclude that the anthracenedione derivatives present in fractions CT01 (5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione; 9,10-Anthracenedione, 2-methyl- and 1-Hydroxy-4-methylanthraquinone) and CT02 (9,10- Anthracenedione, 2-ethyl-) were responsible for the cytotoxic action on the DU-145 cancer cells and the inhibition of the human topoisomerase II enzyme.

The manner in which anthracenedione based drugs work can be explained by describing the importance of topoisomerase II on the human cellular system (McClendon and Osheroff, 2007) (Figure 59). The formation of topoisomerase II- DNA cleavage complexes are transient in nature and are required for the enzyme to perform its essential cellular functions. If the level of cleavage complexes becomes too low, cells are unable to undergo chromosome segregation and this leads to cell death as a result of mitotic failure (left arrow). If the level of cleavage complexes becomes high, the actions of the DNA tracking system (replication and transcription) causes the conversion of these transient complexes to permanent double-

stranded breaks in the genetic material (right arrow). These events result to the inhibition of essential DNA processes that initiate multiple recombinant/repair pathways as well as generate chromosomal translocations and other DNA aberrations. If the DNA strand breaks overwhelm the cells, apoptotic pathways are triggered. This mechanism is the basis for the cytotoxic action of anthracenedione based drugs and several widely prescribed anticancer drugs. If the topoisomerase II enzyme mediates a too low concentration of DNA breaks to cause the cells to be overwhelmed then the surviving population may inherit chromosomal translocations. This can lead to the formation of leukemias which involve the mixed lineage leukemia (*MLL*) gene.

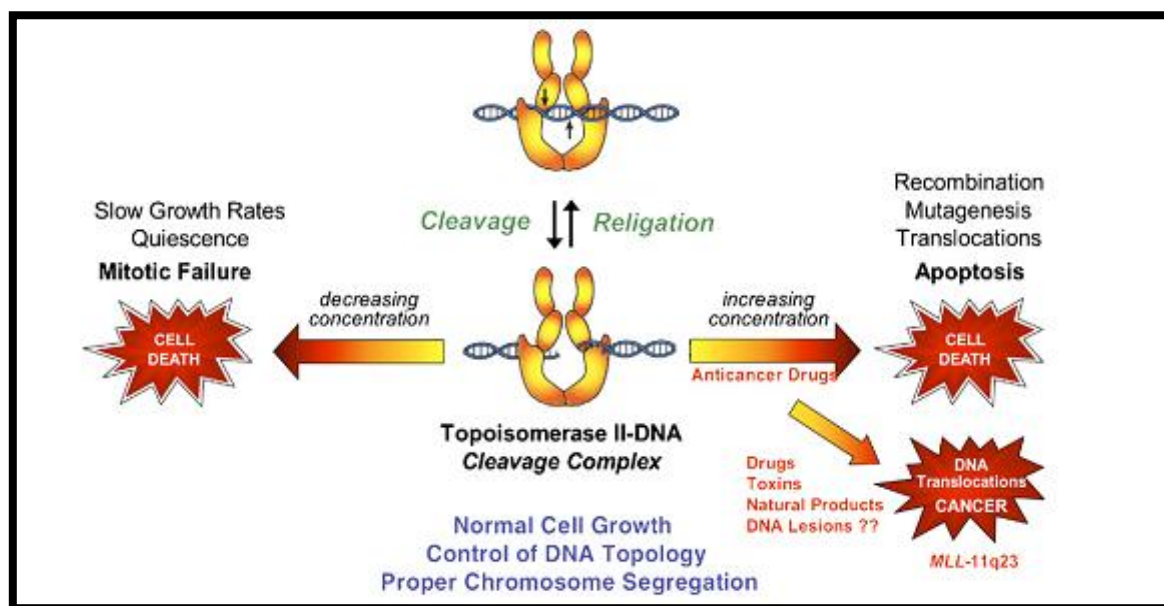


Figure 59: The effects of increased and decreased topoisomerase II-DNA cleavage complexes in the human cellular system (McClendon and Osheroff, 2007).

CHAPTER 5: SUMMARY AND GENERAL CONCLUSION

C. triloba is an indigenous plant that is widespread in Southern Africa (Smithies, 2000). In a previous study, three structurally similar anthraquinones; 9,10-Anthracenedione, 1-Hydroxy-4-methylanthraquinone and 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione and one steroid; Androst-5-ene-3, 17, 19-triol were isolated from the root extracts of *C. triloba* (Mohanlall *et al.*, 2011). The anthraquinones have shown to inhibit the activity of the human topoisomerase II enzyme which transforms supercoiled DNA to linear DNA. This mechanism is currently the basis of many anticancer compounds. Due to the low production of anthraquinones in the field roots, a recent study was conducted whereby cell suspension cultures of *C. triloba* were established for the production of anthraquinones by methyl jasmonate elicitation; however low yields of anthraquinones were produced (Naicker *et al.*, 2010). Therefore this study was undertaken to produce these compounds using *in vitro* plant systems with particular emphasis on cell suspension and *A. rhizogenes* transformed hairy root cultures of *C. triloba*.

Since the establishment of *C. triloba in vitro* plant systems required a source of sterile explants, a protocol (30 % NaClO for 10 minutes) was optimized for the germination of sterilized seeds which provided seedlings that were generated into shoot cultures on MS medium. Subsequently the leaf explants of the shoot cultures were used to induce orange-yellow calli. These were transferred into MS liquid medium to form cell suspension cultures that turned from orange to brown after one month and the total biomass increased from 4 g to 134.18 g (wet weight) after two months. The stem explants from shoot cultures were successfully transformed by infection with a low cell density inoculum of *A. rhizogenes* and hairy roots were induced at a transformation efficiency of 73.33%. Thereafter the use of 500 mg.l⁻¹ and 200 mg.l⁻¹ of cefotaxime decontaminated the hairy roots. It is well known that hairy root cultures grow rapidly in hormone free medium, however *C. triloba* hairy roots did not grow well. Therefore MS medium was supplemented with hormones and the mean hairy root biomass was 1.03 g in the control culture (without hormone) and 23.91 g and 46.13 g in the cultures containing IAA or NAA, respectively.

Accomplishment of high biomass yields allowed us to analyze the secondary metabolites and anthraquinones produced in *C. triloba* plant cell and tissue cultures (hairy root, shoot and cell suspension cultures) by using chromatographic analyses. The TLC profile of the natural root and plant cell and tissue culture extracts differed with most of the secondary metabolites being present in the extract obtained from the hairy root culture supplemented with NAA. TLC analysis also showed that band D (fraction CT02) had a R_f value of 0.55 which correlated to the R_f values (0.55 and 0.60) of the anthraquinone standards. In addition three other bands; A, B and C (Fraction CT01) had R_f values (0.71, 0.70 and 0.64 respectively) similar to that of the standards. The four bands of interest were predominant in the extract from the hairy roots cultivated in the presence of NAA.

HPLC analysis revealed three important results: the first is that hairy root cultures supplemented with IAA ($125.03 \mu\text{g} \cdot \text{mg}^{-1}$ [intra-cellular extract]) or NAA ($98.25 \mu\text{g} \cdot \text{mg}^{-1}$ [intra-cellular extract]) produced a higher concentration of anthraquinones compared to the control culture ($13.33 \mu\text{g} \cdot \text{mg}^{-1}$ [intra-cellular extract]). This was a 9.38 and a 7.37 fold increase, respectively. The second is that hairy root cultures supplemented with IAA or NAA produced a higher concentration of anthraquinones compared to the field roots ($33.51 \mu\text{g} \cdot \text{mg}^{-1}$) of *C. triloba*. This was a 3.73 and a 2.93 fold increase, respectively. The third is that shoot ($3.23 \mu\text{g} \cdot \text{mg}^{-1}$) and cell suspension cultures ($13.17 \mu\text{g} \cdot \text{mg}^{-1}$) produced a lower concentration of the anthraquinones in comparison to the field roots and hairy roots (supplemented with hormones). Hence with the use hormone supplementation, the production of anthraquinones in hairy root cultures is a more feasible approach compared to shoot and cell suspension cultures.

Individual anthraquinones could not be identified by HPLC analysis due co-elution. Therefore PTLC was performed with the extract obtained from the hairy roots cultivated in the present of NAA and six fractions (CT01, CT02, CT03, CT04, CT05 and CT06) were scraped off the TLC plate and the compounds were identified by EI-LC-MS. Using spectral libraries, one acridone derivative; 5-Methoxy-2-nitro-10H-acridin-9-one, one naphthoquinone derivative; 2H-Naphto[2,3-b]pyran-5,10-dione,3,4-dihydro-2,2-dimethyl- and five anthracenedione derivatives; i) 9,10-Anthracenedione, 2-methyl-, ii) 9,10- Anthracenedione, 2-ethyl-, iii) 1,5-Diaminoanthraquinone, iv) Phenanthrene, 3,6-dimethoxy-9-methyl- and v) 9,10-Anthracenedione,1,4-dimethyl- were identified in hairy root culture extract of *C. triloba* (Figure 60).

(Figure 60). This finding was the highlight of the study as these compounds were not identified in the field root extracts of *C. triloba*.

Also two anthraquinones, 5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione and 1-Hydroxy-4-methylanthraquinone that were isolated from the hairy root culture extract were structurally identical to those found in *C. triloba* field roots by Mohanlall *et al.* (2011) (Figure 60).

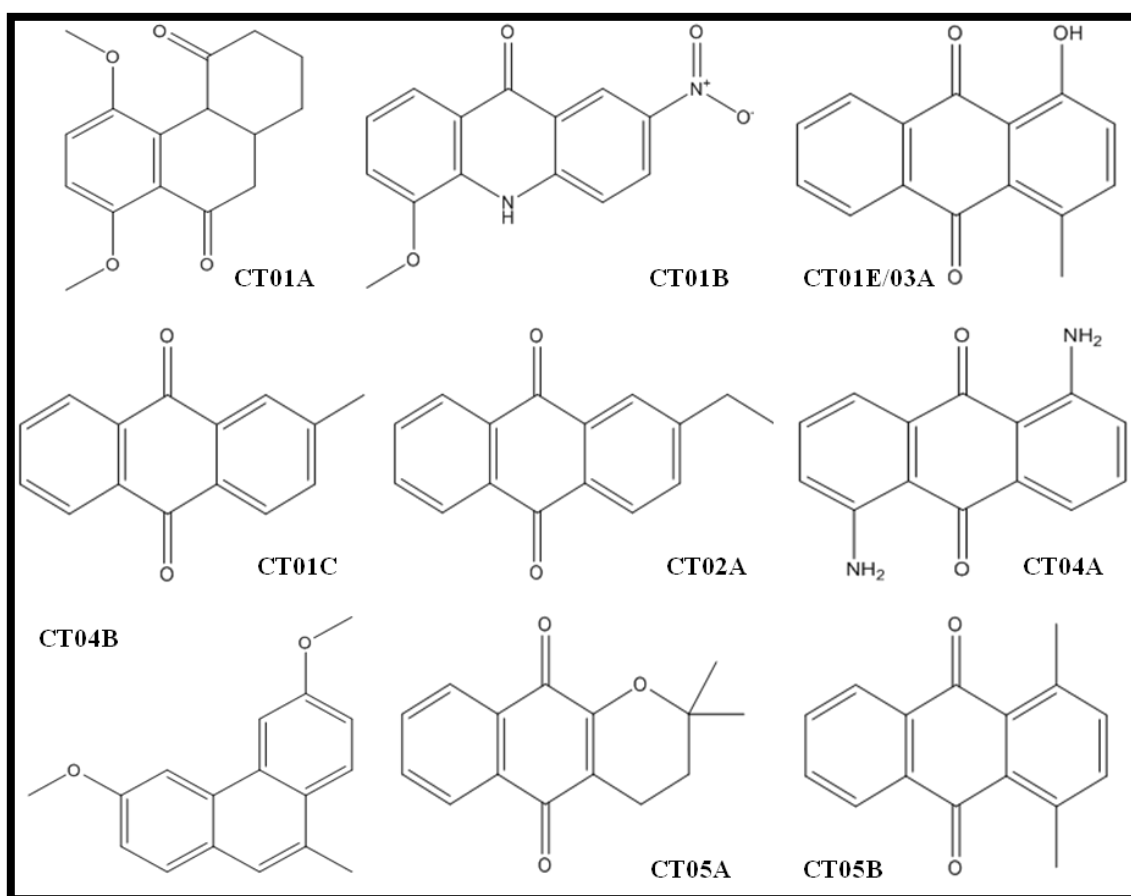


Figure 60: Compounds isolated from *C. triloba* hairy roots. CT01A-5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, CT01B-5-Methoxy-2-nitro-10H-acridin-9-one, CT01C-9,10-Anthracenedione, 2-methyl-, CT01E/03A-1-Hydroxy-4-methylanthraquinone, CT02A-9,10-Anthracenedione, 2-ethyl-, CT04A-1,5-Diaminoanthraquinone, CT04B-Phenanthrene, 3,6-dimethoxy-9-methyl-, CT05A-2H-Naphto[2,3-b]pyran-5,10-dione, 3,4-dihydro-2,2-dimethyl-, CT05B-9,10 Anthracenedione, 1,4-dimethyl-

Six other compounds were also identified in the hairy root culture extract, these were: 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl, n-Hexadecanoic acid, 1,2-Benzenedicarboxylic acid, diisooctyl ester, Octadecanoic acid, 9,12-Octadecadienoic acid (Z,Z)- and 6-Octadecenoic acid, (Z)-.

The acridone, naphthoquinone and anthracenedione derivatives that have been isolated from *C. triloba* hairy root cultures are similar or identical in structure to those that have reported in literature (Werner *et al.*, 1979; Huang *et al.*, 2005; Kumar *et al.*, 2011). Thus these compounds may have the potential to be used in pharmaceutical applications as broad spectrum agents of biological activities such as effects on cancer, on bacteria and fungi, on inflammatory responses, and also potent antioxidants.

In our study we showed that *C. triloba* field root and hairy root extracts as well as the isolated fractions, CT01 and CT02 were cytotoxic to the DU-145 cancer cell line. The order of cytotoxicity for the extracts and fractions at a 1000 $\mu\text{g}\cdot\text{ml}^{-1}$ concentration was: hairy root extract (from the culture supplemented with IAA) > hairy root extract (from the culture supplemented with NAA) > fraction CT01 > fraction CT02 > field root extract, respectively. Currently synthetic anthracenedione derivatives such as mitoxantrone and doxorubicin are being used in the treatment of cancer. However a number of cell lines have developed resistance to these drugs (Dietel *et al.*, 1990; Taylor *et al.*, 1991; Nakagawa *et al.*, 1992; Futscher *et al.*, 1994; Hazlehurst *et al.*, 1999; Doyle and Ross, 2003; Ma, and Wink, 2010). Thus this work provides a biological method (through *C. triloba* hairy root cultures) of producing anthracenedione derivatives that contain different side chains (methyl, ethyl, hydroxyl and amino groups) which could have a higher biological potency and efficacy.

Since there is evidence that anthracenedione based drugs (for example, mitoxanthrone) are topoisomerase inhibitors; from the results of the anti-topoisomerase assay we concluded that the anthracenedione derivatives present in fractions CT01 (5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, 9,10-Anthracenedione, 2-methyl- and 1-Hydroxy-4-methylanthraquinone) and CT02 (9,10- Anthracenedione, 2-ethyl-) were responsible for the inhibition of the human topoisomerase II enzyme. This result was vital since topoisomerases are targeted in chemotherapy in order to trigger the apoptotic (cell death) pathway in cancer cells. Also this study validates the previous findings which indicated that *C. triloba* root derived 9,10-Anthracenedione and 1-hydroxy-4-

methylanthraquinone are topoisomerase inhibitors.

In conclusion this is the first study that describes the transformation of *C. triloba* by *A. rhizogenes* and the production of anthraquinones from *C. triloba* hairy root cultures. Protocols have been developed for the surface sterilization and germination of *C. triloba* seeds for the cultivation shoot cultures to serve as a source of explants for transformation. Hairy root cultures were induced by employing a low cell density inoculum of *A. rhizogenes* and antibiotic decontamination to induced cultures. Improvement of the biomass yield of the hairy root cultures by hormone supplementation; allowed for the comparison of the TLC profiles of the hairy root culture extracts to the natural root, shoot and cell suspension culture extract profiles. Also the highest yield of anthraquinones was produced in hairy root cultures supplemented with NAA. This yield exceeded the amount of anthraquinones found in *C. triloba* field roots. EI-LC-MS analysis revealed that the essential chemical constituents present in the hairy root hexane extract were seven anthracenedione derivatives, one acridone derivative and one naphthoquinone derivative. The bioactivity of these compounds is yet to be determined however their close structural relationship with other chemical derivatives indicates their pharmaceutical potential. Most importantly the anthracenedione derivatives from fractions CT01 (5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione, 9,10-Anthracenedione, 2-methyl- and 1-Hydroxy-4-methylanthraquinone) and CT02 (9,10-Anthracenedione, 2-ethyl-) have already depicted cytotoxic activity on cancer cells and displayed the anticancer mechanism of inhibiting the topoisomerase enzyme.

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Appendix 2: EI-LC-MS Library matches

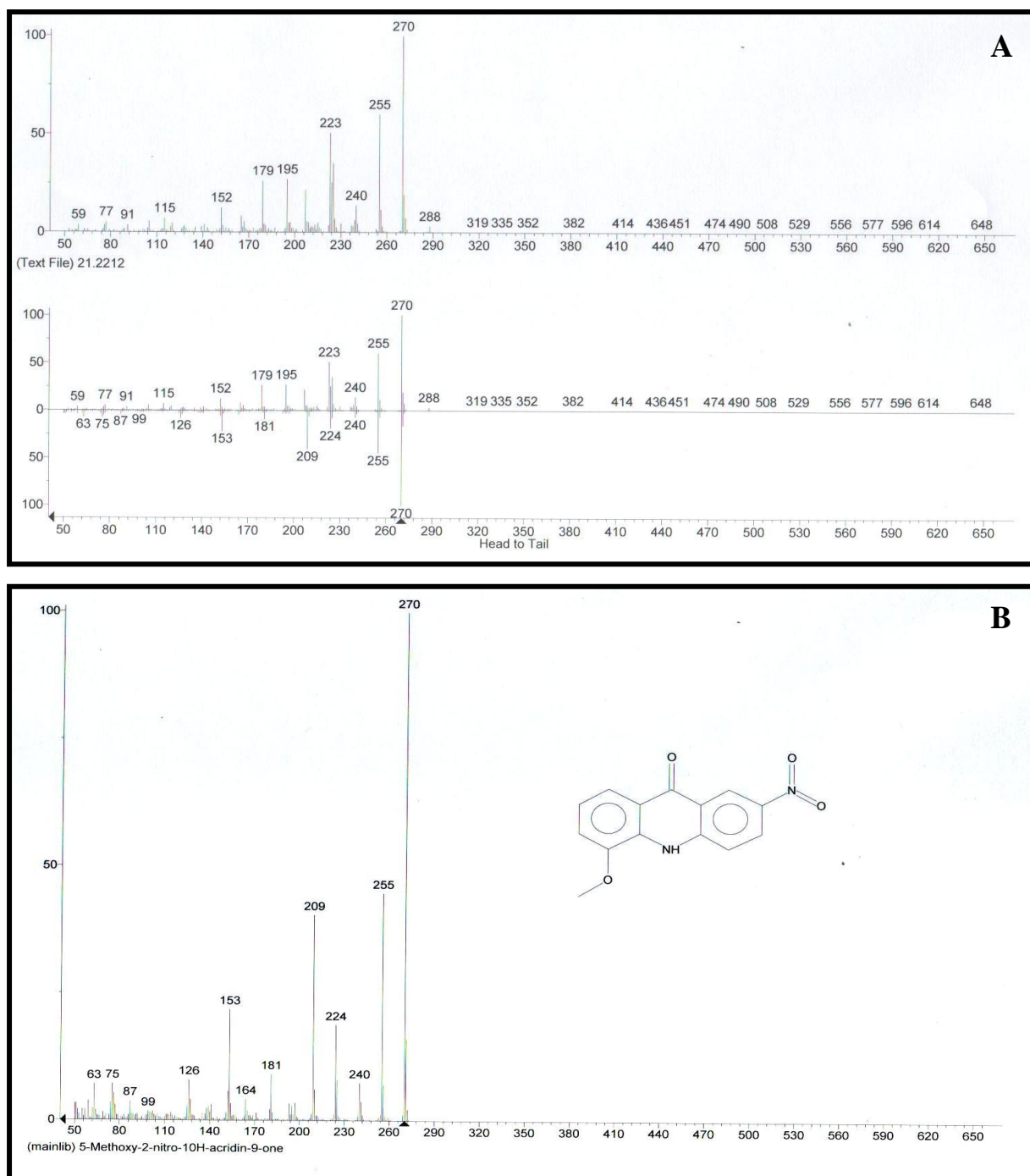


Figure A1: Library match of 5-Methoxy-2-nitro-10H-acridin-9-one (compound CT01B) isolated from *C. triloba* hairy roots. A-Library template of 5-Methoxy-2-nitro-10H-acridin-9-one and B-isolated 5-Methoxy-2-nitro-10H-acridin-9-one from *C. triloba* hairy roots.

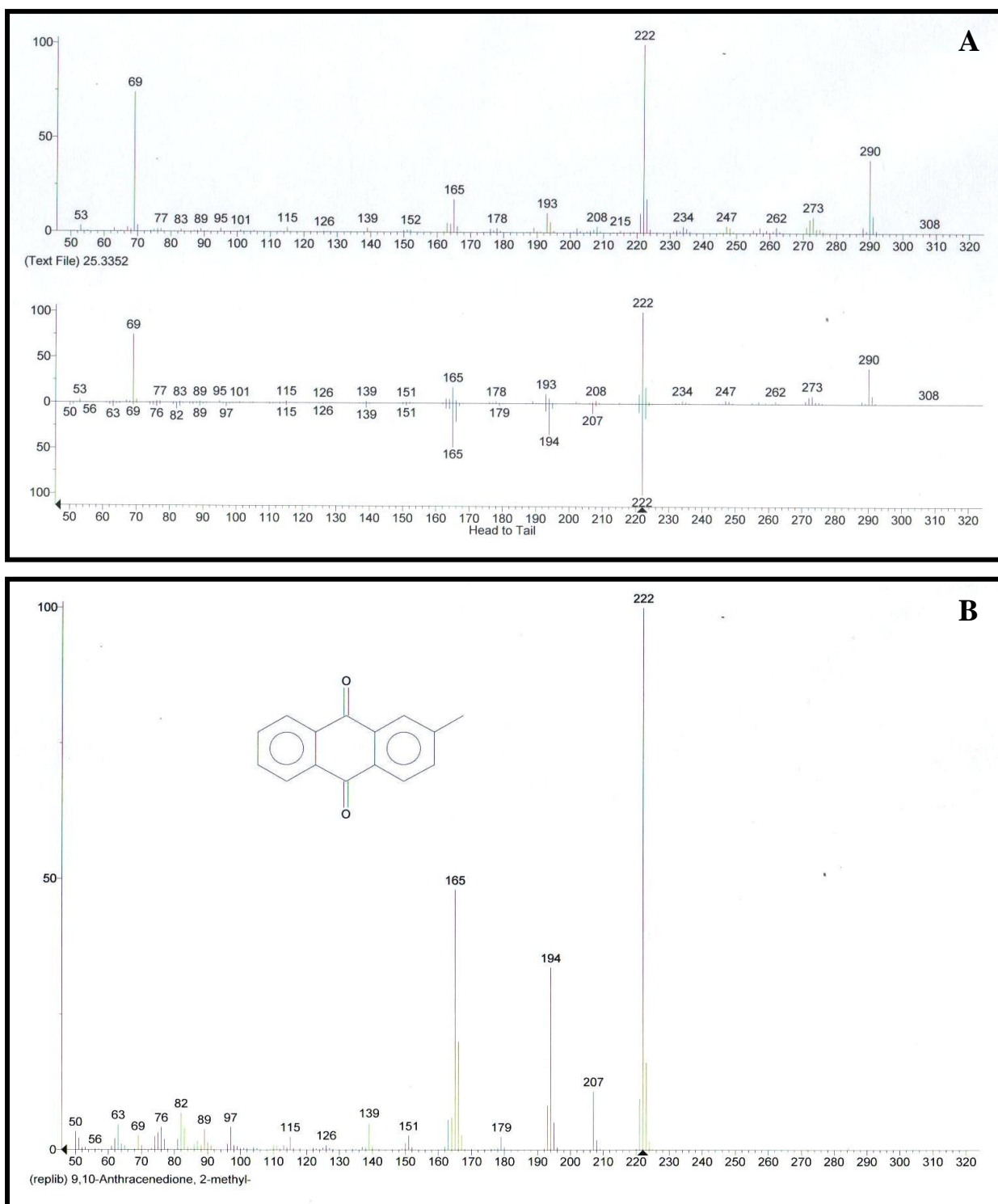


Figure A2: Library match of 9,10-Anthracenedione, 2-methyl- (compound CT01C) isolated from *C. triloba* hairy roots. A-Library template of 9,10-Anthracenedione, 2-methyl- and B-isolated 9,10-Anthracenedione, 2-methyl- from *C. triloba* hairy roots.

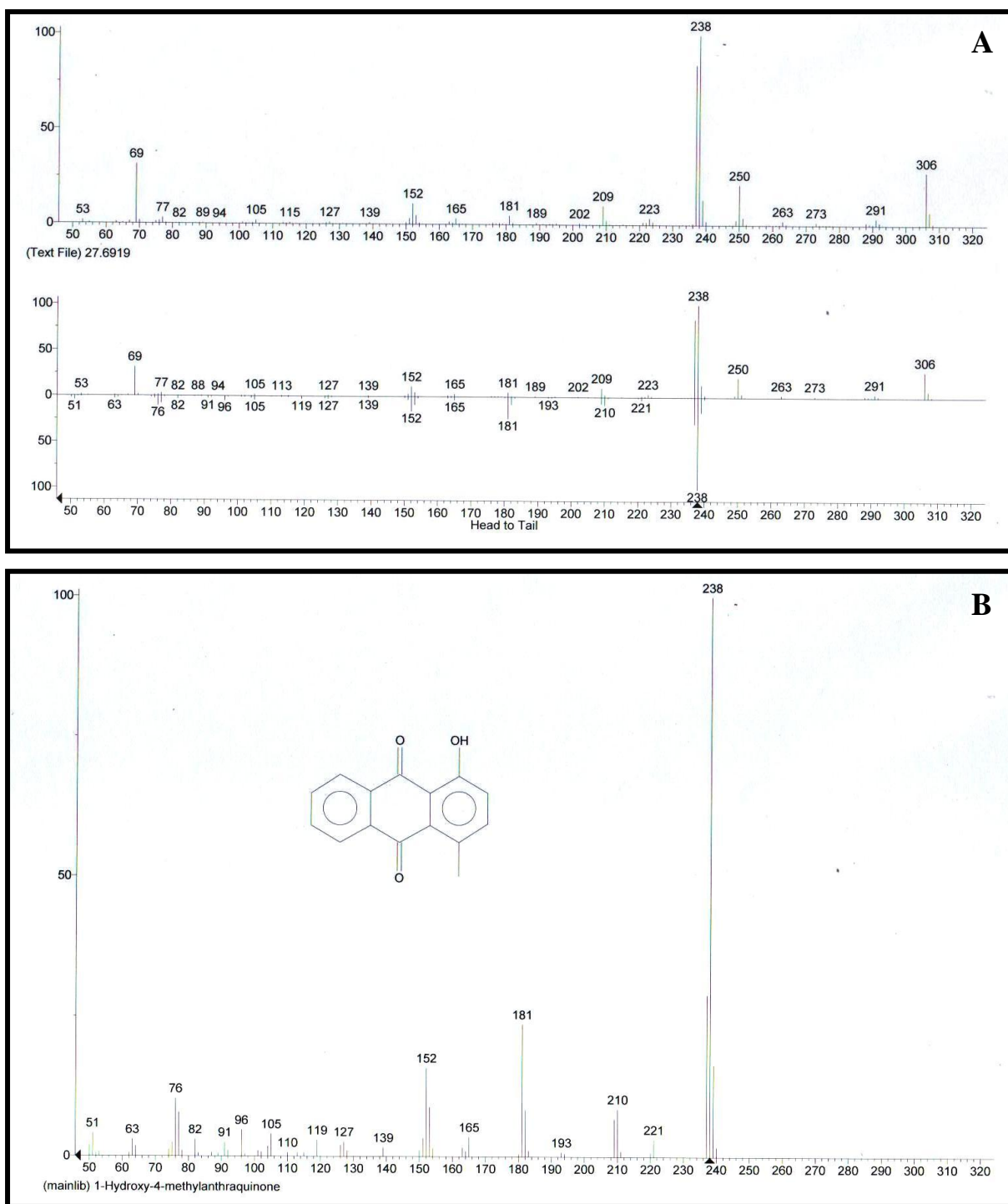


Figure A3: Library match of 1-Hydroxy-4-methylantraquinone (compound CT01D) isolated from *C. triloba* hairy roots. A-Library template of 1-Hydroxy-4-methylantraquinone and B-isolated 1-Hydroxy-4-methylantraquinone from *C. triloba* hairy roots.

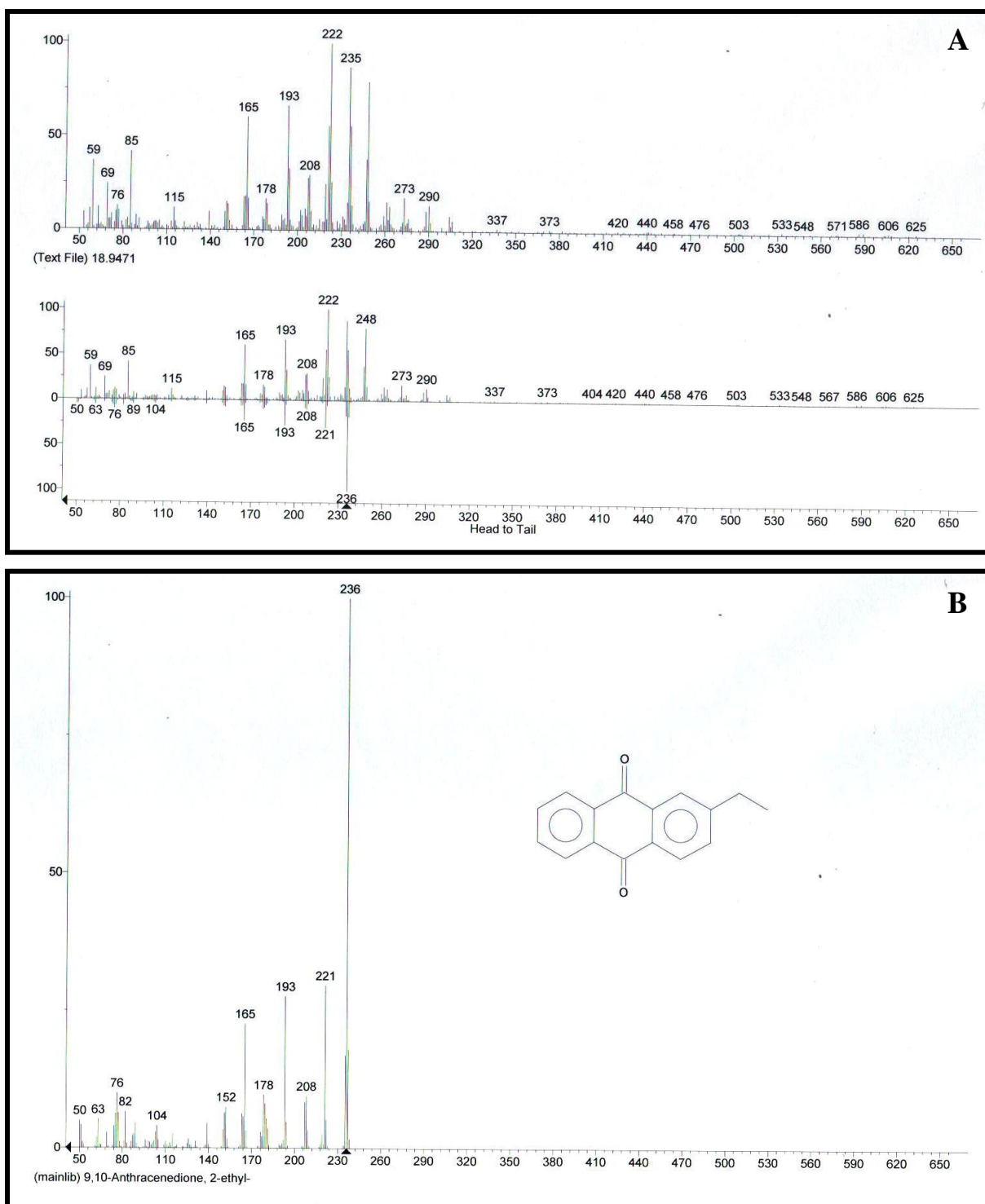


Figure A4: Library match of 9,10- Anthracenedione, 2-ethyl- (compound CT02A) isolated from *C. triloba* hairy roots. A-Library template of 9,10- Anthracenedione, 2-ethyl- and B-isolated 9,10- Anthracenedione, 2-ethyl- from *C. triloba* hairy roots.

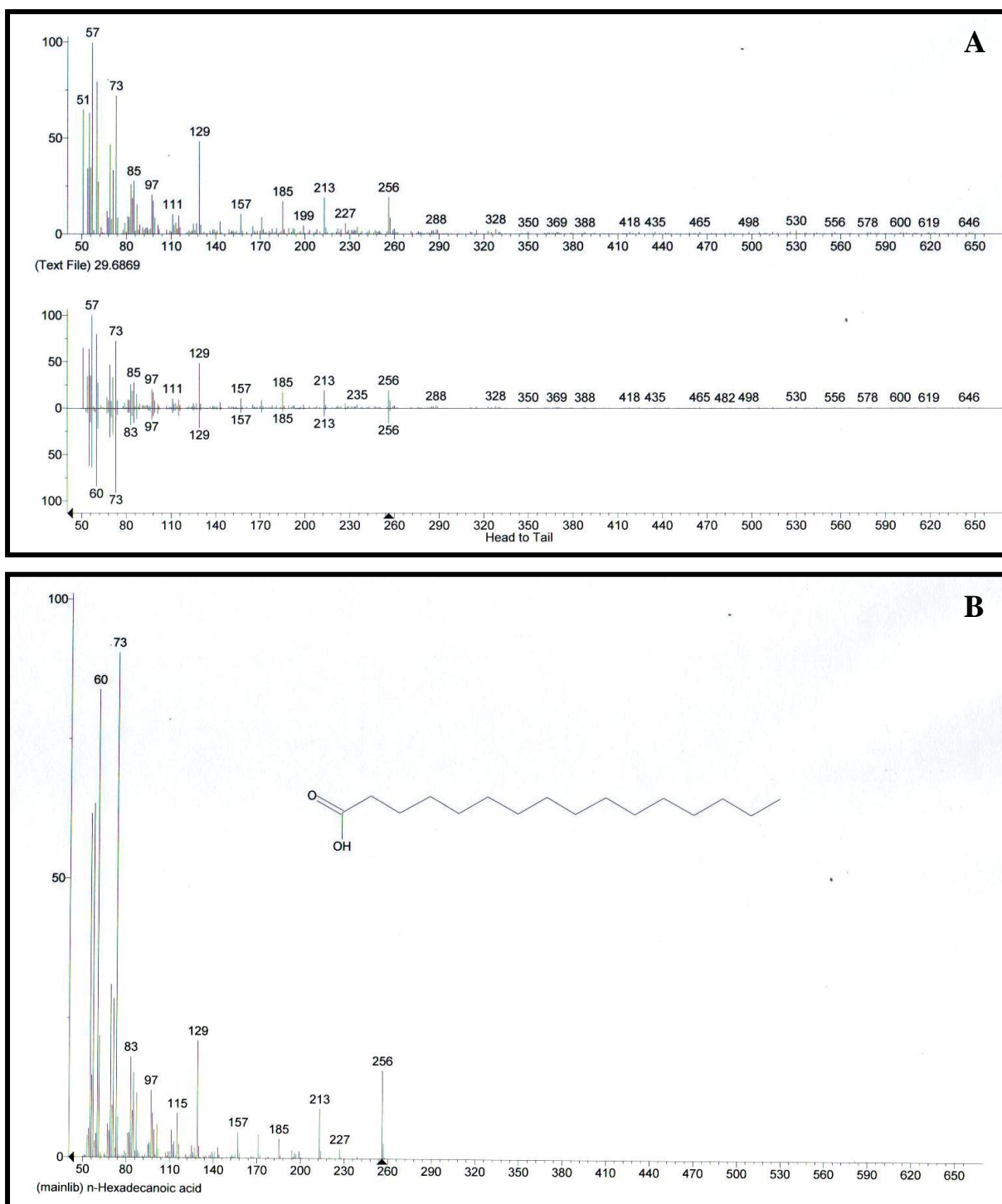


Figure A5: Library match of n-Hexadecanoic acid (compound CT02B) isolated from *C. triloba* hairy roots. A-Library template of n-Hexadecanoic acid and B-isolated n-Hexadecanoic acid from *C. triloba* hairy roots.

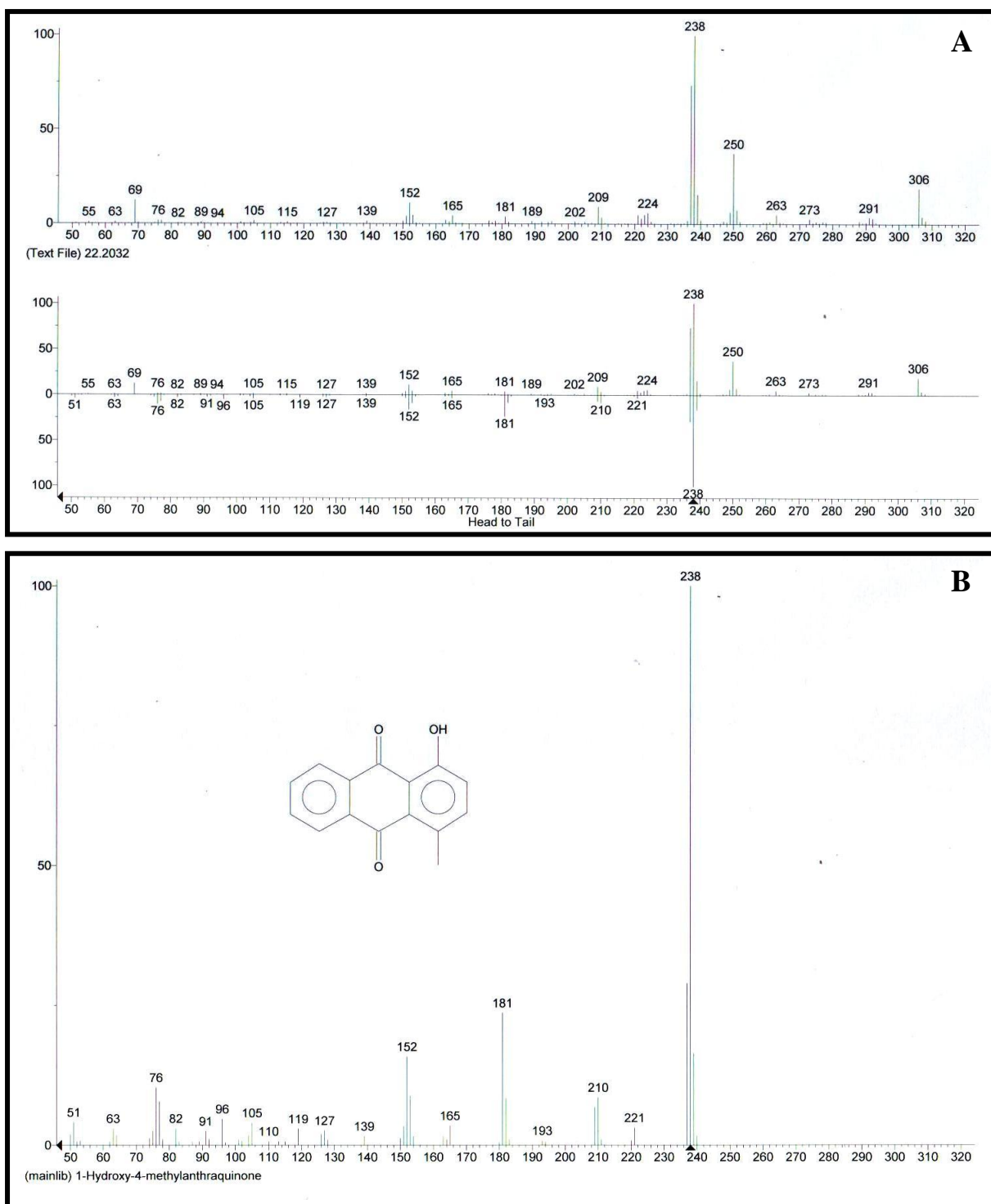


Figure A6: Library match of 1-Hydroxy-4-methylantraquinone (compound CT03A) isolated from *C. triloba* hairy roots. A-Library template of 1-Hydroxy-4-methylantraquinone and B-isolated 1-Hydroxy-4-methylantraquinone from *C. triloba* hairy roots.

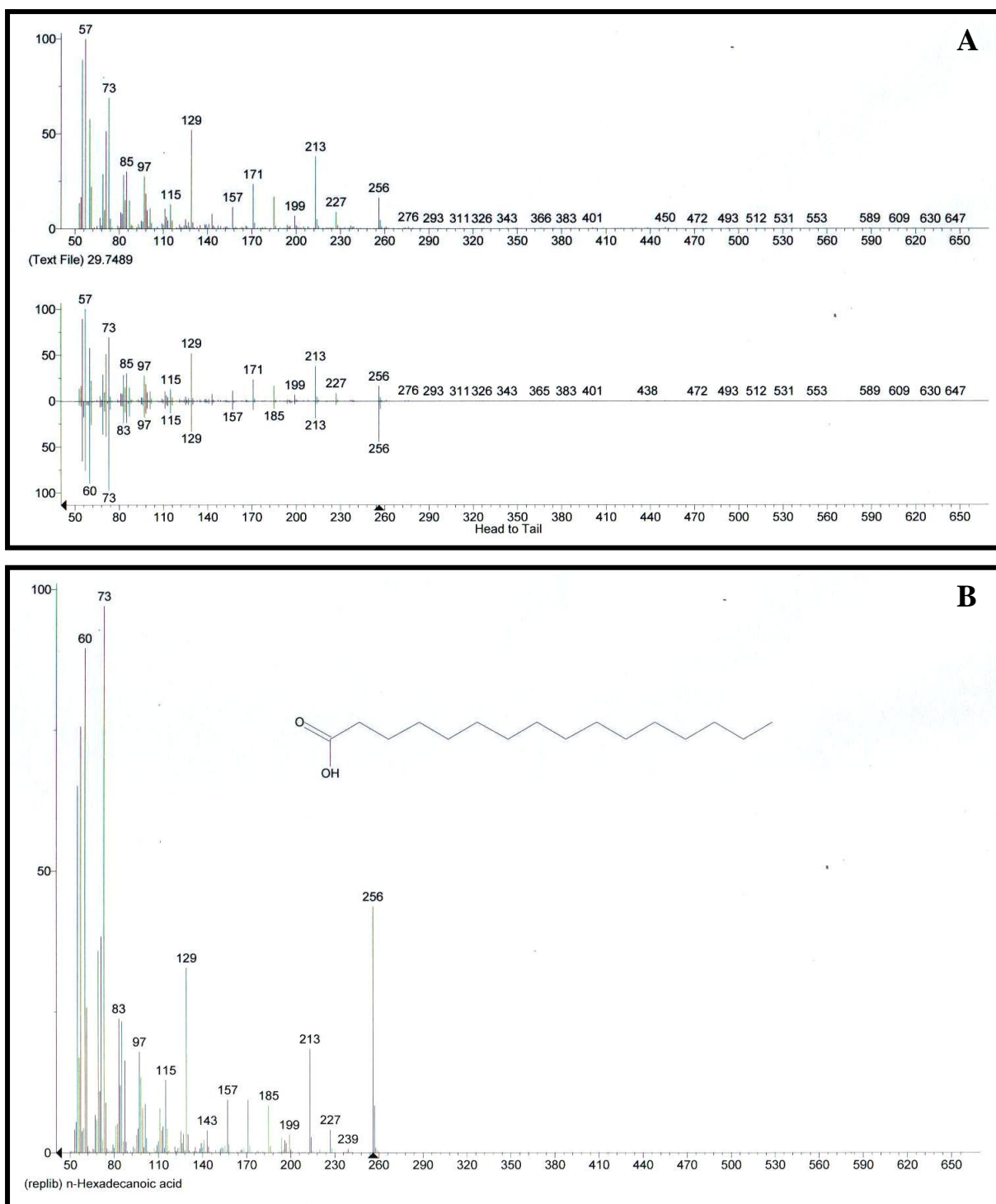


Figure A7: Library match of n-Hexadecanoic acid (compound CT03B) isolated from *C. triloba* hairy roots. A-Library template of n-Hexadecanoic acid and B-isolated n-Hexadecanoic acid from *C. triloba* hairy roots.

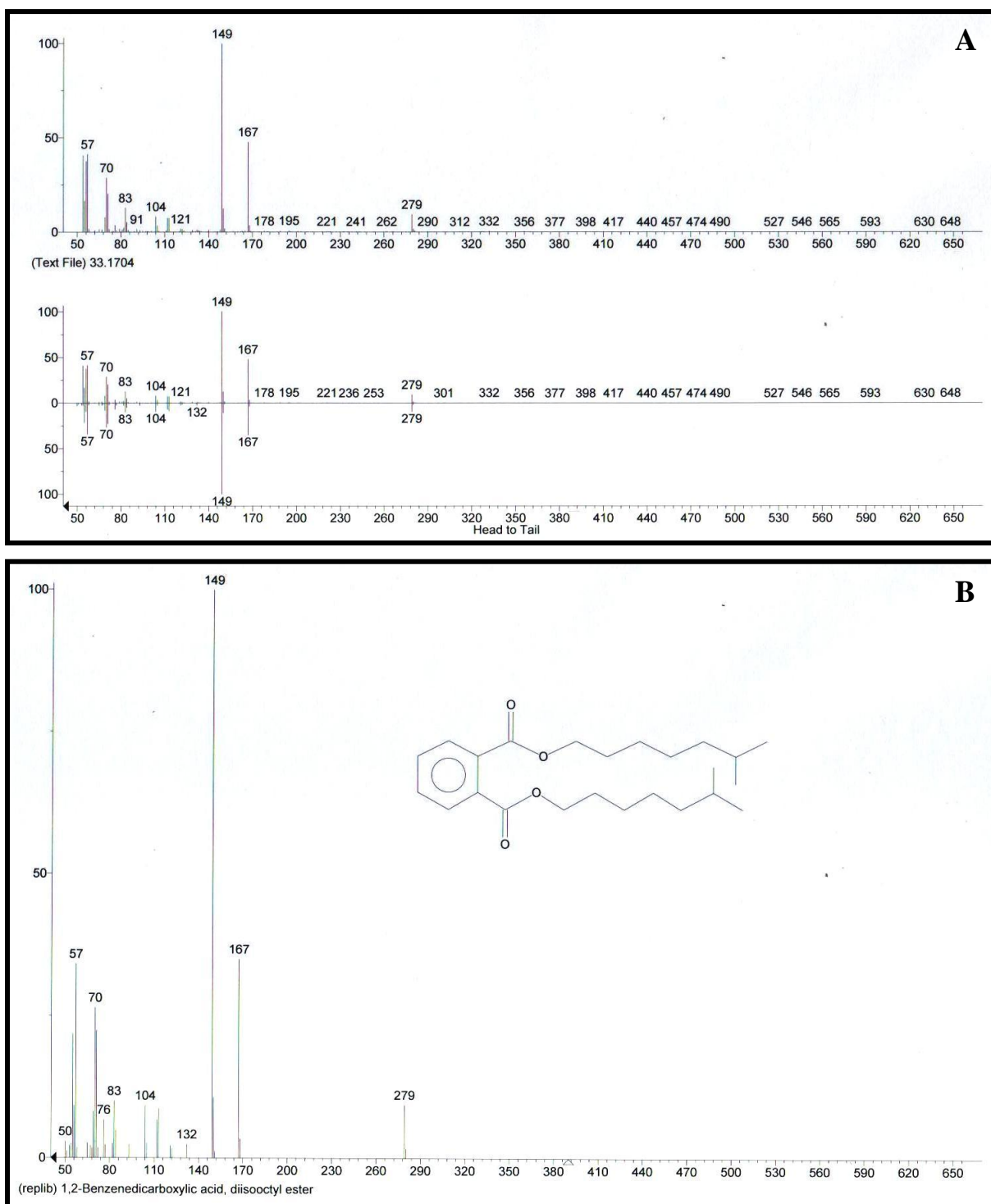


Figure A8: Library match of 1,2-Benzenedicarboxylic acid, diisooctyl ester (compound CT03C) isolated from *C. triloba* hairy roots. A-Library template of 1,2-Benzenedicarboxylic acid, diisooctyl ester and B-isolated 1,2-Benzenedicarboxylic acid, diisooctyl ester from *C. triloba* hairy roots.

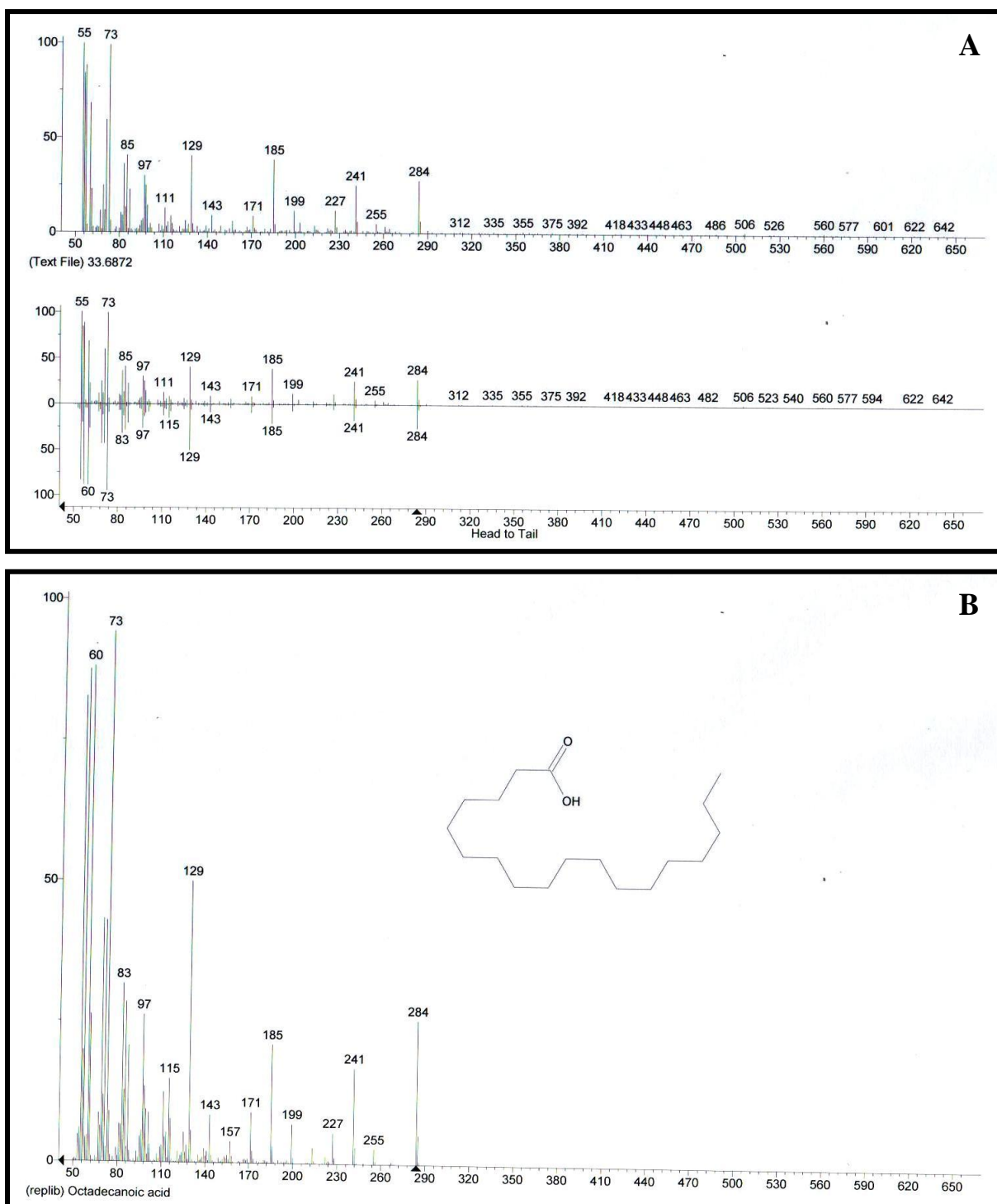


Figure A9: Library match of Octadecanoic acid (compound CT03D) isolated from *C. triloba* hairy roots. A-Library template of Octadecanoic acid and B-isolated Octadecanoic acid from *C. triloba* hairy roots.

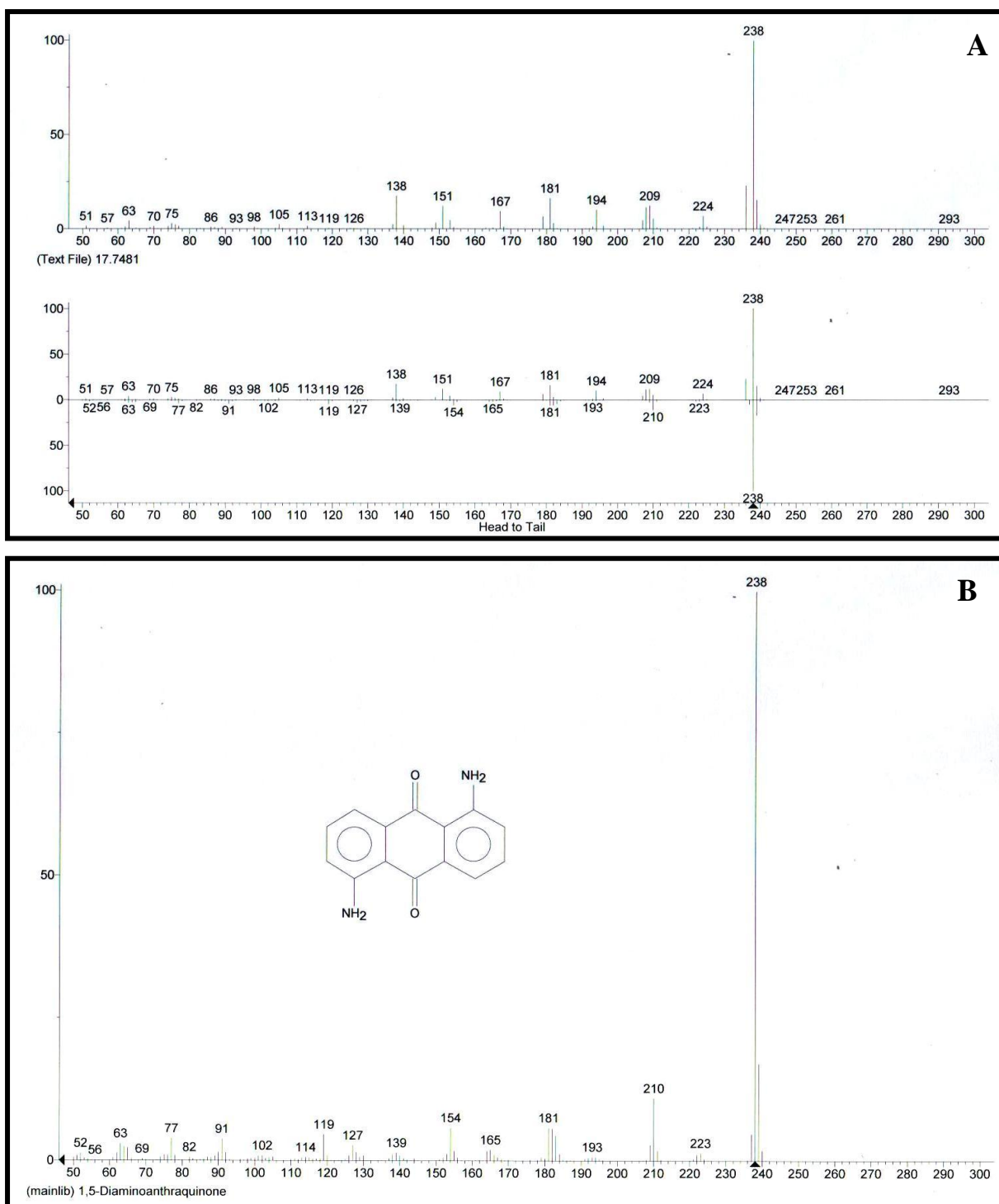


Figure A10: Library match of 1,5-Diaminoanthraquinone (compound CT04A) isolated from *C. triloba* hairy roots. A-Library template of 1,5-Diaminoanthraquinone and B-isolated 1,5-Diaminoanthraquinone from *C. triloba* hairy roots.

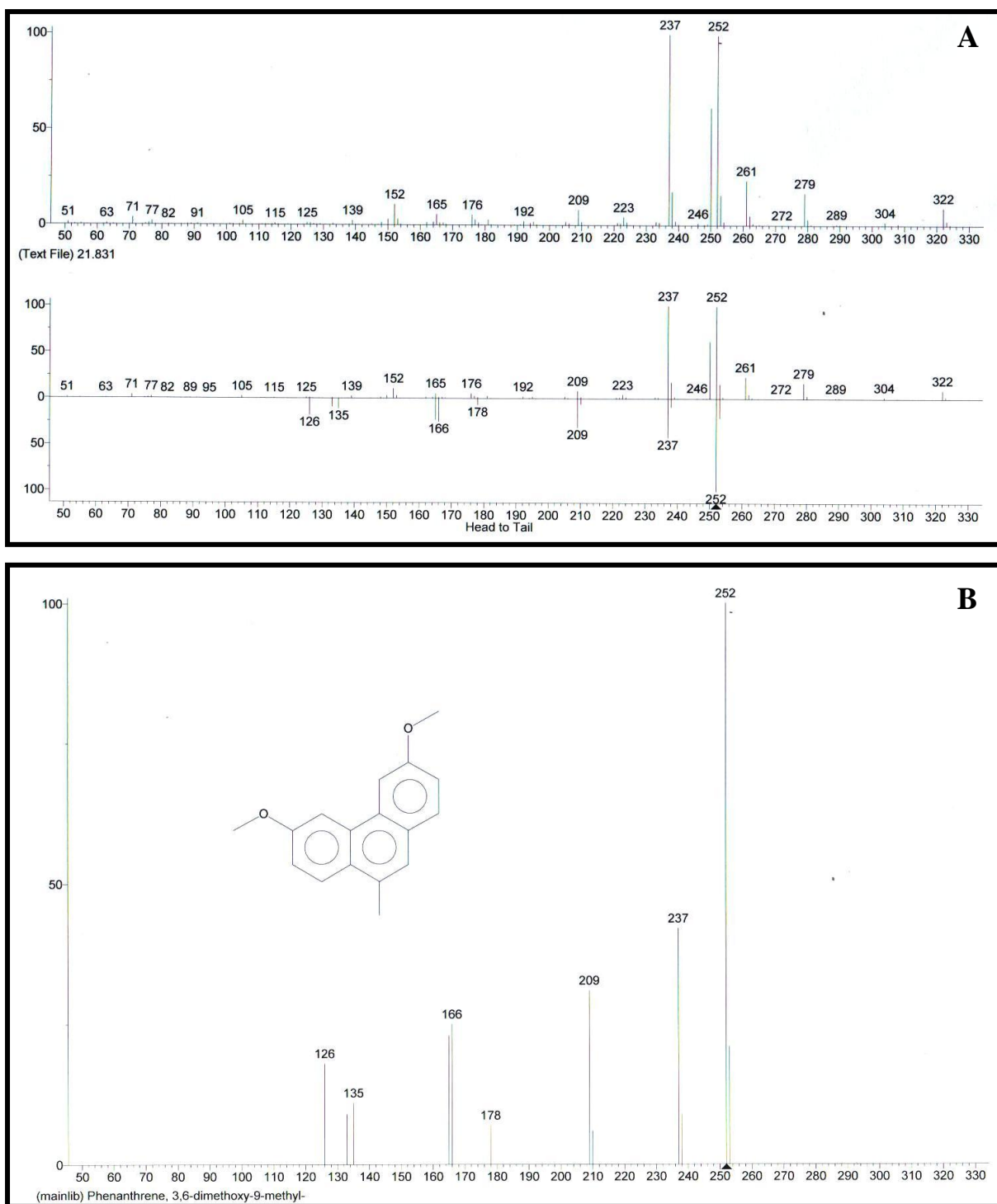


Figure A11: Library match of Phenanthrene, 3,6-dimethoxy-9-methyl- (compound CT04B) isolated from *C. triloba* hairy roots. A-Library template of Phenanthrene, 3,6-dimethoxy-9-methyl- and B-isolated Phenanthrene, 3,6-dimethoxy-9-methyl- from *C. triloba* hairy roots.

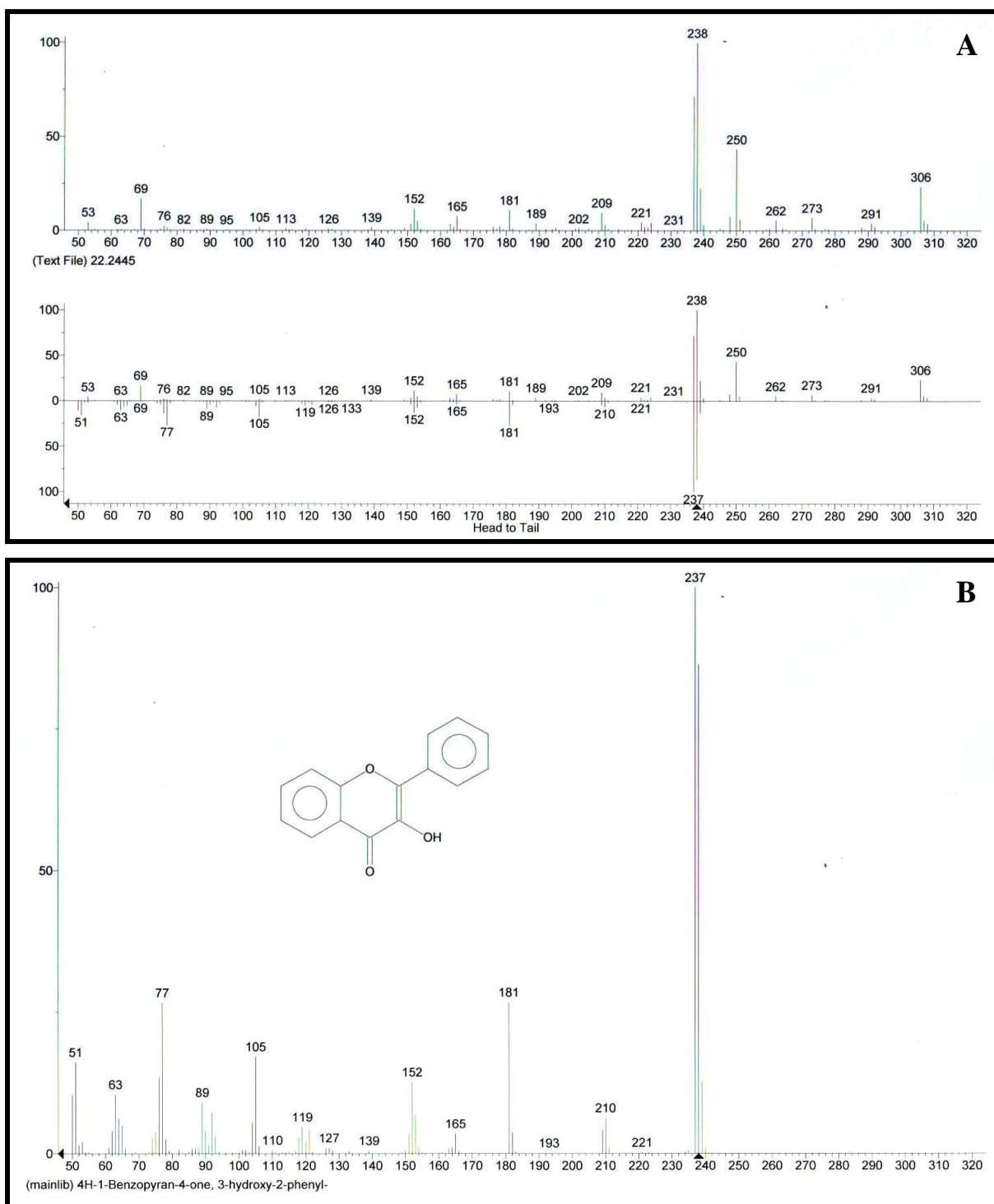


Figure A12: Library match of 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl (compound CT04C) isolated from *C. triloba* hairy roots. A-Library template of 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl and B-isolated 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl from *C. triloba* hairy roots.

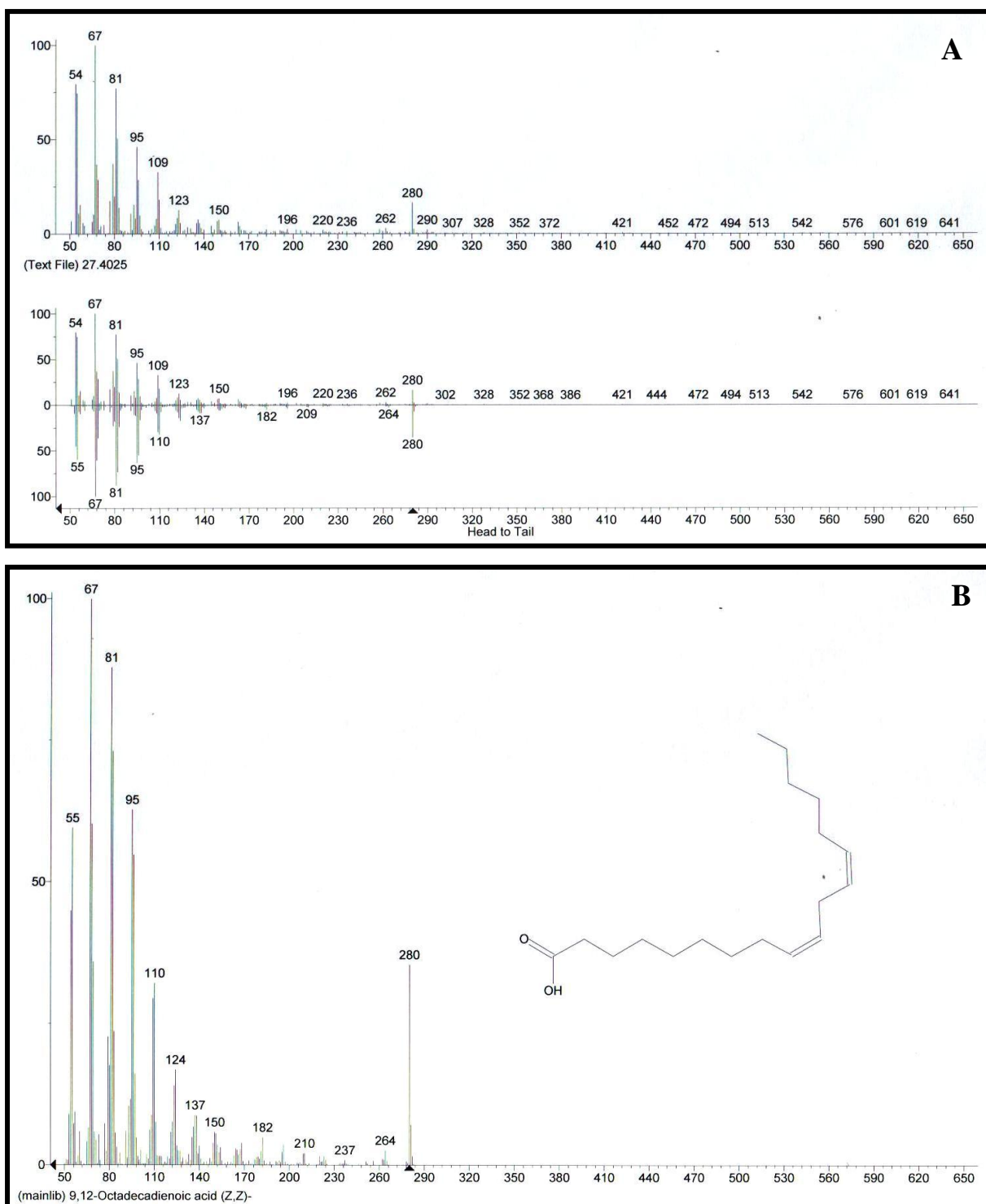


Figure A13: Library match of 9,12-Octadecadienoic acid (Z,Z)- (compound CT04D) isolated from *C. triloba* hairy roots. A-Library template of 9,12-Octadecadienoic acid (Z,Z)- and B-isolated 9,12-Octadecadienoic acid (Z,Z)- from *C. triloba* hairy roots.

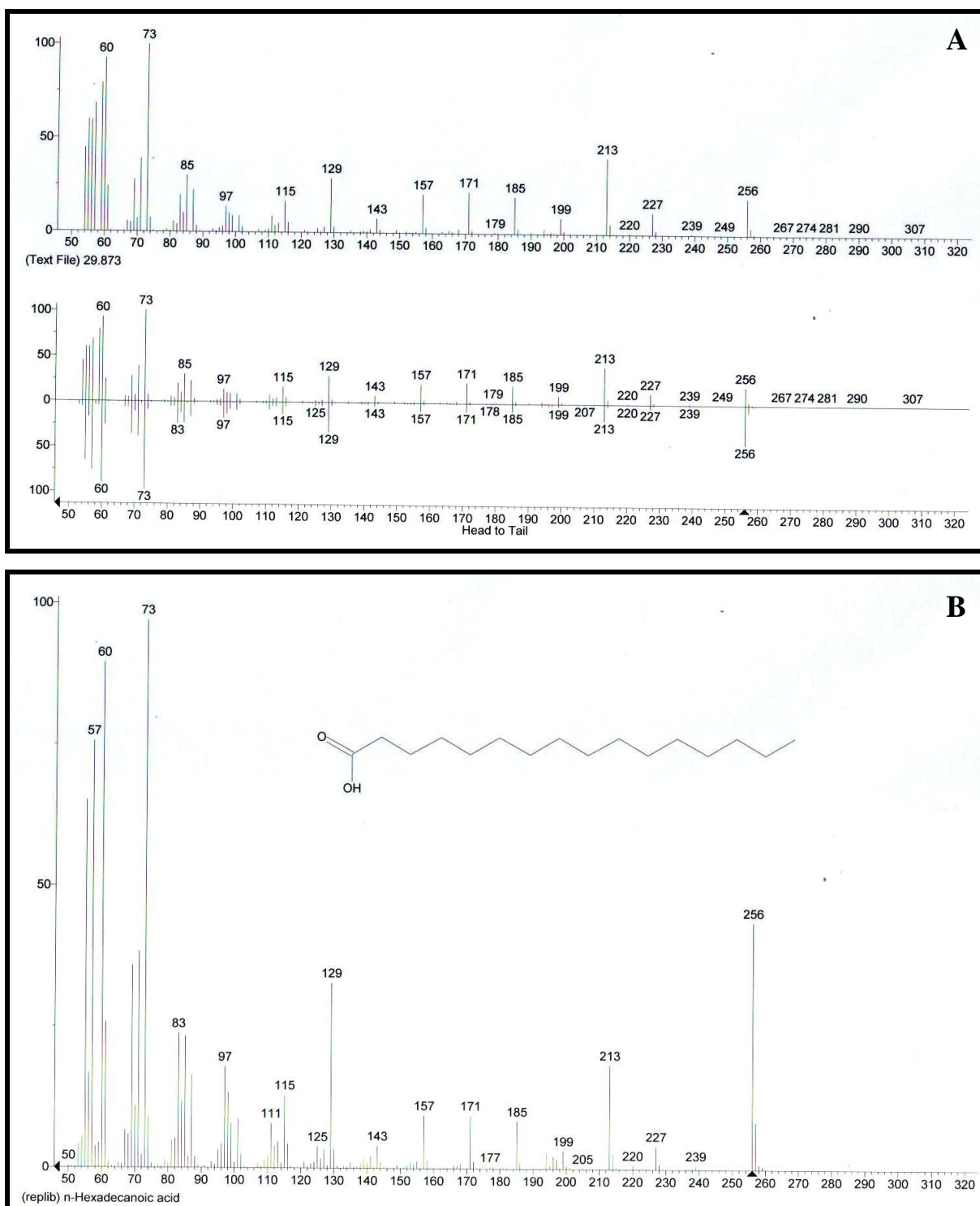


Figure A14: Library match of n-Hexadecanoic acid (compound CT04E) isolated from *C. triloba* hairy roots. A-Library template of n-Hexadecanoic acid and B-isolated n-Hexadecanoic acid from *C. triloba* hairy roots.

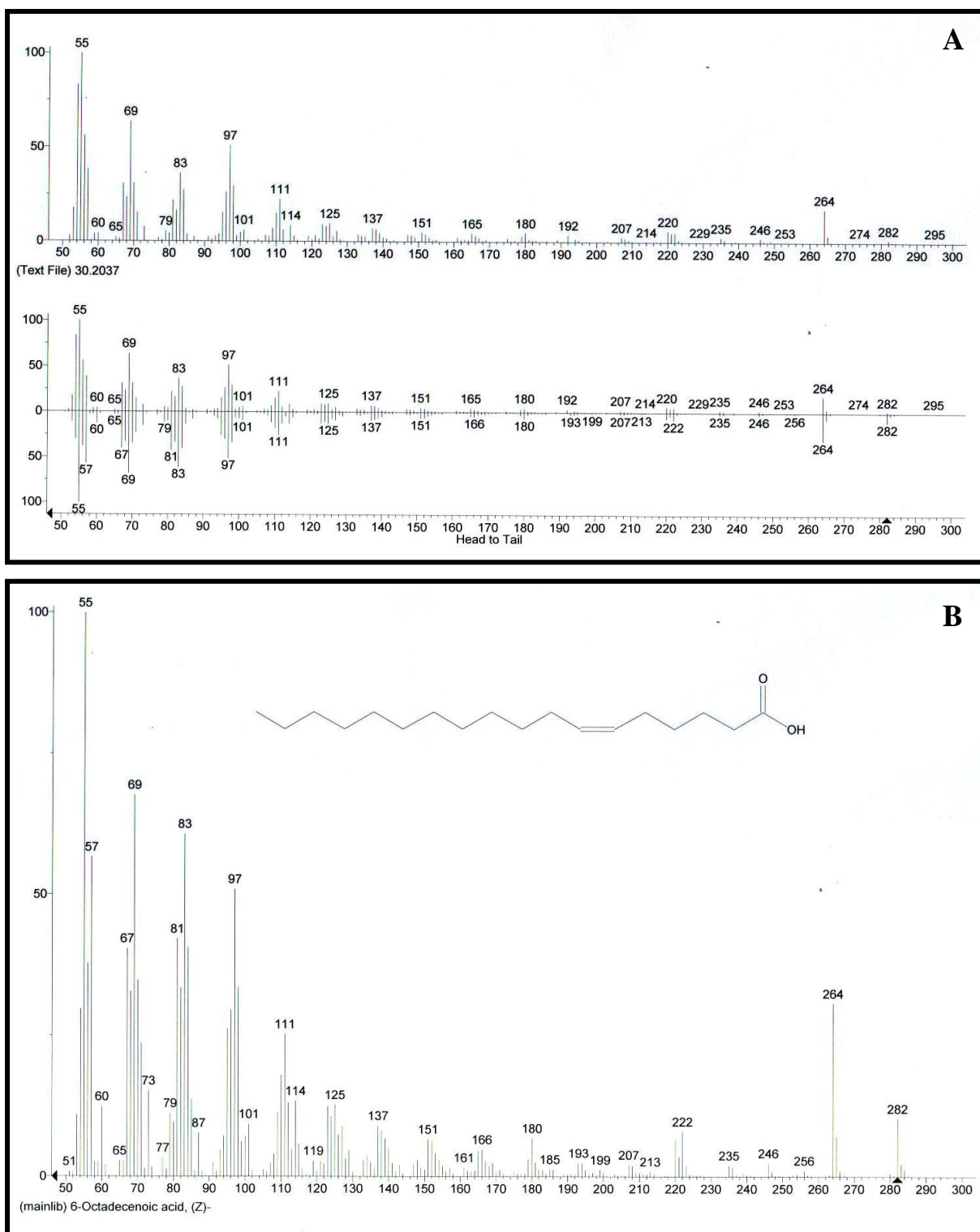


Figure A15: Library match of 6-Octadecenoic acid, (Z)- (compound CT04F) isolated from *C. triloba* hairy roots. A-Library template of 6-Octadecenoic acid, (Z)- and B-isolated 6-Octadecenoic acid, (Z)- from *C. triloba* hairy roots.

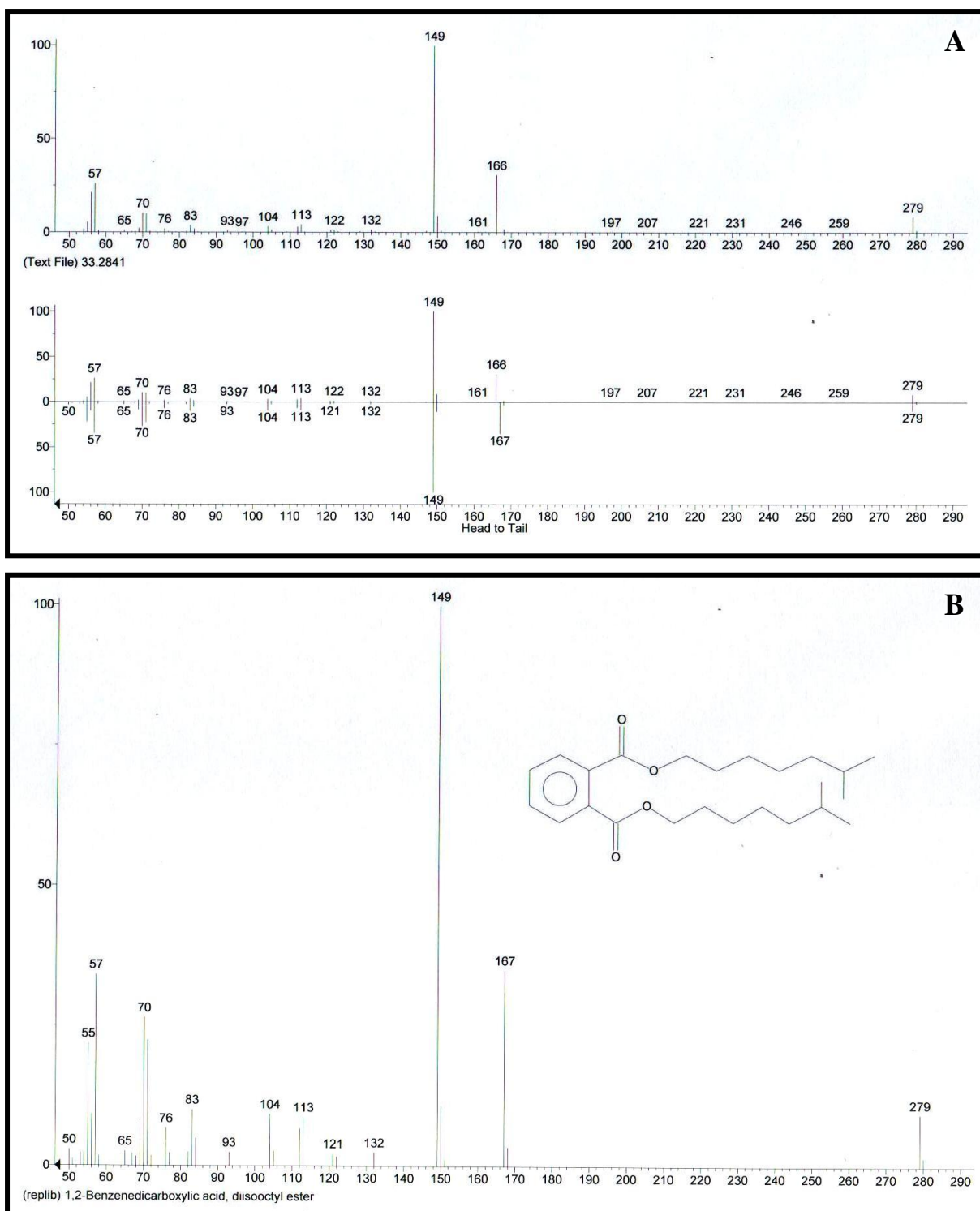


Figure A16: Library match of 1,2-Benzenedicarboxylic acid, diisooctyl ester (compound CT04G) isolated from *C. triloba* hairy roots. A-Library template of 1,2-Benzenedicarboxylic acid, diisooctyl ester and B-isolated 1,2-Benzenedicarboxylic acid, diisooctyl ester from *C. triloba* hairy roots.

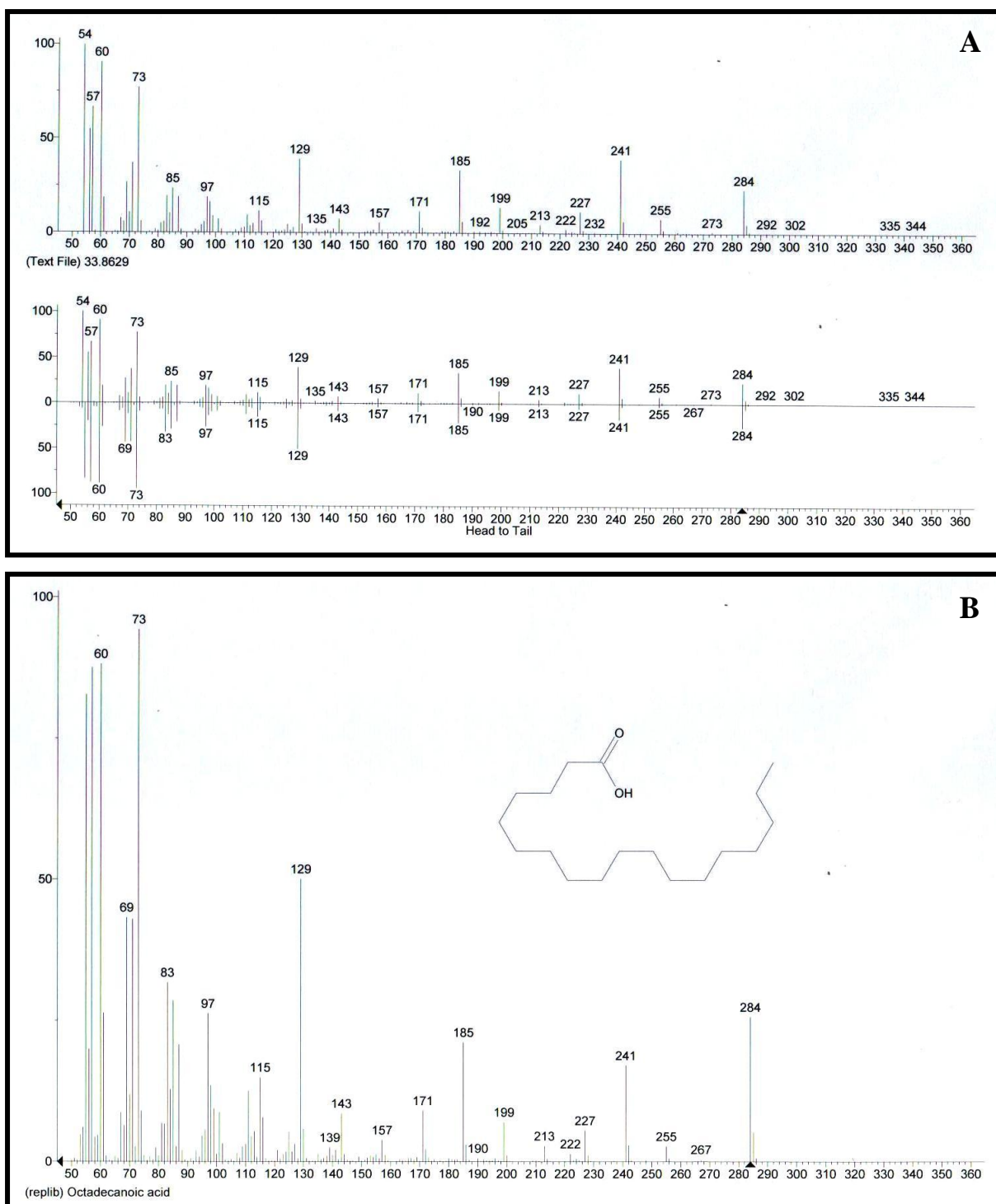


Figure A17: Library match of Octadecanoic acid (compound CT04I) isolated from *C. triloba* hairy roots. A-Library template of Octadecanoic acid and B-isolated Octadecanoic acid from *C. triloba* hairy roots.

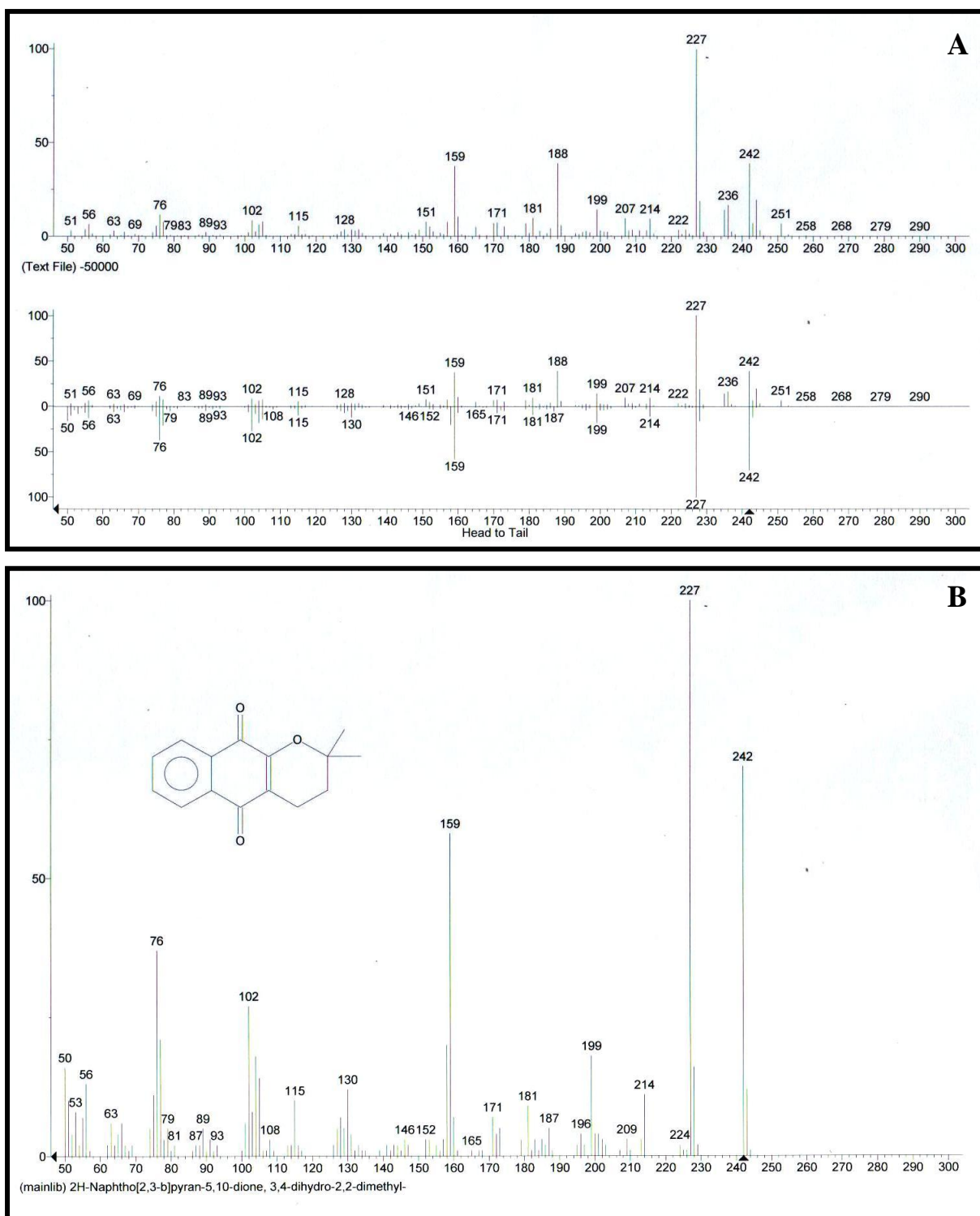


Figure A18: Library match of 2H-Naphto [2,3-b]pyran-5,10-dione, 3,4-dihydro-2,2-dimethyl- (compound CT05A) isolated from *C. triloba* hairy roots. A-Library template of 2H-Naphto[2,3-b]pyran-5,10-dione, 3,4-dihydro-2,2-dimethyl- and B-isolated 2H-Naphto[2,3-b]pyran-5,10-dione, 3,4-dihydro-2,2-dimethyl- from *C. triloba* hairy roots.

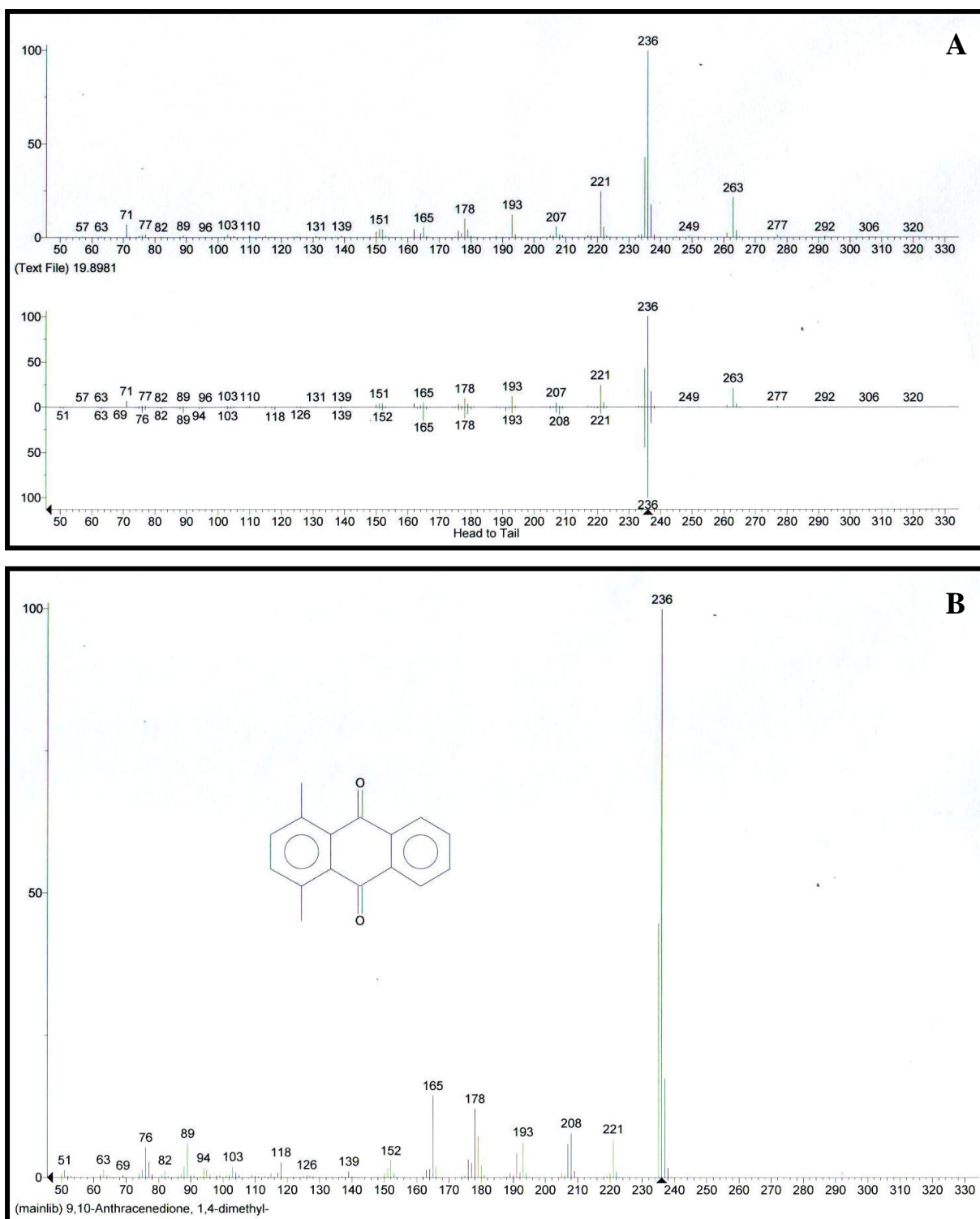


Figure A19: Library match of 9,10-Anthracenedione, 1,4-dimethyl- (compound CT05B) isolated from *C. triloba* hairy roots. A-Library template of 9,10-Anthracenedione, 1,4-dimethyl- and B-isolated 9,10-Anthracenedione, 1,4-dimethyl- from *C. triloba* hairy roots.

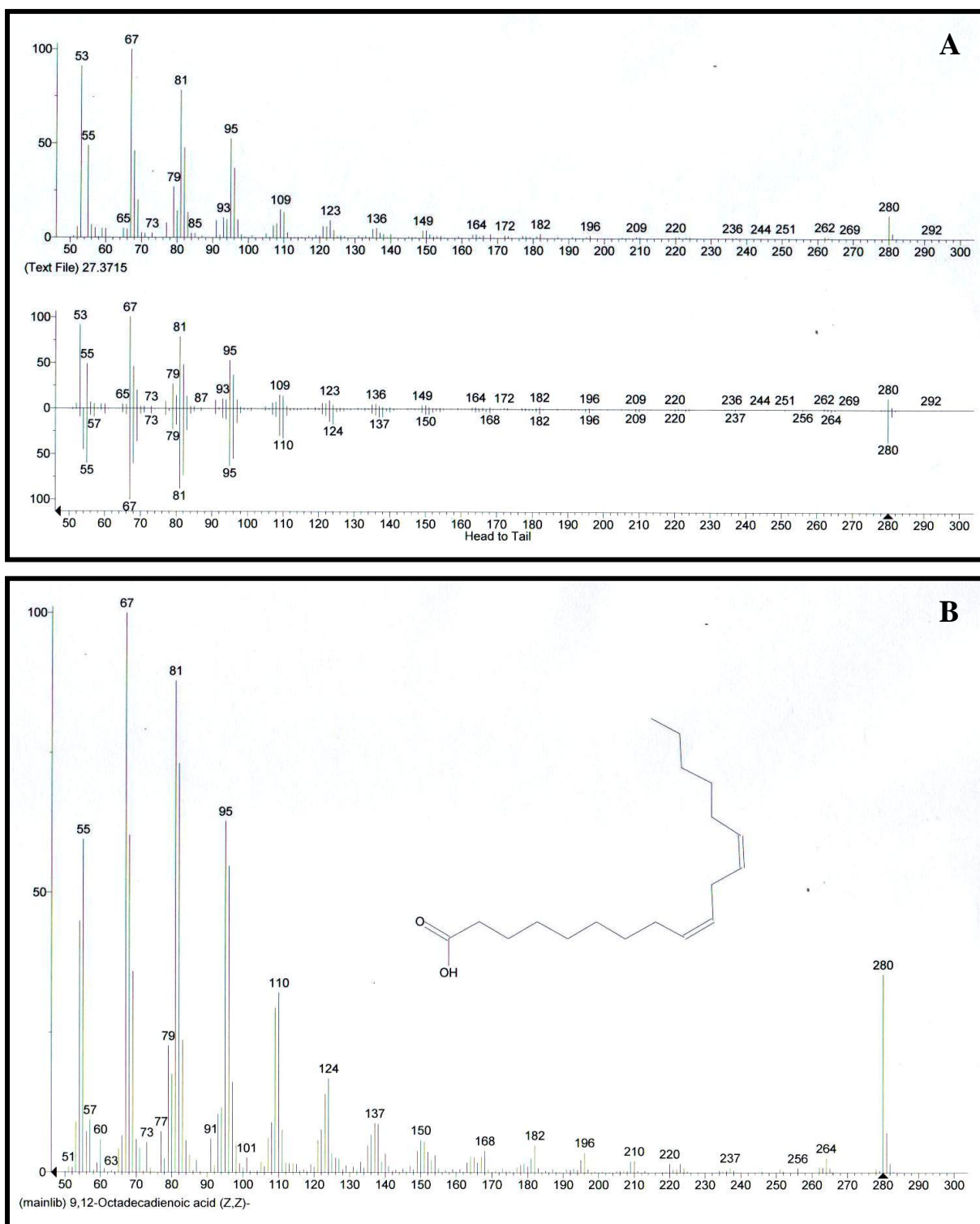


Figure A20: Library match of 9,12-Octadecadienoic acid (Z,Z)- (compound CT05C) isolated from *C. triloba* hairy roots. A-Library template of 9,12-Octadecadienoic acid (Z,Z)- and B-isolated 9,12-Octadecadienoic acid (Z,Z)- from *C. triloba* hairy roots.

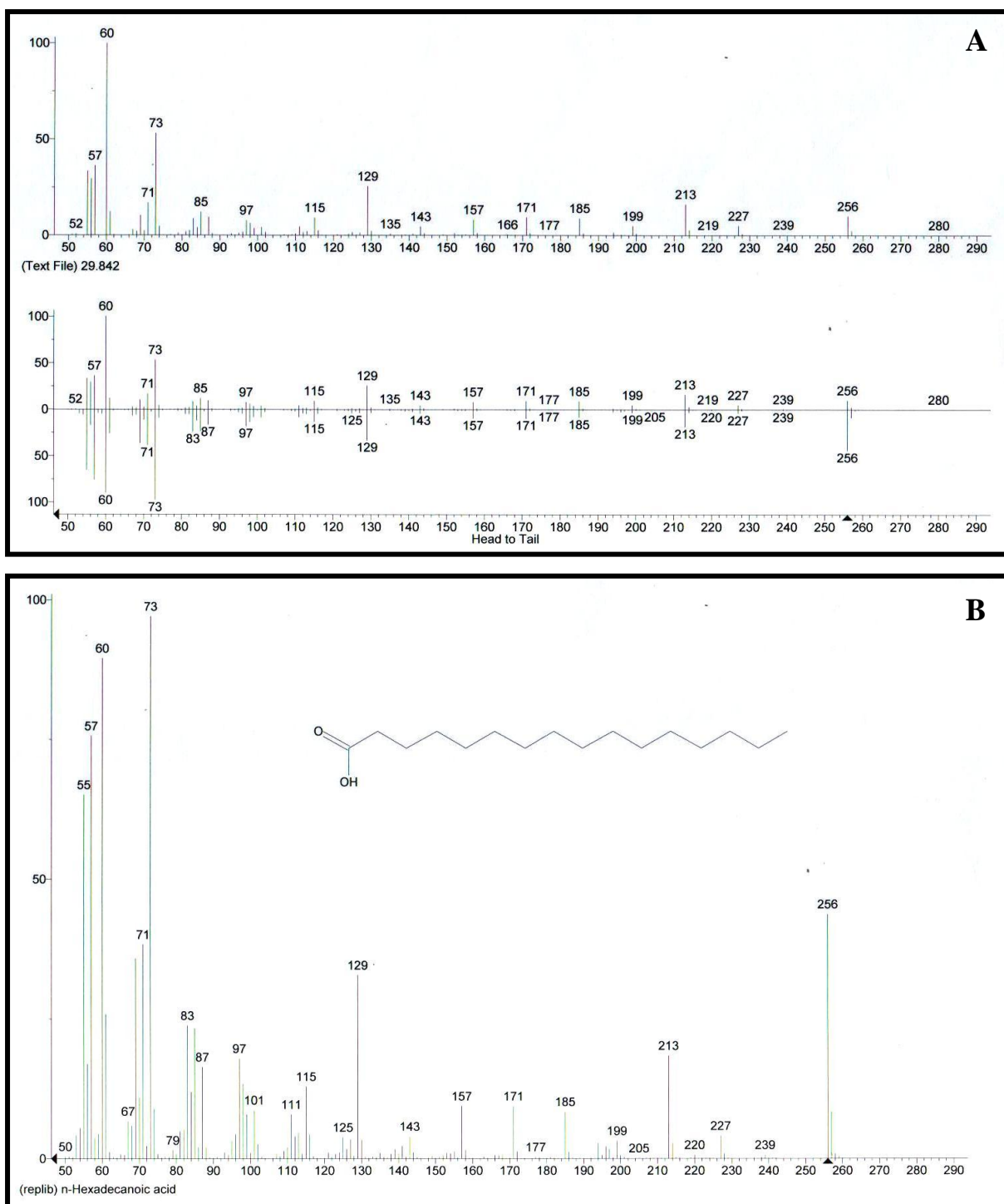


Figure A21: Library match of n-Hexadecanoic acid (compound CT05D) isolated from *C. triloba* hairy roots. A-Library template of n-Hexadecanoic acid and B-isolated n-Hexadecanoic acid from *C. triloba* hairy roots.

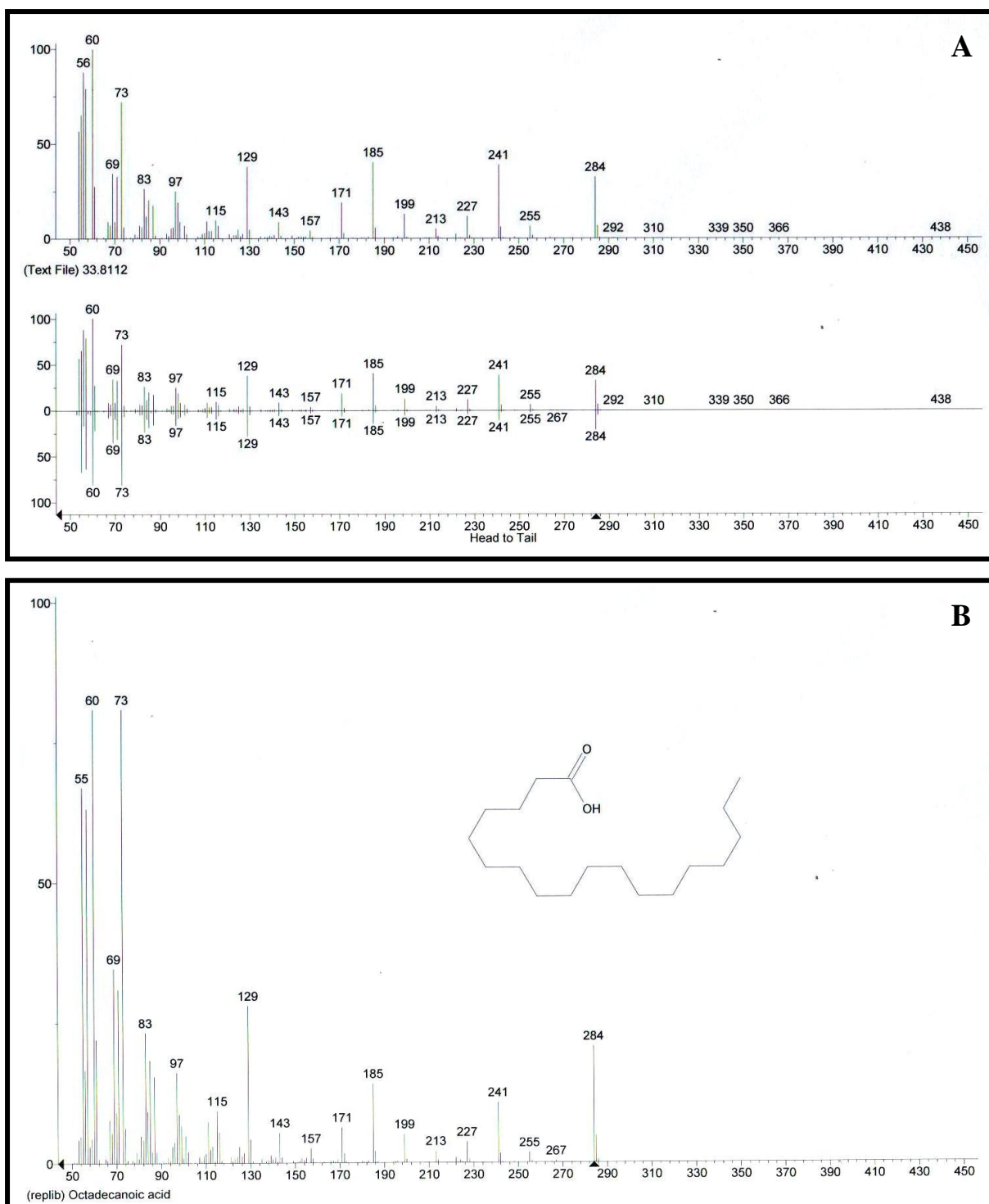


Figure A22: Library match of Octadecenoic acid (compound CT05E) isolated from *C. triloba* hairy roots. A-Library template of Octadecenoic acid and B-isolated Octadecenoic acid from *C. triloba* hairy roots.

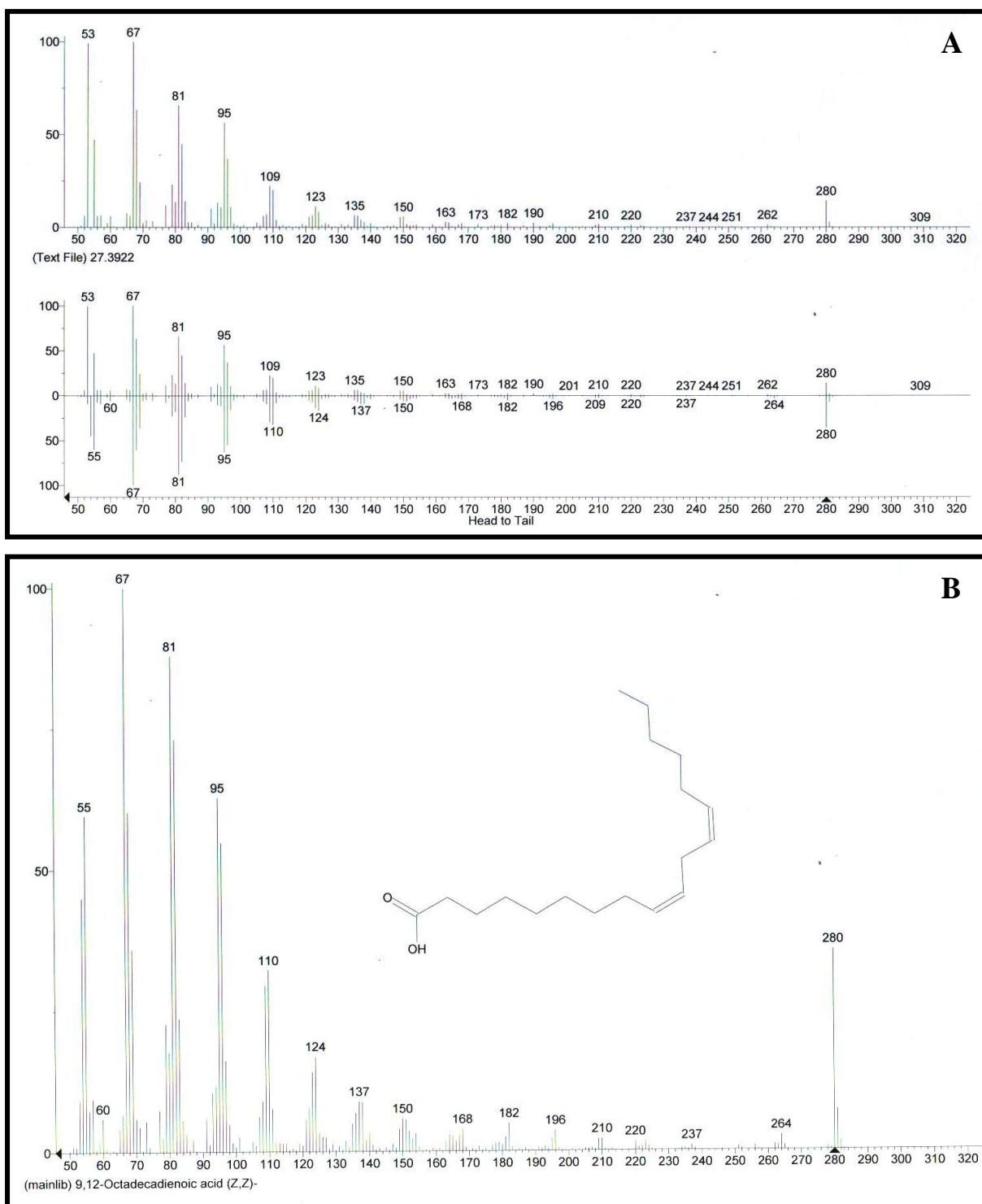


Figure A23: Library match of 9,12-Octadecadienoic acid (Z,Z)- (compound CT06A) isolated from *C. triloba* hairy roots. A-Library template of 9,12-Octadecadienoic acid (Z,Z)- and B-isolated 9,12-Octadecadienoic acid (Z,Z)- from *C. triloba* hairy roots.

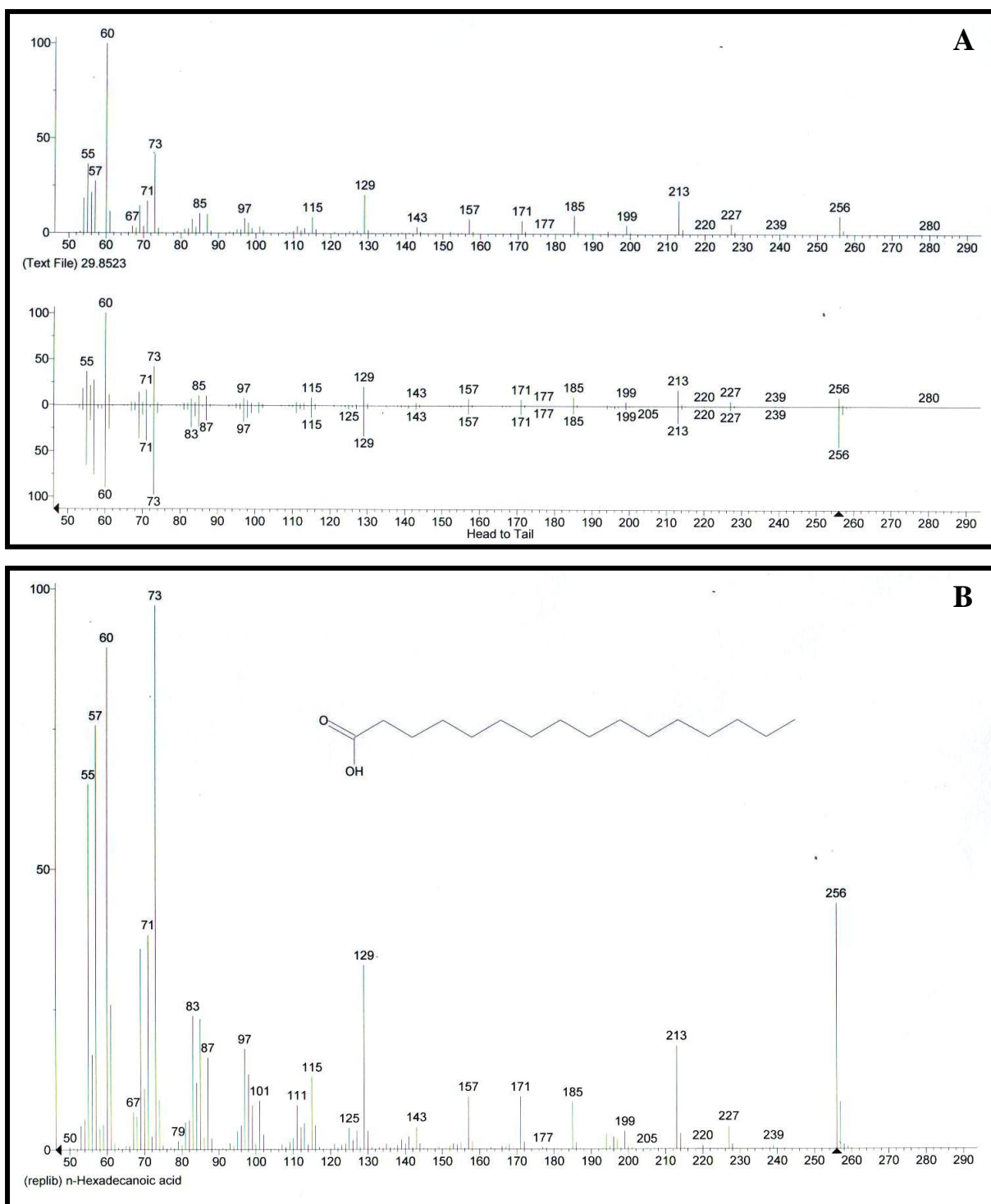


Figure A24: Library match of n-Hexadecanoic acid (compound CT06B) isolated from *C. triloba* hairy roots. A-Library template of n-Hexadecanoic acid and B-isolated n-Hexadecanoic acid from *C. triloba* hairy roots.

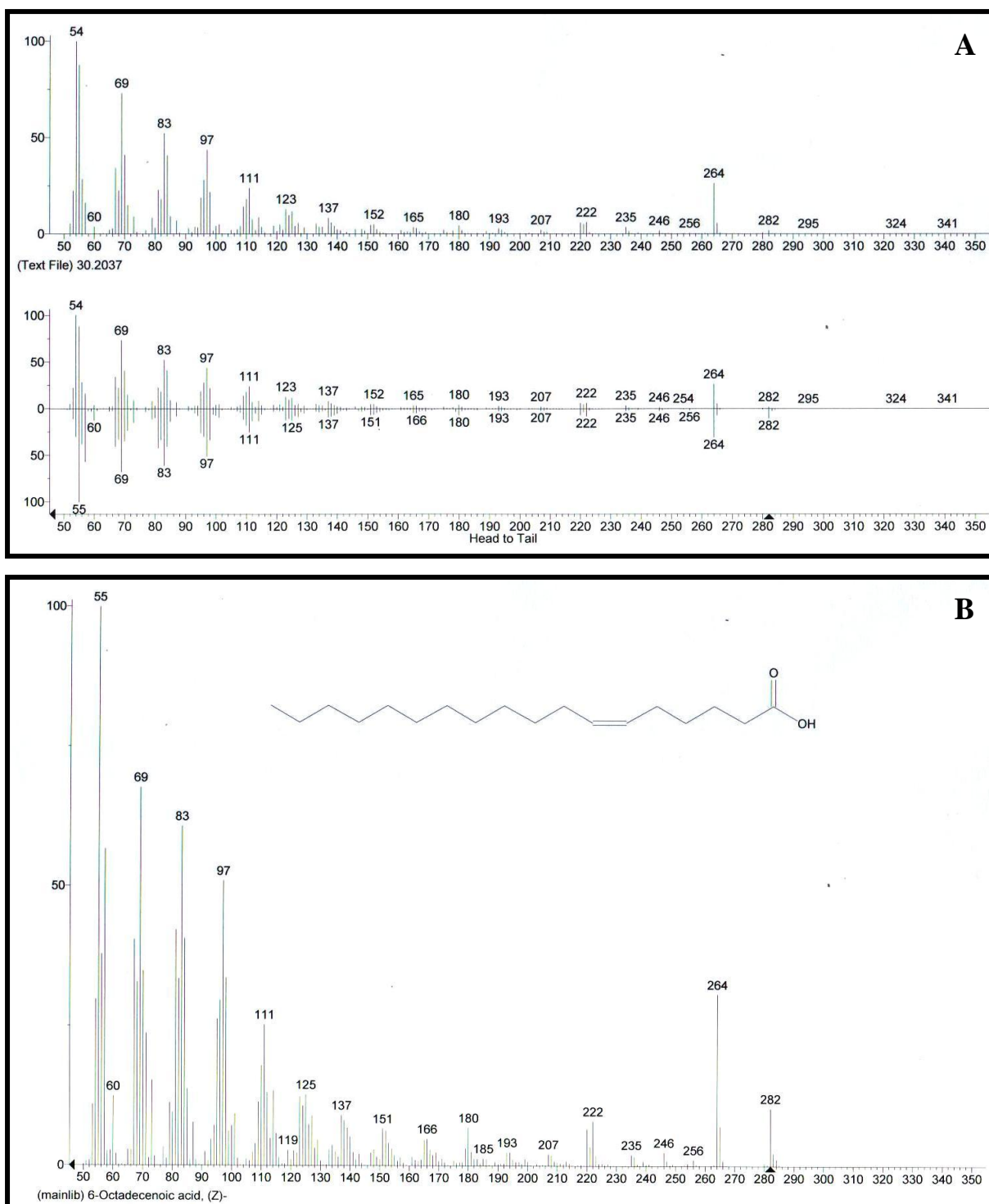


Figure A25: Library match of 6-Octadecenoic acid, (Z)- (compound CT06C) isolated from *C. triloba* hairy roots. A-Library template of 6-Octadecenoic acid, (Z)- and B-isolated 6-Octadecenoic acid, (Z)- from *C. triloba* hairy roots.

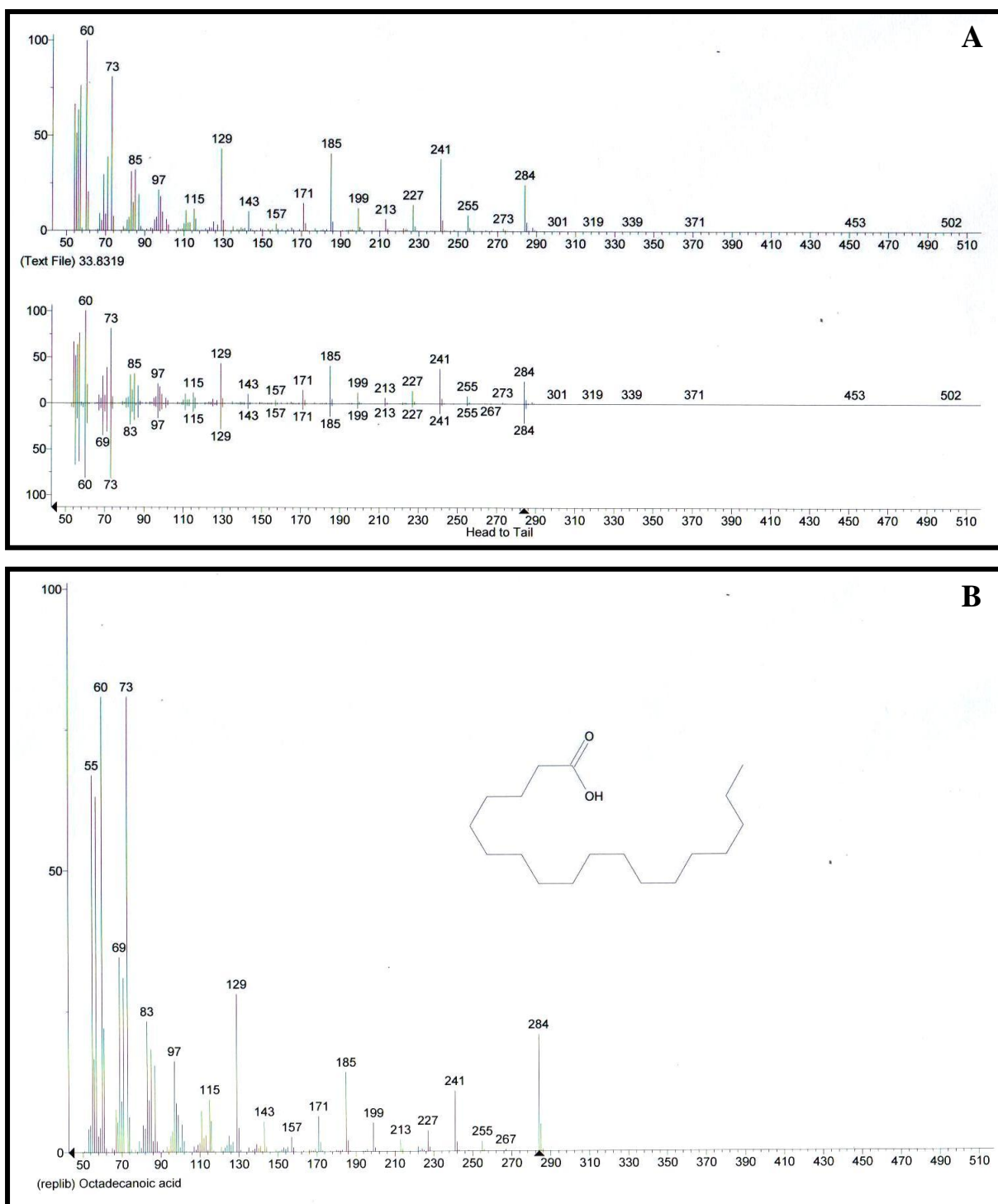


Figure A26: Library match of Octadecenoic acid (compound CT06D) isolated from *C. triloba* hairy roots. A-Library template of Octadecenoic acid and B-isolated Octadecenoic acid from *C. triloba* hairy roots.