Effects of *Catharanthus roseus* and *Centella asiatica* leaf extracts on enzymes of glutamine catabolism in human colon carcinoma (Caco-2) cell line and in enterocytes from male Sprague-Dawley rats.

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Discipline of Biochemistry

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Declaration

Original Work

I, **Thobekile Precious Dladla**, hereby declare that this study represents original, unaided work that was done by the author under the supervision of **Dr. B. Masola and Dr. M Singh**. It has not been submitted in any form for any degree or diploma to another tertiary institution. The research described in this dissertation was carried out in the Discipline of Biochemistry; School of Life Sciences; College of Agriculture, Engineering and Science; University of KwaZulu-Natal (Westville campus) from February 2013 to December 2015.

References

The use of work by others has been duly acknowledged and referenced in the text. I understand that copywriting or plagiarism could lead to my degree being disqualified.

Ethical Clearance

This study investigated both *in vitro* and *in vivo* properties and hence it required the use of animals. Training on how to work with animals was obtained at the Biomedical Resource Unit (BRU) UKZN, Westville Campus. Ethical clearance from relevant University ethical Committee was thus obtained (**055/ 14 ANIMAL**). A commercially available cell line was also used.

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COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 1 – PLAGIARISM

I, Thobekile Precious Dladla, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
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3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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"Akunanto Jesu, engangisusa othandweni lwakho"

"Rejoice in the Lord always: and again I say, Rejoice. Let your moderation be known unto all men. The Lord is at hand. Be careful for nothing; but in every thing by prayer and supplication with thanksgiving let your requests be made known unto God. And the peace of God, which passeth all understanding, shall keep your hearts and minds through Christ Jesus."

Abstract

Phosphate-dependent glutaminase (PDG) is a key enzyme in several physiological processes and hence has become a target of anti-cancer drug development because of the role of glutamine in providing energy for rapidly dividing cells. This study investigated the effects of the *Centella asiatica* and *Catharanthus roseus* leaf extracts on enzymes of glutamine catabolism in human colon carcinoma (Caco-2) cell line and in enterocytes from Sprague-Dawely rats both treated with plant extracts. Our hypothesis was that these extracts would arrest the growth of Caco-2 cells by inhibiting glutaminase but have less deleterious effects on normal cells due to the former being more avid consumers of glutamine. The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to assess the cytotoxic effects, and hence the anti-cancer potential of extracts from medicinal plants *C. asiatica* and *C. roseus* against Caco-2 and human embryonic kidney (HEK 293) cell lines. These *in vitro* effects were assessed using various doses of plant extracts (0- 16 mg/ml) and different exposure periods of 24, 48 and 72 h. Results show that the cytotoxicity effect of the *C. asiatica* extract to the caco-2 cell line is dose dependent whilst *C. roseus* treatment decreased the Caco-2 cells viability at all the exposure times but this was not dose-dependent. In contrast, both extracts significantly increased cell proliferation of the HEK 293 cells compared to the controls. The PDG and ALT activities were decreased in Caco-2 cells whilst the HEK293 cells were largely unaffected. In the *in vivo* studies, the activities of phosphate-dependent glutaminase (PDG), glutamate dehydrogenase (GDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined in the enterocytes. Results show that the activities of GDH remained constant whilst there was a decrease in ALT and a slight increase in AST activities in the enterocytes in both the *C. asiatica* and *C. roseus* treated groups. The PDG enterocytes activity, on the other hand, was lower in both the treated groups compared to the control group. Expression of both PDG and AST assessed by dot blots in the enterocytes increased in both plant extract treated groups. Induction of apoptosis was also investigated using cytochrome c. The plant extracts were screened for presence of phytocomponents that may be causing changes in Caco-2 cell growth and in enzymes of glutamine catabolism using Gas Chromatography-Mass Spectrometry (GC-MS) and qualitative phytochemical analysis. The extracts revealed the presence of anticancer and many other compounds. We conclude that the both extracts have potent anti-cancer cell activity that leaves normal cells unaffected, in fact stimulating their growth.

List of Abbreviations

Abbreviation	Description
Α	Alpha
Β	Beta
α-KG	α-Ketoglutarate
°C	Degrees Celsius
Abs.	Absorbance
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APS	Ammonium persulfate
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCIP- NBT	5-Bromo-4-Chloro-3-Indolyl Phosphate Nitroblue Tetrazolium
BRU	Biomedical Research Unit
BSA	Bovine serum albumin
C. asiatica	*Centella asiatica*
CO_2	Carbon dioxide
C. roseus	*Catharanthus roseus*
DCM	Dichloromethane
DMEM	Dulbecco's modified medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EA	Ethyl acetate
EDTA	Ethylene diamine tetra acetic acid
EMEM	Earle's minimum essential medium
et al	and others
FCS	Foetal calf serum
G	Grams

GCMS	Gas Chromatography Mass Spectroscopy
GDH	Glutamate dehydrogenase
H_2O	Water
HCl	Hydrochloric acid
HEK293	Human embryonic kidney cell line
HEPES	N-(2- hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
KCl	Potassium chloride
kDa	Kilo Dalton
Kg	Kilogram
L	Litre
LDH	Lactate dehydrogenase
M	Molar
MDH	Malic dehydrogenase
MEM	Minimum essential medium;
Mg^{2+}	Magnesium
Mol	Mole
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MW	Molecular weight
Na^+	Sodium
NaCl	Sodium chloride
NAD^+	Beta-Nicotinamide adenine dinucleotide
NADH	Beta-Nicotinamide adenine dinucleotide, reduced form
$NADP^+$	Beta-Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NCI	National Cancer Institute
NH_4^+	Ammonium
Nm	Nanometres
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PDG	Phosphate Dependent Glutaminase

PMSF	Phenylmethanesulfonylfluoride
Ppm	Parts per million
Rpm	Rotation per minute
PS	Phosphatidylserine
ROS	Reactive oxygen species
SANBI	South African National Biodiversity Institute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of means
sp.	Species
TBST	Tris buffered saline- tween
TCA	Tricarboxylic acid cycle
TEA	Triethanolamine
TEMED	N, N, N, N –Tetramethylethylenediamine
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl) aminomethane
UKZN	University of KwaZulu-Natal
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
M	Micro

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CHAPTER 1

Literature review

1. Preamble

Many drugs that are used in modern medicine have been directly isolated from plants or are derived from templates of compounds from plant sources (Coopoosamy and Naidoo, 2012; Jena *et al.*, 2012). Medicinal plants are an important part of the South African cultural heritage hence are considered as a health source for the majority of people because of their therapeutic properties (Van Wyk *et al.*, 1997). South Africa has an abundance of medicinal plants which are used in the traditional treatment of various diseases which include diabetes, intestinal and skin disorders, oral, urinary tract infections (UTIs) (Coopoosamy and Naidoo, 2012). Apart from treating physical diseases, medicinal plants are also used to meet the psychological health needs in traditional medicine (Iwu, 2014).

Due to the limitations and cost of current anticancer drugs, there is a need to identify safe, easily available and effective anticancer drugs to combat and prevent cancer particulary for poor communities. Plant extracts are known to contain compounds that possess anti-tumour activitiy and some act by hindering the activation of the cell cycle inducing cell death. Examples of anticancer drugs isolated from medicinal plants are taxol and vinblastine which have been in use for decades (Jena *et al.*, 2012; Pan *et al.*, 2012).

Rapidly dividing cells appear to be addicted to glutamine utilization (Souba, 1993; Medina, 2001). The cancer cells in culture are avid consumers of glutamine and metabolize glutamine at higher rates compared to other amino acids (Erickson and Cerione, 2010). Glutamine is said to be a good substrate for oxidation by cancer cell mitochondria hence the high glutaminase activity. Tumour cells utilize large quantities of glutamine and compete with the host for circulating glutamine simply because glutamine is essential for tumour growth (Suzannec Klimberg and McClellan, 1996). The key enzyme that is responsible for initiating glutamine oxidation is phosphate-dependent glutaminase (Erickson and Cerione, 2010) but the enzyme is also the target of anti-cancer drugs that are being developed (DeBerardinis and Cheng, 2010). Since PDG is required for normal cell function it is important to assess the

effect of anti-cancer medicinal plant extracts and drugs on enzyme in both cancerous and normal cells. Not much is known about the effect of medicinal plant species such as *Catharanthus roseus* and *Centella asiatica* on enzymes of glutamine catabolism in such cells hence our study on Caco-2 cell line and enterocytes.

1.1 What is cancer?

Cancer is the generic term for a group of diseases characterized by uncontrolled proliferation and growth of abnormal cells (Kleinsmith, 2006). Cancer cells divide in a haphazard manner in which the balance between cell division and cell differentiation is disrupted when compared to normal cells. These cells are large, have irregularly shaped nuclei, prominent nucleoli, large nuclear- cytoplasm volume, show variations in shape and cell sizes and visible loss of normal tissue organization (Hanahan and Weinberg, 2011). The result is that there is a progressive pile up of a non-structured mass called a tumour (Hanahan and Weinberg, 2011; Munoz-Pinedo *et al.*, 2012). If the spread is not controlled, it can result in death (Nishida *et al.*, 2006; Crawford and Ferrara, 2009). Cancer cells invade surrounding tissues, enter the circulatory system and metastasize to distant sites. This invasion is facilitated by decreased cell-cell adhesion, increased motility and production of enzymes responsible for degradation of the extracellular matrix and basal lamina (Kleinsmith, 2006; Weaver, 2006; Friedl and Alexander, 2011).

1.2 The causes of cancer

The known causes of cancer include external factors namely, tobacco use, ionizing and ultraviolet radiation exposure etc. (Soto and Sonnenschein, 2010) and internal factors which include inherited mutations, viruses and DNA mutations triggered by chemical carcinogens (Siegel *et al.*, 2015). These causal factors, illustrated in Figure 1.1, may act together or in sequence to initiate or promote carcinogenesis (Boyle and Levin, 2008).

It is estimated that 15% of cancers diagnosed are a result of hereditary factors (Kleinsmith, 2006; Hanahan and Weinberg, 2011; Siegel *et al.*, 2015) and 9% are caused by carcinogens in the environment (Boyle and Levin, 2008; Soto and Sonnenschein, 2010). A further 5% can be attributed to viral infections (Hepatitus B & C / Human Papillomavirus) and 1% can be due to other factors (Woodman *et al.*, 2007). Poor lifestyle choices further enhance cancer risks. It is documented that 5% of cancers occur as a result of overweight and not getting enough exercise (Kolonel *et al.*, 2004), 3% consuming too much alcohol and 20 – 25% using tobacco or tobacco products (Proctor, 2001; Siegel *et al.*, 2015). If healthy lifestyles can be adapted, cancer risks can be reduced significantly by approximately 30% (Siegel *et al.*, 2015).

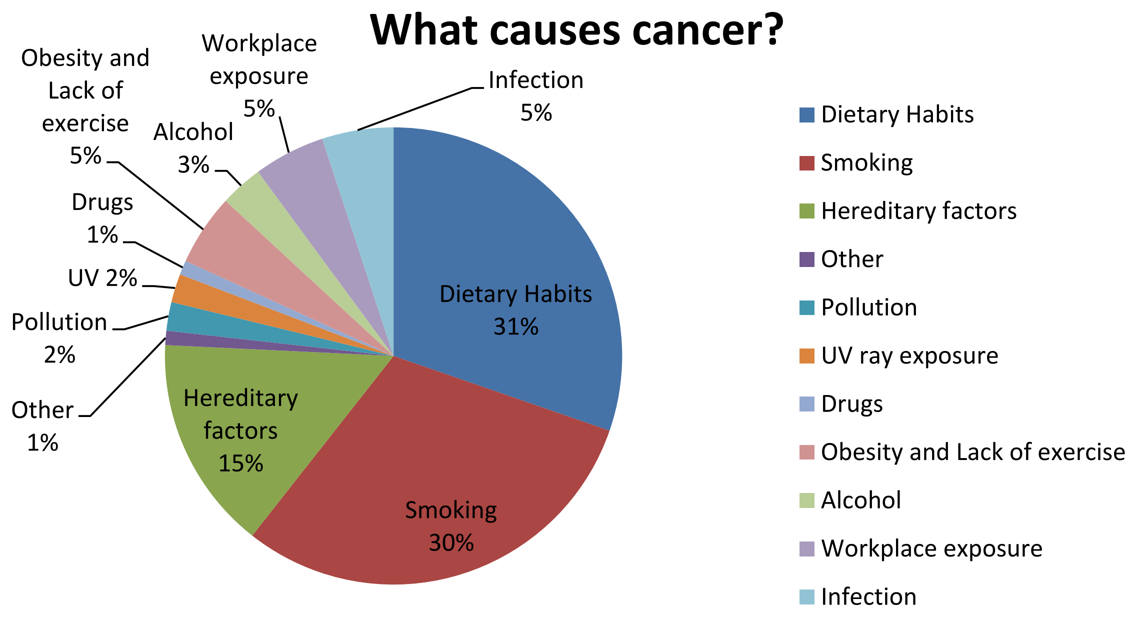

Figure 1.1: Pie chart showing causes of cancer as percentages.

1.2.1 Genetics

From one generation to the next, abnormal genes can be passed which then ultimately cause cancer to give rise to what is referred to as *inherited* cancer (Kleinsmith, 2006). The cancer itself is not inherited but an abnormal gene that leads to cancer is what is inherited (Hanahan and Weinberg, 2011). According to (Kleinsmith, 2006; Ferlay *et al.*, 2015) about 5% to 15% of all cancers result directly from gene defects (called *mutations*) inherited from a parent. Chemical carcinogens trigger DNA mutations through a multistep process involving initiation (DNA mutation), promotion (the initiated cell is stimulated to proliferate) and tumor progression (mutations and changes in gene expression create variant cells exhibiting enhanced growth rates and aggressive properties (Proctor, 2001; Kleinsmith, 2006; Soto and Sonnenschein, 2010; Hanahan and Weinberg, 2011; IARC, 2012).

1.2.2 Tobacco

As thoroughly documented, cigarette smoking causes more cancer mortality [death] worldwide than any other causative factor (Ferlay *et al.*, 2015; Siegel *et al.*, 2015). Side stream smoke (smoke from the lighted end of a cigarette, pipe or cigar) is believed to have higher concentrations of cancer-causing agents (carcinogens) and is more toxic than mainstream smoke (the smoke exhaled by a smoker) (Proctor, 2001). Tobacco smoke is said to contain a mixture of more than 7,000 chemical compounds, 250 of these chemicals are known to be harmful, and about 69 are known to cause cancer (Proctor, 2001). Second hand smoke causes

lung cancer, heart disease, worsens asthma and asthma related problems and lower respiratory tract infections (Proctor, 2001).

1.2.3 Sun, UV and Radiation

Ionizing radiation removes electrons from molecules generating highly reactive ions that create DNA damage (Soto and Sonnenschein, 2010). Ultraviolet radiation (UV) causes skin cancer by damaging DNA mainly after exposure to sunlight for long hours (Pfeifer and Besaratinia, 2012). This radiation is absorbed by the skin and triggers the formation of pyrimidine dimer which causes distortion of the double helix due to improper base pairing during replication (Kleinsmith, 2006; Soto and Sonnenschein, 2010; Hanahan and Weinberg, 2011). For example, P53 genes have been shown to be mutated in many human cancers and in skin cancer cells the gene shows CC-TT distinctive UV signature (Kleinsmith, 2006).

1.2.4 Other carcinogens (workplace, pollution and infections)

Other carcinogens include cancer-causing viruses and infectious agents which act by creating tissue destruction thereby indirectly stimulating cell proliferation (You and Jones, 2012). A gene whose presence can cause cancer and arises from normal cellular genes called proto-oncogene by point mutation, gene amplification, local DNA rearrangements and/or insertional mutagenesis is called oncogene (Futreal *et al.*, 2004; Hanahan and Weinberg, 2011). On the other hand, tumor suppressor genes can lead to cancer when they are lost or inactivated (Futreal *et al.*, 2004).

1.2.5 Obesity and lack of exercise

Obesity presents a risk to health in the sense that it is associated with an increased risk of cancers. In South Africa, there is an alarming increase of individuals who are obese. Obesity in women is associated with cancers of the reproductive system i.e. breast, cervix etc. as excess body fat results in excess production of oestrogen by adipose stromal cells whereas in men it is associated with cancer of the rectum, colon and prostate in men (Futreal *et al.*, 2004; Kolonel *et al.*, 2004; Boyle and Levin, 2008; Ferlay *et al.*, 2015).

1.3 Classification of cancers

Cancer cells begin to develop in almost any organ. There are several different types of categories into which cancers are grouped, depending on the type of cell from which they originate, histology and the extent of the disease (Table 1.1).

Table 1.1: The major five major categories of cancers.

Category	Origin
Carcinoma	Originates in the skin or in tissue that line or cover internal organs. For example, breast, prostate, lung and colon cancer.
Central nervous system cancers	Begin in the tissues of the brain and spinal cord.
Leukemia	Starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.
Lymphoma and myeloma	Originate in the cells of the immune system.
Sarcoma	Originates in supportive and connective tissues such as bones, cartilage, fat, muscle and blood vessels.

1.4 Epidemiology: cancer and its impact

Cancer is a serious health concern worldwide as it affects all people irrespective of age, gender, race as well as socio-economic status. The International Agency for Research on Cancer (IARC), in the GLOBOCAN series, reported that every year not less than 11 million people are diagnosed as living with cancer world-wide and of this number 100 000 are South Africans (Ferlay *et al.*, 2015). It is estimated that there will be 16 million new cases every year by 2020. About 12.5% of all deaths worldwide are caused by cancer (IARC, 2015). Cancers arise with differing frequencies in different parts of the world. Japanese have stomach cancer (Haga *et al.*, 2013) whilst in the United States the breast cancer is prominent (DeSantis *et al.*, 2014) and liver cancer is common in Africa and Southeast Asia (Khong, 2014; Ladep *et al.*, 2014). Cancer has long been the second leading cause of death in the United States following heart disease (Siegel *et al.*, 2015). It accounts for about a quarter of all deaths. With cancer taking such a toll on the global population, the search of effective anticancer drugs has become very important.

Table 1.2: South Africa's top ten cancers in both the adults and children (non-melanoma skin cancers excluded), (National Cancer Registry, 2009).

SI No.	Top ten Adult Cancers in SA		Top ten Children's Cancers in SA	
	Men	**Women**	**Boys**	**Girls**
1.	Prostate Cancer LR 1:26	Breast Cancer LR 1:35	Leukaemia (all leukaemias combined) – TD: 85	Leukaemia (all leukaemias combined) – TD: 55
2.	CUP * LR 1:85	Cervical Cancer LR 1:42	Hodgkin's Lymphoma -TD: 47	Kidney Cancer TD: 48
3.	Lung Cancer LR 1:91	CUP * LR 1:120	Bone Cancer TD : 44	Bone Cancer TD: 36
4.	Kaposi Sarcoma LR 1:197	Kaposi Sarcoma LR 1:303	Kidney Cancer TD: 36	Brain & CNS Cancers -TD: 34
5.	Colorectal Cancer LR 1:115	Colorectal Cancer LR 1:199	Non-Hodgkin's Lymphoma TD: 35	Connective Tissue Cancers TD: 32
6.	Oesophageal Cancer LR 1:131	Uterine Cancer (uterus) LR 1:176	Connective Tissue Cancers TD: 34	Non-Hodgkin's Lymphoma TD: 28
7.	Non-Hodgkin Lymphoma LR 1:244	Oesophageal Cancer LR 1:208	Brain & CNS Cancers TD: 29	Kaposi Sarcoma TD: 22
8.	Bladder Cancer LR 1:199	Non-Hodgkin Lymphoma - LR 1:376	Kaposi Sarcoma TD: 27	Eye Cancers TD: 18
9.	Malignant Melanoma LR 1:277	Lung Cancer LR 1:251	Eye Cancers TD: 23	Malignant Melanoma TD: 18
10.	Stomach Cancer LR 1:226	Malignant Melanoma LR1:342	CUP * TD: 23	CUP * TD: 16

***CUP- means that it is not possible to determine where the cancer originated in the body. LR- lifetime risk, TD- total diagnosis.**

1.5 Colorectal cancer

The fourth most common type of cancer amongst both South African men and women is colorectal cancer (colon cancer). This cancer affects the colon, the large intestine or bowel. Colon cancer occurs as a result of a process whereby the normal replacement of lining cells become abnormal and cause mucosal cell division mutations to occur (Burkitt, 1977; Parkin *et al.*, 2014). Figure 1.2 below show that these mutated cells grow and divide and ultimately lead to growths within the colon (polyps) that vary in type i.e. adenomas and hyperplastic polyps. These polyps become slow growing precancerous tumours. The growth of precancerous tumours through the tube results in the invasion of other layers of the large intestine. When this takes place, a cancerous precancerous polyp results (http://www.cansa.org.za/files/2015/05/Fact-Sheet-Colorectal-Cancer-May-2015.pdf, CANSA, 2015). The National Institute for Occupational Health documented a publication on South African cancer statistics showed that cases of colorectal cancer in males and females is 1295 and 1132 and the lifetime risks of 1:114 and 1:182 respectively (http://www.nioh.ac.za/assets/files/NCR_Final_2010_tables(1).pdf). Researchers firmly believe that diet and lifestyle play an important role in its prevention.

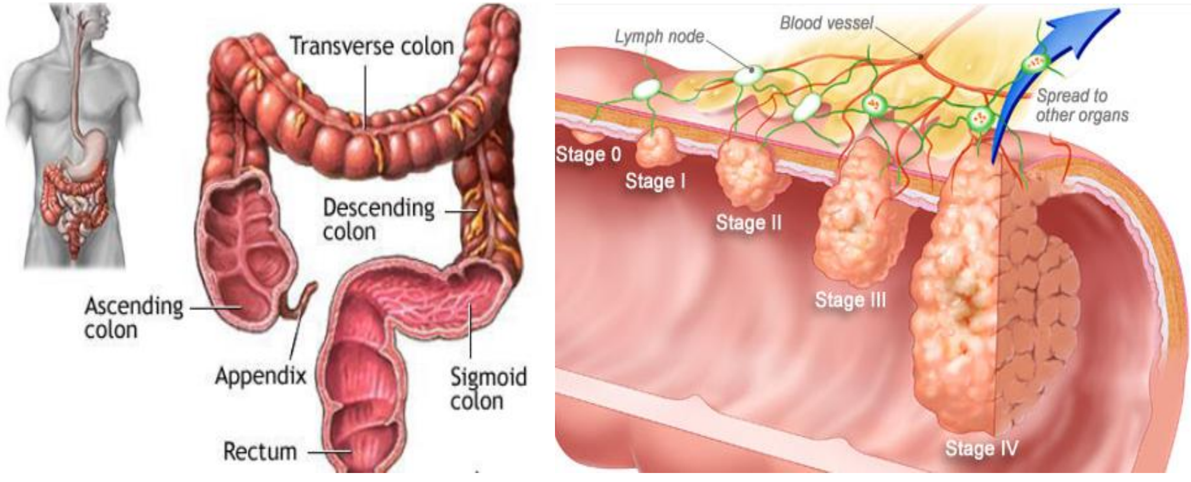

Figure 1.2: Anatomy of the colon and the stages of colon cancer. Stage 0 - Cancer has grown only in the innermost lining (mucosa) of the colon. Stage I - Cancer has grown into the thin muscle layer (muscularis mucosa) of the colon. Stage II - Cancer has grown into or through the outermost layer and fibrous tissue beneath the thick muscle layer (muscularis propria) of the colon. Stage III - Cancer then grows through musclaris propia and it starts to spread to one or more lymph nodes in the area. Stage IV - Cancer is attached to and colon and invades other parts of the body nearby (https://www.nlm.nih.gov/medlineplus/ency/article/000262.htm).

1.5.1 Colorectal cancer diagnosis

Digital rectal examination (DRE) is a physical examination that is commonly used for checking lumps inside the rectum (Maas *et al.*, 2015). In about 50-80% cases of colorectal cancer, a noticeable rectal lump is found (Hewitt *et al.*, 2003). The DRE is done by placing a well lubricated and gloved middle finger into the anus then up into the rectum. Other commonly used diagnosis tests are sigmoidoscopy and colonoscopy (Brenner *et al.*, 2014).

1.6 Cancer management and therapy

The methods that are currently used for the treatment of cancer include surgery (Kennedy *et al.*, 2014), chemotherapy (Mariette *et al.*, 2014), radiation (Milano *et al.*, 2014), hormonal therapy (Hait *et al.*, 2015), targeted therapy and immunotherapy (Kleinsmith, 2006). Of all the conventional cancer therapy methods, surgery has been the most successful cancer treatment for many years and nowadays there are less invasive methods i.e. removing cancerous cells whilst the non-affected cells maintain normal functions (Kennedy *et al.*, 2014).

There are more than 100 chemotherapeutic drugs administered to patients which are often distributed throughout the body to reach cancer cells location (Hardin *et al.*, 2012; Mariette *et al.*, 2014). These drugs are sometimes combined or used alone (Lane and Chabner, 2009). The problem is often their side effects. There are different types of chemotherapy drugs (chemo drugs) which are grouped according to how they work, chemical structure and interaction with other drugs for example, alkylating agents, antimetabolites, anti-tumor antibiotics, topoisomerase inhibitors, mitotic inhibitors, corticosteroids, miscellaneous etc (Hanahan and Weinberg, 2011; Galluzzi *et al.*, 2012). These chemo-drugs only take advantage of the rapid division of cancer cells or cell cycle (Chabner and Roberts, 2005). Other drugs and biological treatments are used to treat cancer which target properties of cancer cells that are different from normal cells. Lastly, there are some other drugs that are given to cancer patients in order to stimulate their immune-compromised systems to recognize and attack cancer cells (Luo *et al.*, 2009).

An exposure to chemotherapeutic drug results in a situation wherein the resistant cancer cells emerge following treatment. This then calls for approaches that will target proliferating cancer cells and leaving the normal cell unaffected. Alternative cancer treatment involves the use of medicinal plants that possess anti-tumour activitiy (Jena *et al.*, 2012; Pan *et al.*, 2012). On the other hand, a new anticancer strategy involves inhibiting glutamine metabolism by proliferative cells.

1.7 Glutamine

1.7.1. Role of glutamine in normal mammalian cells

Glutamine is the most abundant amino acid in the blood, constituting 20-25% of the total amino acid pool. Being synthesized *de novo* from glutamate in the presence of ammonia and ATP by glutamine synthetase (E.C 6.3.1.2) (Reaction 1), glutamine plasma concentration ranges between 0.6-0.9 mM (Souba, 1993; Medina, 2001) and is about 20 mM in some tissues (Souba, 1993). While listed as a non-essential amino acid, it participates as the substantial energy source for intestinal epithelial cells as it regulates cell proliferation of the human colon and ileum (Windmueller, 1982; Wise and Thompson, 2010). Rapidly dividing cells such as intestinal mucosa, lymphocytes, fibroblasts and cancer cells appear to be more reliant to glutamine utilization (Souba, 1993; Suzannec Klimberg and McClellan, 1996; Papaconstantinou *et al.*, 1998b). According to (Windmueller, 1982; Yoshida *et al.*, 2001; Newsholme *et al.*, 2003; DeBerardinis and Cheng, 2010), the principal organs and cells that utilize glutamine most are enterocytes of the small intestines, macrophages, kidneys, lymphocytes and liver.

$$\text{Glutamate} + NH_4^+ + \text{ATP} \xrightarrow{\text{Glutamine synthetase}} \text{Glutamine} + \text{ADP} + \text{Pi}$$

(Reaction 1)

This amino acid is a crucial requirement for synthesis of nucleic acids (purines and pyrimidines), proteins and lipids and plays a central role in nitrogen metabolism (Watford, 2000; Häussinger and Sies, 2012). The skeletal muscle is the main tissue that produces glutamine endogenously and has a glutamine concentration of more than 20 mM. It accounts for most of the glutamine released into the blood (Pinkus and Windmueller, 1977; Meister, 1984; Chen *et al.*, 1993; McCauley *et al.*, 1999) (Figure 1.3). Glutamine is essential for promoting glucose uptake (Matés *et al.*, 2002), enhancing the hydration state of muscle (Chen *et al.*, 1993) and maintaining acid-base homeostasis during intense trainings or workouts (Matés *et al.*, 2009). Furthermore, this amino acid is essential for the maintenance of gut homeostasis and health (Rhoads and Wu, 2009) as it has beneficial effects such as protection of the small intestine from various harmful agents (Pinkus and Windmueller, 1977; Suzannec Klimberg and McClellan, 1996), improvement of nitrogen retention and keeping up defences against microbes (Wise and Thompson, 2010).

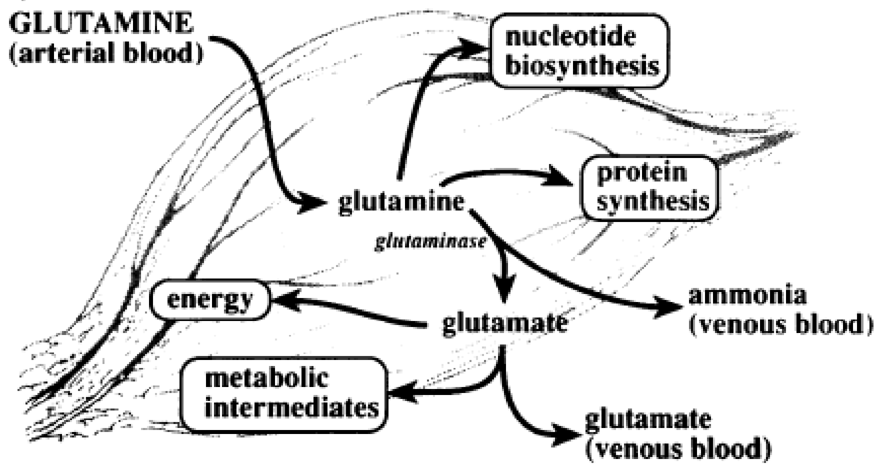

Figure 1.3: Illustartion of skeletal muscle as the main source of glutamine, its uptake and fate. Adopted from (Souba, 1993).

1.7.2 Role of glutamine in cancer cells

Since cancer cells are highly proliferating cells, they require a great deal of energy in the form of glutamine. They then utilize glutamine more rapidly than the normal cells resulting in rapid cancer cells growth as the host glutamine depletion occurs. As the cancerous cells use glutamine as a substrate, there is a relative increase in the specific phosphate-dependent-glutaminase activity (Souba, 1993; Erickson and Cerione, 2010; Nathiya *et al.*, 2012). The utilization of glutamine by cancer cells is directly proportional to phosphate-dependent glutaminase activity (Linder-Horowitz *et al.*, 1969; Kovačević and Morris, 1972). Nucleic acid and protein synthesis is intensified as well as that of glucose (Suzannec Klimberg and McClellan, 1996). Therefore the study of the regulation of glutamine metabolism in cancer cells is most crucial as well as the evaluation of its impact in the tumor-bearing state. Cancer cells have been reported to have increased glutaminase activity and glutamine synthatase inhibition (Erickson and Cerione, 2010; Munoz-Pinedo *et al.*, 2012).

1.7.3 Glutamine metabolism in the small intestine and cancer cells

The small intestine plays a key role in digestion and absorption of nutrients. Recent literature reveals that the function of the small intestine is much more complex (McCauley *et al.*, 1999; Grant *et al.*, 2015). The bowel (multicellular organ) is composed of absorptive enterocytes, and other cells namely secretory, immune and neuroendocrine cells that perform a number of essential physiological functions including secretion of hormones (Pinkus and Windmueller, 1977; Petras, 2013). There are three subdivisions in the small intestine, that is, the duodenum, jejunum and ileum. The duodenum receives the chime (processed food) in the stomach and

is the part of the gastrointestinal tract where most digestion happens, whereas the jejunum is the part of the entire gastrointestinal tract where the most absorption of nutrients occurs and the last part of the gastrointestinal tract is the ileum wherein there is some important absorption of nutrients that takes place e.g. absorption of vitamins etc. Other organs such as liver and gall bladder are said to be important as they deliver bile (composed of bile salts and bile pigments) to the duodenum (DeSesso and Jacobson, 2001; Petras, 2013). In addition to the above, there is also a pancreas which delivers some important enzymes for digestion (Reed and Wickham, 2009). The duodenum has also the brush border enzymes that are very important for activation of certain enzymes and for digestion of several nutrients (DeSesso and Jacobson, 2001; Reed and Wickham, 2009).

Figure 1.4 shows the wall of the intestine which consists of several layers namely the mucosa, submucosa, muscularis and serosa. The mucosa is the epithelial lining that faces the lumen of the gut and the connective tissue underlies it. The submucosa, fibrous connective tissue, contains blood and lymph vessels as well as nerve networks. Lastly, the muscle layer (also called muscularis propia) is covered by the outermost serosa (DeSesso and Jacobson, 2001; Petras, 2013). The intestinal wall has numerous infoldings that help increase the surface area. These finger-like projections of the intestinal lining are called villi. The villus has a lacteal which is a specialized lymphatic channel that absorbs and transport nutrients (Petras, 2013). Furthermore, electron microscopic examination revealed the presence of more microscopic projections in the villi that play a further important function aiding better digestion due to 14 to 40 fold surface area increase and these are termed microvilli (singular: microvillus) (Reed and Wickham, 2009; Petras, 2013). Additionally, there are brush border enzymes which are present on the villi and microvilli. The epithelium of the villi and microvilli is lined by a continuous sheet of absorptive cells called the enterocytes.

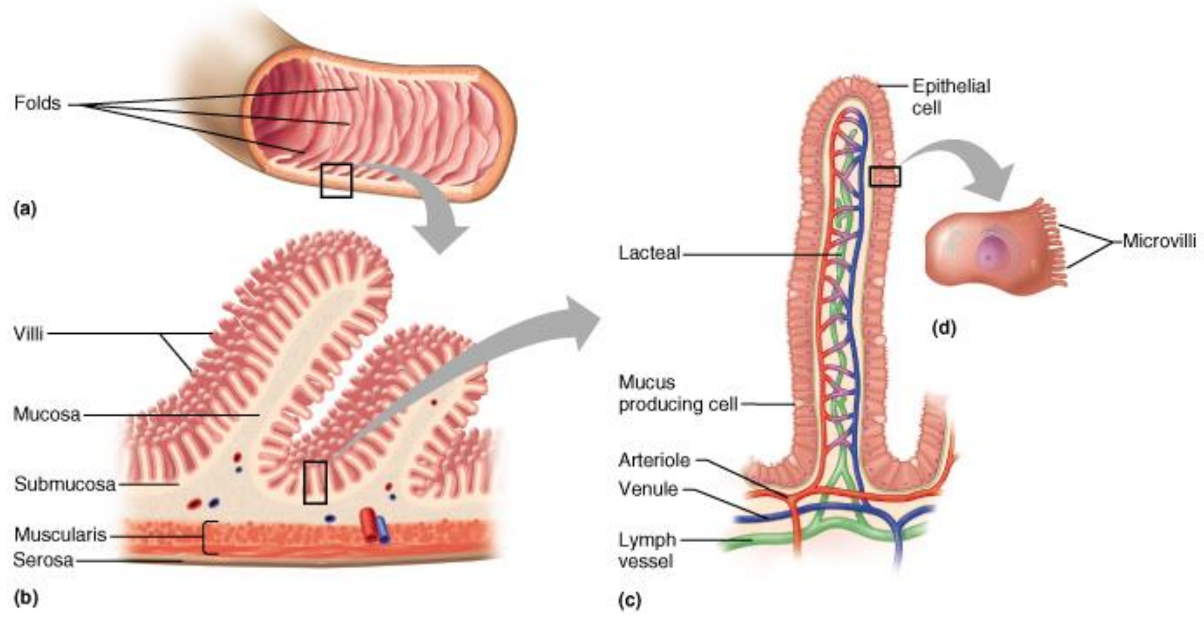

Figure 1.4: Anatomy of the small intestine (http://sciencelearn.org.nz).

Glutamine is the most preferred respiratory substrate in the small intestine (Pinkus and Windmueller, 1977; Windmueller, 1982; James *et al.*, 1998) and plays a role in the functioning and structure of the mucosa. Different organs such as spleen, kidneys, liver, pancreas and stomach have been found to utilize circulatory glutamine (Windmueller and Spaeth, 1974). Glutamine is taken up in the post-absorptive state by the intestine and other organs such as the kidneys which also exhibit net glutamine uptake in the same state (Häussinger and Sies, 2012). The metabolism of glutamine in the body occurs by two functional pathways (Figure 1.5). The first pathway involves the use of glutamine-derived amide nitrogen in amino sugar, purine biosynthesis via glutamine phophoribosylpyrophosphate amidotransferase (GPAT) and pyrimidine biosynthesis via carbamoyl phosphate synthetase 2 (CPS 2). The second pathway involves the utilization of glutamine-derived carbon chain for energy production and α-amino group for the synthesis of other amino acids (Windmueller and Spaeth, 1974; Meister, 1984; Watford, 2000; Newsholme *et al.*, 2003). The former pathway occurs in the cytoplasm while the latter is initiated in the mitochondria (Watford, 1993; Watford, 2000) to produce glutamate the immediate product of glutamine metabolism in most cells (Newsholme *et al.*, 2003)(Figure 1.5).

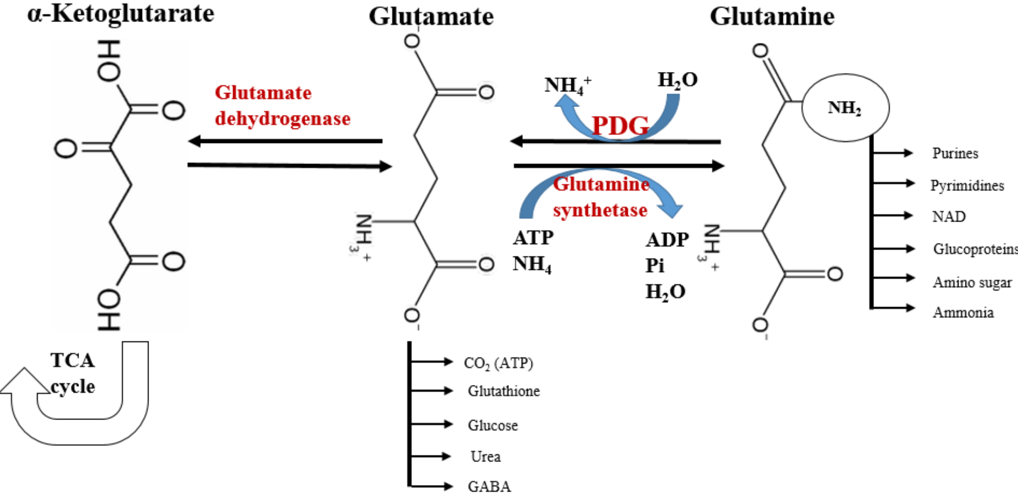

Figure 1.5: Pathways and fate of glutamine metabolism in the body. Adapted from (Papaconstantinou *et al.*, 1998a; Watford, 2000).

The Figure 1.6 below shows the metabolism of glutamine in the intestinal mucosa where it serves as an energy source. Endogenous glutamine from the blood stream enters into the mitochondria for energy production. In the intestinal mucosa, glutamine is converted to glutamate and ammonia catalysed by the mitochondrial enzyme called phosphate-dependent glutaminase (PDG; E.C 3.5.1.2) also known as L-glutamine amidohydrolase as shown in reaction 2. The glutamate produced from the glutamine catabolism is converted into α-ketoglutarate primarily via transamination reactions. During these transamination reactions, alanine and aspartate are produced catalysed by alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively. These two transaminases are present in both mitochondria and cytoplasm (Masola *et al.*, 1985). The α-ketoglutarate then enters the tricarboxylic acid (TCA) cycle where it is oxidized. NADH and $FADH_2$ are produced which are then oxidized by the electron transport chain in the mitochondria to synthesize ATP (Curthoys and Watford, 1995; Watford, 2000; Newsholme *et al.*, 2003).

$$\text{Glutamine} + H_2O \xrightarrow{\text{Phospahte-dependent Glutaminase}} \text{Glutamate} + NH_3 \qquad \text{(Reaction 2)}$$

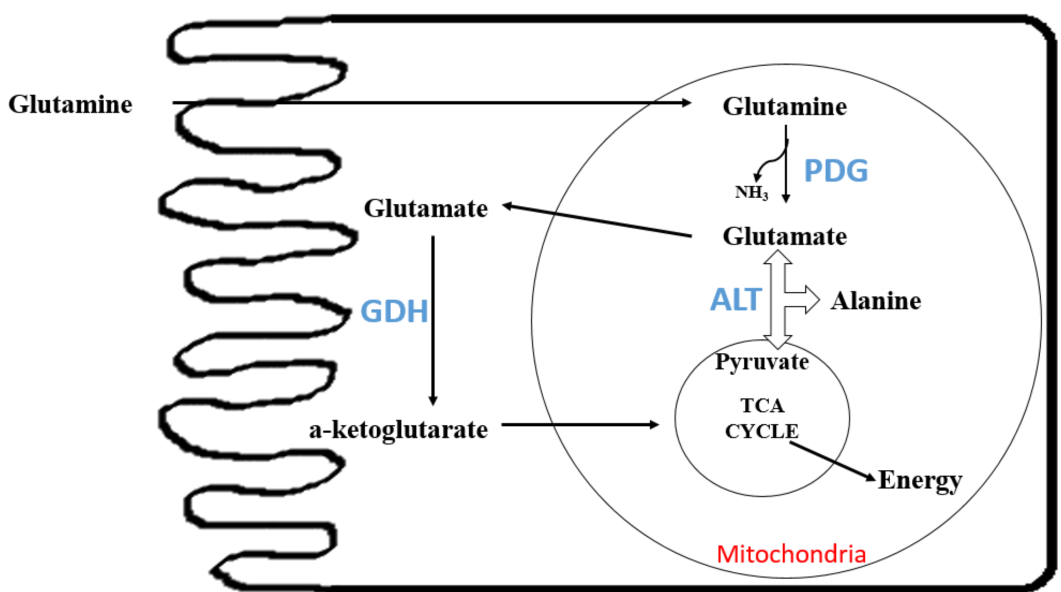

Figure 1.6: Illustration of glutamine metabolism in the small intestine enterocytes. PDG, phosphate-dependent glutaminase; GDH, Glutamte dehydrogenase; ALT, Alanine aminotransferase.

Glutamine is taken up by the intestine in the post-absorptive state and its deficiency in the intestine leads to intestinal conditions such as diarrhoea, intestinal necrosis, acute necrotizing colitis, gut mucosal ulcerations and mild villous atrophy (Papaconstantinou *et al.*, 1998a). Therefore, this reinforces the requirement that it should be maintained at a constant concentration. (Windmueller and Spaeth, 1974; Watford *et al.*, 2002). Also, it has been demonstrated that apoptosis is induced in intestinal cells due to glutamine deprivation (Papaconstantinou *et al.*, 1998a). Glutamine can be used as a therapeutic supplement to restore and improve health in mammalian cells (Suzannec Klimberg and McClellan, 1996; Rao and Samak, 2012). It was further shown that glutamine supplements increase splanchnic circulation and reduce mucosal infections as well as restore the balance between cell death and proliferation (Hall and Heel, 1996). There is an ongoing debate whether the traditional classification of glutamine as a non-essential amino acid should be re-visited (Watford, 2015).

Cancer cells grow at faster rates thus requiring great amount of energy for continued growth and survival. Cancer glutamine metabolism is quite complicated to study since there is flexibility of the metabolic systems as well as the number of extracellular free nutrients to which the cells have access to simultaneously (Erickson and Cerione, 2010; Katt *et al.*, 2012). The cancer cells in culture are avid consumers of glutamine and metabolize glutamine at

higher rates compared to other amino acids (Souba, 1993; Medina, 2001). Glucose, on the other hand, is said to be the major energy fuel playing a crucial role in cancer cell metabolism (Vander Heiden *et al.*, 2009) followed by glutamine (Eagle, 1955; Kvamme and Svenneby, 1960) and both are needed for proliferating cancer cells for bioenergetics and synthesis of intermediates for macromolecules (DeBerardinis and Cheng, 2009; Macintyre and Rathmell, 2013). The Warbug's effect states that most cancer cells rely aerobic glycolysis to generate energy for cellular processes which yields less adenosine 5′-triphosphate (ATP). In contrast, the normal cells depend on mitochondrial oxidative phosphorylation. Many studies have shown that glutamine contributes essentially to most metabolic tasks of proliferating cancer cells (Souba, 1993; Medina, 2001; Macintyre and Rathmell, 2013) thus reflecting its significant resourcefulness as both the nutrient and mediator of other metabolic processes (DeBerardinis and Cheng, 2009).

Cancer cells have large intracellular pools of glutamate (Newsholme *et al.*, 2003) which is the product of breakdown of glutamine facilitated by the activity PDG, an enzyme highly expressed in cancer cells hence the tumor PDG activity is very high (DeBerardinis and Cheng, 2009; Erickson and Cerione, 2010; Daye and Wellen, 2012) resulting in sizeable production of glutamate. This then highlights the correlaation of PDG activity with tumor growth. Experimental designs in which PDG activity was lowered demonstrated decreased growth rates of tumor cells (Newsholme *et al.*, 2003). Through the activities of transaminases, especially alanine and aspartate aminotransferases, the glutamine's α-nitrogen is transported into pools of other nonessential amino acids (DeBerardinis and Cheng, 2010).

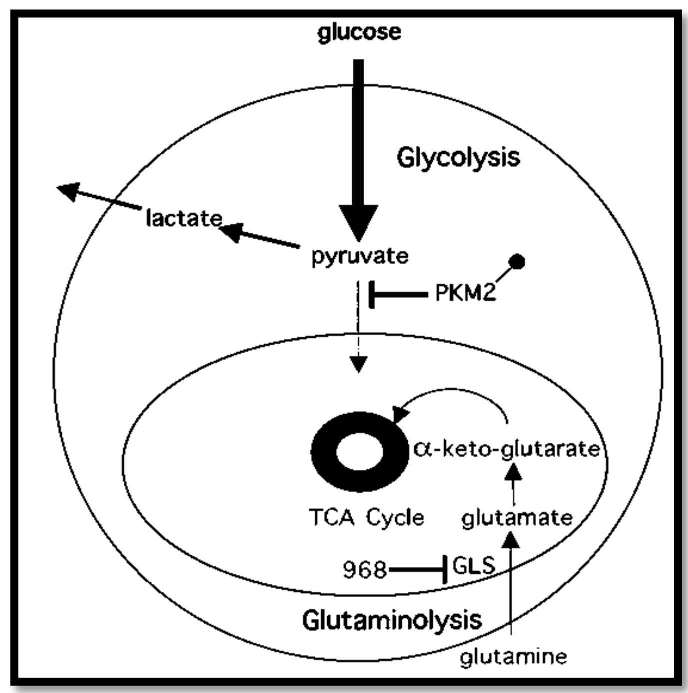

Figure 1.7: Illustration of glutamine metabolism in cancer cells. Adapted from (Erickson and Cerione, 2010). PKM2, The M2 isoform of pyruvate kinase; TCA, Tricarboxylic acid; GLS, glutaminase; 968, anti-tumour drug.

There is an increased glutamine metabolism in cancer cells through hydrolysis of glutamine to glutamate followed by the subsequent conversion of glutamate to α-ketoglutarate. The resulting sizeable α-ketoglutarate is poised to maintain the TCA cycle in cancer cells whereas in normal cells pyruvate is converted to acetyl-CoA which then maintains TCA cycle. Glycolysis, in cancer cells, results in lactate and its secretion. Another major change is the presence of pyruvate kinase isoform (PKM2), an enzyme expressed in cancer cells. This pyruvate kinase isoform becomes tyrosine phosphorylated leading to attenuation of pyruvate acetyl-CoA conversion (Figure 1.7).

1.8 Enzymes of glutamine catabolism

1.8.1 Phosphate-dependent glutaminase (PDG)

The phosphate-dependent glutaminase (PDG; E.C 3.5.1.2) is the enzyme which hydrolyzes glutamine at high rates yielding glutamate and ammonium ions generating the metabolic intermediates required for cell growth (shown in Reaction 2)(McCauley *et al.*, 1999). In the middle 1980's, Shapiro and colleagues (Shapiro *et al.*, 1985) studied the orientation of this mitochondrial enzyme and suggested that it is located in either the matrix or on the inner face of the inner mitochondrial membrane. Other studies challenged this idea and concluded

that this enzyme is found on the outer face of the inner mitochondrial membrane (Kvamme *et al.*, 1991; Roberg *et al.*, 1995; Kvamme *et al.*, 2001). Studies by (Haser *et al.*, 1985; Shapiro *et al.*, 1991; Srinivasan *et al.*, 1995) have fully isolated and characterized *in vitro* expression of glutaminase encoding cDNA.

1.8.1.1 Isoforms of phosphate-dependent glutaminase

There are two types of glutaminases known as the liver (hepatic) type (LGA) and the kidney type (KGA). The kidneys, intestinal epithelial cells, lymphocytes as well as rapidly proliferating cells have been shown to express KGA (Curthoys and Watford, 1995; Thangavelu *et al.*, 2012) whereas LGA is expressed in the hepatocytes and pancreas (Aledo *et al.*, 2000). Human tissues can simultaneously express both isoforms.(Aledo *et al.*, 2000; Campos-Sandoval *et al.*, 2007). There is noticeable 86% similarity in LGA and KGA glutaminases amino acid sequence (McCauley *et al.*, 1999), but separate genes encode these isozymes. Apparently, the most noticeable difference between the two isoenzymes is that KGA is strongly inhibited by glutamate (end-product) whereas the LGA is not inhibited (Márquez *et al.*, 2006).

There are two genes that encode the LGA and KGA glutaminases which are located on different chromosomes. The KGA gene is found on chromosome 2 whilst the LGA gene seems to be located on chromosome 12 (Aledo *et al.*, 2000; Márquez *et al.*, 2006). It is believed that glutaminase genes have evolved over time and they have a common ancestral gene (Aledo *et al.*, 2000).

1.8.2 Glutamate dehydrogenase

Glutamate dehydrogenase (GDH) (EC 1.4.1.3) catalyzes the reversible conversion of glutamate to α-ketoglutarate and ammonia (Spanaki and Plaitakis, 2012) using NADP(H) and NAD(H) as cofactors. Various tissues such as mammalian liver, brain, kidney, pancreas and intestines are known to possess high GDH activity (Fang *et al.*, 2002; Mastorodemos *et al.*, 2005). In periportal hepatocytes, GDH yields increased quantities of ammonia which are in turn utilized for urea synthesis. The GDH activators are ADP and leucine whereas GTP and ATP are the inhibitors (Li *et al.*, 2012). During the synthetic reaction (high $NADH/NAD^+$ ratio), α-ketoglutarate is converted to glutamate via the GDH action. The resultant glutamate can be further converted to glutamine and other amino acids (Frigerio *et al.*, 2008). Conversely, at low $NADH/NAD^+$ ratio, the action of GDH results in the production of α-ketoglutarate which is channelled to the TCA cycle (Frigerio *et al.*, 2008; Li *et al.*, 2012).

1.8.3 Alanine and aspartate aminotransferases (ALT and AST)

Alanine aminotransferase (EC 2.6.1.2) is an enzyme present in tissues such as liver, skeletal muscle kidney and heart. It catalyzes a reversible transamination reaction between alanine and α-ketoglutarate yielding pyruvate and glutamate (Reaction 3). Aspartate aminotransferase (EC 2.6.1.1) catalyzes a reversible transamination reaction between aspartate and α-ketoglutarate resulting in oxaloacetate and glutamate (Reaction 4). Two isoforms of AST have been reported so far, that is, the cytoplasmic and mitochondrial. Hepatocellular injury often results in an increase of serum ALT activity (Tikkanen *et al.*, 2013).

$$\text{Alanine} + \alpha\text{-Ketoglutarate} \xrightleftharpoons{\text{Alanine aminotransferase}} \text{Pyruvate} + \text{Glutamate}$$

(Reaction 3)

$$\text{Aspartate} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{Aspartate aminotransferase}} \text{Oxaloacetate} + \text{Glutamate}$$

(Reaction 4)

1.9 Medicinal plants used in the treatment of cancer

Medicinal plants have a prehistoric use in the primary health care of individuals. They are being used because of their phytoconstituents that are reputed to have curative properties (Van Wyk *et al.*, 1997; Howes *et al.*, 2003). Modern medicine utilizes drugs that have been primarily derived from medicinal plants (Jena *et al.*, 2012; Street and Prinsloo, 2012) implying that plants are primary source of most medicines and still continue to provide new remedies. To be effective, some phytoconstituents may be combined with phytoconstituents from the same plant or from a different plant for enhancement of the activity of the phytocompounds (Howes *et al.*, 2003). The medicinal plant derived extracts aid in healing and prevention of various diseases. South Africa is rich in plant biodiversity with over 30 000 species well documented (Van Wyk *et al.*, 1997; Street and Prinsloo, 2012). Of these about 3 000 species are used as medicines (Coetzee *et al.*, 1999; Street and Prinsloo, 2012). The potential of using plants as anticancer agents was recognized in the 1950s by the U.S. National Cancer Institute (NCI) under the leadership of the late Dr. Jonathan Hartwell (Cragg *et al.*, 2012).

Trichomes are secretory structures found in plants. Early microscopy studies recognized these structures are among other anatomical features of plants. These secretory structures differ in morphology and arise directly as hairs on the adaxial and abaxial leaf surfaces (Wagner, 1991). Trichomes have a number of functions (Wagner *et al.*, 2004) including secretion of primary and secondary metabolites that are widely used in pharmaceuticals, fragrances, food coloring and many more (Van Wyk *et al.*, 1997). Along with medicinal properties, the trichomes are being recognized in plant biology research as a source of essential oil, terpenes alkaloids and aromatic biosynthesis.

Examples of phytoconstituents from medicinally recognized plant species of the genus Podophyllum include epipodophyllotoxin known as the active antitumor agent extracted from the roots as well as etoposide, a topoisomerase inhibitor drug class, are widely used in the treatment of lung cancer, testicular cancer and leukaemia (Holthuis, 1988)(Figure 1.8). Natural products chemists have identified chemical groups from plant species that possess antitumor properties, namely: alkaloids, phenylpropanoids, terpenoids, triterpenoids, sesquiterpene lactones, flavonoids and coumarins (Wu *et al.*, 2012). Sesquiterpene lactones pharmacological activities include cholinergic, hypoglycemic, and anti-inflammatory activity and are in species of the Asteraceae, Magnoliaceae and Apiaceae (McKinnon *et al.*, 2014).

Figure 1.8: Chemical structures of Etoposide (left) and epipodophyllotoxin (right) from a genus *Podophyllum*. Adapted from (Shi *et al.*, 2001).

1.9.1 Plant species used in this research project

The two plant species used for this research were *Centella asiatica* and *Catharanthus roseus*.

1.9.1.1 Plant classification

Figure 1.9: Plant species *Catharanthus roseus* (left) and *Centella asiatica* (right) used in the current project.

Domain:	Eukarya	
Kingdom:	Plantae	
Phylum:	Magnoliophyta	
Class:	Magnoliopsida	
Order:	Gentianales	Apiales
Family:	Apocynaceae	Apiaceae
Genus:	*Catharanthus* G. Don	*Centella* L.
Species:	*Catharanthus roseus (L.)* G. Don	*Centella asiatica* (L.) Urb.

1.9.1.2 Description of the plant species

1.9.1.2.1 Centella asiatica

Centella asiatica (L.) Urban also known as Gotu-Kola and Pennywort, is one of the widely used plant species in Malaysia, China, India, Southeast Asia, Sri Lanka and South Africa (Brinkhaus *et al.*, 2000; Alfarra and Omar, 2013). Centella has about 50 species and grows well in humid conditions. *C. asiatica* is a small, herbaceous annual plant. The leaves are kidney-shaped and sometimes circular, are swaps over and are clustered simultaneously at the nodes. *C. asiatica* has trichomes present in both young and adult leaves. *C. asiatica* has many beneficial effects for human health and has been utilized as a revitalizing herb that restores youth and retards aging (Brinkhaus *et al.*, 2000; James and Dubery, 2009). It is reputed to strengthen nervous function and memory and treats skin-associated diseases

(Orhan, 2012). The herb also prevents dementia and has anticonvulsant, antidiabetic, anxiolytic and sedative properties (Rahman *et al.*, 2012). People with mental illness and epilepsy are usually given the herb concoction. Previous studies have suggested that *C. asiatica* may have anti-inflammatory effects since it has been used a as tonic for rheumatism and inflammation (Babu *et al.*, 1995; Brinkhaus *et al.*, 2000; Zainol *et al.*, 2003; Bunpo *et al.*, 2010). The plant's essential oil is said to contain monoterpenes such as α- pinene, β- pinene and γ- terpinene and is reported to inhibit acetylcholinesterase (AChE) (Miyazawa *et al.*, 1997). Furthermore, phytoconstituents such as alkaloids flavones, triterpenoid, pectin, sterols and polyacetylenes are found in *C. asiatica* extracts. Asiatic acid and medacassic acid have been detected and isolated and are known from their therapeutic activities. Also, amino acids such as glutamate, alanine, serine have been detected (Howes and Houghton, 2003; Howes *et al.*, 2003). In mice, an extract of *C. asiatica* leaf administered orally at 100 mg/kg increased speed rats chronic wound healing. Some other studies conducted regarding the use of *C. asiatica* extracts in treating symptoms of depression and anxiety in Alzhiemer's disease were successful and effective (Howes *et al.*, 2003). In managing epileptic seizure healing, the extracts of *C. asiatica* were found to be proficient (Howes and Houghton, 2003).

1.9.1.2.2 Catharanthus roseus

Catharanthus roseus (L.) G. Don, is commonly known as *Vinca rosea* and Madagascar periwinkle. *C. roseus* has received a lot of attention for its therapeutic abilities in treating various kinds of diseases. The plant is native to Madagascar, Caribbean Basin, Europe, India and other parts of the world. *C. roseus* is utilized for conditions such as headache, diabetes and to treat various cancers (Farnsworth *et al.*, 1967; Gajalakshmi *et al.*, 2013). *C. roseus* extracts from dried flowers and leaves have been applied to wounds (Nayak and Pereira, 2006) and the plant has potential efficacy in memory-enhancement (Gajalakshmi *et al.*, 2013). *C. roseus* extracts, amongst other plant extracts, is also known for its anti-cancer properties with no adverse results recorded (Farnsworth *et al.*, 1967; Widowati *et al.*, 2010). It also possess hypoglycemic properties (Mohan *et al.*, 2015) thus it is being used for the treatment of diabetes.

Figure 1.10 below shows the *Vinca* alkaloids in *C. roseus.* Vinblastine is known in the treatment of many forms of leukaemia and has anti-inflammatory effects. Proportionately, the vincristine is also used as an anticancer agent (Gajalakshmi *et al.*, 2013).

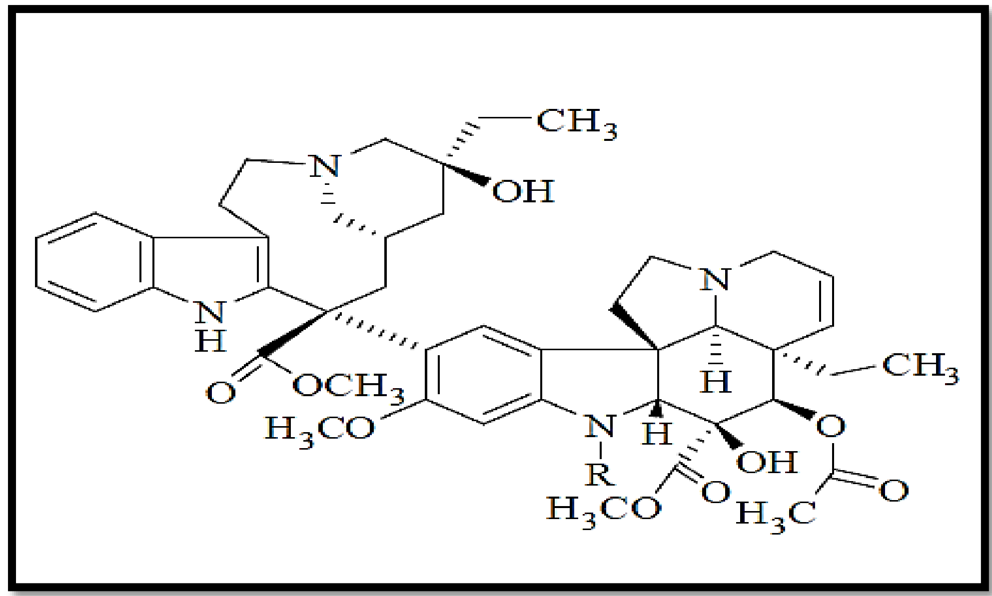

Figure 1.10: Chemical structures of Viblastine, R= CH_3 and Vincristine, R= CHO which are the two major active compounds isolated from *C. roseus*.

1.10 Phytochemical screening techniques

1.10.1 Phytochemistry

Phytochemical tests are used to detrermine the presence of active phytoconstituents such as glycosides, terpenoids and steroids, alkaloids, carbohydrates flavonoids tannins and many more. Basic laboratory testing methods are done using commercially available stains and solutions to detect these compounds (Harborne, 1973; Joshi *et al.*, 2011).

1.10.2 Gas Chromatography- Mass Spectrometry

Gas Chromatography – Mass Spectrometry (GC-MS) is a single system as a result of integration of gas chromatography as well as mass spectrometry. Gas chromatography (GC) is a partitioning and analysis technique of volatile compounds (McNair and Miller, 2011) separating mixture components. There is a mobile phase (carrier gas) and stationery phase (liquid or solid). On the other hand, mass spectrometry (MS) provide a plot of mass-to-charge ratio (*m/z*) versus ion abundance produced from sample. A bar graph or table can be used to represent mass spectra results. The ion abundance, often in percentages (y-axis), represents the number of ions and the mass-charge ratio (x-axis) are separated and detected ions (De Hoffmann and Stroobant, 2007).

1.11 Cell culture studies

In cell culture, cells are grown under well monitored (controlled) conditions. However, these conditions can be manipulated depending on the types of cells grown and their nutritional

demands. *In vitro* studies play a crucial role in testing whether cytotoxicity exists and are then followed by animal studies (Li *et al.*, 2004b). The present study employed 3-4,5dimethylthiazol-2-yl 2,5diphenyltetrozolium bromide (MTT) assay which is a method quantifying metabolically viable cells and monitors cytotoxicity (Price and McMillan, 1990) to assess whether *C. asiatica* and *C. roseus* exhibited any toxicity in the Caco-2 cell lines. Nowadays, biomedical research in different fields including cell biology, drug discovery, genetics, HIV testing/treatment, medicine and vaccine development utilizes cell lines before a specific drug is passed and administered to animals and human (Li *et al.*, 2004a).

1.11.1 Cell Viability: MTT assay

Viable cells reduce a soluble yellow tetrazolium salt (MTT) producing a blue-purple formazan crystals catalysed by succinate dehydrogenase (E.C 1.3.5.1), a mitochondrial enzyme. In the resultant solution, the formed crystals are dissolved and spectrophotometrically quantified by measuring the solution absorbance (Tolosa *et al.*, 2015).

1.12 Cell lines

1.12.1 Caco-2 cell line

Caco-2 cell line (heterogeneous human epithelial colorectal adenocarcinoma) are derived from a colon carcinoma as the name suggest. Under growth conditions, these cells differentiate forming a polarized epithelial cell monolayer. Caco-2 cells phenotype, morphologically and functionally resembles the enterocytes in the small intestine (Hidalgo *et al.*, 1989; Bédrine-Ferran *et al.*, 2004).

1.12.2 HEK293 cell lines

HEK293 cells are derived from human embryo kidney cells grown in tissue culture (Ararat and Graham, 2002). These cells are widely used for transfection and cytotoxicity studies. However, this study used HEK293 cell line for comparative purposes and the enzyme of interest which is PDG is also found in the kidneys.

1.13 Project

1.13.1 Justification of the present study

Medicinal plants extracts have proven to be a potential alternative to manage cancer despite the current use of conventional anti-cancer drugs such as chemotherapeutic drugs. The study assessed the effect of extracts on Caco-2 cell line growth and viability. This particular cell line (Caco-2) was investigated since it is a good model for assessing the effects of anticancer drugs and has the ability to differentiate in culture forming a polarized monolayer similar to that of the small intestine. Treatments targeting this cell line may provide a therapeutic

strategy for treatment of intestinal cancers and possibly cancers involving other tissues. PDG, an enzyme that has become a focus of anti-cancer drug development was the major object of investigation in this project. However, the enzyme is also required for normal enterocyte function hence our study not only assessed the effects of the plant extracts on the enzyme in Caco-2 cells *in vitro* but also assesed the effect of the extracts in vivo on rat enterocytes. Other downstream enzymes of glutamine catabolism were also investigated as was cytochrome c release from cells.

1.13.2 Aim and Objectives

Aim: To investigate the effects of *Catharanthus roseus* and *Centella asiatica* extracts on enzymes of glutamine metabolism in Caco-2 cell line and enterocytes of male Sprague-Dawley rats.

Objectives

1. Evaluate the effect of the extracts on Caco-2 cell line growth and viability as indicators of cytotoxicity.
2. Assessment of the PDG activity and related enzymes in the Caco-2 cell line and in rat isolated enterocytes following treatment with medicinal plant extracts.

Specific objectives

1. To determine the effect of the plant extracts on Caco-2 cells by performing MTT assay.
2. To measure the activities of PDG, GDH, ALT and AST in both Caco-2 cell line and enterocytes isolated from rats after treatment with medicinal plant extracts using enzyme assays.
3. To determine the effect of medicinal plant extract administration on the expression of PDG and AST enzyme using SDS-PAGE Western Blot analysis.
4. Phytochemical analysis of both *C. roseus* and *C. asiatica* extracts using preliminary phytochemical screening and GC-MS.

1.13.3 Research questions

1. How is cell growth and viability affected after treatment with the plant extract?
2. Are there any effects of the plant extracts on the glutaminase activity and expression in Caco-2 cell lines?
3. Are there any effects of the plant extracts on the glutaminase activity in enterocytes of Sprague-Dawley rats treated with plant extracts?

4. Are there any effects of the plant extracts on downstream enzymes of glutamine metabolism e.g. aspartate and alanine aminotransferases (ALT and AST)?

1.13.4 Potential benefits of the project

The potential benefit is to develop new and alternative sources of drugs for the treatment of cancer in humans.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

The following drugs and chemicals were sourced from SIGMA- ALDRICH (St. Louis, Missouri, USA): 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Acrylamide, Adenosine monophosphate, Benzamidine hydrochloride hydrate, Bovine serum albumin (BSA), Bromophenol blue, , Dimethyl sulfoxide (DMSO), Di-Potassium hydrogen orthophosphate (K_2HPO_4), Ethylene diaminetetraacetic acid (EDTA), Methanol (CH_3OH), N'N'-bis-methylene-acrylamide, Phenylmethanesulfonylfluoride (PMSF), Potassium chloride (KCl), Potassium dihydrogen phosphate (KH_2PO_4), Sodium chloride (NaCl), Sodium hydroxide (NaOH), Triton-X 100, TRIZMA base (tris), Tween-20, sodium bicarbonate ($NaHCO_3$) and β-Mercaptoethanol. Copper sulphate ($CuSO_4.H_2O$), Disodium hydrogen phosphate (Na_2HPO_4), Hydrochloric acid (HCl), Potassium iodide (KI), Potassium sodium tartate ($KNaC_4H_4O_4.H_2O$), Sodium hydroxide (NaOH), Sodium sulphate ($NaSO_4$) and Sulphuric acid were purchased from MERCK (Johannesburg, South Africa). 10X Tris/Glycine/SDS, N, N, N, N -Tetramethylethylenediamine (TEMED), Sodium dodecyl sulphate (SDS), TRIZMA base (tris), Ammonium persulphate (APS) and precision plus protein standards (molecular weight marker) we purchased from BIORAD, South Africa. The kidney-type glutaminase antibody (KGA) H-45: sc 366447 was bought from Santa Cruz Biotechnology, Inc., USA. The secondary antibody Goat anti-Rabbit IgG Alkaline phosphatase conjugate were purchased from BIORAD, South Africa. Halothane was from Fluorothane® (AstraZeneca Pharmaceuticals, South Africa). Earle's minimum essential medium (EMEM) was from Gibco BRL (Inchinnan, Scotland), Foetal bovine serum were purchased from Whittaker Bioproducts (Walkersville, Maryland, USA). 95% Oxygen: 5% Carbon dioxide gas was bought from AFROX (South Africa).

All chemical reagents were of analytical grade and deionized water was used to prepare all solutions.

2.1.2 Animals

Ethical clearance for animal studies was obtained from the Animal Ethics Committee of the University of KwaZulu-Natal (reference 055/14 Animal).

Male Sprague-Dawley rats, body weight (230-250 g), were bred and housed in the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal, Westville Campus. For the entire experimental period, the animals were maintained under standard laboratory conditions and were fed a standard rat chow (Meadows, Pietermaritzburg, South Africa) *ad libitum.* Also, drinking water was provided for all the animals *ad libitum*. The animals handling rules and regulations of the University of KwaZulu-Natal (UKZN) Animal Ethics Committee were followed.

2.1.3 Plant Material

The *Catharanthus roseus* and *Centella asiatica* plant species were harvested from the oval residence cricket field around the fence within University of KwaZulu-Natal, Westville Campus. Photographic Voucher specimens (Dladla 2) showing plant characteristics was prepared and deposited in the Ward Herbarium of the University of KwaZulu-Natal, Westville Campus.

2.2 Methods

2.2.1 Preparation of extracts

Leaves of *Catharanthus roseus* and whole plant of *Centella asiatica* plant species were freshly harvested and air-dried in the laboratory. The dried leaf material was then ground to coarse using a mortar and pestle and then to fine powder using Phillips Mini food processor with blender (HR7625). The ground material of each plant species was soaked in 1000 ml methanol at room temperature for 3 days and overnight at 220 rpm in Infors AG CH- 4103 BOTTMINGEN shaker (United Scientific, South Africa). Both extracts in the solvent were collected separately in pre-weighted glass bottles and filtered using Whattman no.1 paper. The resultant filtrates were concentrated to dryness under reduced pressure below 40˚C in a rotary evaporator to remove the entire methanol. The dry methanolic extracts were stored sealed at 4˚C for further use.

2.2.2 Experimental Design

This study involved both *in vitro* and *in vivo* experiments (Figure 2.1). In the *in vitro* studies, Caco-2 and HEK293 cells were utilized. These studies evaluated the effects of medicinal plant derived extracts on growth and viability using the human colon carcinoma (Caco-2) cell line to establish whether the extracts exhibited any toxicity. The cultured cells were treated with the plant extracts and the effects of extracts on Caco-2 cell growth and viability parameters were determined using MTT assay to measure cytotoxicity (loss of viable cells) of potential medicinal agents and toxic materials. The haemocytometer was used to determine

the concentration of cells in a liquid sample. HEK293 cells were used as control in the MTT assay since they are normal cells derived from kidney and the PDG enzyme is found in the kidneys. The i*n vivo* studies investigated the effects of medicinal plant derived extracts on PDG and related enzymes in the enterocytes isolated from male Sprague-Dawley rats. Male Sprague-Dawley rats treated with extracts were used as the source of enterocytes. Apoptotic parameters and enzyme activities were also assessed in enterocytes and a comparison made between enterocytes and Caco-2 cells.

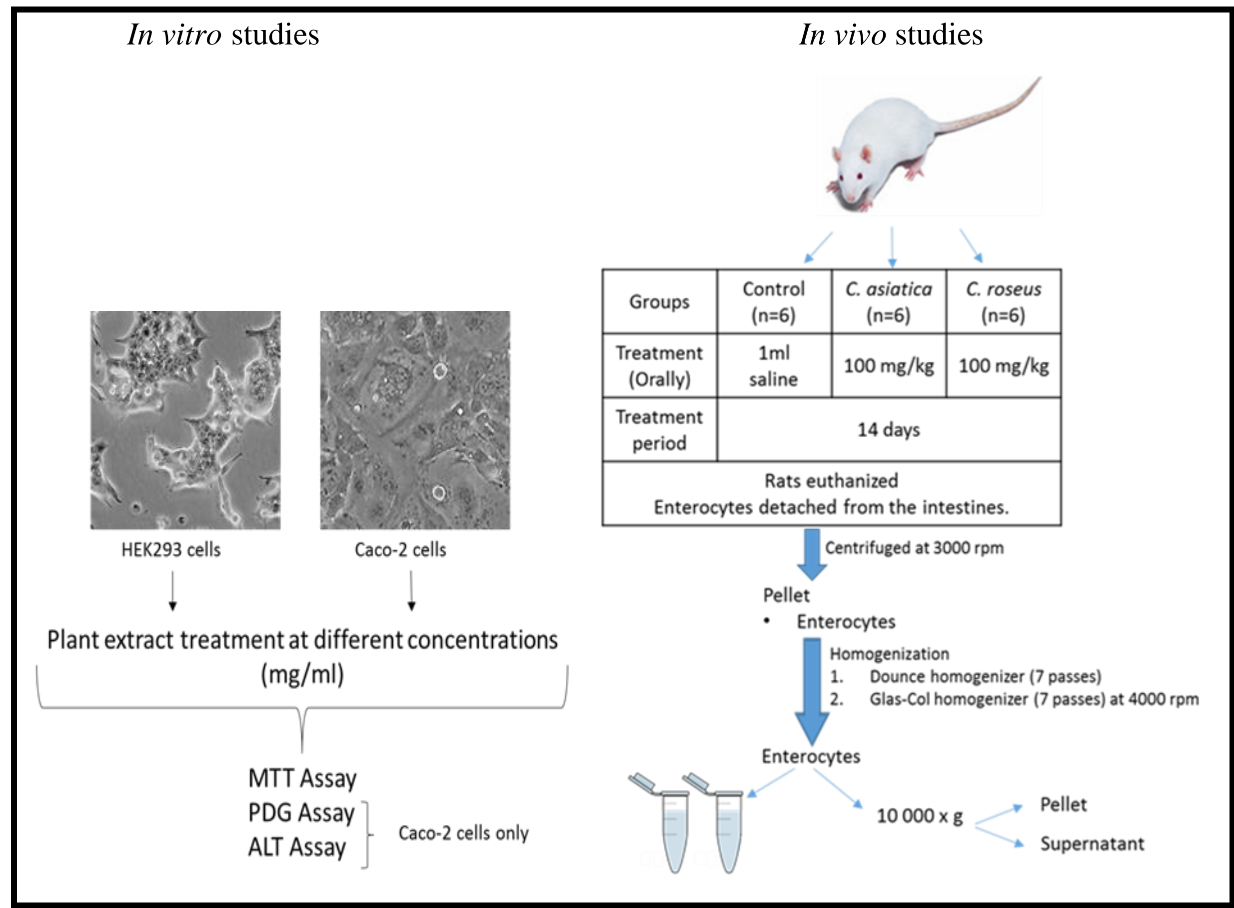

Figure 2.1: Illustration of the experimental design for both *in vitro* and *in vivo* studies.

2.3 The effects of plant extracts on enterocytes isolated from rats intestines

Extracts were dissolved in physiological saline (0.9% or 154 mM NaCl). A 15 to 16 gauge blunt-ended needle cannula about 11 cm in long (bulbed-end) was used to treat the Sprague-Dawley rats with the plant extract. The male Sprague-Dawley rats were divided into three groups of 6 rats each, namely; control (CNTRL), *Centella asiatica* treated (CA) and *Catharanthus roseus* treated (CR). The control group did not receive any treatment but was orally dosed with 1 ml of physiological saline. The treatment groups were orally dosed with

100 mg/kg CA and CR plant extracts. All the rats were euthanized 14 days following treatment and were then used as source of enterocytes. A total of 18 rats (n = 6 per treatment) were used for this study to establish if the extracts had any effect over a 14-day treatment period *in vivo.*

Table 2.1: Representation of the different treatments groups for the *in vivo* study.

Male Sprague-Dawley rats			
Groups	Control n = 6	*C. asiatica* n = 6	*C. roseus* n = 6
Treatments (orally)	1 ml phys. Saline	100 mg/kg	100 mg/kg
Period	14 days		
Rats euthanized after 14 days treatment period and used as source of enterocytes.			

2.3.1 Terminal studies and procedure for isolation of enterocytes

Enterocytes were prepared using a method by (Watford *et al.*, 1979) modified by (Evered and Masola, 1984). Animals were euthanized one at a time using an overdose of halothane (an inhalation anaesthetic) in a 2.3L anaesthetic chamber with 2.17 ml/L of halothane (Fluorothane®, Astra Zeneca 2002) pharmaceuticals (Pty) LTD for approximately 3 minutes. Thereafter the rats were taken out of the chamber and incised on the abdomen. The intestines were recovered and the fatty layer around the small intestine was removed. Intestines were washed to remove digested residues using medium 1 (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.9 mM $MgSO_4.7H_2O$, pH 7.4, saturated with 95% O_2 :5% CO_2). Each intestine was ligated on one end, filled with medium 2 (medium 1 supplemented with 5 mM EGTA and 0.25% BSA) and the other end was ligated as well. They were then placed in a conical flask containing 250 ml of medium 1. This conical flask was incubated at 37˚C for 12 minutes in a water bath and was gassed continuously with 95% O_2: 5% CO_2. Medium 2 was removed from the intestines and the intestines were rinsed with medium 1. The intestines were then three-quarters filled with ice cold medium 3 (medium 1

supplemented with 0.25% BSA) and ligated on the other end. Enterocytes were detached gently by rubbing the intestines along its length using the forefinger and thumb. Once the enterocytes were detached, one end of the intestine was unligated and the detached enterocytes were released into a clean beaker.

2.3.2 Homogenate processing

Enterocytes suspension was divided into four 15 ml centrifuge tubes and centrifuged at 3000 rpm for 5 minutes using a Hettich Zentrifugen D-7200 Tulttingen bench top centrifuge. The supernatant was discarded and the remaining pellet in the centrifuge tubes was washed twice with homogenizing buffer containing 50 mM Tris and 120 mM KCl, pH 7.4 with protease inhibitors (0.5 mM Benzamidine hydrochloride hydrate and 0.5 mM PMSF). Enterocytes were then homogenized in a buffer using Dounce homogenizer by 7 passes while chilled on ice. These were then re-homogenized using a motor driven Glas-Col homogenizer (Terre Haute, USA) by 7 passes at 4000 rpm. The homogenates were divided to a total volume of 15 ml and topped up with 50 mM Tris, 120 mM KCl buffer, pH 7.4 containing protease inhibitors. These were then collected into the eppendorf tubes and stored in the deep freezer at -20 °C until further use. Some of the homogenate were centrifuged at 10 000 x *g* for 10 minutes at 4 °C a using Beckman Coulter J26XPI centrifuge (USA) and both the supernatant and pellet were collected and made up to 15 ml volume with homogenizing buffer, pH 7.4. These were collected to for assessment of cytochrome c leakage from mitochondria for testing apoptotic parameters.

2.4 Assays of enzymes of glutamine catabolism

The activities of phosphate-dependent glutaminase, glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase were assayed.

2.4.1 Phosphate-dependent glutaminase (PDG) assay

PDG (EC 3.5.1.2) was assayed at 37°C in a two stage procedure as described by (Curthoys and Weiss, 1974) but with modifications (Masola and Zvinavashe, 2003). The amount of glutamate produced by PDG was determined through its oxidation by glutamate dehydrogenase (GDH). The GDH reaction is accompanied by the reduction of NAD^+ to NADH monitored at 340 nm (Figure 2.2).

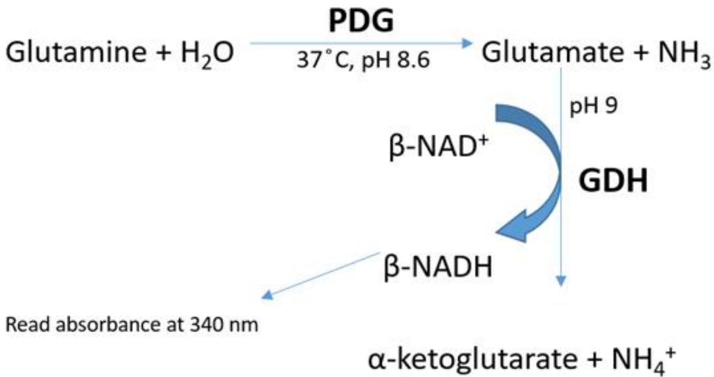

Figure 2.2: Phosphate-dependent glutaminase activity assay.

The assay consisted of solution 1 made up of 10 mM phosphate buffer, 50 mM Trizma base and 120 mM KCl and 0.020 M glutamine at pH 7.4. On the other hand, solution 2 had 50 mM Tris, 0.31 hydrazine, 2 mM NAD^+ and 0.25 mM ADP at pH 9. 50 µl of the enzyme (enterocytes homogenate or its fractions) was added to 200 µl Solution 1 (containing freshly prepared glutamine to initiate the reaction) and incubated at 37 ˚C for 15 minutes. The reaction was stopped by addition of 120 µl of 30% Perchloric acid solution to each test tube at intervals of 30 seconds with test tubes being placed on ice for 5 minutes. An aliquot of 225 µl of cold 3N KOH was added to each test tube to neutralize the acid followed by the addition of 2 ml of Solution 2. The test tubes were centrifuged at 3000 rpm (5 minutes) using a Hettich Zentrifugen D-7200 bench top centrifuge. Clean test tubes were used to transfer a clear supernatant followed by addition of 10 µl GDH enzyme in 50% glycerol (13 U). The test tubes were incubated at room temperature for 45 minutes. The absorbance was read at 340 nm using a Varian Cary 50 UV/Vis Spectrophotometer, USA.

2.4.2 Glutamate Dehydrogenase (GDH) assay

A Varian Cary 50 UV/Vis Spectrophotometer, USA fitted with a temperature controlled cuvette holder was used to take absorbance measurements at 340 nm. The GDH activity in enterocytes was assayed as described by (Schmidt and Schmidt, 1974) by monitoring NADH oxidation at 25℃ (Figure 2.3).

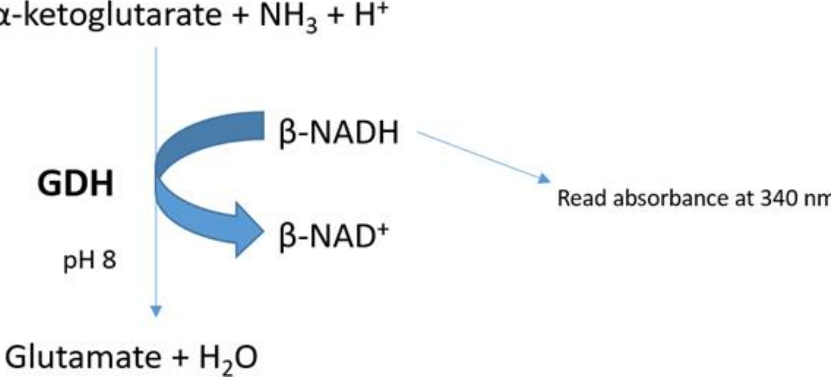

Figure 2.3: Glutamate dehydrogenase assay.

A buffer consisting of 50 mM Triethanolamine (TEA), 100 mM Ammonium acetate (NH_4^+) and 2.5 Ethylene diamine tetra acetic acid (EDTA), pH 8 was made. A freshly prepared 7 mM α-ketoglutarate and 0.2 mM NADH + 1 mM ADP solution were made in buffer and pH adjusted to 8. A 100 µl of enterocytes sample was added to a clean cuvette with 2700 µl TEA, EDTA and NH_4^+ solution and 100 µl ADP + NADH solution. A 0.1 % Triton-X was added to solubilize the enzyme and 10 µl lactate dehydrogenase (LDH) (2.86 units/ml) enzyme was also added to remove any interfering pyruvate. The absorbance was read until all pyruvate had been consumed and a reaction was started by addition of 100 µl of 7 mM α-ketoglutarate in buffer and monitored again at 3-5 minutes.

2.4.3 Alanine and Aspartate aminotransferase (ALT and AST) assays

Alanine and aspartate aminotransferases were assayed in coupled assays as described by (Volman-Mitchell and Parsons, 1974). Alanine aminotransferase catalyzes the reaction between alanine and α-ketoglutarate which generates pyruvate and glutamate. The pyruvate produced is reduced to lactate catalysed by lactate dehydrogenase (LDH). The LDH reaction is accompanied by the oxidation of NADH to NAD^+ monitored at 340 nm (Figure 2.4).

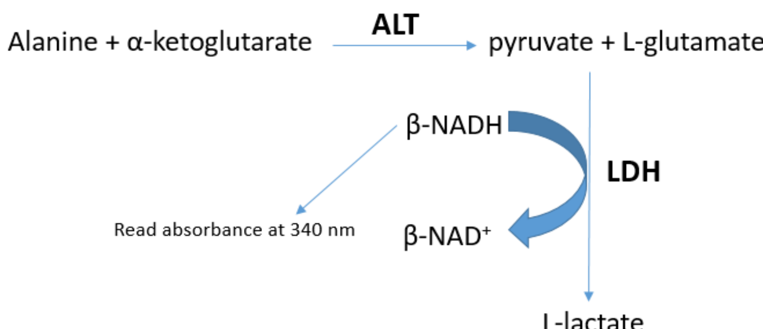

Figure 2.4: Alanine aminotransferase assay.

The reaction mixture for ALT consisted of 0.1 M Sodium phosphate buffer pH 7.6 ($NaH_2PO_4.2H_2O$ and Na_2HPO_4), 7 mM α-ketoglutarate in buffer, 0.2 mM NADH in buffer and 8.6 units/ml LDH in 50% glycerol. A 0.1 ml of sample was then added on a cuvette with the above mentioned mixture. After monitoring the non-specific oxidation of NADH, the reaction was then started by adding 33 mM L-alanine in buffer in a final reaction volume of 3ml. On the other hand, aspartate aminotransferase catalyzes the reaction between aspartate and α-ketoglutarate which generates oxaloacetate and glutamate. The oxaloacetate produced is reduced to malate catalysed by malate dehydrogenase (MDH). The MDH reaction is accompanied by the oxidation of NADH to NAD^+ monitored at 340 nm (Figure 2.5).

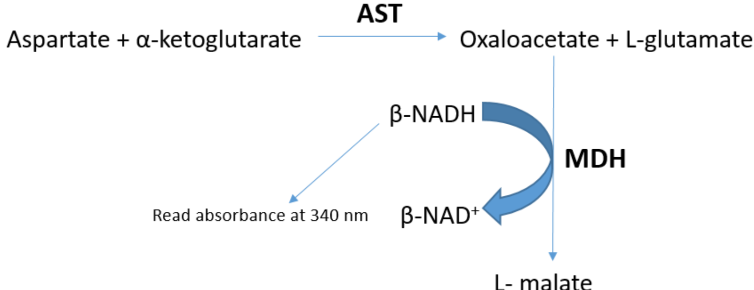

Figure 2.5: Aspartate aminotransferase assay.

The reaction mixture for AST consisted of 0.1 M Sodium phosphate buffer pH 7.6, 7 mM α-ketoglutarate in buffer, 0.2 mM NADH in buffer and 19 units/ml MDH in 50% glycerol

followed by addition of 0.1 ml of sample. The reaction was started by addition of 33 mM L-aspartate in buffer after monitoring non-specific oxidation of NADH.

2.5 The effect of medicinal plant extract administration on the expression of PDG enzyme

2.5.1 Protein Determination

Protein content of enterocytes was measured using Buiret assay. Bovine serum albumin (BSA) was used as a protein standard (Gornall *et al.*, 1949). The biuret reagent consisted of 3 g of cupric sulfate pentahydrate ($CuSO_4.H_2O$) and 9 g of Sodium potassium tartate tetrahydrate ($KNaC_4H_4O_4.H_2O$) in 500 ml of 0.2 M Sodium hydroxide (NaOH). A 5 g of Potassium Iodide (KI) was added and volume made up to 1 litre with 0.2 M NaOH. A 3 ml of the biuret reagent was added to 2 ml of protein sample (enterocytes, supernatant and/or pellet sample) in the test tubes. This was vortexed and incubated for 10 minutes at 37 ^{0}C in a water bath. The test tubes were left on the bench to cool and centrifuged for 5 minutes using the Hettich Zentrifugen D-7200 bench top centrifuge at 1 000 rpm. The absorbance was read at 540 nm using a Varian Cary 50 UV/Vis Spectrophotometer, USA. The protein standards were plotted against their known protein concentrations in the range of 0-10 mg/ml. A standard curve was drawn and the protein concentrations calculated.

The Lowry method (Lowry *et al.*, 1951) was also used to determine protein concentration. Alkaline sodium carbonate reagent (20 g/litre Na_2CO_3 in 0.1 M NaOH) and copper sulphate-sodium potassium tartrate solution (5 g/litre $CuSO_4.5H_2O$ in 10 g/litre Na, K tartate) were prepared. A 0.5 ml of sample was added in a test tube containing 5 ml alkaline reagent (prepared by mixing alkaline sodium carbonate solution and copper sulphate–sodium potassium tartrate solution 50:1) and incubated at 40 °C for 15 min. Thereafter, the Folin-Ciocalteu reagent was freshly prepared by diluting stock 1:2 with deionized water. Samples were cooled followed by the addition of 0.5 ml Folin Ciocalteu reagent. These were then vortexed and incubated at room temperature for 30 minutes. Using a Varian Cary 50 UV/Vis Spectrophotometer, USA, absorbances were read at 600 nm. BSA was used as a standard range (20 - 100 µg). From the standard curve graph, the concentrations of samples were extrapolated.

2.5.2 SDS-PAGE and Western Blot analysis

2.5.2.1 Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as per instructions in the BIORAD manual. The resolving Gel Buffer – 1.5 M Tris-HCl, pH 8.8 and the stacking Gel Buffer – 0.5 M Tris-HCl, pH 6.8. Proteins were denatured by heating them in buffer containing sodium dodecyl sulfate (SDS) and a thiol reducing agent 2-mercaptoethanol. The resultant denatured polypeptides take on a rod-like shape and a uniform charge-to-mass ratio proportional to their molecular weights. Proteins were separated according to their molecular weight. The sample proteins were separated on a 15% SDS-PAGE gel [2.4 ml (Distilled water) 5.0 ml (30% degassed Acrylmide/Bis) 2.5 ml (Gel Buffer) 0.1 ml (10% w/v SDS)] in the presence of sodium dodecyl sulphate using a Bio-Rad Mini - PROTEAN 3 Electrophoresis cell, BIORAD (South Africa). The samples were electrophoresed in Tris/Glycine/SDS buffer, pH 8.3 run at 90 V and increased to 110 V when the samples have moved through the stacking gel. The gels were coomasie stained to visualize the proteins. Proteins were visualized using a Syngene GBOX Chem XR5 imager (Vacutec ,South Africa). Images were captured using the GeneSys software.

2.5.2.2 Western-Blot

Western blot analysis was carried out as follows: The samples containing equal amount of proteins were subjected to electrophoresis in 15% (w/v) polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS-PAGE) using a Bio-Rad Mini- PROTEAN 3 Electrophoresis cell (BIORAD, South Africa). A Tris/Glycine/SDS buffer, pH 8.3 was used to carry out the electrophoresis at 110 V. To keep the blotting apparatus cool, an ice cooling unit with water was stored in a deep freezer until ready to use. A Tris/Glycine/Methanol transfer buffer pH 8.3 was also prepared. The prepared gel was equilibrated with transfer buffer and the PVDF membrane, filter paper as well as fibre pads were soaked in transfer buffer prior the assemblage of the gel sandwich. A Bio-Rad Trans-Blot Electrophoretic Transfer Cell (BIORAD, South Africa) assembled with ice-cooling unit, run at 100 V for 2 hours at $4^{0}C$ in transfer buffer pH 8.3 to transfer the proteins from gel to PVDF membrane. The membranes were blocked overnight with 20 mM Tris, 500 mM NaCl, 0.05% Tween 20 (TBS-T) buffer, pH 7.4 containing 5% (w/v) non-fat dried milk at $4^{0}C$ (which is a blocking buffer). The primary IGg rabbit anti-glutaminase antibody was diluted (1: 500) in Tris buffered saline containing 0.05% Tween (TBS-T) buffer and overlaid on PVDF membrane with shaking for 1 hour at room temperature. Thereafter, the PVDF membrane was washed

twice for 10 minutes each with (Tris-buffered saline) TBS-T buffer and once with blocking buffer containing 5% (w/v) non-fat dried milk for 10 minutes. The membranes were probed with a secondary antibody Goat anti-rabbit IgG conjugated with alkaline phosphatase, diluted 1: 1500 in blocking buffer, for 1 hour with shaking at room temperature. Membranes were washed thrice for 10 minutes in TBS-T buffer. A substrate, premixed BCIP- NBT solution, was used to reveal the antibody-bound proteins. These were then visualized using a Syngene GBOX Chem XR5 (Vacutec South Africa), images were captured using the GeneSys software and the bands were quantified using GeneTools analysis software.

Dot blots were conducted as follows: For PDG and AST, 15 µg of proteins from each treatment groups were spotted on strrips of PVDF membrane and air dried at room temperature. The blocking buffer and probing with antibodies were added for 30 min each. Thereafter, the PVDF membranes were washed as described above. A Syngene G-BOX Chem XR5 (Vacutec South Africa), was used to visualize the dots blotted.

2.6 The effect of the plant extracts on caco-2 cells

2.6.1 Caco-2 and HEK 293 cells

The Caco-2 and HEK293 cell lines were purchased from Highveld Biological (Johannesburg, South Africa). Cell lines came in 25 cm^2 flasks (Bibby-Sterilin, Staffordshire, England) at 37 ^oC in Earle's minimum essential medium (EMEM) (Gibco BRL, Inchinnan, Scotland) containing 10% (v/v) foetal bovine serum, 20 mM HEPES, 10 mM $NaHCO_3$, 100 U/ml penicillin and 100 µg/ml streptomycin (Whittaker Bioproducts, Walkersville, Maryland, USA) at pH 7.5. Cells were divided and stored in a bio-freezer (-80 ^oC) in complete medium containing 10% dimethyl sulphoxide (DMSO).

2.6.2 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Cell viability was assessed using the MTT assay originally described by (Mosmann, 1983). Cells were trypsinized and seeded into 48-well plates (Bibby-Sterilin, Staffordshire, England) at a seeding density of 2.4 x 10^4 cells/well (Caco-2) and 2.8 x 10^4 cells/well (HEK293) and incubated for 24 h to permit attachment and growth of cells to semi-confluency. Thereafter, the medium (0.5 mL) was replaced and plant extracts (0, 2, 4, 8 and 16 mg/ml) was added to the wells and cells incubated at 37 ºC for 24, 48 and 72 h respectively. After each incubation period, the medium was removed and MTT solution (5mg/ml in phosphate buffered saline, 200 µL) and new medium was added to wells. The cells were incubated for 4 h to allow for the formation of blue formazan crystals. The MTT solution was then replaced with DMSO

(200 µl/well) and absorbance measured at 540 nm in a UV-visible spectrophotometer (Thermo Scientific Biomate, Cambridge, UK).

The percentage cell viability was then calculated as follows:

$$\frac{Abs. 540\ nm\ (Treated\ cells)}{Abs. 540\ nm\ (Control\ cells)}\ x\ 100$$

Technique: Viable cells have active mitochondrial dehydrogenase enzymes which reduce MTT dye by cleaving the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystal (Bernas and Dobrucki, 2002). These crystals are largely impermeable to cell membranes and accumulate within healthy cells. They are also insoluble in an aqueous solution but dissolved in DMSO. When DMSO is added, it solubilizes formazan crystals into a purple colour. The number of surviving cells is directly proportional to the level of the purple formazan product (Bernas and Dobrucki, 2002). This coloured solution can be quantified by measuring it's absorbance at a certain wavelength (between 500 and 600 nm) using a spectrophotometer. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated cells, the effectiveness of the agent in causing death or changing metabolism of cells can be deduced through calculation of percentage cell viability using the above mentioned formula.

2.6.3 Caco-2 cells Protein Determination: Bicinchoninic Acid (BCA)

Caco-2 cells protein concentration was determined using the BCA method as first introduced by (Smith *et al.*, 1985). A working solution was prepared as follows: 49 ml of BCA and 1 ml Copper (II) sulfate pentahydrate 4% (w/v) solution. A 0.1 ml of sample and 2.0 ml working solution were added in a test tube and mixed well then incubated at 37 ℃ for 30 minutes. The test tubes were then cooled for 5 minutes and absorbance read at 562 nm. BSA was used as a standard range (20 – 100 µg). From the standard curve, the concentration of each sample was extrapolated.

2.7 Phytochemical analysis of the plant extracts

2.7.1 Gas Chromatography- Mass Spectrometry (GC-MS)

GC-MS was performed using Perkin Elmer Gas Chromatography Clarus 580 (PE Autosystem GC with built-in auto-sampler). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was used as a carrier gas at a flow rate of 1.0 ml/min. The injector and MS transfer line temperature were set at 250 ˚C and 280 ˚C

respectively. Oven temperature program was 50-240 ˚C at 5 ˚C min $^{-1}$. Split ratio was 1:10, whereas split flow was 30.7-ml/ min $^{-1}$. Mass scan range 50 to 500 amu. One µl of sample (dissolved in hexane 100% v/v) was injected into the system. The relative % of each component was calculated by comparing its average peak area to the total area. Software adopted to handle mass spectra and chromatograms was a Turbomass. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST). The name, molecular weight and structure of the components of the test materials were ascertained and compound identification was based on the comparison of retention indices, mass spectra and the NIST spectrometer data bank as well as comparison with literature data.

2.7.2 Phytochemistry

Phytochemical tests

The phytochemical components were screened by using the methods of (Harborne, 1973; Joshi *et al.*, 2011). The phytochemical analyses were solely based on crude extracts obtained above and were conducted as follows:

Detection of Alkaloids

Dragendroff's test: 2 drops of Dragendroff's reagent were added to 1 ml of crude extract. Formation of reddish/orange precipitate indicates the presence of alkaloids.

Detection of Amino acids and proteins

Ninhydrin test: 2 drops of Ninhydrin reagent were added to 2 ml of dilute extract. A characteristic deep brown colour change indicates the absence of amino acids.

Detection of carbohydrates

Bennedict's test: 1 ml of Bennedict's reagent (Solution 1: made up of 173 g sodium citrate and 100 g sodium carbonate in 800 ml water, filtered and made up to 850 ml was added to a second solution composed of 17.3 g copper sulphate in 100 ml distilled water) was added to 1 ml of extract and heated for 2 minutes. No reddish-brown precipitate indicates the absence of carbohydrates.

Molisch's test: 2 ml of crude extract was added with 2 drops of alcoholic solution of α-naphthol. The mixture was shaken well and 1 ml of concentrated sulphuric acid was added along the sides of the test tube and allowed to stand. No violet or purple ring indicates the absence of carbohydrates.

Detection of flavones and flavonones

Aqueous sodium hydroxide: 1.5 ml of extract was added with 1ml of aqueous sodium hydroxide solution. Blue to violet colour indicates the presence of anthocynins while yellow to orange colour indicates the presence of flavones. An orange-crimson colour change indicates the presence of flavanones.

Detection of glycosides, steroids and terpenoids

Ferric chloride and H_2SO_4 test: 2.5 ml of extract was mixed with 1 ml glacial acetic acid. 2 drops of ferric chloride was added followed by the addition of concentrated sulphuric acid such that the acid remained underneath. The presence of glycosides is indicated by the formation of a brown ring on the junction of the 2 layers and a blue-green ring at the upper surface.

Concentrated Sulphuric acid and chloroform test: 5 ml of the crude extract was mixed with 2 ml of chloroform. 3 ml of concentrated sulphuric was then carefully added to the mixture forming distinct layers. The presence of steroids/terpenoids is indicated by the formation of a reddish-brown colour at the interface between the two solutions.

Detection of saponins

Foam test: 5 ml of extract was diluted with distilled water to 20 ml. About 2 ml was then poured in a test tube, for 15 minutes the solution was shaken. The presence of saponins in the crude extract is indicated by the formation of a persistence 2 cm foam layer.

2.8 Analysis of data

All results are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using Graph Pad InStat Software (GraphPad ® Prism version 5.0, San Diego, California, USA). One-way analysis of variance (1- way ANOVA) followed by Tukey-

Kramer multiple comparisons test was used for statistical comparisons of data between groups. A p-value of less than 0.05 was considered as statistically significant ($p < 0.05$).

CHAPTER 3

Results

3.1 Plant material

The fine powdered plant species of *C. asiatica* and *C. roseus* weighed 2490.80 g and 2703.97 g, respectively. The resultant dry weight of each of the crude extract after preparation is shown in Table 3.1.

Table 3.1: Dry weight and percentage yield of the medicinal plant extracts.

Plant species	Resultant dry weight	Percentage yield
C. asiatica	102.36 g	4.11 %
C. roseus	102.38 g	3.79 %

3.2 The effect of the plant extracts on Caco-2 cells

Cell growth and viability Assessment of the anti-cancer potential of extracts from medicinal plants *Centella asiatica* (L.) Urb. and *Catharanthus roseus* [L] G.D involved *in vitro* cytotoxic tests against Caco-2 and human embryonic kidney (HEK293) cell lines. Cell viability was assessed using the MTT assay. *In vitro* effects of various doses of plant extracts (0, 2, 4, 8, 16 mg/ml) were evaluated in isolated HEK293 and Caco-2 cell lines which were exposed to the extracts for 24, 48 and 72 h.

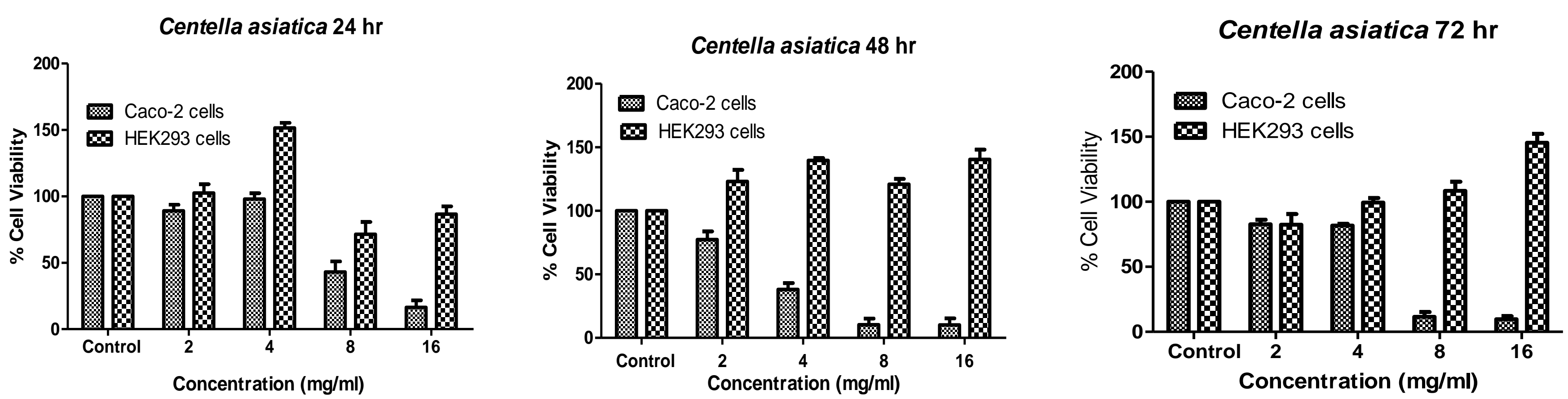

Figure 3.1: The effects of *Centella asiatica* extract on the cell viability of HEK 293 and Caco-2 cells *in vitro* after exposure to various concentrations of extract for 24, 48 and 72 h. Values are presented as means and vertical bars indicate SEM (n = 3).

The percentage cell viability of Caco-2 and HEK293 cell lines treated with *C. asiatica* plant extract is shown in figure 3.1. The cell viability of HEK293 cells first increased at 2 and 4 mg/ml and then decreased at 8 and 16 mg/ml plant extract concentrations after 24 h exposure period when compared to the control. After 48 and 72 h exposure periods, the cell viability increased significantly and it remained above that of the control. *C. asiatica* treatment significantly decreased ($p < 0.05$) the cell viability of Caco-2 cells in a dose dependent manner after 24, 48 and 72 h exposure periods in comparison to the control. Of note is that, the *C. asiatica* plant extract significantly decreased the cell viability of Caco-2 cells at 8 and 16 mg/ml concentrations across all three exposure periods. A slight increase in the cell viability of both caco-2 and HEK293 cell lines is notable after 24 h exposure at 4 mg/ml plant extract concentration when compared to the control.

The percentage cell viability of Caco-2 and HEK293 cell lines treated with *C. roseus* plant extract is shown in figure 3.2 below. The cell viability of HEK293 cells increased at 4 and 8 mg/ml plant extract concentrations and there was a decrease of cell viability at 16 mg/ml plant concentration after 24 h exposure period when compared to the control. Following 48 and 72 h exposure periods, the HEK293 cell viability increased significantly when compared to the Caco-2 cell viability. Of note is that, HEK293 cell viability was below that of the control after 24 and 48 h *C. roseus* plant extract treatment whereas after 72 h it remained above that of the control. On the other hand, the *C. roseus* treatment significantly decreased the cell viability of Caco-2 cells at different plant extract concentrations (2, 4, 8 and 16 mg/ml) after 24, 48 and 72 h in comparison to the control. The Caco-2 cell viability remained below 50% or slightly higher across all exposure periods.

Both extracts did not exhibit toxicity to the normal cell line (HEK293) but they significantly decreased the viability of the cancer cell line (Caco-2). These results paved the way for *in vivo* studies of the anti-cancer effects of *C. asiatica* and *C. roseus* extracts since any use of such extracts in treating colon carcinoma or other forms of cancer might also affect PDG essential for normal enterocyte function.

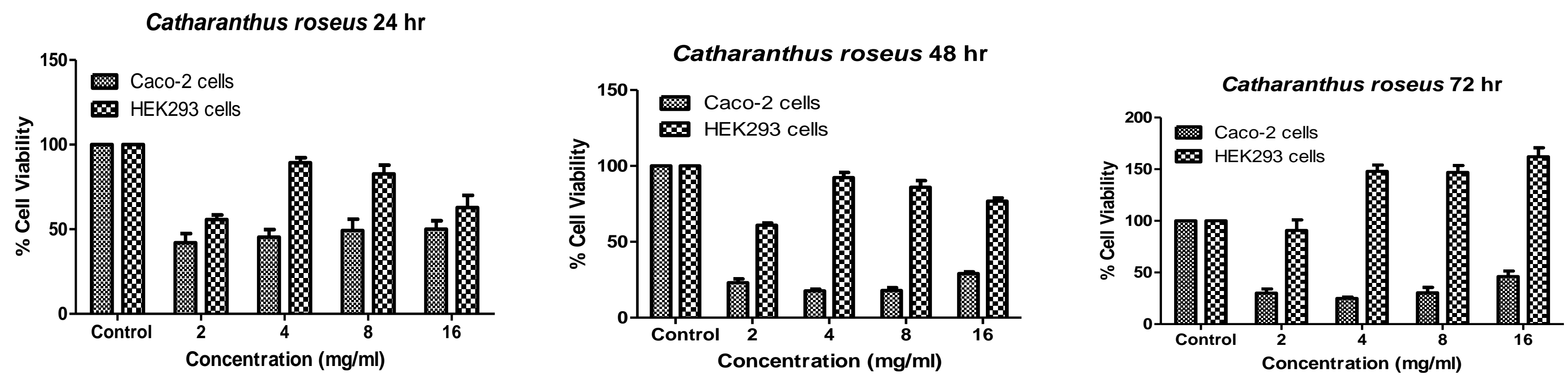

Figure 3.2: The effects of *Catharanthus roseus* extract on the viability and/or metabolic activity of HEK 293 and Caco-2 cells *in vitro* after exposure to various concentrations of extract for 24, 48 and 72 h. Values are presented as means and vertical bars indicate SEM (n = 3).

3.3 Effect of plant extracts on phosphate-dependent glutaminase and alanine aminotransferase in Caco-2 cells

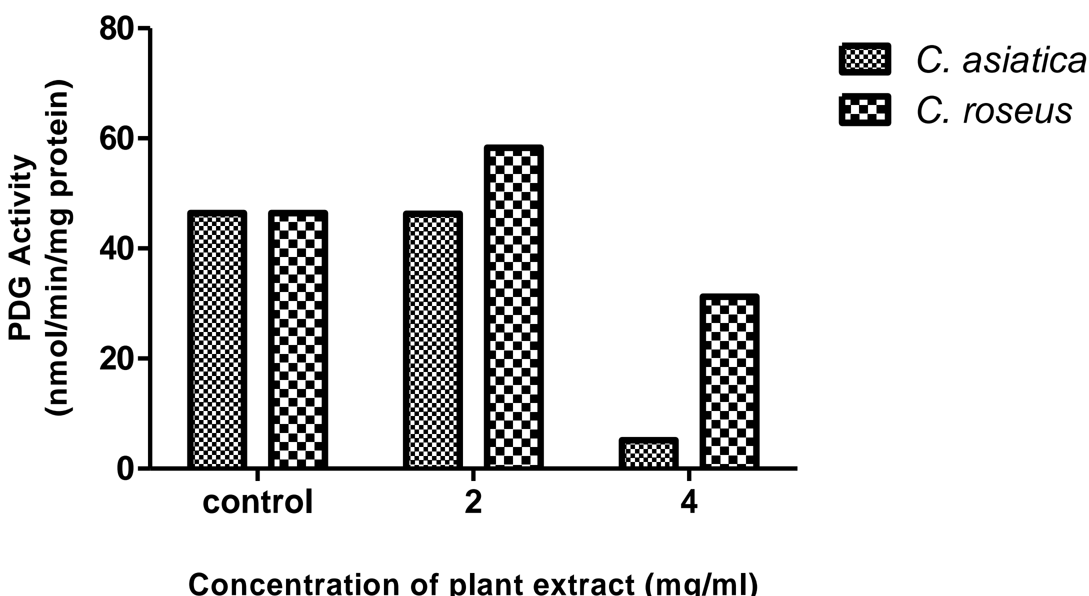

Figure 3.3: Effect of *C. asiatica* and *C. roseus* on phosphate-dependent glutaminase activity in the Caco-2 cell lines at 2 and 4 mg/ml concentrations. Data is represented as means (n = 2).

The PDG activity and related enzymes in the Caco-2 cells were then assessed. The 2 and 4 mg/ml concentration of plant extract were chosen because the % cell viability in these concentrations was greater compared to the subsequent plant extract concentrations. Figure 3.3 above shows that specific activity of phosphate-dependent glutaminase remained the same at 2 mg/ml and then decreased at 4 mg/ml following treatment with *C. asiatica* when compared to the control. Caco-2 cells PDG activity increased at 2 mg/ml after treatment with *C. roseus*. At 4 mg/ml there was a decrease in the PDG activity meaning that both plant extracts decreased PDG activity in Caco-2 cells.

Figure 3.4 below shows the ALT activity of the Caco-2 cells. The untreated Caco-2 cells had an ALT activity of 68 nmol/NADH oxidized/min/mg protein. Treatment with *C. asiatica* resulted in a decrease of ALT activity in a dose-dependent manner at both 2 and 4 mg/ml plant concentrations. On the other hand, the ALT activity of Caco-2 cells increased after treatment with *C. roseus* at mg/ml plant concentration and there was a decrease at 4 mg/ml plant concentration..

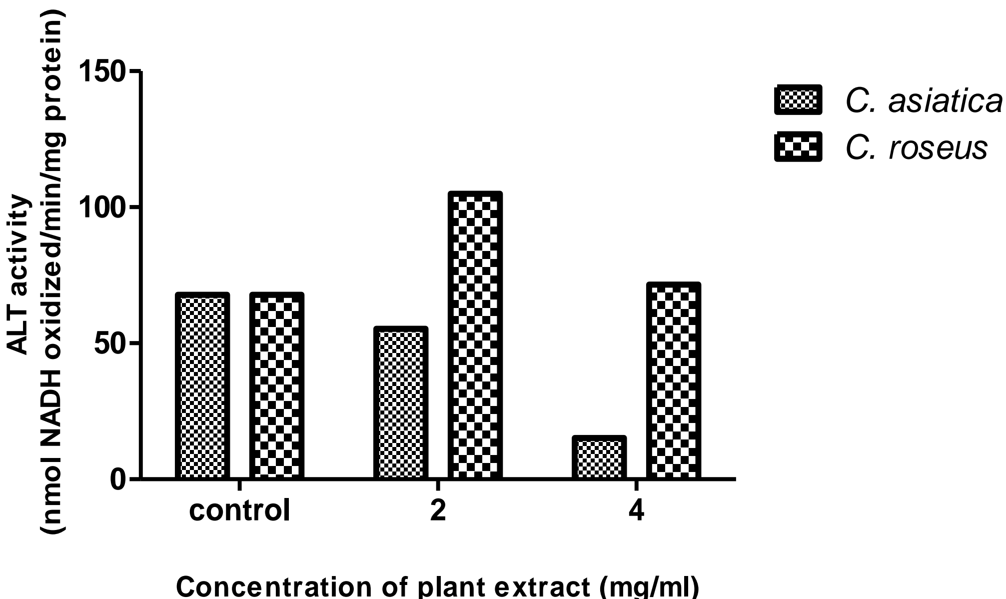

Figure 3.4: Effect of *C. asiatica* and *C. roseus* at 2 and 4 mg/ml plant extract concentrations on ALT activity in the Caco-2 cell line. Data is represented as means (n = 2).

3.4 The effect of plant extracts on enterocytes isolated from rat intestines

Assays on enzymes of glutamine metabolism were then conducted in rat isolated enterocytes. PDG, GDH, ALT and AST assays was performed on the three treatment groups, namely; control, *C. asiatica* and *C. roseus*. The PDG specific activity was lower in both the *C. asiatica* and *C. roseus* treatment groups when compared to the control group. However, the difference was not significant ($p > 0.05$) between all three treatment groups (Figure 3.5). A mean difference of 4.337 and 4.765 was obtained in *C. asiatica* and *C. roseus*, respectively.

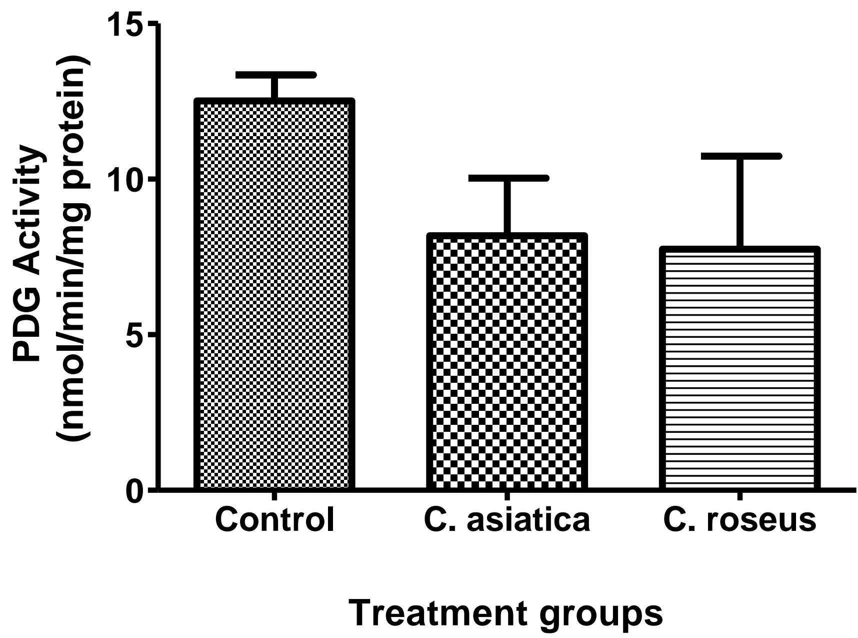

Figure 3.5: Phosphate-dependent glutaminase activities in the enterocytes isolated from rat intestines following treatment with 100 mg/kg *C. asiatica* and *C. roseus.* Values are presented as means and the vertical bars indicate SEM (n = 6).

The GDH, ALT and AST activities in homogenates (enterocytes), pellets and supernatants were also performed as shown in Figure 3.6, Figure 3.7 and Figure 3.8 respectively. There was no significant difference of the GDH activity in homogenates and pellet fractions ($p > 0.05$). In the case of GDH activity in supernatant fraction, a significant difference was observed in the *C. roseus* treatment group ($p > 0.05$) when compared to the control group (Figure 3.6).

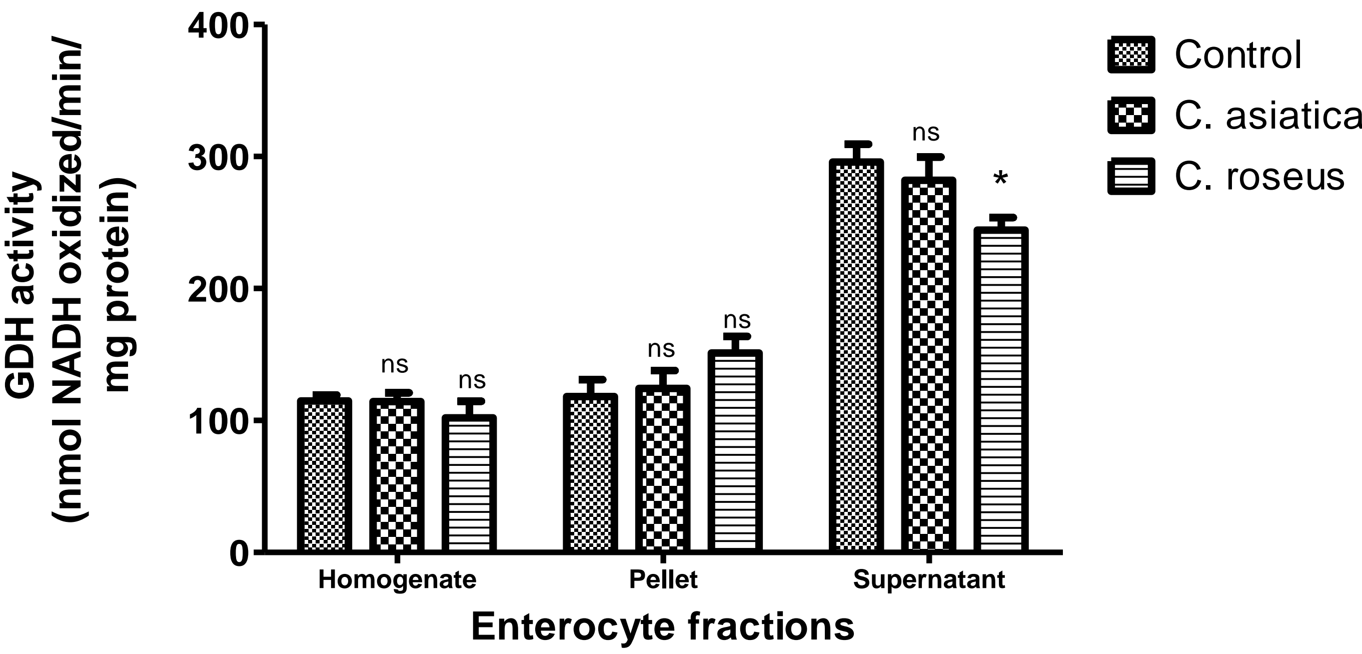

Figure 3.6: Glutamate dehydrogenase (GDH) activity in fractions of enterocytes from Sprague-Dawley rats treated with plant extracts. The fractions were the homogenate, 10 000 *g* pellet (mitochondrial fraction) and 10 000 g supernatant (cytosolic fraction). Each data point is shown as mean ± SEM (n=6). * $p < 0.05$ was considered significant, ns means not significant when activities were compared with the respective controls.

With the exception of supernatant ALT, there were no significant differences ($p > 0.05$) in the ALT and AST activities of homogenates, pellet and supernatants when compared to the untreated group. There was a slight increase in the AST activity of the homogenates (Figure 3.8). Although the differences remained insignificant, there is a decrease in the ALT activity of the homogenates (Figure 3.7). Both the ALT and AST activities revealed reduced levels in the pellets fractions. In the supernatant, the ALT activities show a significant difference ($p > 0.05$) in the *C. asiatica* group when compared to the control. There was a an increase in the AST activity of the supernatant when compared to the control but this was not significant.

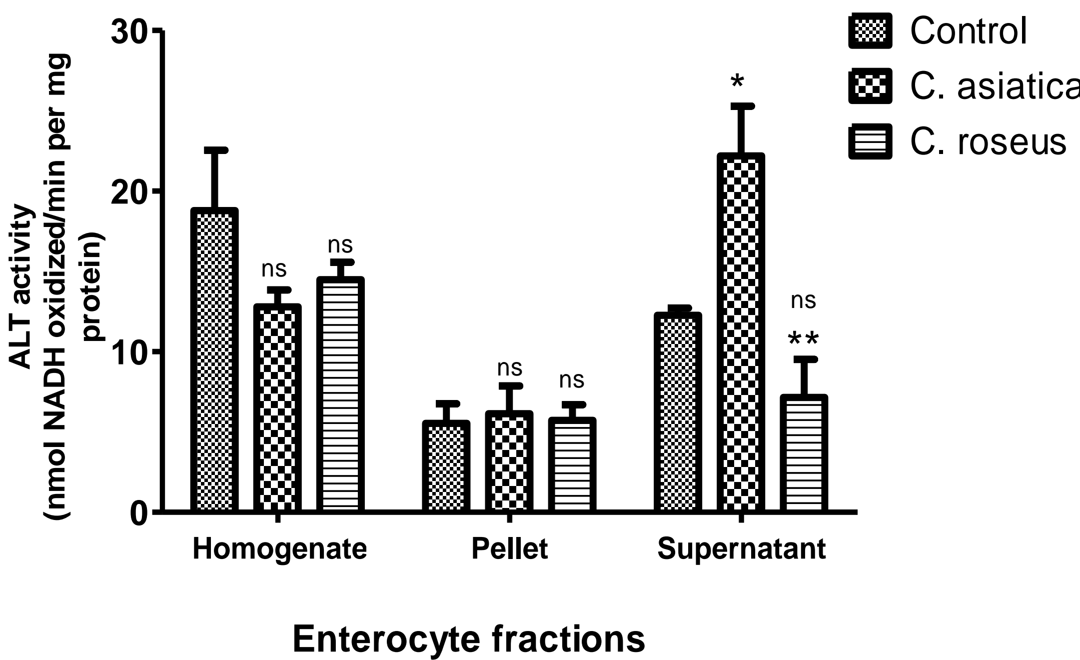

Figure 3.7: Alanine aminotransferase (ALT) activity in enterocytes fractions from Sprague-Dawley rats treated with plant extracts. The fractions were the homogenate, 10 000 *g* pellet (mitochondrial fraction) and 10 000 *g* supernatant (cytosolic fraction). Each data point is

shown as mean ± SEM (n=6). * $p < 0.05$ was considered significant, ns means not significant when activities were compared with the respective controls and ** $p < 0.05$ means significant when *C. asiatica* is compared with *C. roseus*.

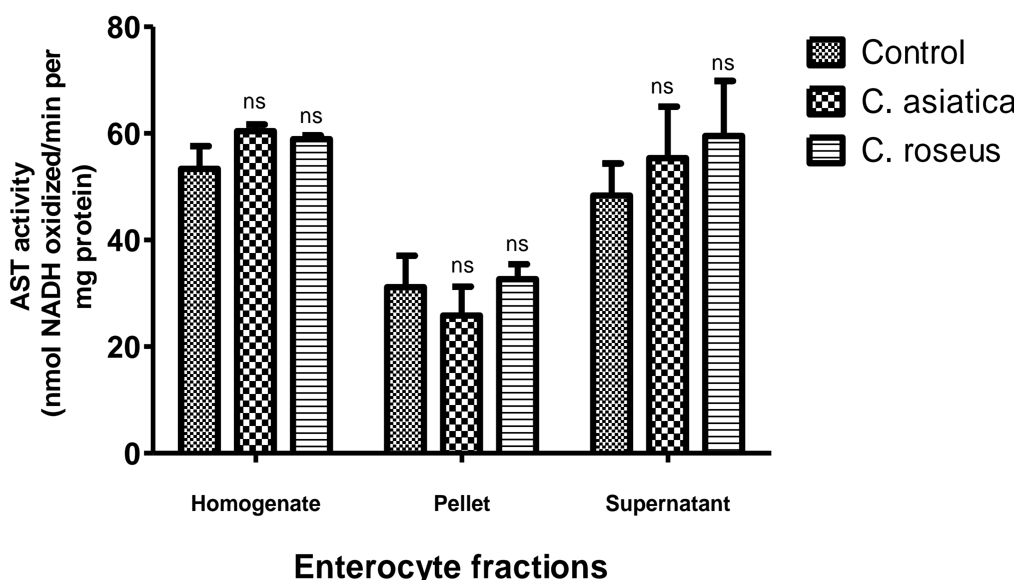

Figure 3.8: Aspartate aminotransferase (AST) activity in enterocytes fractions from Sprague-Dawley rats treated with plant extracts. The fractions were the homogenate, 10 000 *g* pellet (mitochondrial fraction) and 10 000 *g* supernatant (cytosolic fraction). Each data point is shown as mean ± SEM (n= 6). ns means not significant when activities were compared with the respective controls.

3.5 The effect of medicinal plant extracts on the expression of PDG enzyme

3.5.1 SDS-PAGE gels

3.5.1.1 Caco-2 cells

Figure 3.9 below shows Caco-2 cells protein distribution in a Commassie stained 15% polyacrylamide SDS-PAGE gel. The gel shows extremely faint molecular weight proteins in Caco-2 cells which are almost undetectable. Due to the little protein content in the Caco-2 cells, it was then impossible to carry on with the Western blot analysis.

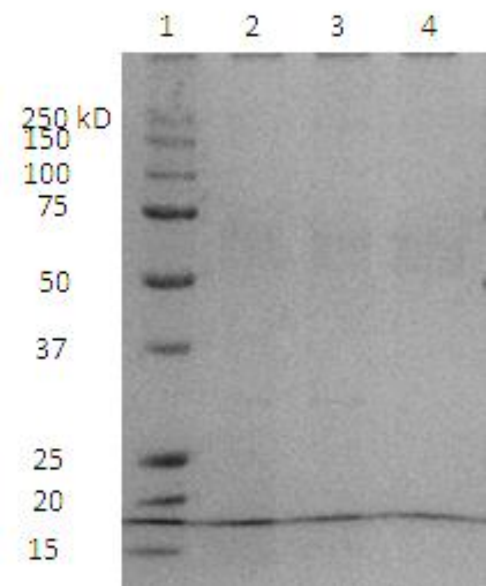

Figure 3.9: Representative Coomassie stained 15% polyacrylamide SDS-PAGE gel of Caco-2 cells. Lane 1: (Molecular weight marker), 2 (Controls), 3 (*C. asiatica*), 4 (*C. roseus*). 5 µg of protein was loaded for each treatment group.

3.5.1.2 Rat intestinal enterocytes

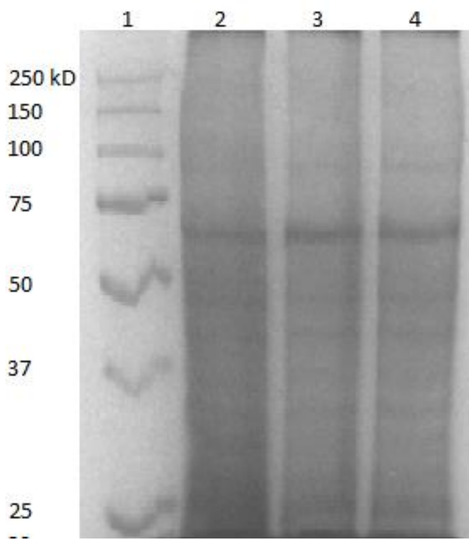

Figure 3.10: Representative Commassie stained 15% polyacrylamide SDS-PAGE gels of rat isolated enterocytes. Lane 1 (Molecular weight marker), 2 (Control), 3 (*C. asiatica*), 4 (*C. roseus*). 50 µg of protein was loaded.

3.5.2 Western Blot

3.5.2.1 Cytochrome c

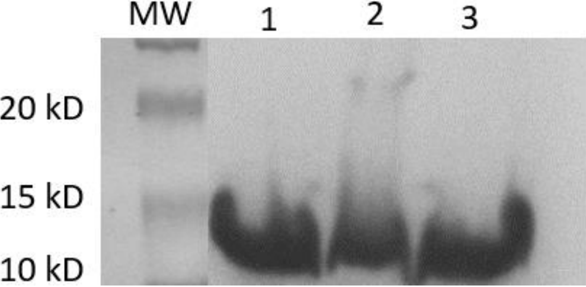

Figure 3.11: Representative Western blot of Cytochrome of rat isolated enterocytes. Lane 1 (Molecular weight marker), 2 (Control), 3 (*C. asiatica*), 4 (*C. roseus*). 100 µg of protein was loaded.

This study attempted to quantify the expression of PDG and AST using western blot since there was adequate protein distribution in the samples of enterocytes (Figure 3.10). The problem in carrying out the Western blots of PDG and AST related to the antibodies which had low cross-activity due to aging and this did not allow successful band formation on the PVDF membrane. Therefore, strips of PVDF membrane were used to carry out dot blots which allow for

concentrated primary antibody loading. On the positive side, the western blot of cytochrome c was successful (Figure 3.11). The bands were skewed which did not allow densitometry analysis to be possible hence the dot blots were conducted instead.

3.5.3 Dot Blots

3.5.3.1 Caco-2 cells

The effects of *C. asiatica* and *C. roseus* on expression of phosphate-dependent glutaminase enzyme in Caco-2 cell lines were not studied due to low protein concentrations.

3.5.3.2 Rat intestinal enterocytes

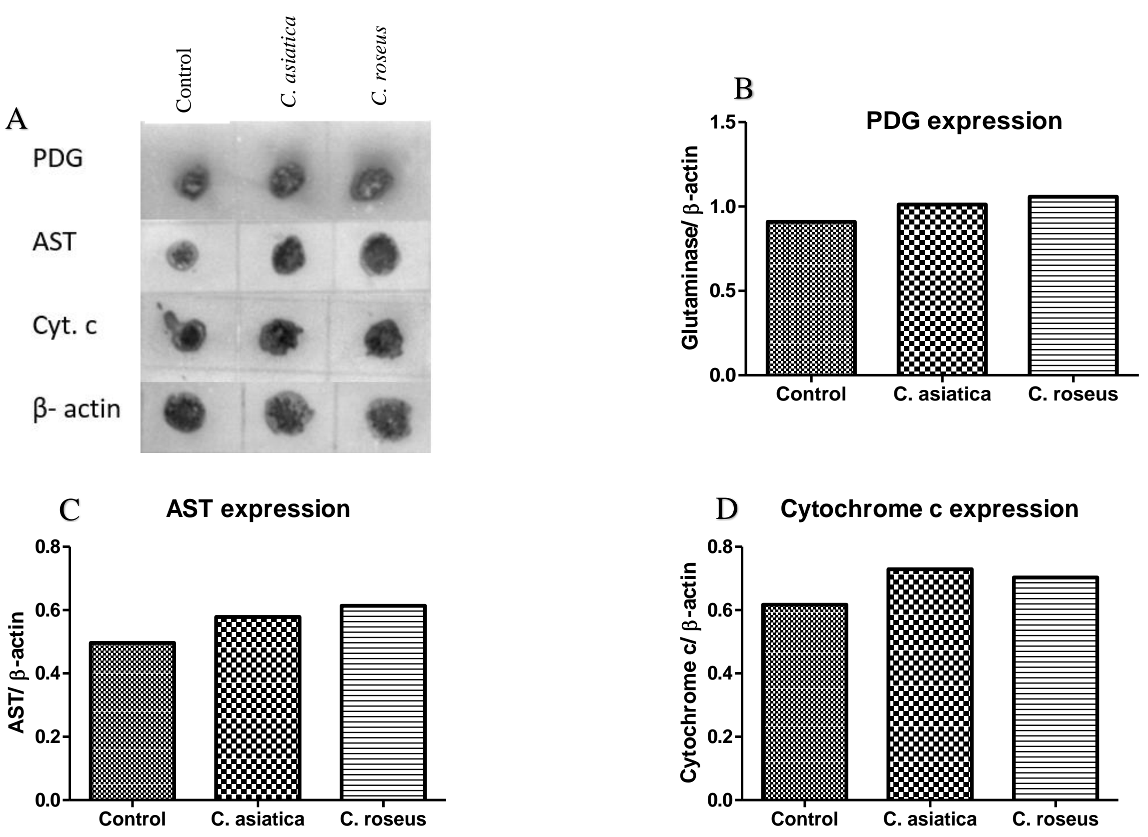

Figure 3.12: Dot blots (A) of PDG, AST and Cytochrome c. β-actin was used as loading control. 15 µg of protein was loaded for PDG, AST and β-actin expression studies. Cytochrome c had 5 µg protein loaded. Densitometry values were obtained and used to show the expression of PDG (B), AST (C) and Cyt. C (D) wherein β-actin was used for normalization.

The study also investigated the effects of medicinal plant extract administration on the expression of PDG and AST enzymes using Dot blot analysis. Induction of apoptosis was also investigated using cytochrome c. We used β-actin, a house keeping gene, to normalize the expression of the above enzymes. Figure 3.12 intensity of the PDG and AST expression in the *C. asiatica* and *C. roseus* treated groups increased when compared to the untreated group (indicated in Graph B and C). In the Cytochrome c (indicated in graph D) expressions, there was a marginal increase in intensities in both the *C. asiatica* and *C. roseus* treated groups but these increase was not different when compared to respective control.

3.6 Phytochemical screening

3.6.1 Phytochemistry

Table 3.2 below represents the preliminary phytochemical screening of *C. asiatica* and *C. roseus* methanolic extracts analysed qualitatively. The phytochemical tests showed the presence of the terpenoids, flavones, essential fatty oils, alkaloids, glycosides, saponins and carbohydrates in the *C. asiatica* plant extract. The alkaloids, amino acids and proteins, carbohydrates, flavones, glycosides, terpenoids and saponins were all detected in *C. roseus* plant extract. Flavones and saponins were not present in *C. asiatica* extract as negative reactions resulted.

Table 3.2: *C. asiatica* and *C. roseus* methanolic leaf extracts preliminary phytochemical constituents.

Phytochemical test	Target compounds	Plant extracts	
		C. asiatica	***C. roseus***
Control			
Dragendroff's	Alkaloids	+	+
Ninhydrin	Amino acids	+	+
Bennedict's and Molisch's	Carbohydrates and proteins	+	+
Aqueous sodium hydroxide	Flavones	-	+
Ferric chloride and H_2SO_4 **H_2SO_4 and Chloroform**	Glycosides Steroids/terpenoids	+ +	+ +
Foam test	Saponins	-	+

Key: + present, - absent

3.6.2 GC-MS

Profiling of *C. asiatica* and *C. roseus* extracts was performed by utilizing GC-MS. This analysis indicated the presence of sugars, carotenoids, anti-oxidants, saponins, antibacterial, terpenes and anti-tumour compounds in both the plant extracts as shown in Table 3. 3 (a and b). The chromatography and mass spectra with identified compounds structures are found in the appendices (Few compounds structures are not clear due to a problem encounted with the printer). A total of 33 phytocomponents were detected in *C. asiatica* and 36 phytocomponents in *C. roseus*. Table 3.4 presents the identified anticancer phytocomponents that have not been found in both *C. asiatica* and *C. roseus* leaf extracts but are known to have anticancer properties. These are marked with asterisks in Table 3.3 (a and b).

Table 3.3 a: Phytocomponents detected in *C. asiatica* leaf extract by GC-MS.

SI No.	Retention Time	Compound name	Formula	Peak area%	Molecular weight
1	5.41	R-Limonene	$C_{10}H_{16}O_3$	0.80	184
2	5.33	Thiofanox	$C_9H_{18}N_2O_2S$	0.38	218
3	4.93	Deoxyspergualin	$C_{17}H_{37}N_7O_3$	0.52	387
4	4.93	Bemegride methyl derivative	$C_9H_{15}NO_2$	0.43	169
5	4.85	Betaxolol	$C_{18}H_{29}NO_3$	0.33	307
6	4.44	Metoprolol	$C_{15}H_{25}NO_3$	0.11	267
7	4.44	*Iberin	$C_5H_9NOS_2$	0.15	163
8	5.33	*Chlorozotocin	$C_9H_{16}ClN_3O_7$	0.25	313
9	4.44	Dihyroxyacetone	$C_3H_6O_3$	32.4	90
10	5.67	*Desulphosinigrin	$C_{10}H_{17}NO_6S$	9.91	279
11	5.65	3.4 Altrosan	$C_6H_{10}O_5$	17.1	162
12	3.86	Aprobarbital	$C_{10}H_{14}N_2O_3$	0.32	210
13	3.86	6-Thioguanosine	$C_{10}H_{13}N_5O_4S$	0.77	299
14	6.25	*Showdomycin	$C_9H_{11}NO_6$	0.21	229
15	8.64	p-Cymen-7-ol	$C_{10}H_{14}O$	1.80	150
16	3.25	Nonanoic acid	$C_9H_{18}O_2$	0.55	158
17	3.25	Undecanoic acid	$C_{11}H_{22}O_2$	4.85	186
18	3.25	Diphenylamine	$C_{12}H_{11}N$	1.40	169
19	3.25	Pheniramine	$C_{16}H_{20}N_2$	15.8	240
20		1-Penten-3-one	C_5H_8O	30.7	84
21	14.74	Phytol	C20H40O	17.58	296
22	14.52	Lysergic acid	$C_{16}H_{16}N_2O_2$	20.03	268

23	3.90	Formic acid, 2-methylhex-3-yl ester	$C_8H_{16}O_2$	7.57	144
24		Pyrrolizidine-3-one-5-ol, ethyl ether	$C_9H_{15}NO_2$	9.99	169
25		Isosorbide dinitrate	$C_6H_8N_2O_8$	24.2	236
26	8.42	Meprobamate	$C_9H_{18}N_2O_4$	0.39	218
27	6.49	Ropivacaine	$C_{17}H_{26}N_2O$	1.40	274
28	6.56	Guanethidine	$C_{10}H_{22}N_4$	0.81	198
29	6.49	Aceclidine	$C_9H_{15}NO_2$	1.09	169
30	8.64	Thymol	$C_{10}H_{14}O$	1.16	150
31	7.52	β- Eucine	$C_{15}H_{21}NO_2$	6.56	247
32	5.3	*Chlorozotocin	$C_9H_{16}ClN_3O_7$	0.59	314
33	8.64	Ascaridole	$C_{10}H_{16}O_2$	1.29	168

* Are anticancer compounds detected.

Table 3.3 b: Phytocomponents detected in *C. roseus* leaf extract by GC-MS.

SI No.	Retention Time	Compound name	Formula	Peak area %	Molecular weight
1	3.07	Pheniramine	$C_{16}H_{20}N_2$	27.1	240
2	3.07	Diphenylamine	$C_{12}H_{11}N$	5.97	169
3	3.05	2-p-Tolylpyridine	$C_{12}H_{11}N$	1.34	169
4	3.67	Norpheniramine acetate	$C_{17}H_{20}N_2O$	0.25	268
5	3.67	*Hydrazinecarboxamide, N,N-diphenyl-	$C_{13}H_{13}N_3O$	3.99	227
6	3.19	Glycyl-l-glutamine	$C_3H_{13}N_3O_4$	0.73	203
7	3.22	Cyclopentobarbital	$C_{12}H_{14}N_2O_3$	0.35	234
8	3.24	(S)-(+)-2-Amino-3-methyl-1-butanol	$C_5H_{13}NO$	6.48	103

9	4.34	Secobarbital	$C_{12}H_{18}N_2O_3$	0.29	238
10	7.69	Valeric acid, 2-tetradecyl ester	$C_{19}H_{38}O_2$	5.08	298
11	7.69	Tetrahydropyranyl ether of citronellol	$C_{15}H_{28}O_2$	1.03	240
12	7.82	Maltol	$C_6H_6O_3$	0.09	126
13	7.82	5-Hydroxymethylfurfural	$C_6H_6O_3$	27.9	126
14	7.82	Spiro[cyclohexanol-3-2´-hexahydropyridine]	$C_{10}H_{19}NO$	4.12	169
15	7.98	3´-Hydroxyquinalbarbitone	$C_{12}H_{18}N_2O_4$	23.3	254
16	7.98	Talbutal	$C_{11}H_{16}N_2O_3$	0.43	224
17	7.98	Kainic acid	$C_{10}H_{15}NO_4$	0.27	213
18	7.98	Thiamylal	$C_{12}H_{18}N_2O_2S$	0.26	254
19	7.98	Butabital	$C_{11}H_{16}N_2O_3$	0.40	224
20	8.60	Pyrrolizidine-3-one-5-ol, ethyl ether	$C_9H_{15}NO_2$	13.6	169
21	8.49	Oleic acid	$C_{18}H_{34}O_2$	1.17	282
22	10.30	Nonanoic acid	$C_9H_{18}O_2$	0.50	158
23	8.60	p-Cymen-7-ol	$C_{10}H_{14}N$	1.04	150
24	8.65	D-Verbenone	$C_{10}H_{14}O$	0.92	150
25	8.65	Thymol	$C_{10}H_{14}O$	1.63	150
26	9.80	9-[2-Deoxy-β-d-ribohexopyranosyl]purin-6-(1H)-one	$C_{11}H_{14}N_4O_5$	25.0	282
27	9.80	*Psicofuranine	$C_{11}H_{15}N_5O_5$	0.11	297
28	9.80	Albuterol	$C_{13}H_{21}NO_3$	0.12	239

29	10.56	*Desulphosinigrin	$C_{10}H_{17}NO_6S$	4.10	279
30	10.22	Solasonine	$C_{45}H_{73}NO_{16}$	0.66	883
31	10.30	Stevioside	$C_{38}H_{60}O_{18}$	3.79	804
32	10.30	Curan-17-oic acid, 19,20-dihydroxy-, methyl ester, (19S)	$C_{20}H_{26}N_2O_4$	0.39	358
33	10.30	Erucic acid	$C_{22}H_{42}O_2$	0.25	338
34	10.30	β-Sedoheptitol	$C_7H_{16}O_7$	0.58	212
35	10.30	Thiofanox	$C_9H_{18}N_2O_2S$	0.49	218
36	10.30	3.4 Altrosan	$C_6H_{10}O_5$	0.49	162

* Are anticancer compounds detected.

Table 3.4: Anticancer compounds detected by GC-MS but not previously identified in *C. asiatica* and *C. roseus* extracts.

C. asiatica	***C. roseus***
Iberin	Showdomycin
Chlorozotocin	Hydrazinecarboxamide, N,N-diphenyl- (a semicarbazide)
Desulphosinigrin	Psicofuranine

CHAPTER 4

Discussion

The aim of the current project was to investigate the effects of *C. roseus* and *C. asiatica* leaf extracts on enzymes of glutamine catabolism in Caco-2 cell line and in enterocytes from male Sprague-Dawley rats. The study first investigated the effects of the medicinal plant leaf extracts on cell growth of the Caco-2 cells in order to stablish whether they exhibit any cytotoxicity towards cancer cells. Secondly, the effects of *C. roseus* and *C. asiatica* leaf extracts on enzymes of glutamine catabolism were also assessed in Caco-2 cells and enterocytes from rat intestines. Furthermore, this project reports on some phytochemical analysis of both *C. roseus* and *C. asiatica* medicinal plant leaf extracts using preliminary phytochemical screening and GC-MS to detect the presence of active compounds making these plant species widely utilized as anticancer agents. GC-MS identified the compounds that may be responsible for changes in cell growth as well as changes in enzymes that could underpin the alterations in cell growth.

The MTT assay results obtained from this present study indicate that the cytotoxicity effects of *C. asiatica* leaf extract to Caco-2 cell line was dose-dependent (Figure 3.1) whilst that of *C. roseus* was not dose dependent (Figure 3.2) as indicated by cell viability. Both the leaf extracts have anticancer potential since they did not exhibit toxicity to the normal cell line (HEK293) but they significantly decreased the viability of the cancer cell line (Caco-2). Previous studies reported that cell cycle arrest was induced in Caco-2 cells following treatment with *C. asiatica* (Bunpo *et al.*, 2010). It was also reported that the proliferation rate of the Caco-2 cells was significantly reduced (Bunpo *et al.*, 2010) in a manner similar to that reported in this present study in which the reduction in growth is concentration and time dependent (Figure 3.1). El-Sayed and Cordell also isolated a new antitumor alkaloid, catharanthamine, from *C. roseus* and they found that this novel alkaloid was cytotoxic in the KB test system *in vitro* and displayed significant activity in the P-388 lymphocytic leukemia test system (El-Sayed and Cordell, 1981). The ideal anticancer therapy is the one that targets the proliferative cancerous cells and leaves the normal cells unaffected following therapy. The HEK293 cell line was used in this study as a control since it is derived from normal cells. This cell line is ideal and suitable for the investigation since the enzyme of interest is also found in kidneys. Treatment with *C. asiatica* and *C. roseus* at different plant extract

concentrations in different exposure times left the HEK293 cells unaffected and their viability was stimulated.

Cancer cells are consumers of glutamine in culture and they require it as an energy source to maintain their proliferative abilities (Rajagopalan and DeBerardinis, 2011). Deamination of glutamine to glutamate catalysed by PDG, an enzyme found in mitochondria, play an important role in providing energy to highly proliferative cells including the enterocytes (Windmueller and Spaeth, 1974). It has been reported that cancer cells require a great deal of energy in the form of glutamine and hence there is high PDG activity observed (Rajagopalan and DeBerardinis, 2011). In this study, we hypothesized that treatment with the extracts would arrest the growth of Caco-2 cells by inhibiting PDG activity but have less deleterious effects on normal cells. The results in this study show that the activity of PDG was decreased following treatment with plant extracts especially at 4 mg/ml (Figure 3.3). The *C. roseus* and *C. asiatica* plant extracts inhibited PDG significantly hence depriving the Caco-2 cells of an energy source hence the reduced viability of the Caco-2 cells. Previous studies have demonstrated that one of the effective means to treat cancer is to target their metabolic substrates (Souba, 1993; Medina, 2001). In this case, we focused our investigations on a mitochondrial enzyme (PDG) that initiates glutamine oxidation. It is also reported that cancer cells rely on mitochondrial respiration to produce ATP, hence mitochondrial enzymes generally play a vital role in cancer cell metabolism (Suzannec Klimberg and McClellan, 1996; Munoz-Pinedo *et al.*, 2012).

When comparing the effects of the extracts on PDG activity in the Caco-2 cell line with the enterocytes, the same effects of the extracts were observed wherein there was a decrease in the PDG activity of the enterocytes from treated animals compared to those from the normal animals (Figure 3.5). However, the drawback in comparisons is that in the *in vivo* studies, the concentration of the plant extract to which the enterocytes are exposed as a result to of the 100 mg/kg oral dose is not known unlike that for Caco-2 cells. If the extracts concentrations are similar, then the comparisons might show which is affected more because this is important in therapy. The PDG activity in Caco-2 cells was 4-fold greater than the PDG activity in the enterocytes in the control groups and 3-6 fold in the extracts treated groups. This also reinforces the finding that Caco-2 cells rely on glutamine for cellular metabolism to maintain their proliferative state (Clark *et al.*, 2003).

The ALT and AST levels have been widely used as biomarkers of cellular damage (Sherman, 1991; Mukorah *et al.*, 1998; Masola *et al.*, 2003) and are more important in the glucose and amino acids metabolism. Hernaez and colleagues evaluated the relationship between liver enzymes, particularly the ALT and AST, and cancer mortality. They found that these enzyme levels were highly elevated in serum in Taiwanese males indicating a positive association or increased cancer risk (Hernaez *et al.*, 2013). In the *in vitro* studies, an increase in ALT activity was seen at 2 mg/ml following treatment with the *C. roseus* which was reduced to control levels by treatment with 4 mg/ml *C. roseus* (Figure 3.4). The observed initial increase in ALT activity in Caco-2 cells is expected due to increased energy demands as a result of treatment when cell viability is still at least 50 % or above at extract concentrations of 2 mg/ml. There was a decrease in ALT activity at both concentrations (2 and 4 mg/ml) *C. asiatica* treated Caco-2 cells (Figure 3.4). The observed main trend was basically a reduction in the ALT activity for both *C. asiatica* and *C. roseus* treated Caco-2 cells. The observed trend actually correlates with the reduction in PDG activity which also occurs at 4 mg/ml (Figure 3.3). Reduction in PDG activity means that there is no substrate coming through to the downstream enzymes. Since ALT is a downstream enzyme and is involved in the energy generation, the current *in vitro* study focused mainly on this specific enzyme and not the AST and GDH. In the intestines, alanine is released as the main amino acid product of glutamine oxidation, therefore, it was important to investigate this transamination reaction. However, with hindsight, AST and GDH should also have been investigated but cell material we had to work with was limited.

The effect of plant extracts on downstream enzymes of glutamine catabolism in enterocytes from rat intestines was assessed particularly GDH, ALT and AST. The changes in GDH activities were not significant (Figure 3.6) and there was a decrease in ALT activity (Figure 3.7) whilst the AST saw a slight increase (Figure 3.8) in the homogenates following treatment with both plant extracts. These are the downstream enzymes of glutamine metabolism and are involved providing substrates for the TCA cycle in energy generation. As the enterocytes were treated with the plant extract, a stress occurred resulting in increased energy demands hence the reduced levels of ALT. AST is mainly involved in generating aspartate for nucleotide synthesis that is why this particular downstream enzyme was slightly activated in enterocytes.

The preliminary phytochemical tests revealed the presence of different plant chemical constituents in both *C. asiatica* and *C. roseus* methanolic extracts, as shown in Table 3.2, which were also shown in previous studies (Rahman *et al.*, 2012; Mohan *et al.*, 2015). Chemical profiling of both the methanolic *C. asiatica* and *C. roseus* plant extracts by GC-MS indicated the presence of phytocomponents such organic acids, alcohols, amino compounds, sugars and monoterpenoids and these were also previously reported (Apichartsrangkoon *et al.*, 2009; Sudha *et al.*, 2011; Mohan *et al.*, 2015). Thirty three and thirty six compounds of leaf extracts of *C. asiatica* and *C. roseus*, respectively, have been identified. Both plant species, since prehistoric times, they have been widely used for their therapeutic properties.

The identified phytochemicals in *C. asiatica* include showdomycin and altrosan which are antibiotics that inhibits bacterial growth (bacteriostat). Previous studies have investigated the antibacterial activities of this plant species (Brinkhaus *et al.*, 2000; Oyedeji and Afolayan, 2005; James and Dubery, 2009). *C. asiatica* has also been found to have sedative, anticonvulsant, anxiolytic and hypnotic properties (Inamdar *et al.*, 1996; Brinkhaus *et al.*, 2000). Aprobarbital which is an anticonvulsant, sedative and hypnotic drug, a lysergenic acid, an alkaloid used as psychedelic drug that improves consciousness and promotes physical and mental healing as well as meprobamate which is an anxiolytic drug have been identified in this present study. *C. asiatica* is regarded as plant with an ability to improve memory (Nalini *et al.*, 1992; Heidari *et al.*, 2012). Metoprolol and isosorbide dinitrate have been used to treat chest pain and angina. These were also detected in this *C. asiatica* plant extract.

Anticancer properties of this plant species are well documented (Babu *et al.*, 1995; Lee *et al.*, 2002; Bunpo *et al.*, 2004; James and Dubery, 2009; Heidari *et al.*, 2012). The current study detected the presence of iberin (induces apoptosis in cancer cell lines, promotes expression of antioxidant and detoxification enzymes), chlorozotocin (used in cancer therapy) and desulphosinigrin (anti-cancer drug). Desulphosinigrin, in particular has been reported to be a new anticancer drug target (Krishnaveni, 2015). Furthermore, many more phytochemicals were detected by GC-MS including the following; R-limonene (a cyclic terpene), aceclidine and betaxolol used for narrow angle glaucoma and hypertension treatment). 6- Thioguanosine (leukemias treatment drug and ulcerative colitis), nonanoic acid (a carboxylic acid used for treatment of seizures), pheniramine

(anticholinergic, treats allergic conditions). Phytol, ascaridole and thymol are acyclic diterpene used in manufacturing of vitamin E and K1, bicyclic monoterpene and monoterpene respectively. Also, β-eucaine and ropivacaine (anaesthetic drugs), p-cymene also known as cuminol (essential oil, powerful antioxidant) (Rahman *et al.*, 2012) and guanethidine (antihypertensive drug) were detected (Table 3.2.1a).

On the other hand, the *C. roseus* has been reported to exhibit hypotensive and tranquilizing properties (Nayak and Pereira, 2006; Agarwal *et al.*, 2011; Gajalakshmi *et al.*, 2013). The current project GC-MS profiling saw the presence of cyclopentobarbital, 3'-Hydroquinalbarbitone, thiamylal and secobarbirtal which are drugs having anticonvulsant, anxiolytic anaesthetic and sedative properties, hence showing this plant's pharmacological properties. *C. roseus* has also received attention due to its ability to treat and prevent cancer as well as its hypotensive abilities (Farnsworth *et al.*, 1967; Gajalakshmi *et al.*, 2013). We found the presence of pheniramine, hydrazinecarboxymide,N,N-diphenyl- a semicarbazide (antineoplastic, anti-infective and antiviral), psicofuramine (antibiotic and antitumor drug) (Evans and Gray, 1959; Slechta, 1960) which may be responsible for the plant being used in cancer treatment. Amongst other world-wide problems is the occurrence of parasitic worms that feed on the host thereby causing intracellular damage to the host (Fumagalli *et al.*, 2010). The kainic acid, an antiworming agent (anthelmintic) as well as a neuroexcitatory amino acid have been detected in the current phytocomponent analysis. The mechanism of action is likely to be targeting the parasitic worm without causing damage to the host.

Other phytocomponents detected in methanolic extracts of *C. roseus* with healing abilities include: glycyl-glutamine, gly-gln (inhibits cardiovascular and respiratory depression)(Cavun *et al.*, 2005), 5-hydromethylfurfural (sickle cell disease treatment), talbutal (schedule 111 drug, induce anesthesia), butalbital (headache and pain treatment when combined with other medications such as paracetamol), pyrrolizidine-3-one-5-ol, ethyl ether, nonanoic acid (a carboxylic acid used for treatment of seizures), p-cymen7-ol also known as cuminol (essential oil, antioxidant), verbenone (naturally plant occurring terpene), thymol (natural monoterpene phenol, cymene and carvacrol derivative), albuterol also known as Ventolin (bronchodilator, treat wheezing and shortness of breath), solasonine (antiproliferative) (Munari *et al.*, 2014). Stevioside is documented to have a

wide range of curative properties as a natural sweetener and has antihyperglycaemic, antihypertension and antitumor effects (Lee *et al.*, 2001; Jeppesen *et al.*, 2002; Gregersen *et al.*, 2004; Paul *et al.*, 2012). Lastly, tetrahydropyranyl ether of citronellol a monoterpenoid, used in perfumes and as insect repellent as well as maltol (flavour enhancer) were also detected by GC-MS analysis.

Of particular interest was the presence of phytocomponents that are known to be anticancer compounds but have not been previously detected in these plant species and are known to be present in other plant species. These phytocomponents are iberin, chlorozotocin and desulphosinigrin in *C. asiatica* as well as showdomycin, hydrazinecarboxymide, N,N-diphenyl- and psicofuramine in *C. roseus* which are anticancer compounds (Table 3.4). Desulphosinigrin was found to be present in both the extracts. Perhaps these anticancer compounds together with the other compounds may be contributing to the anticancer properties of these particular plant species. With regards to this study, these phytocomponents may be causing changes in Caco-2 cell growth and changes in enzymes of glutamine catabolism.

Previous studies have shown that iberin induced cell cycle arrest of some cancer cell lines (Jadhav *et al.*, 2007) and was also found in vegetables of the family Brassicaceae (Munday and Munday, 2004). Chlorozotocin, a nitrosourea and cytostatic agent, that was long discovered and used as an antitumor agent (Anderson *et al.*, 1975; Schein *et al.*, 1976) has been widely utilized in the treatment of cancers particularly of the stomach, large intestine, pancreas and lung (Kannan Elangovan *et al.*, 2014). Kannan and colleagues also investigated the presence of bioactive compounds in the methanolic seed extract of *Memecylon umbellatum* plant species and their GC-MS analysis revealed the presence of chlorozotocin (Kannan Elangovan *et al.*, 2014). The current project showed presence of desulphosinigrin, which has been documented to be found in *Soymida febrifuga* seeds amongst other plant species and is said to be an anticancer target (Krishnaveni, 2015).

Previous *in vitro* cytotoxicity studies revealed that hydrazinecarboxamide, a semicarbazide, had antitumor properties when it was synthesized and evaluated against human solid tumor growths including human non-small cell lung cancer (H1299), lung adenocarcinoma (CL 1-0 and CL 1-5),

prostate cancer (PC-3) and resistant breast cancer (MCF-7) cell growth in vitro (Kakadiya *et al.*, 2010). Although this was synthesized it was also detected in the GC-MS analysis of *C. roseus.* Lasty, the showdomycin and psicofuramine also act as antitumor agents and prevent the growth of L1210 leukemia, HeLa cells and Ehrlich ascites tumor cells (Evans and Gray, 1959; Slechta, 1960).

CHAPTER 5

Conclusion

- Firstly, in *in vitro* studies, cytotoxicity has indicated that *C. asiatica* and *C. roseus* plant extracts inhibited the growth of Caco-2 cells and left the normal cells unaffected. This is the desirable property of these extracts.
- The extracts inhibit the key enzyme that initiate glutamine oxidation (PDG) in Caco-2 cells. This could be the mechanism for the anticancer properties of these extracts.
- The extracts also affects downstream enzymes since there was a decrease in PDG in Caco-2 cells. In normal cells the enzyme PDG was also decreased but there were no drastic changes in the downstream enzymes.
- Great number of compounds were found, as well as several anticancer compounds were detected. However of particular interest are these particular anticancer compounds which have not been previously detected in these plant species namely; iberin, chlorozotocin and desulphosinigrin in *C. asiatica* as well as showdomycin, hydrazinecarboxymide, N,N-diphenyl- and psicofuramine in *C. roseus.*
- We conclude that these plant extracts have anticancer potential but there is need for further studies.
- This study lends scientific credence and validity to the development of new drugs sources from plants for treatment of cancer in humans and reveals the medicinal benefits of consuming the herbal medicines.

Future studies:

Potential future studies may focus on the concentration of the identified anticancer compounds in the intestinal fluid which will give an indication or level in which the extract reaches the intestine.

Also, to measure the final extracts concentration the enterocytes will be exposed to following dosage so that there is a proper comparison with the cultured cells which are the first line of looking at the effects on the enzymes. If the enterocytes are affected less, this will be a breakthrough in cancer treatment given that a critical enzyme, PDG to look at.

It would be ideal to further test the effects of the *C. asiatica* and *C. roseus* in animal that are bearing a cancer status.

CHAPTER 6

Appendices

Appendix 1: Ethical Clearance

12 February 2014

Reference: 055/14/Animal

Miss T Dladla
Biochemistry
School of Life Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Miss Dladla

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2014** on the following project:

"Effects of medicinal plant derived extracts on phosphate-dependent glutaminase (PDG) and related enzymes in the human colon carcinoma (Caco-2) cell line and enterocytes isolated from rats."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar – Mr C Baloyi
Research Office – Dr N Singh
Supervisor – Dr B Masola
Head of School – Prof. S Mukaratirwa
BRU – Dr S Singh

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
Telephone: +27 (0)33 260 5463/35 **Facsimile**: +27 (0)33 260 5105 **Email**: animalethics@ukzn.ac.za **Website**: www.ukzn.ac.za

Founding Campuses: Edgewood, Howard College, Medical School, Pietermaritzburg, Westville

INSPIRING GREATNESS

Appendix 2: School of Life Sciences Poster Presentation

ANTI-CANCER POTENTIAL OF MEDICINAL PLANT EXTRACTS IN THE HUMAN COLON CARCINOMA (CACO-2) CELL LINE

Thobekile P. Dladla[1], Dr. Moganavelli Singh[1] and Dr. Bubuya Masola[1]

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Email Address: 207503282@stu.ukzn.ac.za

INTRODUCTION

Medicinal plant extracts are known to contain compounds that possess anti-tumour activity and act by hindering the activation of cell cycle inducing cell death. Tumor cells utilize large quantities of glutamine (major respiratory fuel for rapidly dividing cells) and compete with the host for circulating glutamine. Phosphate-dependent glutaminase (PDG) enzyme has become a target of anti-cancer drug development.

AIM

To investigate the anti-cancer potential of medicinal plant extracts targeting PDG in human colon carcinoma (Caco-2) cell line.

An initial evaluation anti-cancer potential of extracts from medicinal plants *Centella asiatica* (L.) Urb. and *Catharanthus roseus [L] G.Don* involved *in vitro* cytotoxic effects against Caco-2 and human embryonic kidney (HEK293) cell lines.

Figure 1: Photographs of *C. asiatica* (A) and *C. roseus* (B)

METHODS AND MATERIALS

Freshly harvested leaves of *C. asiatica* and *C. roseus* were collected and authenticated by Prof A. Nicholas at the University of KwaZulu-Natal (UKZN), Durban, South Africa. A voucher specimen of the plant was deposited in the Ward Herbarium of UKZN.

Cell viability was assessed using the MTT assay. *In vitro* effects of various doses of plant extracts (0, 2, 4, 8, 16 mg/ml) were evaluated in isolated HEK293 and Caco-2 cell lines which were exposed to the extracts for 24, 48 and 72 h.

Figure 2: Photograph of cells trypsinised and seeded into 96-well microtitre plates (A) and morphology of Caco-2 cell line at low and high density (B).

ACKNOWLEDGEMENTS

The UKZN College Masters Bursary Award and NRF for funding.

Dr. Masola, Prof. [illegible] and Prof. A. Nicholas for their valuable contribution.

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[illegible] et al. (2010). Apoptosis and Antioxidant Activities of Catharanthus roseus [illegible] Breast Cancer Cell Line. International Journal of Cancer Chemoprevention, [illegible]

[illegible] (1993). Glutamine and cancer. Annals of surgery, [illegible]

[illegible] et al. (2010). Centella asiatica extract induces cell cycle arrest in [illegible] human colon cancer cells. Chiang Mai Medical Journal, [illegible]

RESULTS

Both extracts significantly increased cell proliferation of the HEK293 cells compared to the controls. The cytotoxicity effect of the *C. asiatica* extract to the Caco-2 cell lines was dose dependent as indicated by cell viability. *C.roseus* treatment significantly decreased ($p < 0.05$) the viability of Caco-2 cells in all the exposure times and this was not dose-dependent.

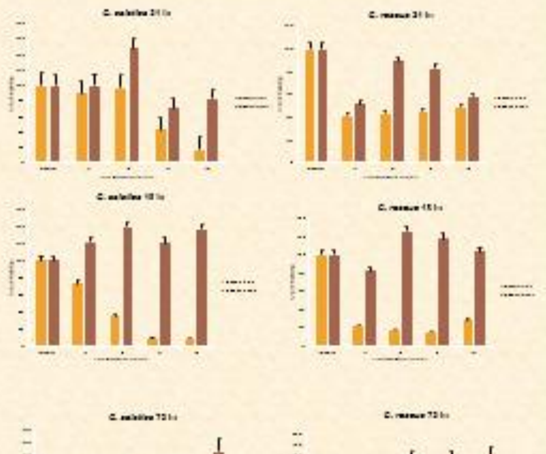
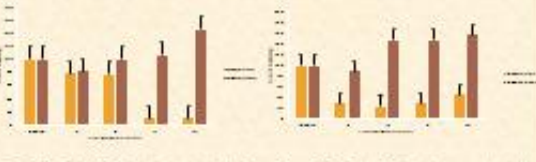



Figure 3. The effects of Centella asiatica extract on the viability and/or metabolic activity of HEK 293 and Caco-2 cells in vitro after exposure to various concentrations of extract for 24 (A), 48 (B) and 72 h (C). Values are presented as means, and vertical bars indicate SEM (n = 6). p< 0.05 by comparison with control.

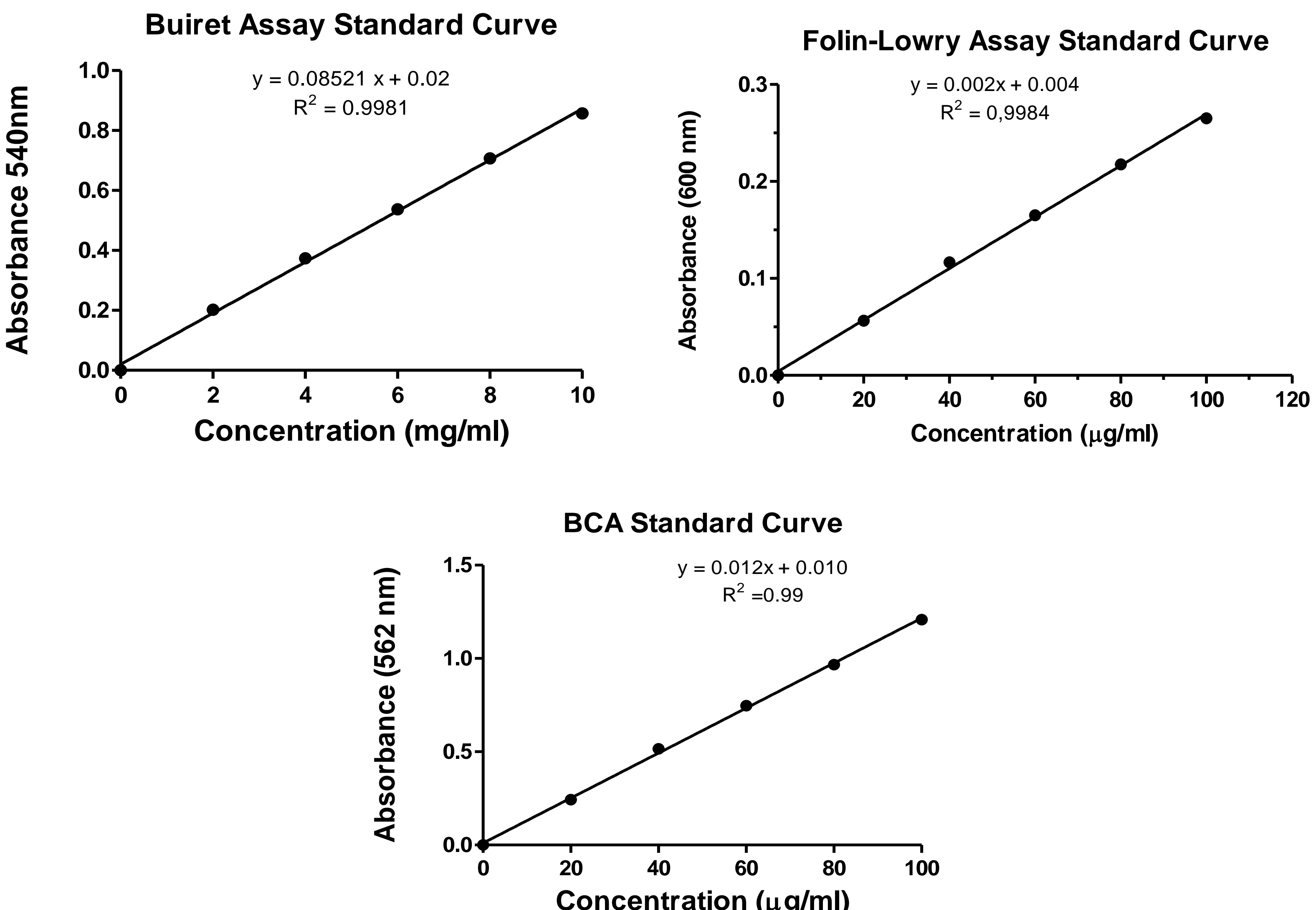
Figure 4. The effects of Catharanthus roseus extract on the viability and/or metabolic activity of HEK 293 and Caco-2 cells in vitro after exposure to various concentrations of extract for 24 (A), 48 (B) and 72 h (C). Values are presented as means, and vertical bars indicate SEM (n = 6). p< 0.05 by comparison with control.

CONCLUSION

Both extracts did not exhibit toxicity to the normal cell line (HEK293) but they significantly decreased the viability of the cancer cell line (Caco-2). These results pave the way for *in vivo* studies of the anti-cancer effects of *C. asiatica* and *C. roseus* extracts since any use of such extracts in treating colon carcinoma or other forms of cancer might also affect PDG essential for normal enterocyte function. Furthermore, *in vivo* experiments will allow for organ toxicity to be assessed. Other studies on mechanisms of the anti-cancer actions of the extracts are ongoing. These concern apoptosis and cell cycle arrests.

UKZN - INSPIRING GREATNESS

Appendix 3: Standard curves



Appendix 4: PDG activity calculations

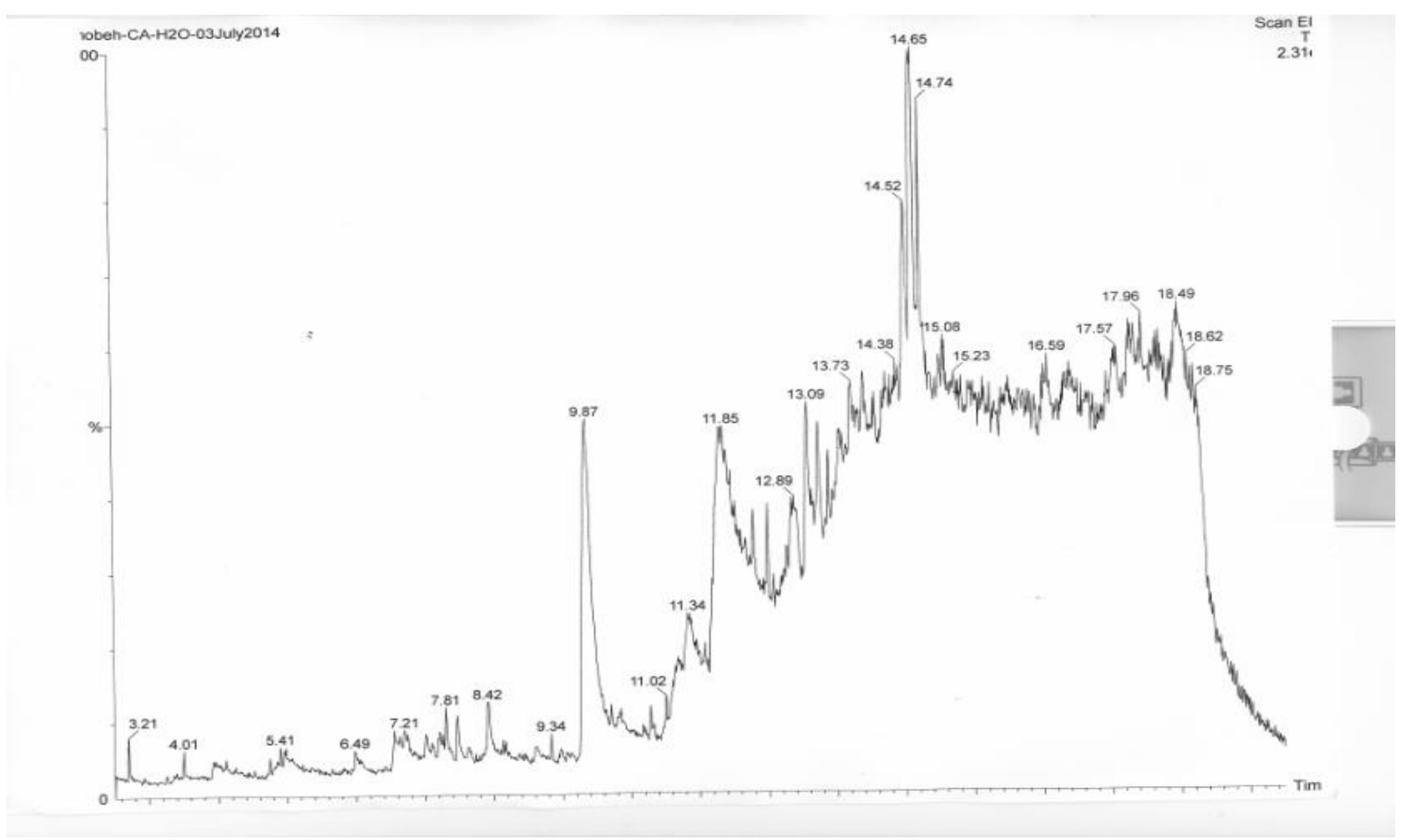
Glutaminase activity = $\frac{\Delta \text{ Abs.} \times \text{ Reaction vol.(ml)} \times 1000 \times 1000}{6.22 \times 1000 \times \text{Time (min)} \times \frac{\text{mg}}{\text{ml}} \text{enzyme}}$

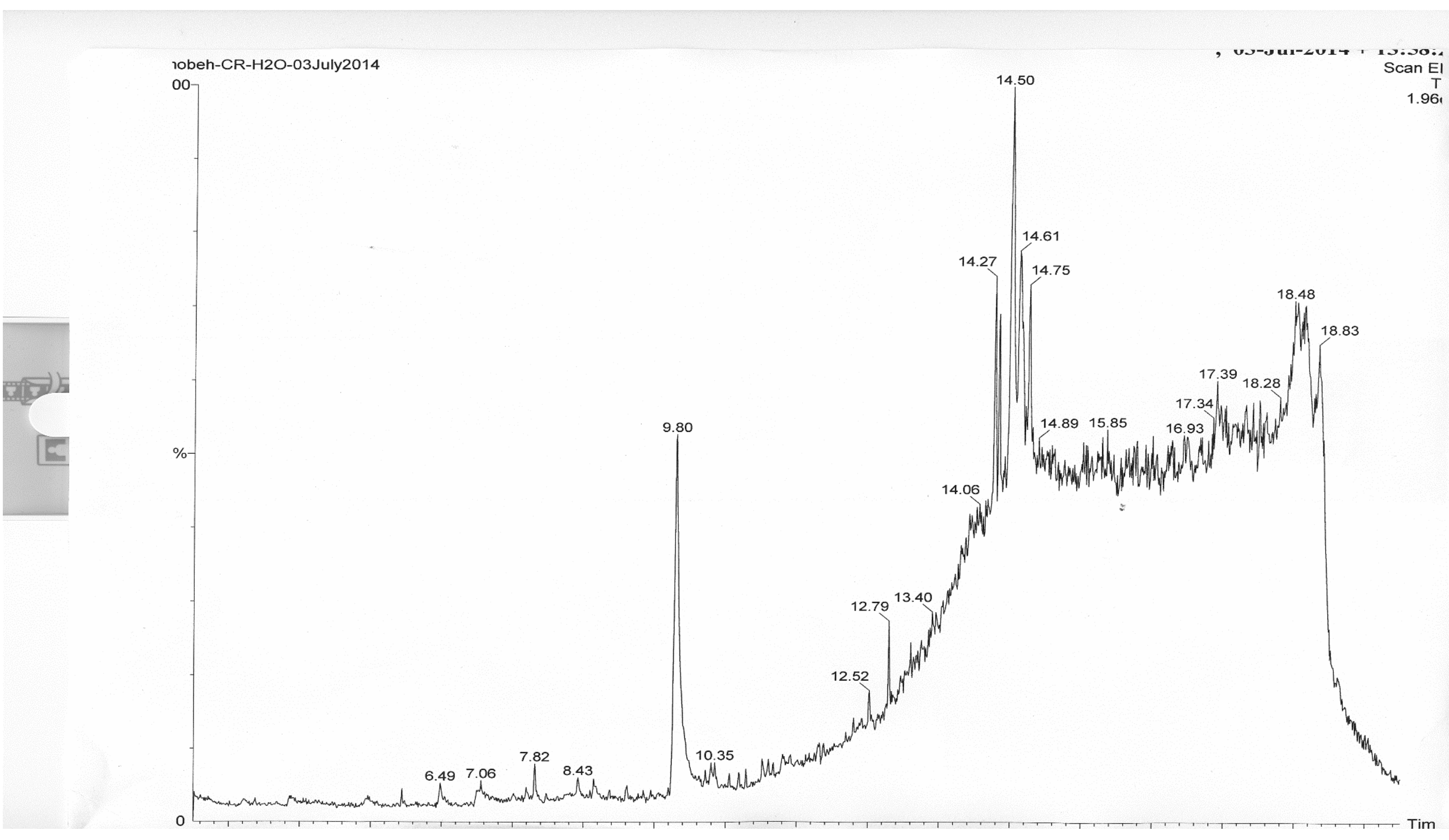
Glutaminase activity is expressed in: nmoles glutamate formed/min/mg protein

Key:

Δ Abs.	= change in absorbance when glutamate is converted to α-ketoglutarate
Reaction vol.	= Final volume of the reaction mixture in ml
× 1000 × 1000	= to express the activity in nano moles
6.22×10^3	= NADH molar extinction coefficient (Moles-1cm-1) at 340 nm
Time (minutes)	= time taken for glutamate dehydrogenase to form β-NADH
mg enzyme	= mg/ml protein as determined using the Lowry's method.

Appendix 5: ***C. asiatica*** **and** ***C. roseus*** **GCMS Chromatography**





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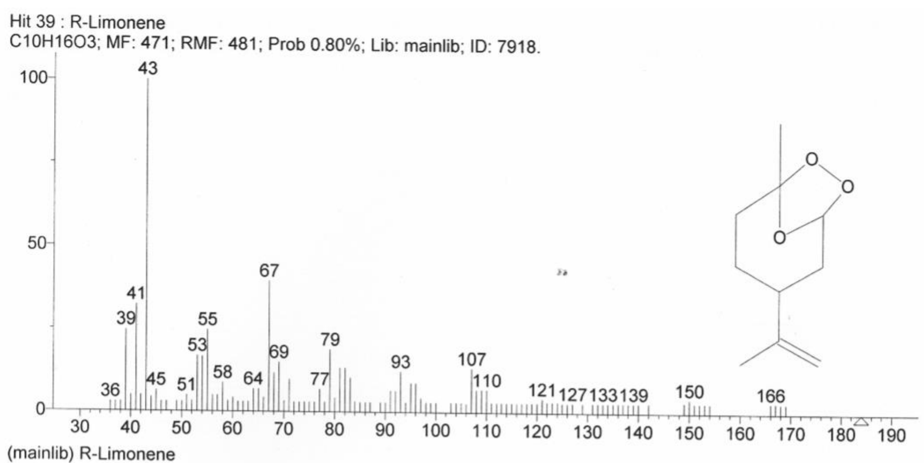
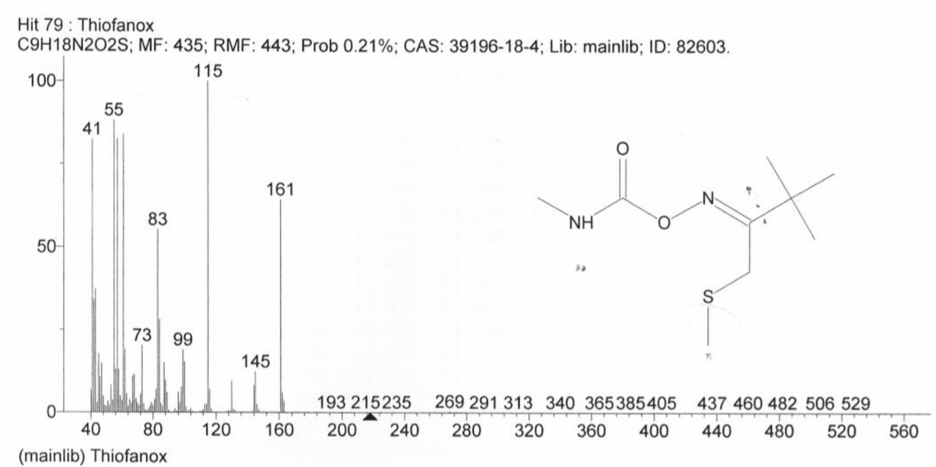
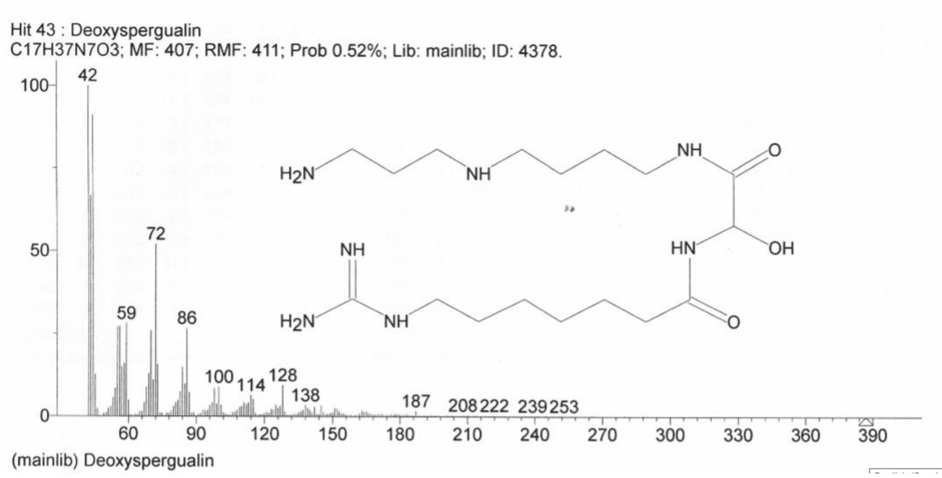
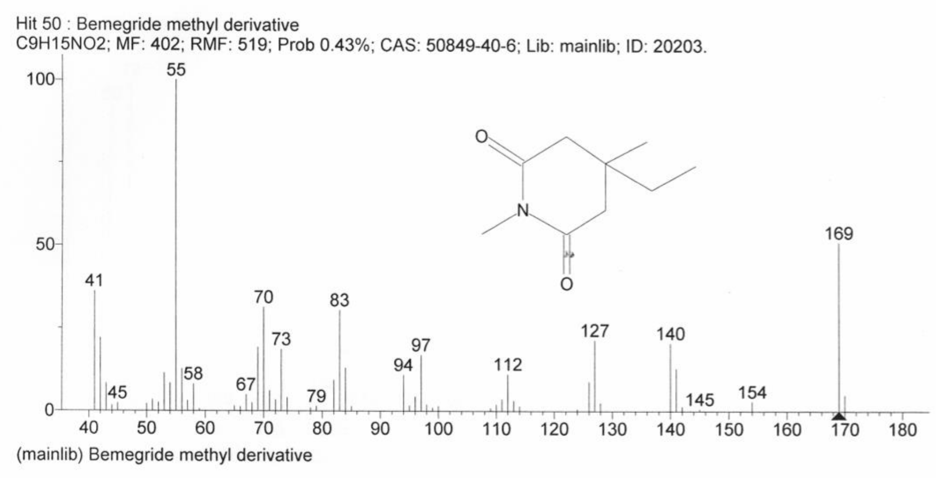
Appendix 6: Phytocomponents of *C. asiatica* spectrums





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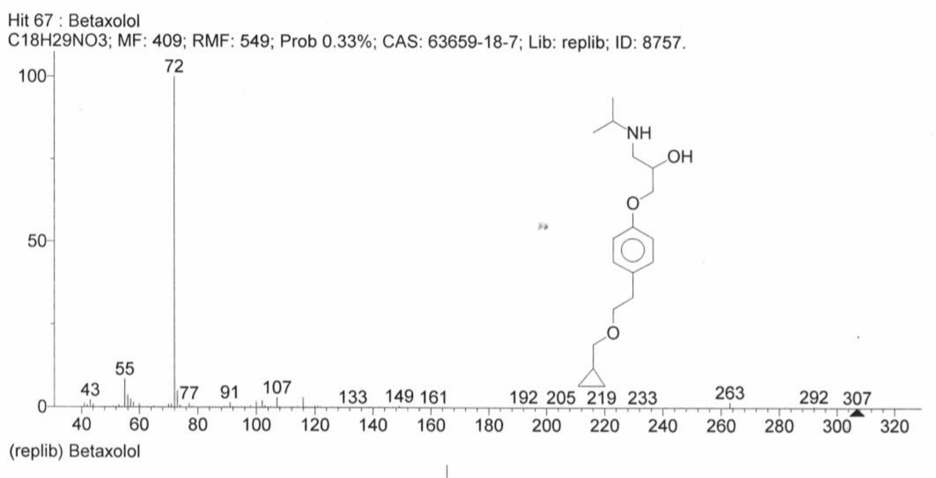
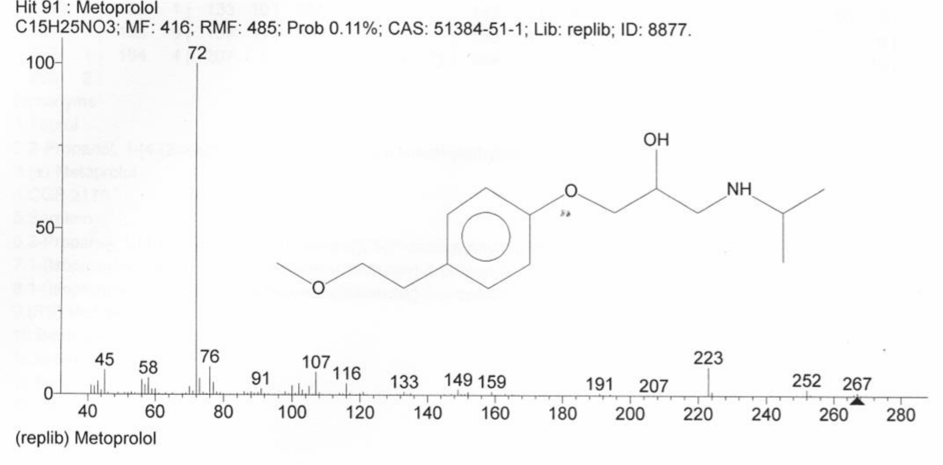
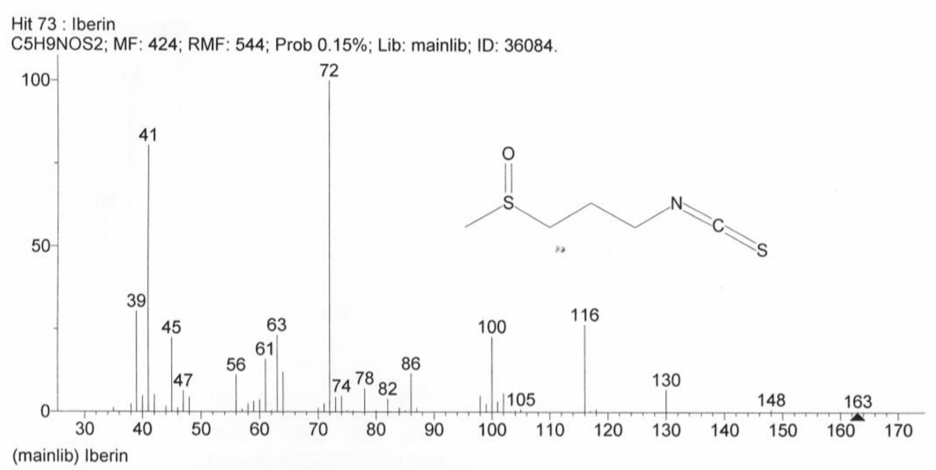
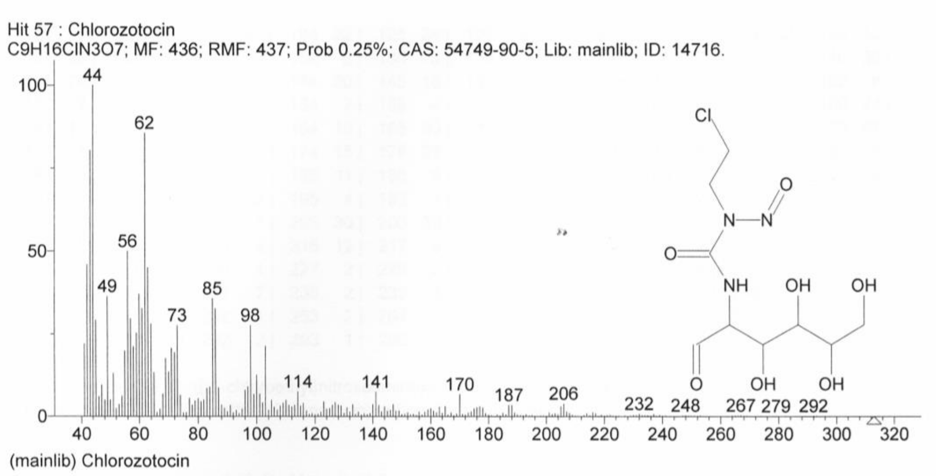
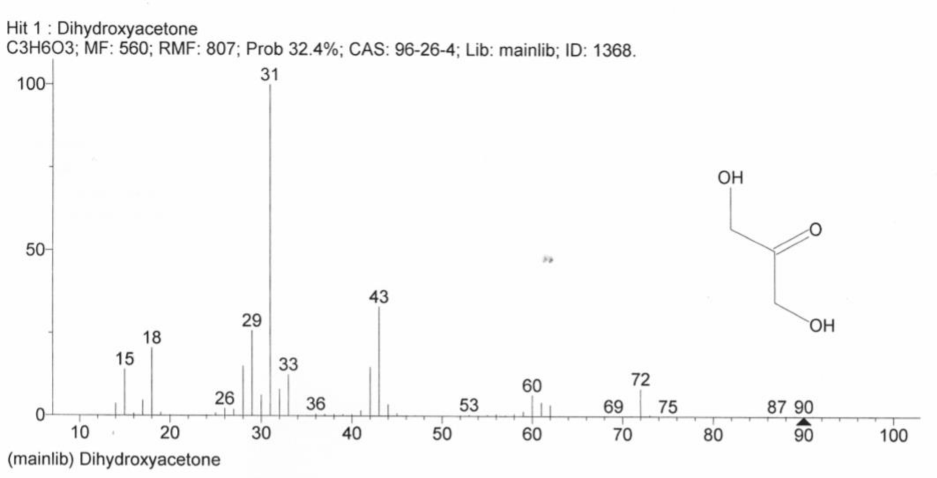
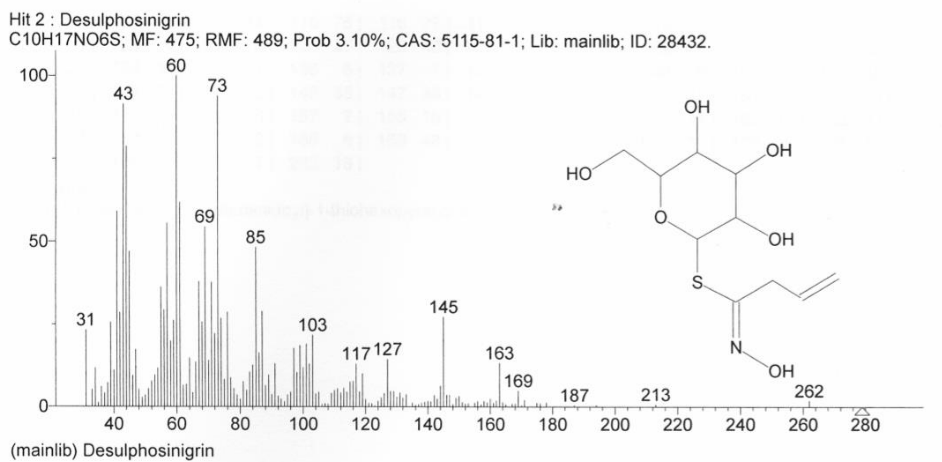

Hit 67 : Betaxolol
C18H29NO3; MF: 409; RMF: 549; Prob 0.33%; CAS: 63659-18-7; Lib: replib; ID: 8757.
(replib) Betaxolol

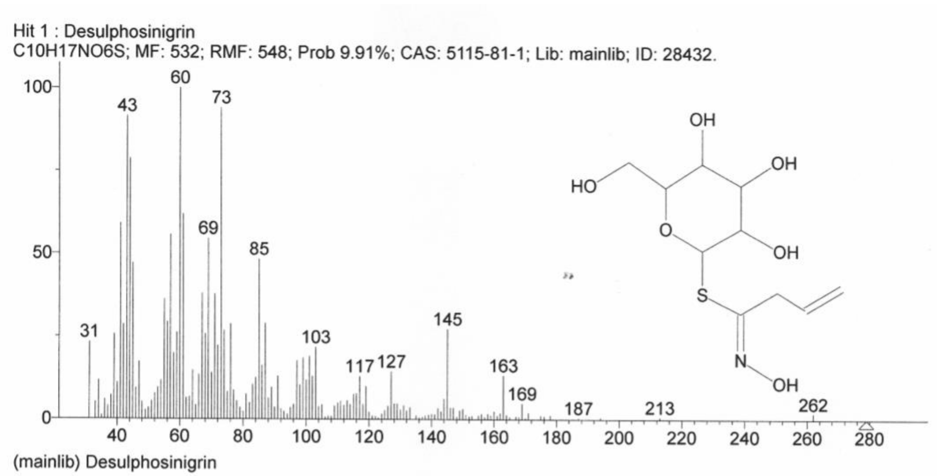

Hit 91 : Metoprolol
C15H25NO3; MF: 416; RMF: 485; Prob 0.11%; CAS: 51384-51-1; Lib: replib; ID: 8877.
(replib) Metoprolol

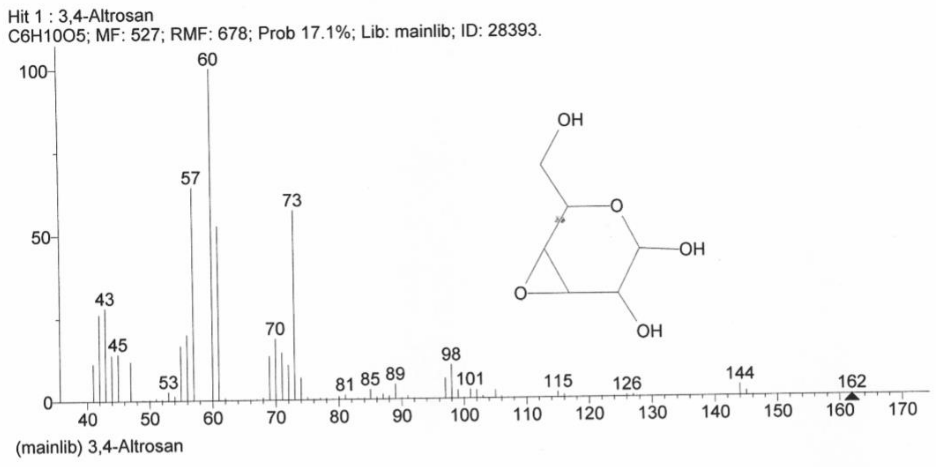

Hit 73 : Iberin
C5H9NOS2; MF: 424; RMF: 544; Prob 0.15%; Lib: mainlib; ID: 36084.
(mainlib) Iberin

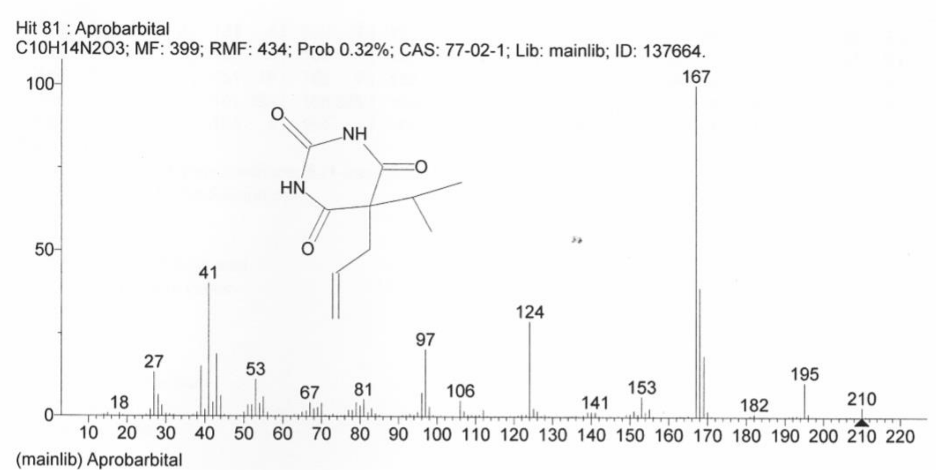

Hit 57 : Chlorozotocin
C9H16ClN3O7; MF: 436; RMF: 437; Prob 0.25%; CAS: 54749-90-5; Lib: mainlib; ID: 14716.
(mainlib) Chlorozotocin

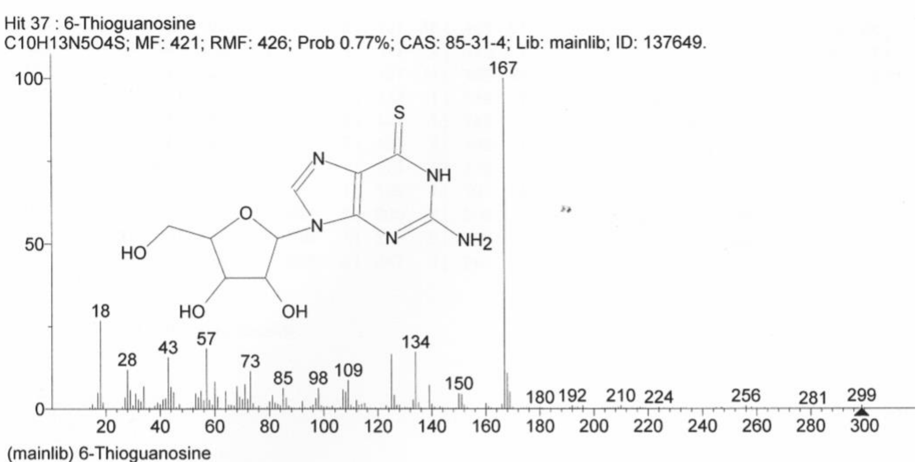

Hit 1 : Dihydroxyacetone
C3H6O3; MF: 560; RMF: 807; Prob 32.4%; CAS: 96-26-4; Lib: mainlib; ID: 1368.
(mainlib) Dihydroxyacetone

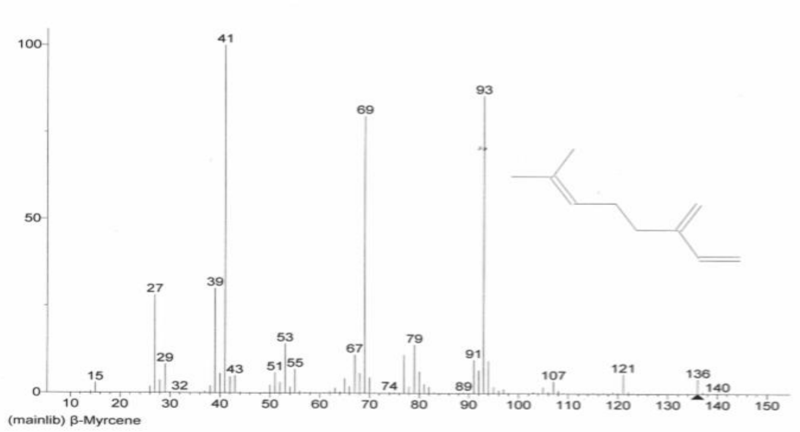

Hit 2 : Desulphosinigrin
C10H17NO6S; MF: 475; RMF: 489; Prob 3.10%; CAS: 5115-81-1; Lib: mainlib; ID: 28432.
(mainlib) Desulphosinigrin

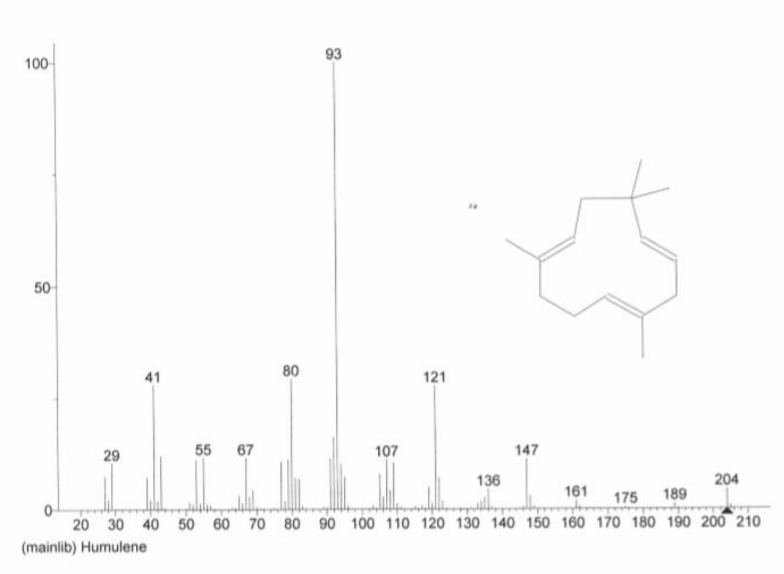

Hit 1 : Desulphosinigrin
C10H17NO6S; MF: 532; RMF: 548; Prob 9.91%; CAS: 5115-81-1; Lib: mainlib; ID: 28432.
(mainlib) Desulphosinigrin


Hit 1 : 3,4-Altrosan
C6H10O5; MF: 527; RMF: 678; Prob 17.1%; Lib: mainlib; ID: 28393.
(mainlib) 3,4-Altrosan

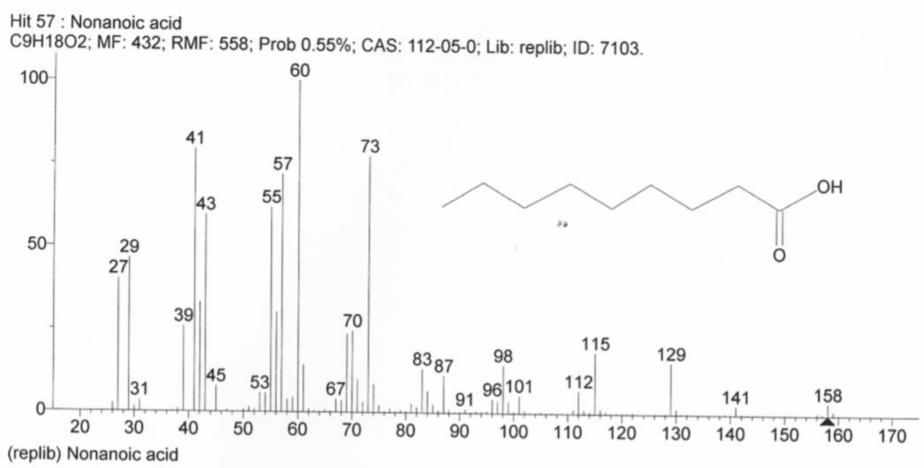

Hit 81 : Aprobarbital
C10H14N2O3; MF: 399; RMF: 434; Prob 0.32%; CAS: 77-02-1; Lib: mainlib; ID: 137664.
(mainlib) Aprobarbital


Hit 37 : 6-Thioguanosine
C10H13N5O4S; MF: 421; RMF: 426; Prob 0.77%; CAS: 85-31-4; Lib: mainlib; ID: 137649.
(mainlib) 6-Thioguanosine

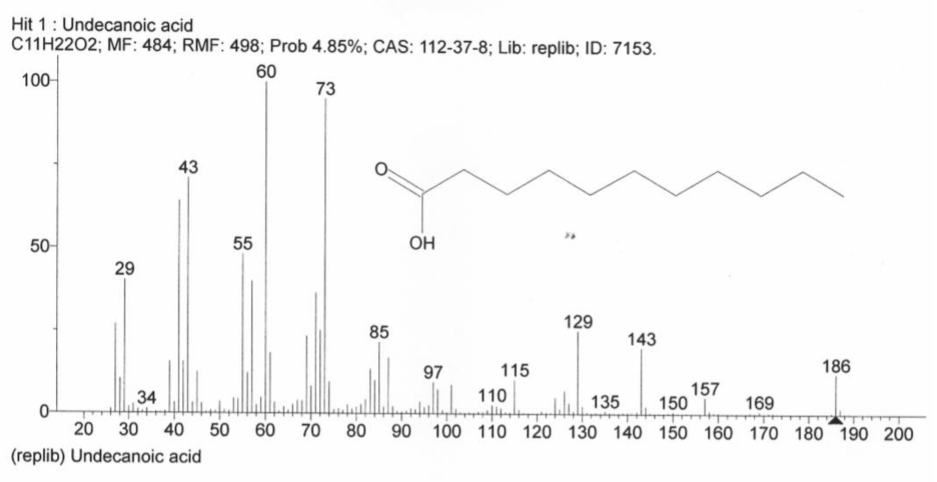

(mainlib) β-Myrcene



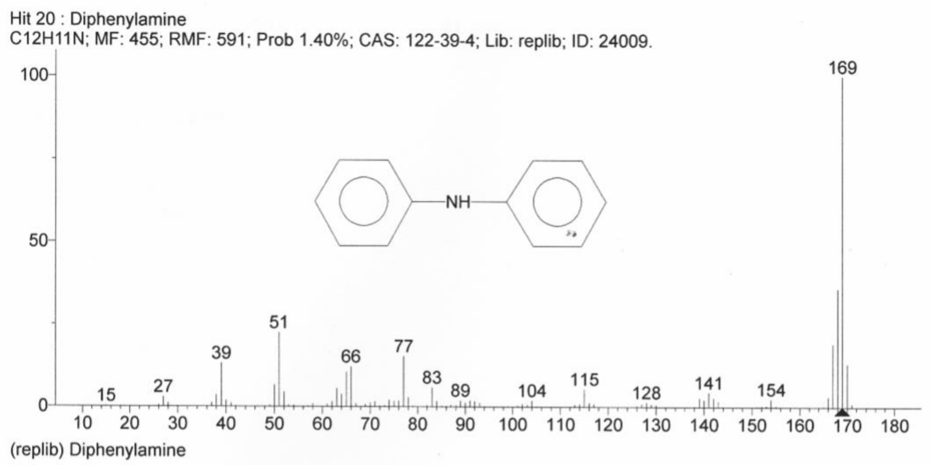
100
50
0
93
41
80
121
29
55
67
107
147
136
161
175
189
204
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210
(mainlib) Humulene

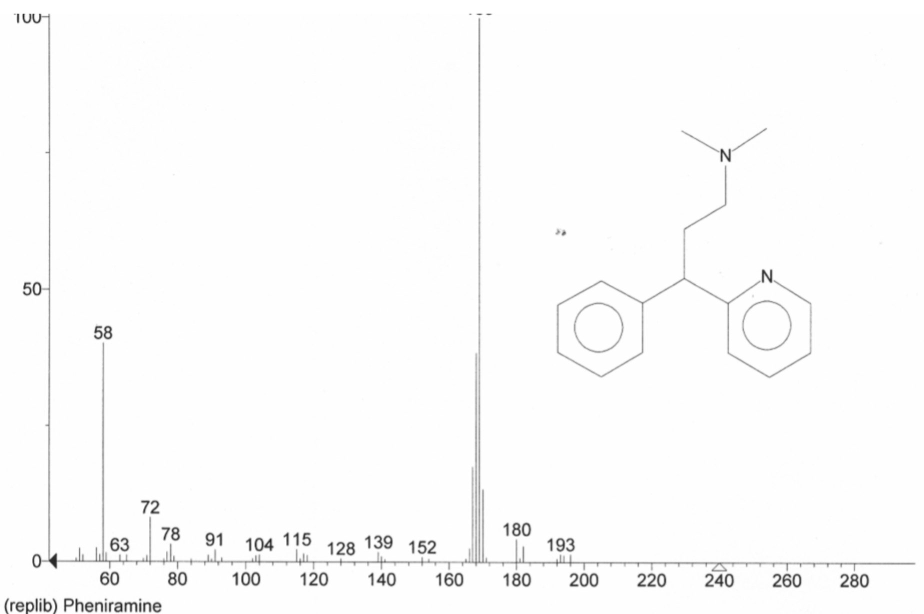


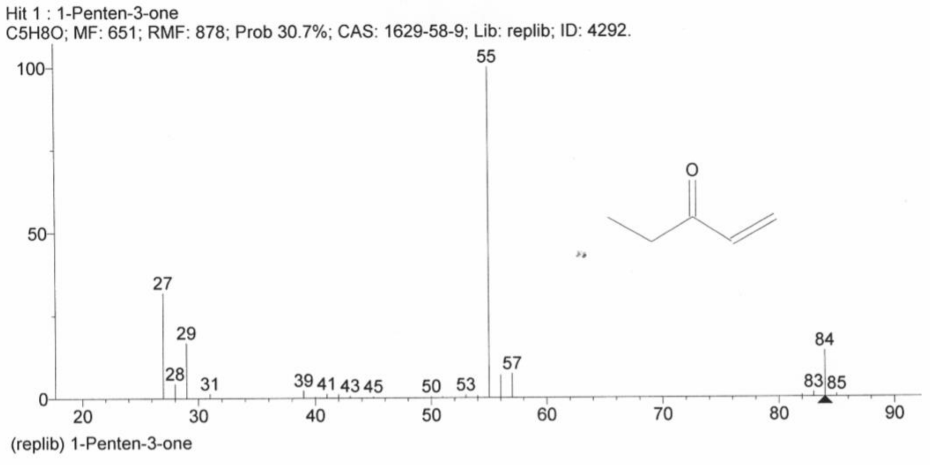
Hit 57 : Nonanoic acid
C9H18O2; MF: 432; RMF: 558; Prob 0.55%; CAS: 112-05-0; Lib: replib; ID: 7103.
100
50
0
60
41
73
57
55
43
29
27
39
70
83
87
98
115
129
31
45
53
67
91
96
101
112
141
158
OH
O
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170
(replib) Nonanoic acid

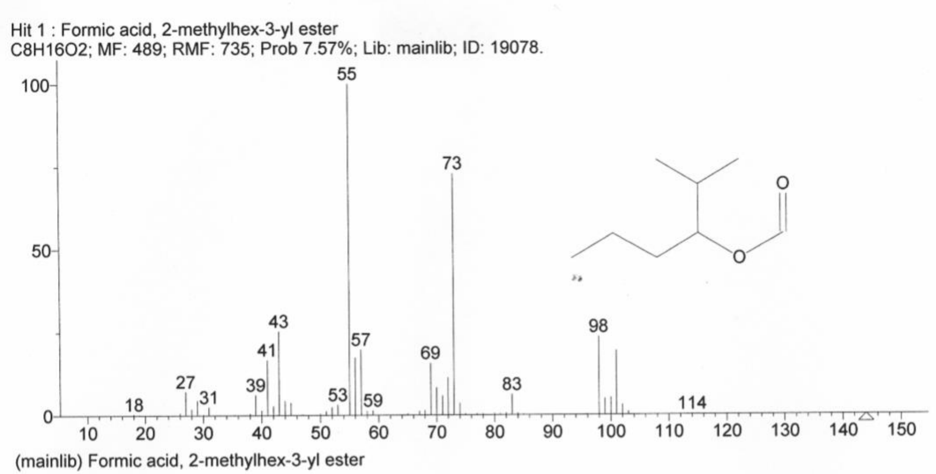


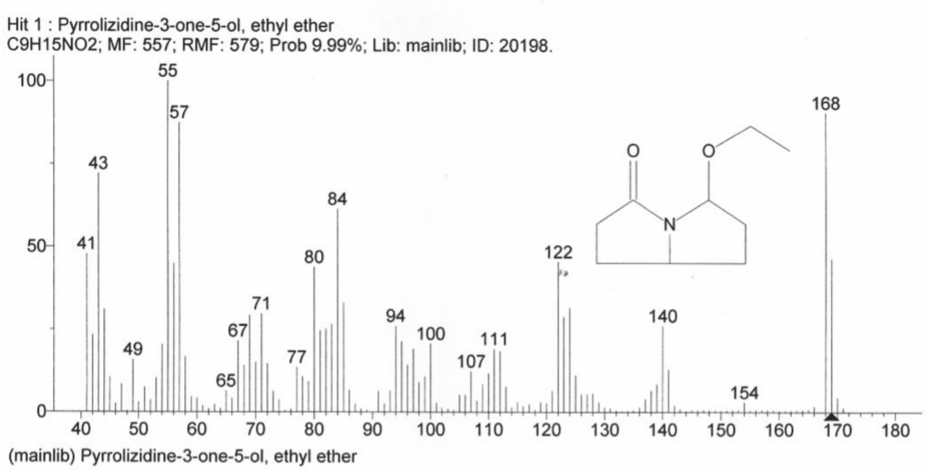
Hit 1 : Undecanoic acid
C11H22O2; MF: 484; RMF: 498; Prob 4.85%; CAS: 112-37-8; Lib: replib; ID: 7153.
100
50
0
60
73
43
55
29
85
129
143
186
34
97
110
115
135
150
157
169
O
OH
20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
(replib) Undecanoic acid

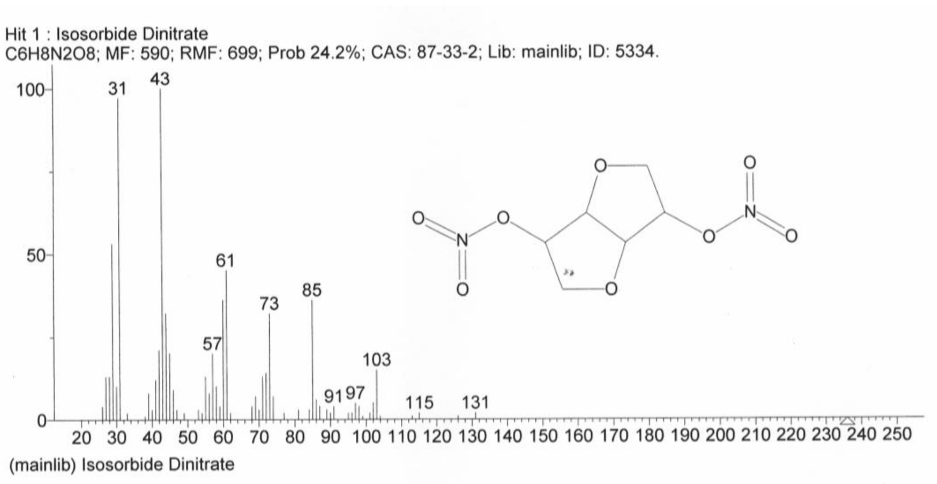

Hit 20 : Diphenylamine
C12H11N; MF: 455; RMF: 591; Prob 1.40%; CAS: 122-39-4; Lib: replib; ID: 24009.
NH
(replib) Diphenylamine

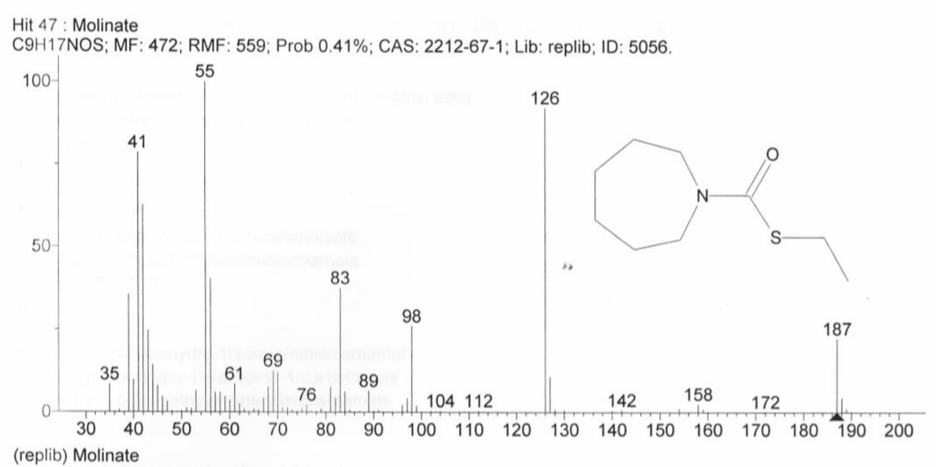

N
N
(replib) Pheniramine

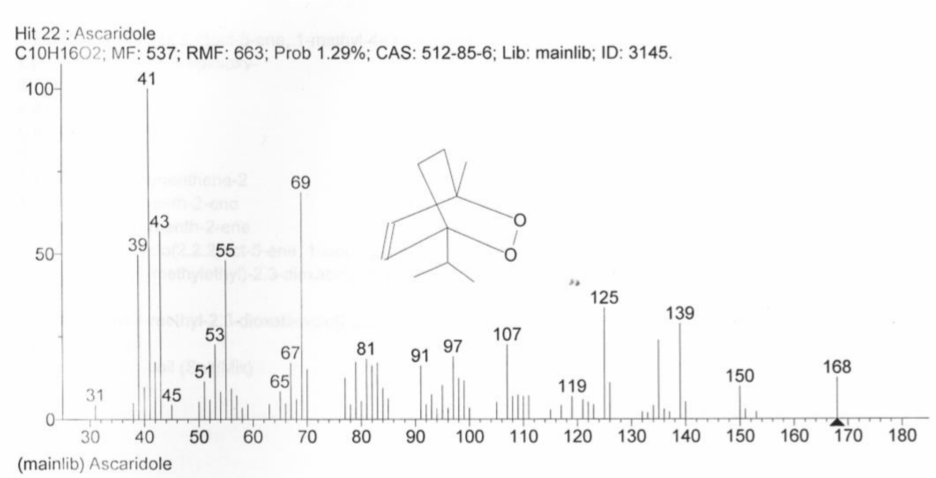

Hit 1 : 1-Penten-3-one
C5H8O; MF: 651; RMF: 878; Prob 30.7%; CAS: 1629-58-9; Lib: replib; ID: 4292.
O
(replib) 1-Penten-3-one

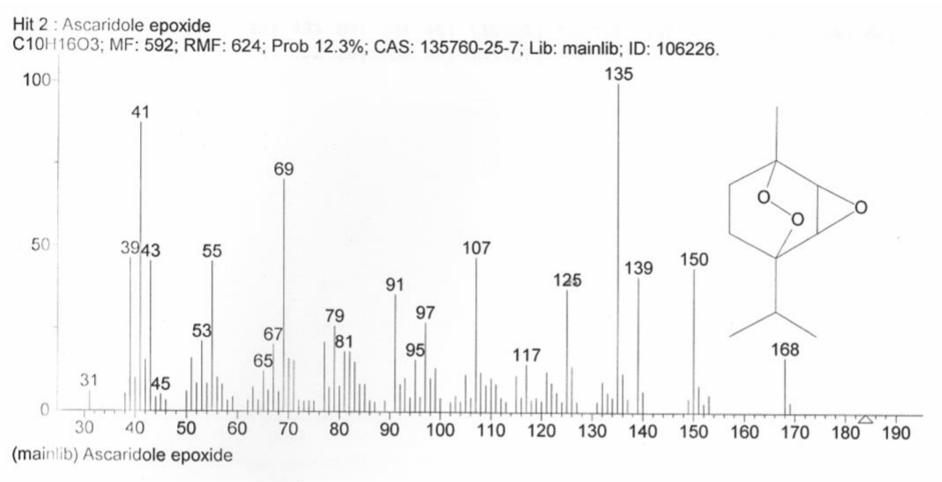

Hit 1 : Formic acid, 2-methylhex-3-yl ester
C8H16O2; MF: 489; RMF: 735; Prob 7.57%; Lib: mainlib; ID: 19078.
(mainlib) Formic acid, 2-methylhex-3-yl ester

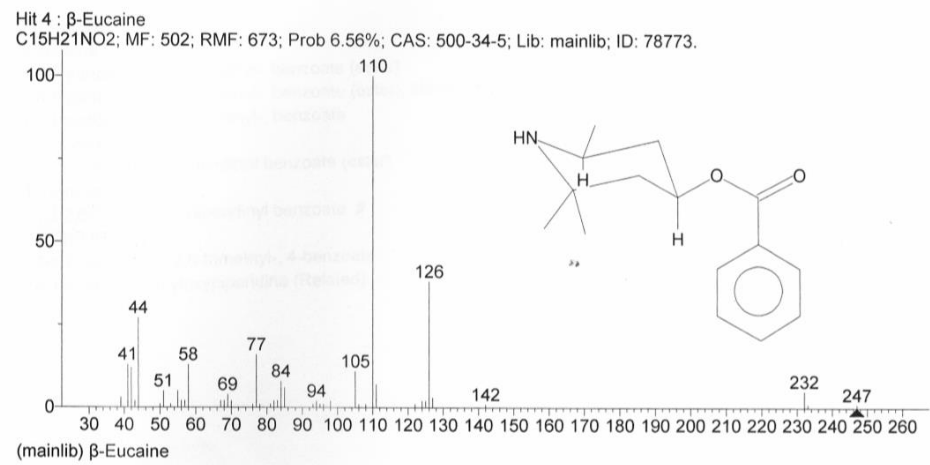

Hit 1 : Pyrrolizidine-3-one-5-ol, ethyl ether
C9H15NO2; MF: 557; RMF: 579; Prob 9.99%; Lib: mainlib; ID: 20198.
(mainlib) Pyrrolizidine-3-one-5-ol, ethyl ether

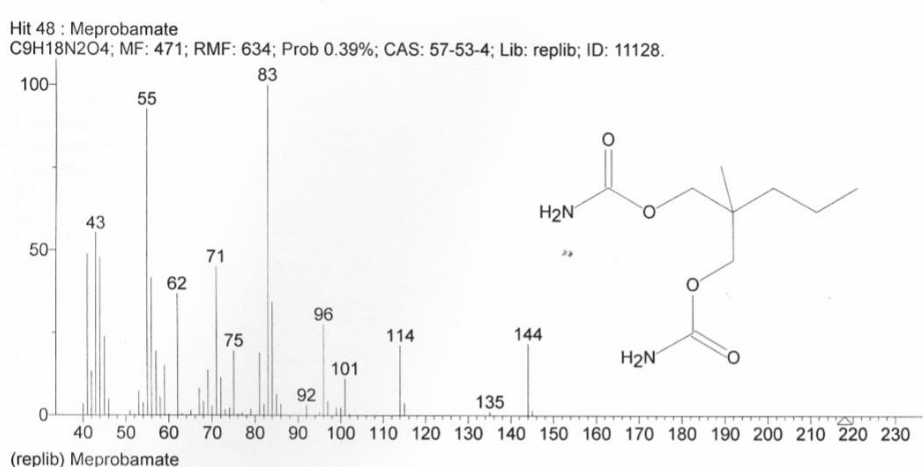

Hit 1 : Isosorbide Dinitrate
C6H8N2O8; MF: 590; RMF: 699; Prob 24.2%; CAS: 87-33-2; Lib: mainlib; ID: 5334.
(mainlib) Isosorbide Dinitrate

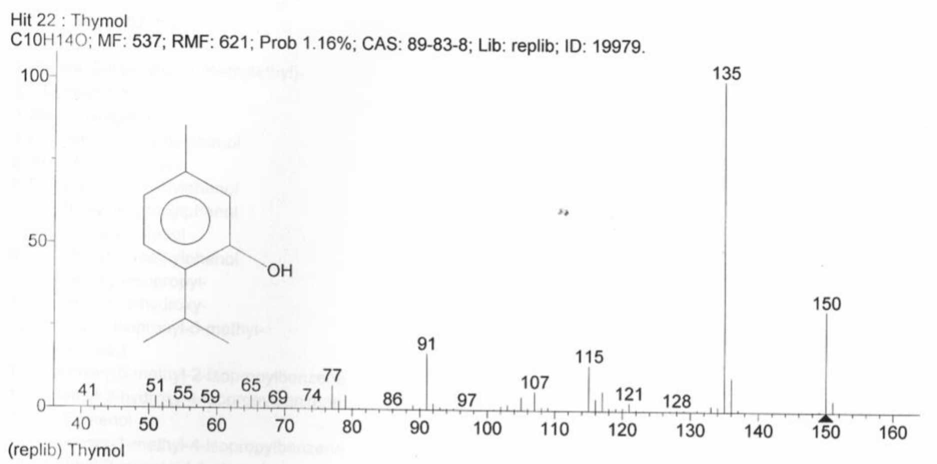

Hit 47 : Molinate
C9H17NOS; MF: 472; RMF: 559; Prob 0.41%; CAS: 2212-67-1; Lib: replib; ID: 5056.
(replib) Molinate
N
O
S

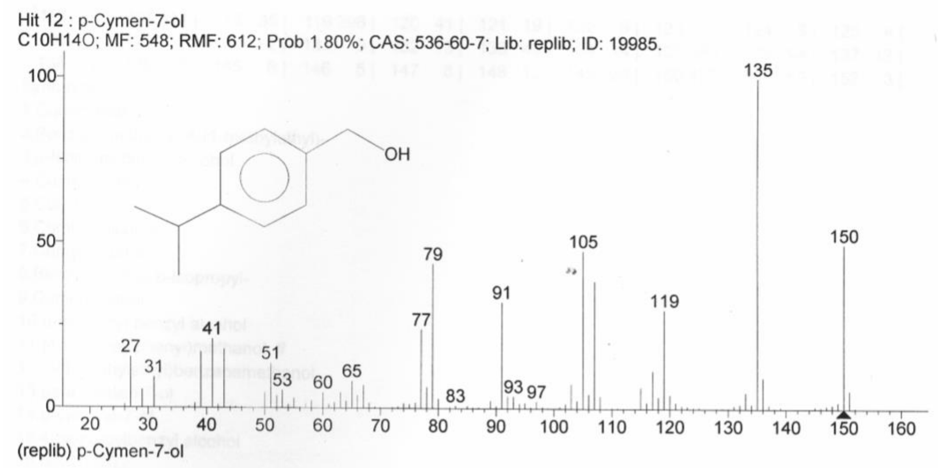

Hit 22 : Ascaridole
C10H16O2; MF: 537; RMF: 663; Prob 1.29%; CAS: 512-85-6; Lib: mainlib; ID: 3145.
(mainlib) Ascaridole
O
O

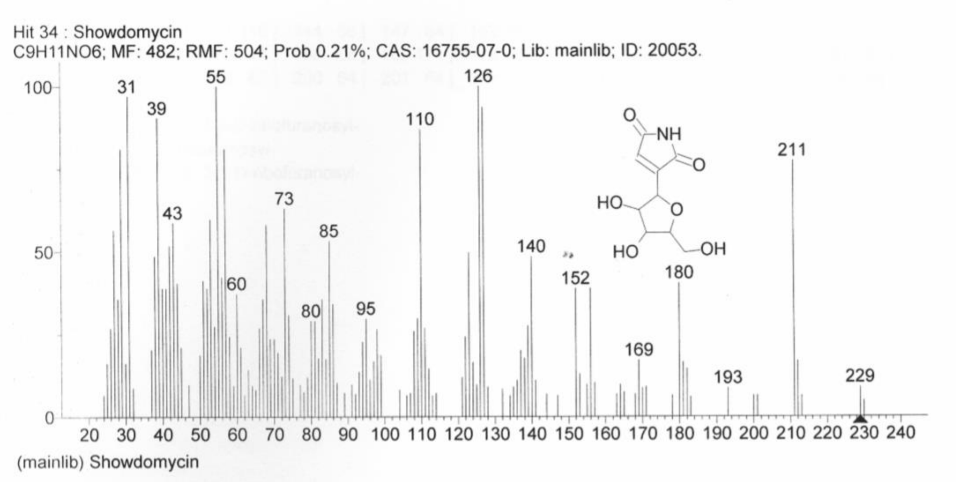

Hit 2 : Ascaridole epoxide
C10H16O3; MF: 592; RMF: 624; Prob 12.3%; CAS: 135760-25-7; Lib: mainlib; ID: 106226.
(mainlib) Ascaridole epoxide
O
O
O

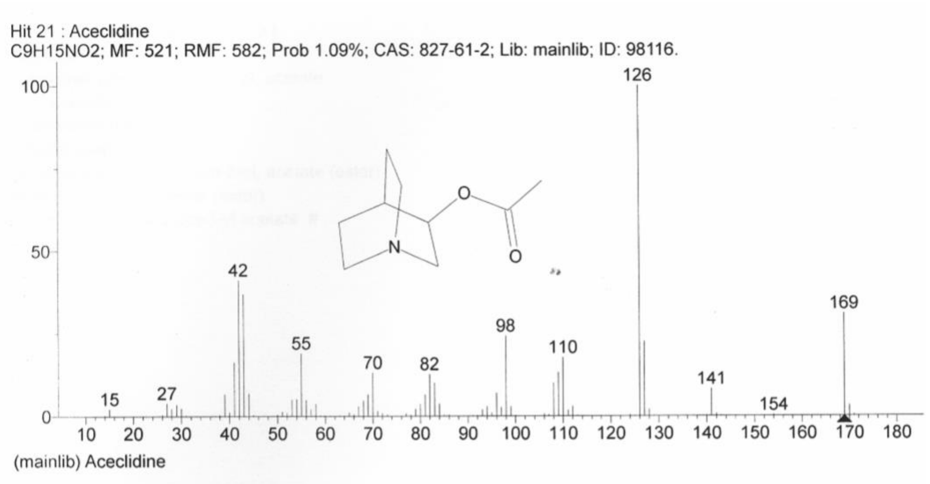

Hit 4 : β-Eucaine
C15H21NO2; MF: 502; RMF: 673; Prob 6.56%; CAS: 500-34-5; Lib: mainlib; ID: 78773.
(mainlib) β-Eucaine

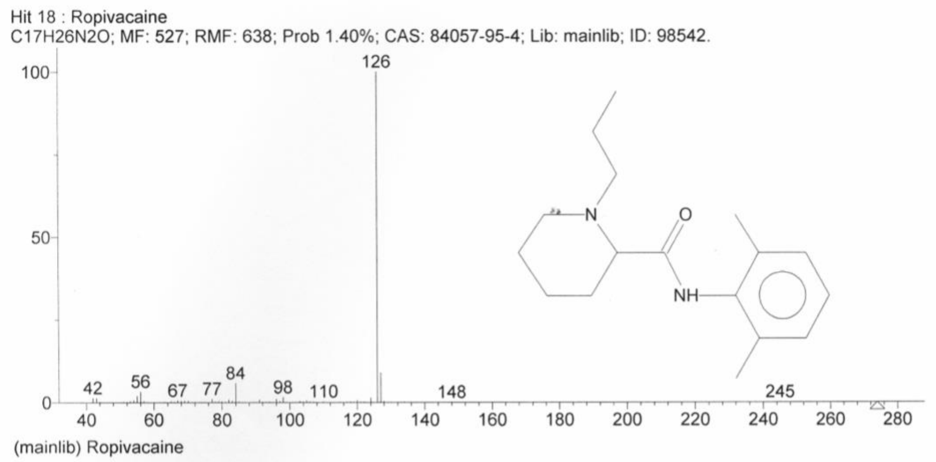

Hit 48 : Meprobamate
C9H18N2O4; MF: 471; RMF: 634; Prob 0.39%; CAS: 57-53-4; Lib: replib; ID: 11128.
(replib) Meprobamate

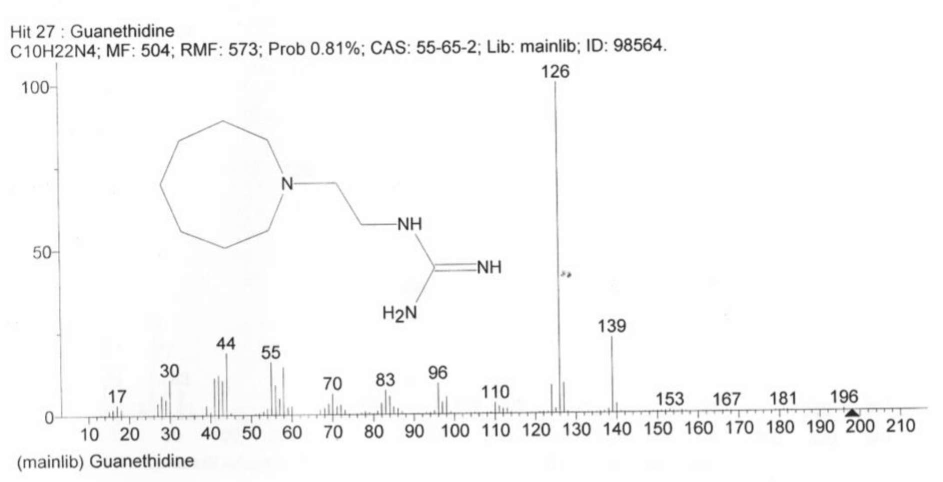

Hit 22 : Thymol
C10H14O; MF: 537; RMF: 621; Prob 1.16%; CAS: 89-83-8; Lib: replib; ID: 19979.
(replib) Thymol


Hit 12 : p-Cymen-7-ol
C10H14O; MF: 548; RMF: 612; Prob 1.80%; CAS: 536-60-7; Lib: replib; ID: 19985.
OH
(replib) p-Cymen-7-ol

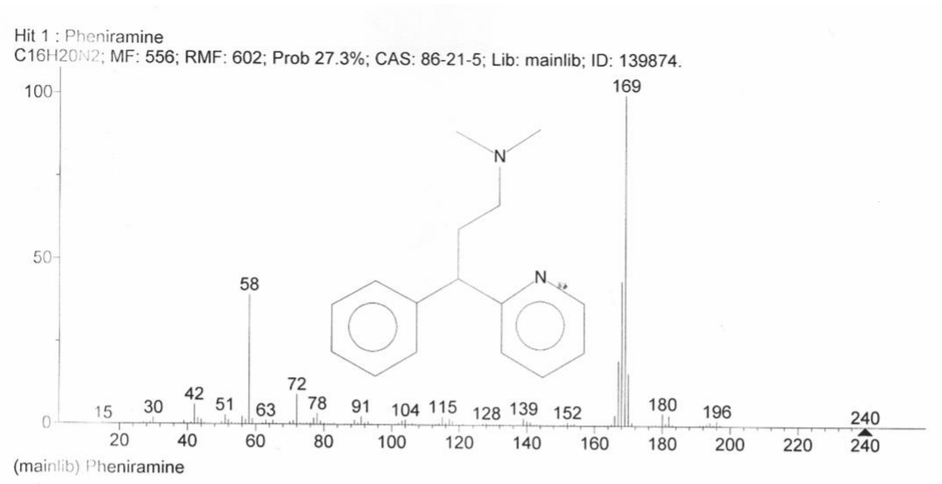

Hit 34 : Showdomycin
C9H11NO6; MF: 482; RMF: 504; Prob 0.21%; CAS: 16755-07-0; Lib: mainlib; ID: 20053.
NH
HO
OH
(mainlib) Showdomycin

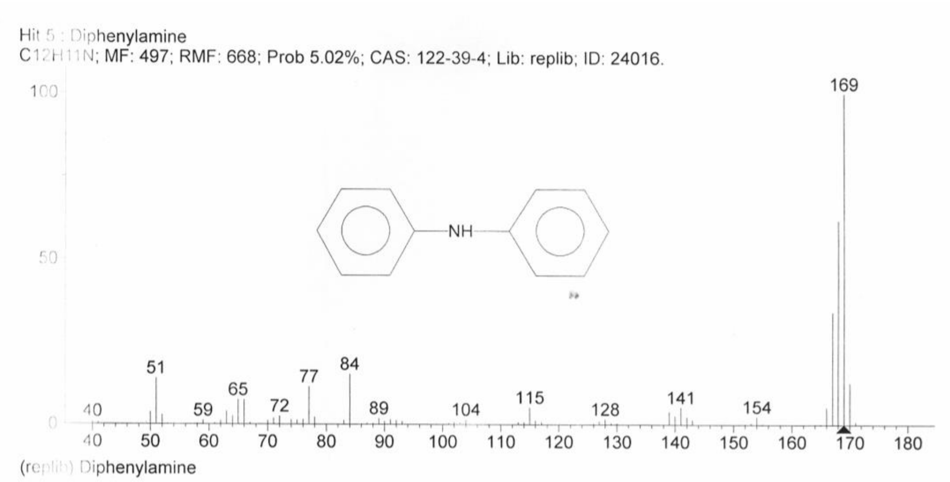

Hit 21 : Aceclidine
C9H15NO2; MF: 521; RMF: 582; Prob 1.09%; CAS: 827-61-2; Lib: mainlib; ID: 98116.
N
(mainlib) Aceclidine

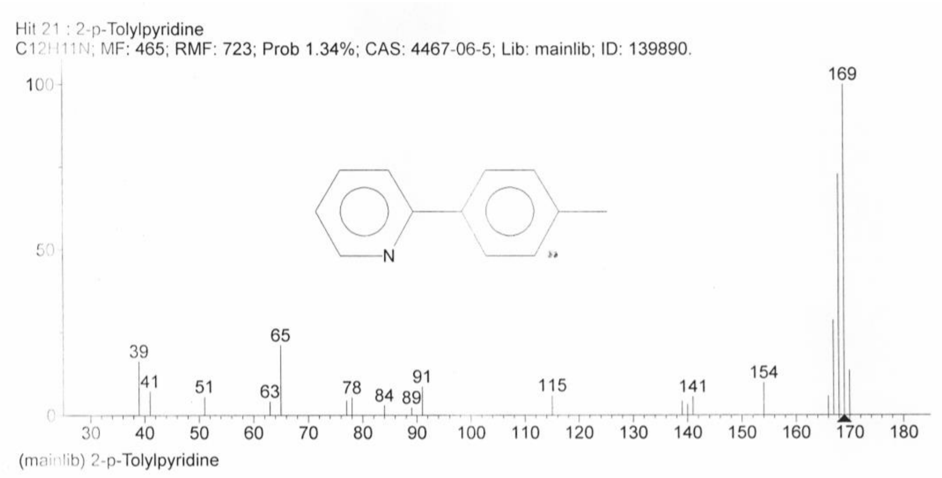

Hit 18 : Ropivacaine
C17H26N2O; MF: 527; RMF: 638; Prob 1.40%; CAS: 84057-95-4; Lib: mainlib; ID: 98542.
126
42
56
67
77
84
98
110
148
245
N
O
NH
(mainlib) Ropivacaine

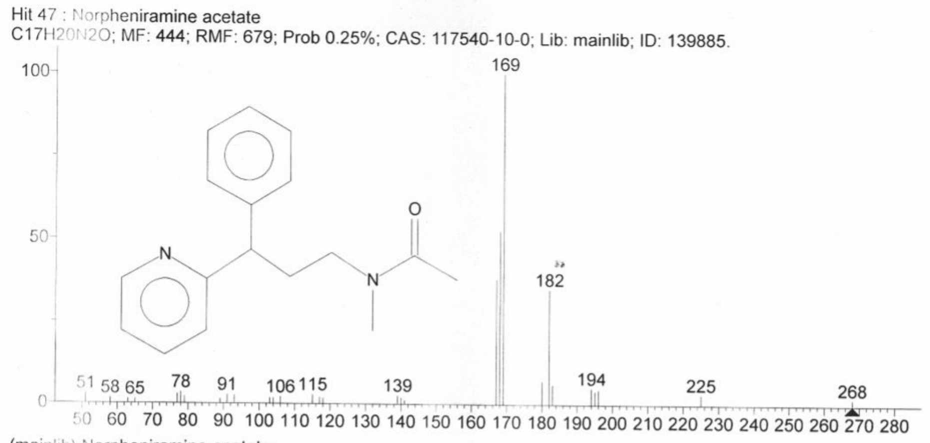

Hit 27 : Guanethidine
C10H22N4; MF: 504; RMF: 573; Prob 0.81%; CAS: 55-65-2; Lib: mainlib; ID: 98564.
126
139
17
30
44
55
70
83
96
110
153
167
181
196
N
NH
NH
H2N
(mainlib) Guanethidine

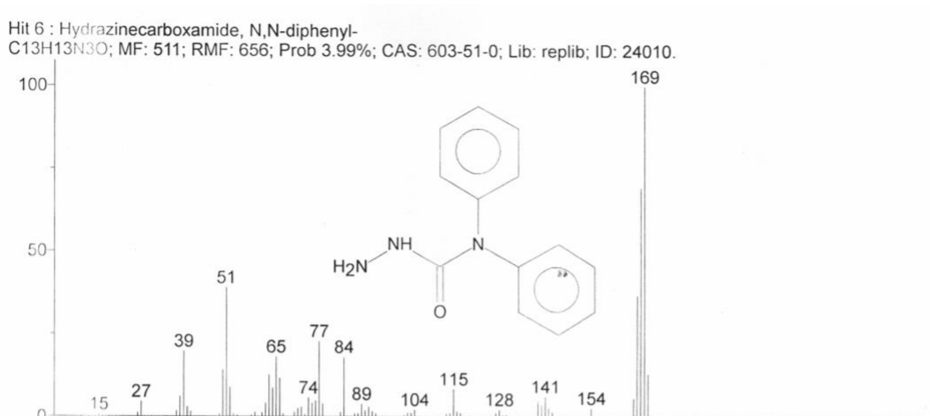
Appendix 7: Phytocomponents of *C. roseus* spectrums

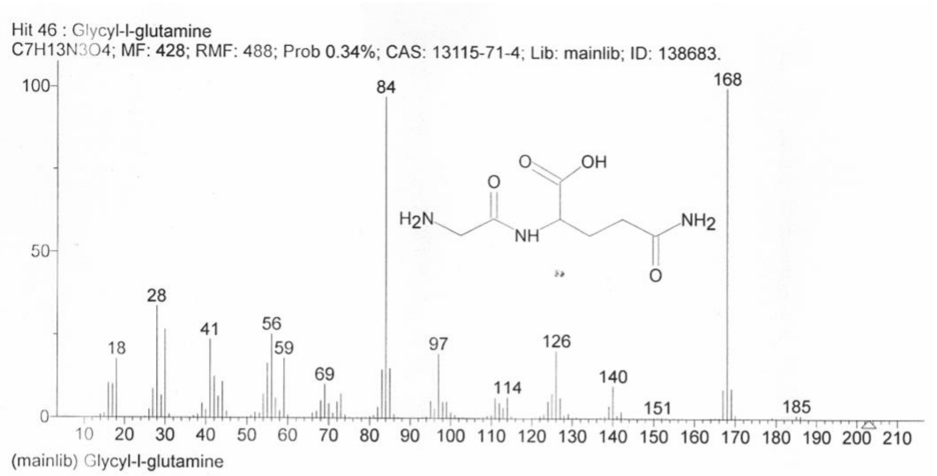


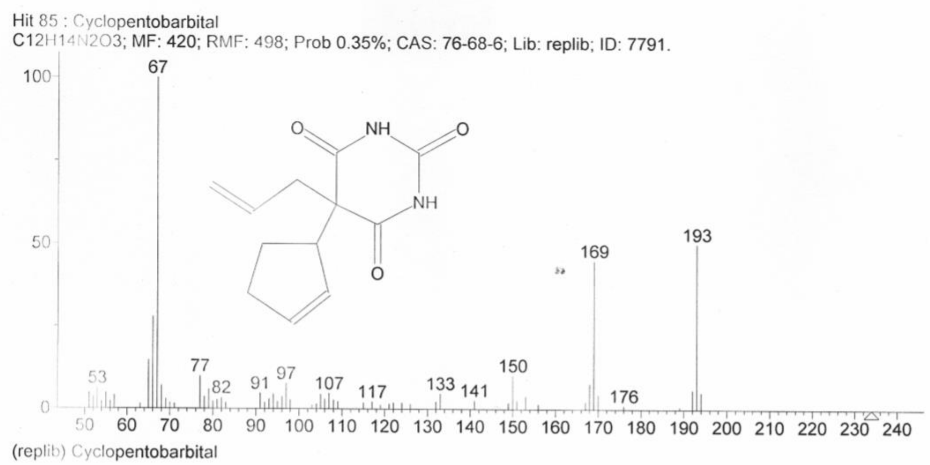


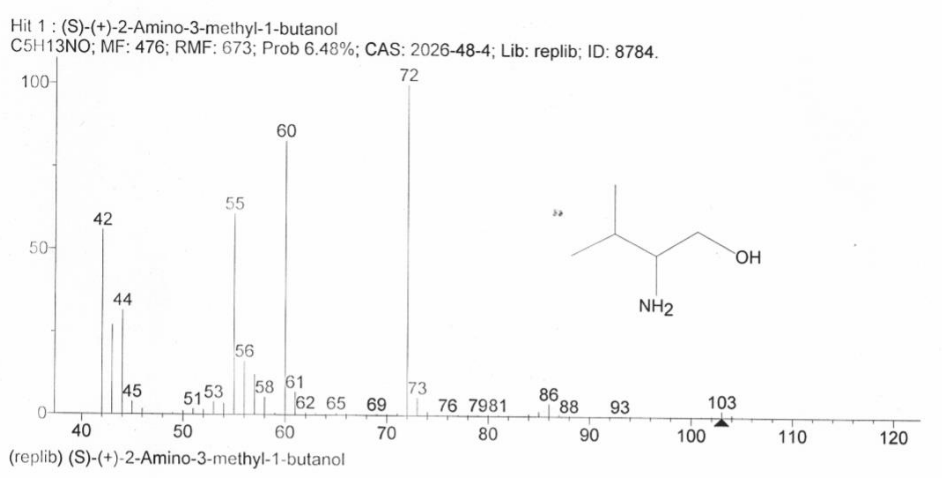


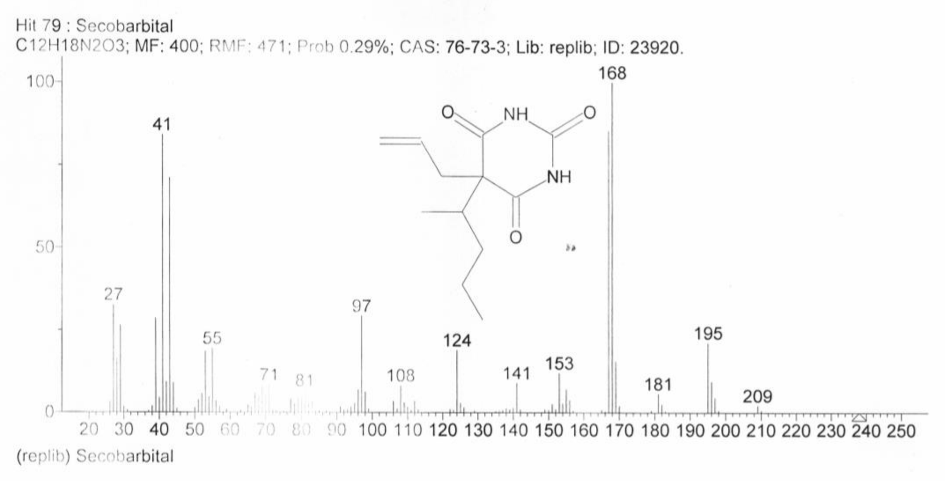

Hit 47 : Norpheniramine acetate
C17H20N2O; MF: 444; RMF: 679; Prob 0.25%; CAS: 117540-10-0; Lib: mainlib; ID: 139885.
169
182
194
225
268
51 58 65 78 91 106 115 139
(mainlib) Norpheniramine acetate

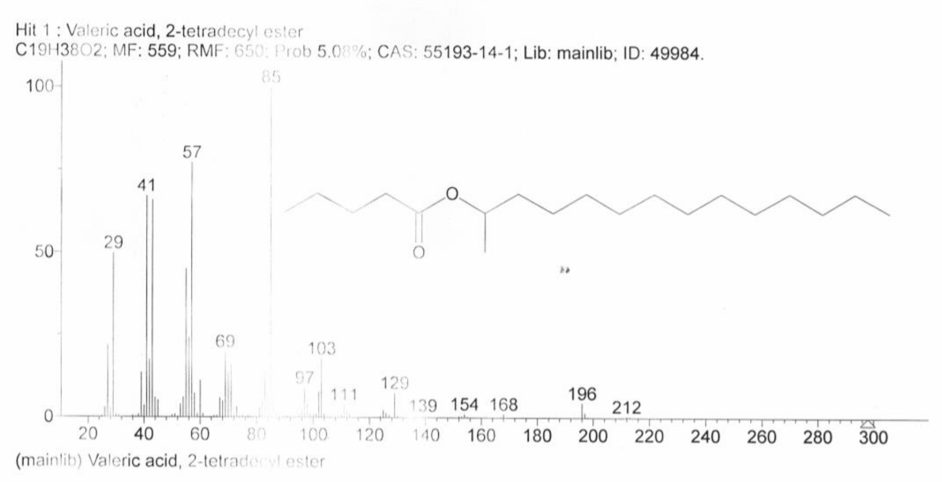

Hit 6 : Hydrazinecarboxamide, N,N-diphenyl-
C13H13N3O; MF: 511; RMF: 656; Prob 3.99%; CAS: 603-51-0; Lib: replib; ID: 24010.
169
H2N
NH
N
O
51
39
65
77
84
15 27 74 89 104 115 128 141 154
(replib) Hydrazinecarboxamide, N,N-diphenyl-

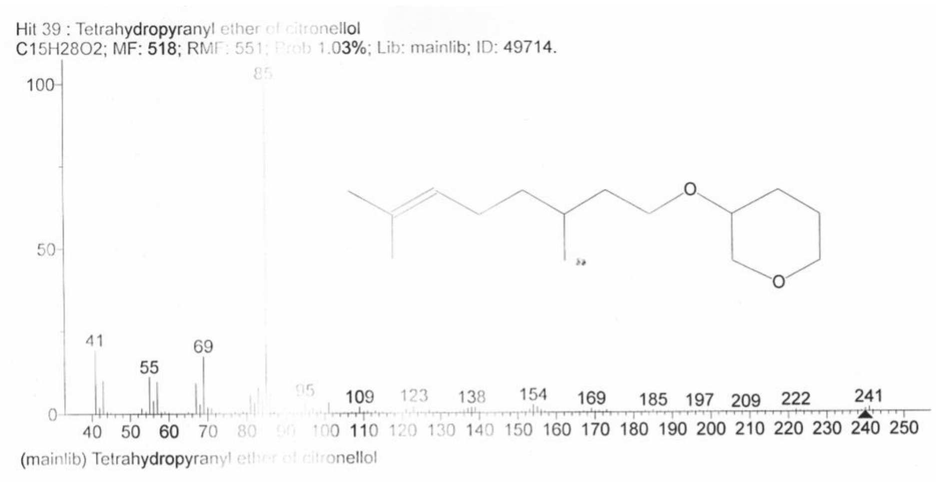

Hit 46 : Glycyl-l-glutamine
C7H13N3O4; MF: 428; RMF: 488; Prob 0.34%; CAS: 13115-71-4; Lib: mainlib; ID: 138683.
84
168
OH
H2N
NH
NH2
28
18 41 56 59 69 97 126 114 140 151 185
(mainlib) Glycyl-l-glutamine

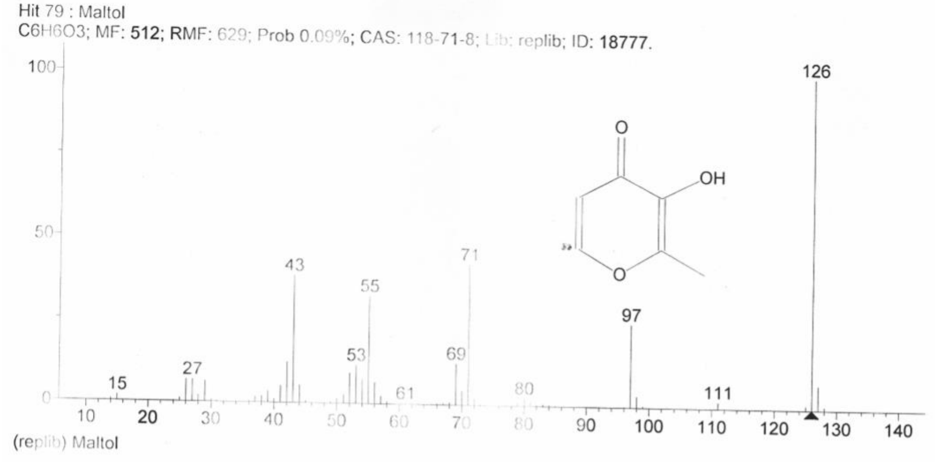

Hit 85 : Cyclopentobarbital
C12H14N2O3; MF: 420; RMF: 498; Prob 0.35%; CAS: 76-68-6; Lib: replib; ID: 7791.
(replib) Cyclopentobarbital

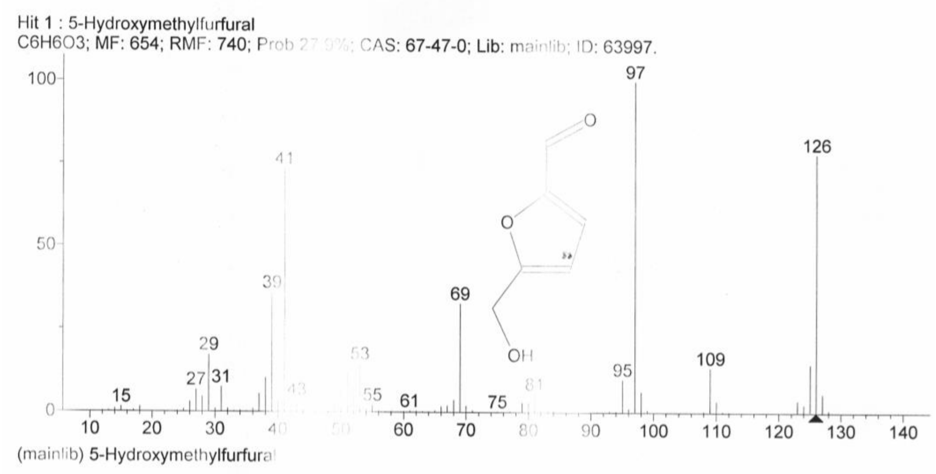

Hit 1 : (S)-(+)-2-Amino-3-methyl-1-butanol
C5H13NO; MF: 476; RMF: 673; Prob 6.48%; CAS: 2026-48-4; Lib: replib; ID: 8784.
(replib) (S)-(+)-2-Amino-3-methyl-1-butanol

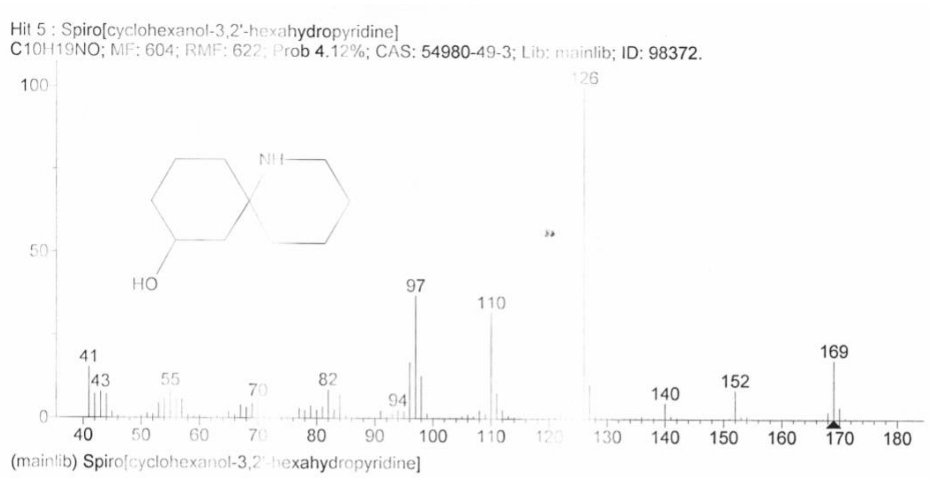

Hit 79 : Secobarbital
C12H18N2O3; MF: 400; RMF: 471; Prob 0.29%; CAS: 76-73-3; Lib: replib; ID: 23920.
(replib) Secobarbital

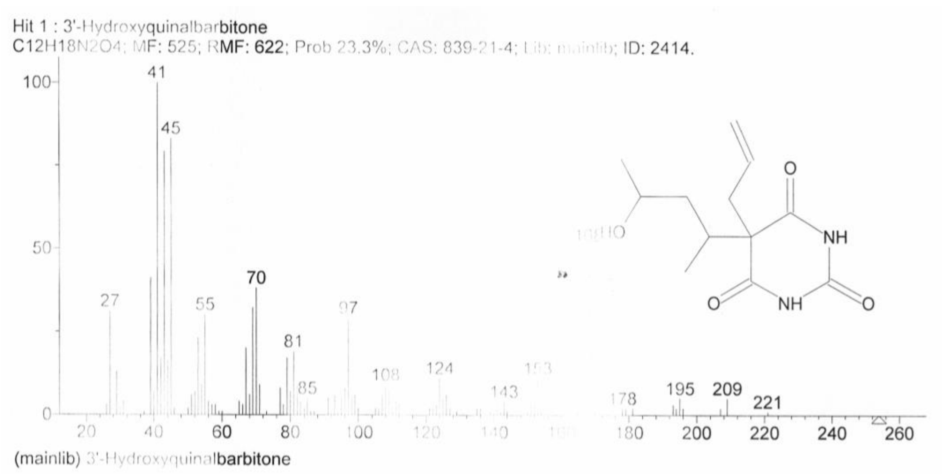

Hit 1 : Valeric acid, 2-tetradecyl ester
C19H38O2; MF: 559; RMF: 650; Prob 5.08%; CAS: 55193-14-1; Lib: mainlib; ID: 49984.
(mainlib) Valeric acid, 2-tetradecyl ester

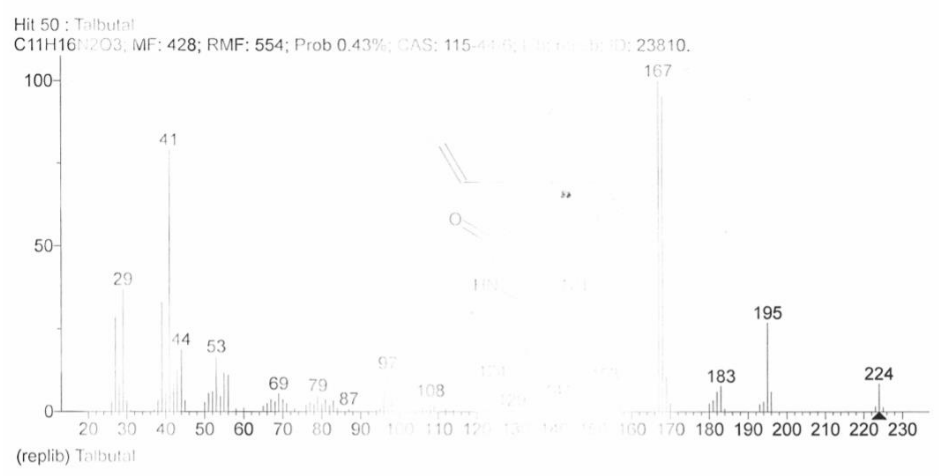

Hit 39 : Tetrahydropyranyl ether of citronellol
C15H28O2; MF: 518; RMF: 551; Prob 1.03%; Lib: mainlib; ID: 49714.
(mainlib) Tetrahydropyranyl ether of citronellol

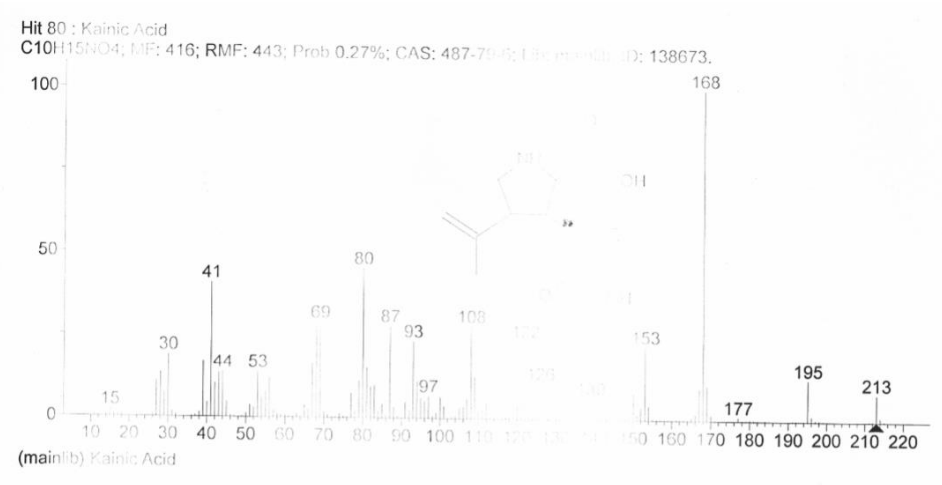

Hit 79 : Maltol
C6H6O3; MF: 512; RMF: 629; Prob 0.09%; CAS: 118-71-8; Lib: replib; ID: 18777.
(replib) Maltol

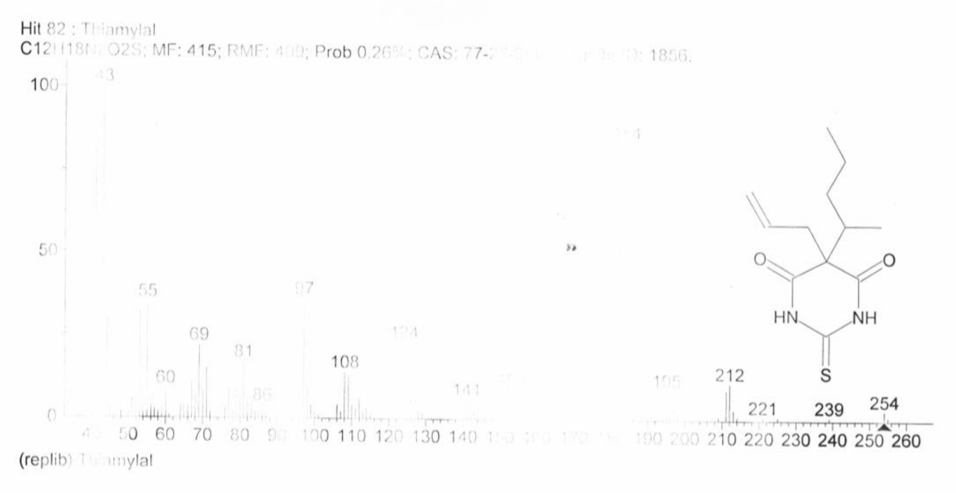

Hit 1 : 5-Hydroxymethylfurfural
C6H6O3; MF: 654; RMF: 740; Prob 27.9%; CAS: 67-47-0; Lib: mainlib; ID: 63997.
(mainlib) 5-Hydroxymethylfurfural

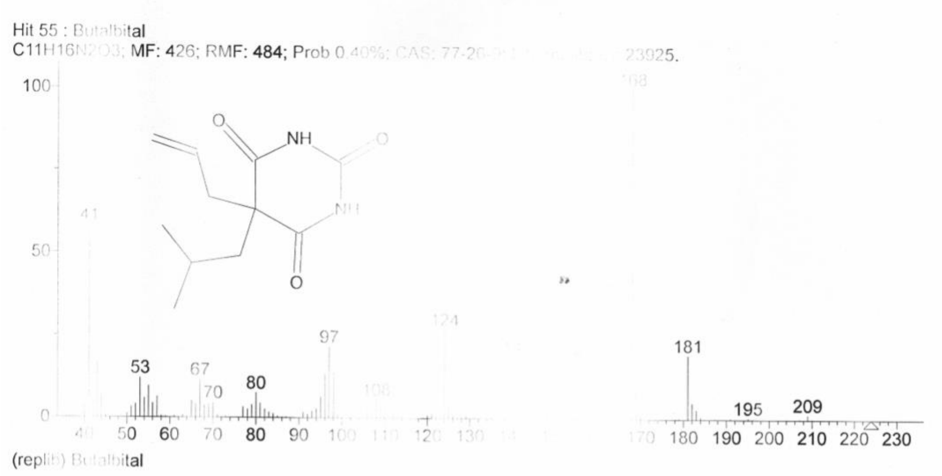

Hit 5 : Spiro[cyclohexanol-3,2'-hexahydropyridine]
C10H19NO; MF: 604; RMF: 622; Prob 4.12%; CAS: 54980-49-3; Lib: mainlib; ID: 98372.
(mainlib) Spiro[cyclohexanol-3,2'-hexahydropyridine]

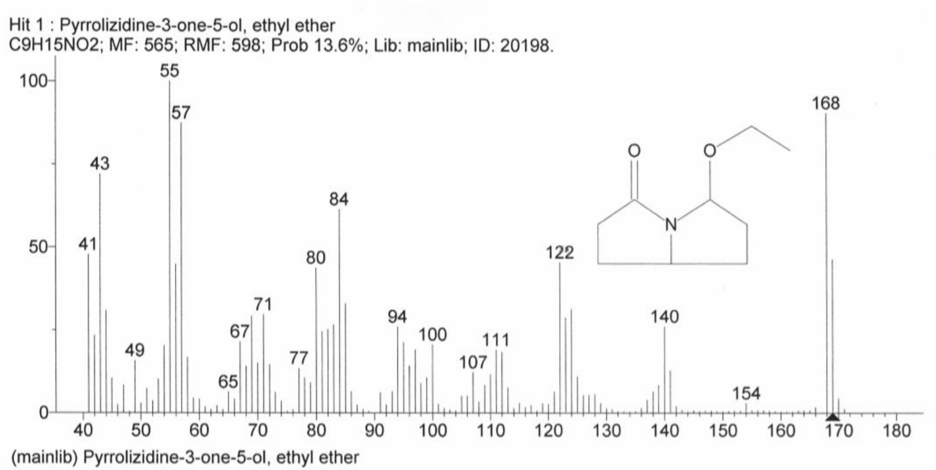

Hit 1 : 3'-Hydroxyquinalbarbitone
C12H18N2O4; MF: 525; RMF: 622; Prob 23.3%; CAS: 839-21-4; Lib: mainlib; ID: 2414.
(mainlib) 3'-Hydroxyquinalbarbitone

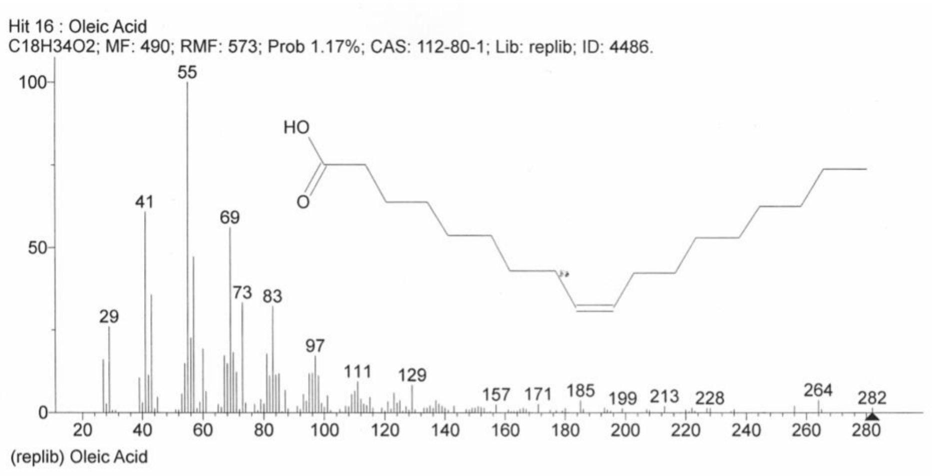

Hit 50 : Talbutal
C11H16N2O3; MF: 428; RMF: 554; Prob 0.43%; CAS: 115-44-6; ID: 23810.
(replib) Talbutal

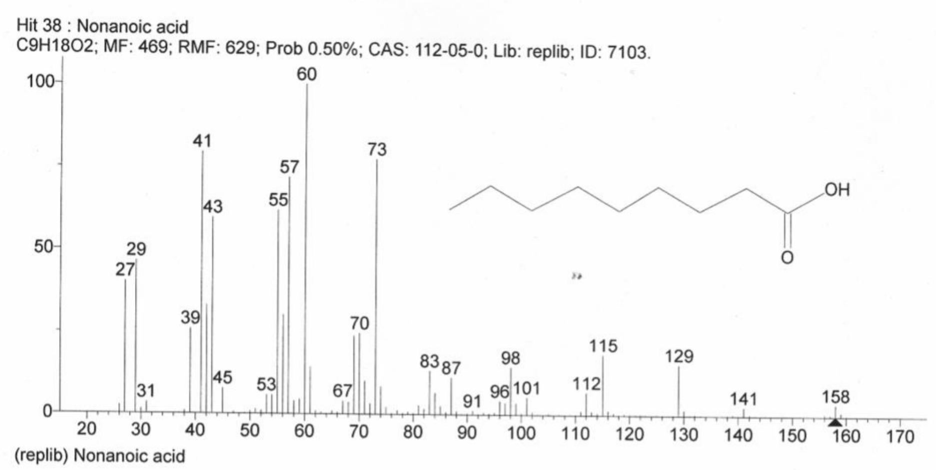

Hit 80 : Kainic Acid
C10H15NO4; MF: 416; RMF: 443; Prob 0.27%; CAS: 487-79-6; ID: 138673.
(mainlib) Kainic Acid

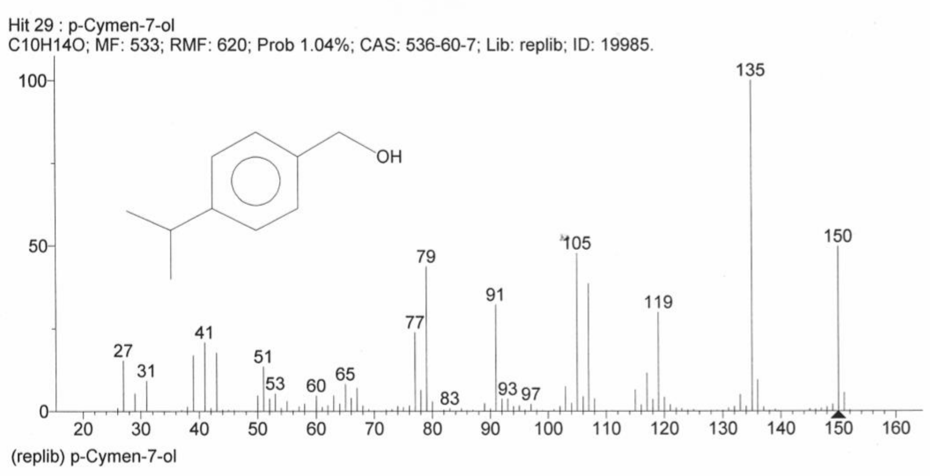

Hit 82 : Thiamylal
C12H18N2O2S; MF: 415; RMF: 409; Prob 0.26%; CAS: 77-27-0; ID: 1856.
(replib) Thiamylal

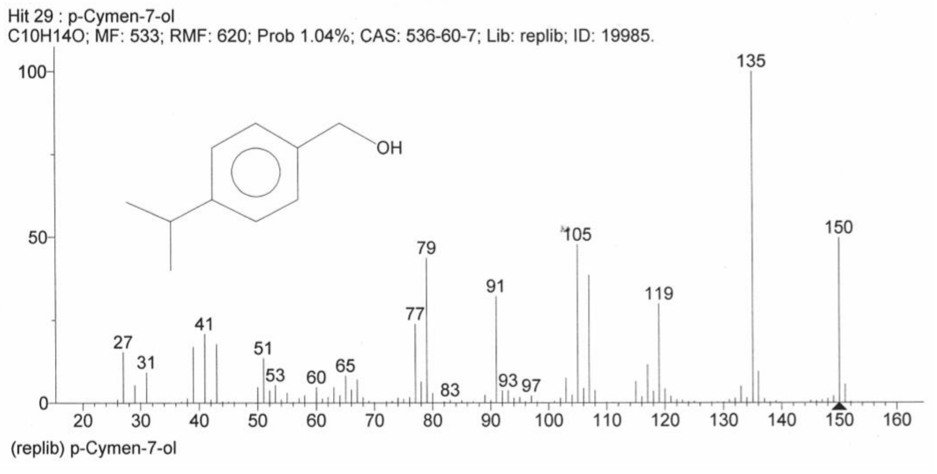

Hit 55 : Butalbital
MF: 426; RMF: 484; Prob 0.40%;
23925.
100
50
0
NH
O
O
O
41
53
67
70
80
97
124
181
195
209
50
60
70
80
90
180
190
200
210
220
230
(replib) Butalbital

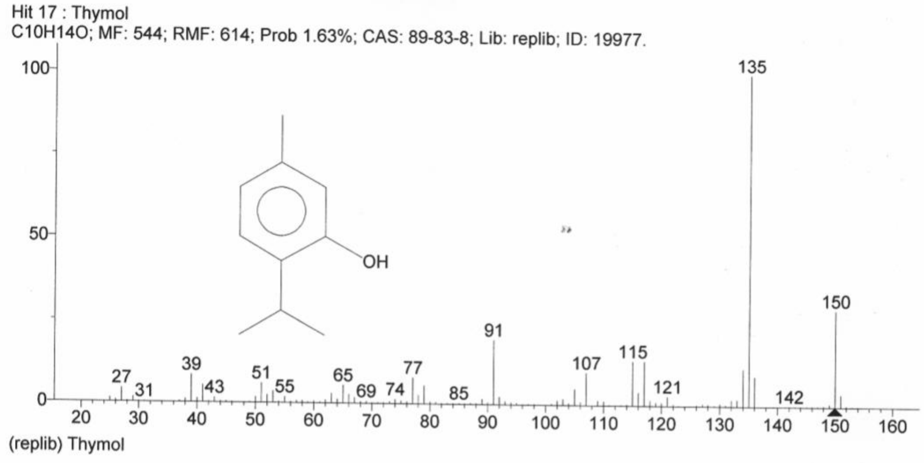

Hit 1 : Pyrrolizidine-3-one-5-ol, ethyl ether
C9H15NO2; MF: 565; RMF: 598; Prob 13.6%; Lib: mainlib; ID: 20198.
100
50
0
55
57
43
41
49
65
67
71
77
80
84
94
100
107
111
122
140
154
168
O
O
N
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
(mainlib) Pyrrolizidine-3-one-5-ol, ethyl ether

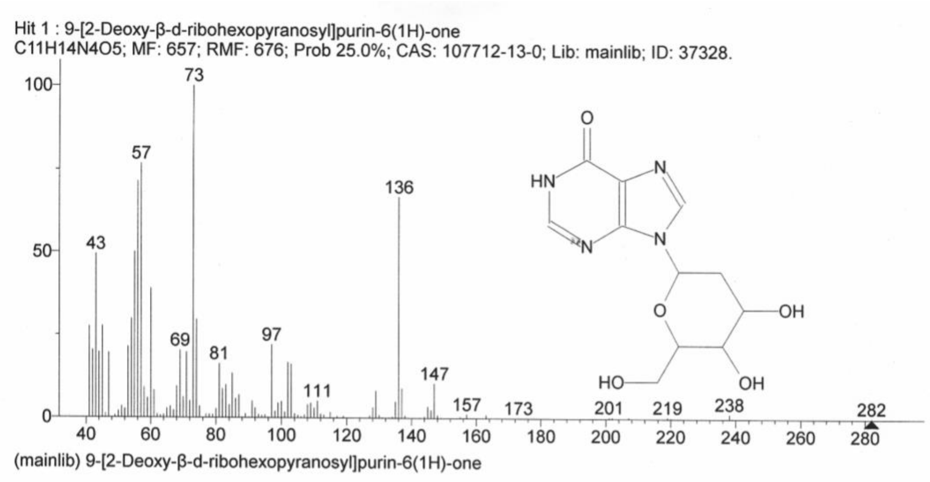

Hit 16 : Oleic Acid
C18H34O2; MF: 490; RMF: 573; Prob 1.17%; CAS: 112-80-1; Lib: replib; ID: 4486.
100
50
0
55
41
69
73
83
29
97
111
129
157
171
185
199
213
228
264
282
HO
O
20
40
60
80
100
120
140
160
180
200
220
240
260
280
(replib) Oleic Acid

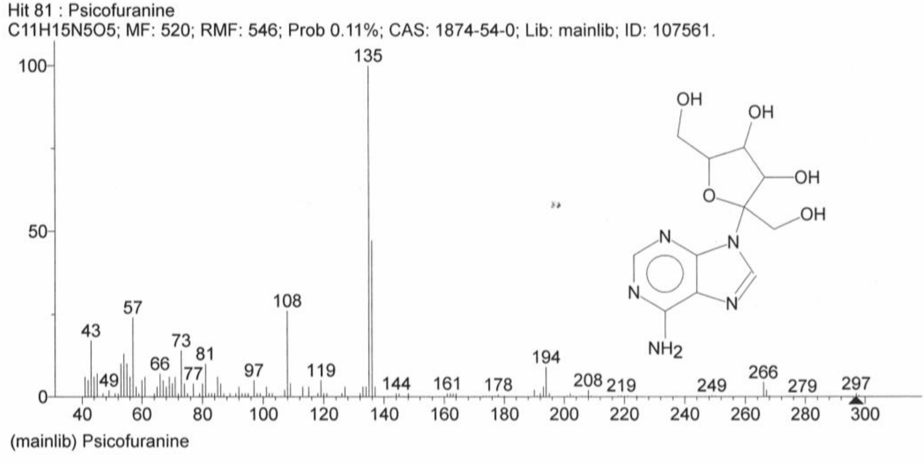

Hit 38 : Nonanoic acid
C9H18O2; MF: 469; RMF: 629; Prob 0.50%; CAS: 112-05-0; Lib: replib; ID: 7103.
OH
O
(replib) Nonanoic acid

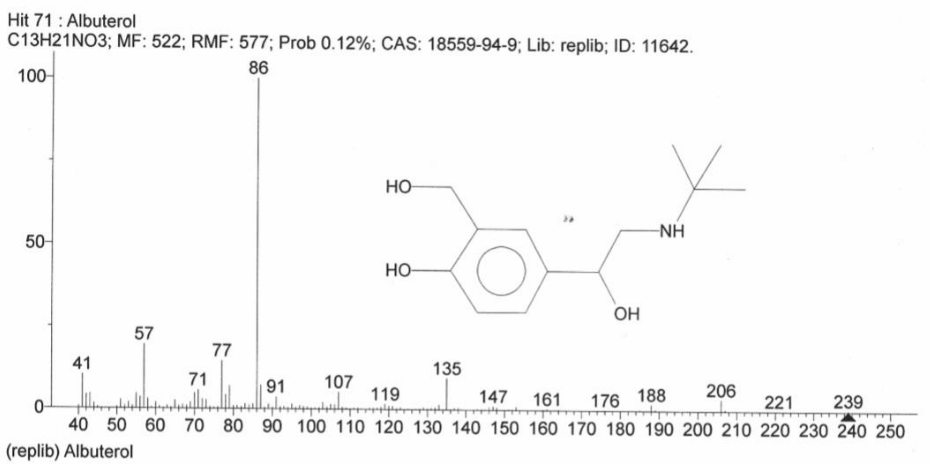

Hit 29 : p-Cymen-7-ol
C10H14O; MF: 533; RMF: 620; Prob 1.04%; CAS: 536-60-7; Lib: replib; ID: 19985.
OH
(replib) p-Cymen-7-ol

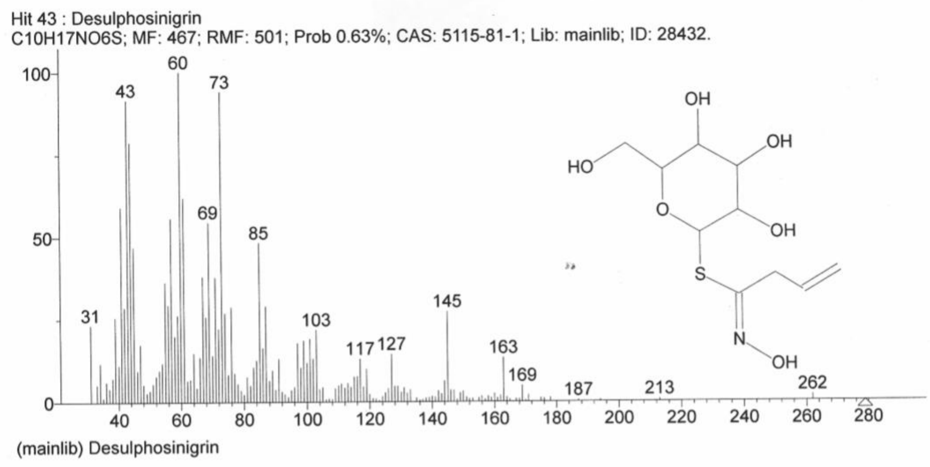

Hit 29 : p-Cymen-7-ol
C10H14O; MF: 533; RMF: 620; Prob 1.04%; CAS: 536-60-7; Lib: replib; ID: 19985.
OH
(replib) p-Cymen-7-ol

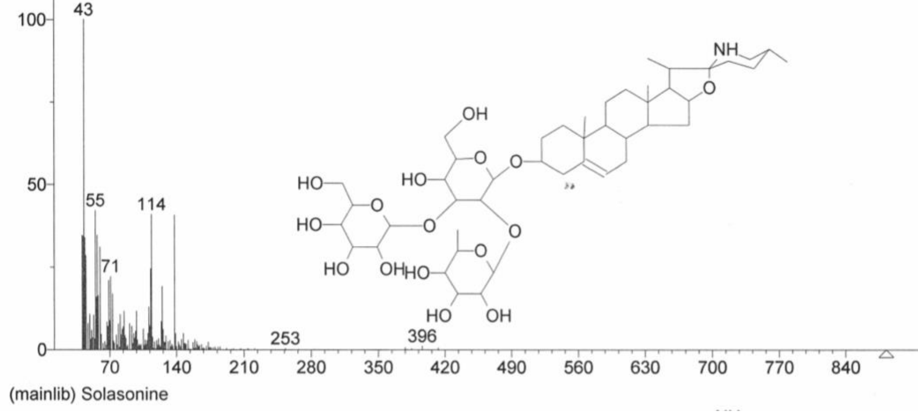

Hit 17 : Thymol
C10H14O; MF: 544; RMF: 614; Prob 1.63%; CAS: 89-83-8; Lib: replib; ID: 19977.
OH
(replib) Thymol

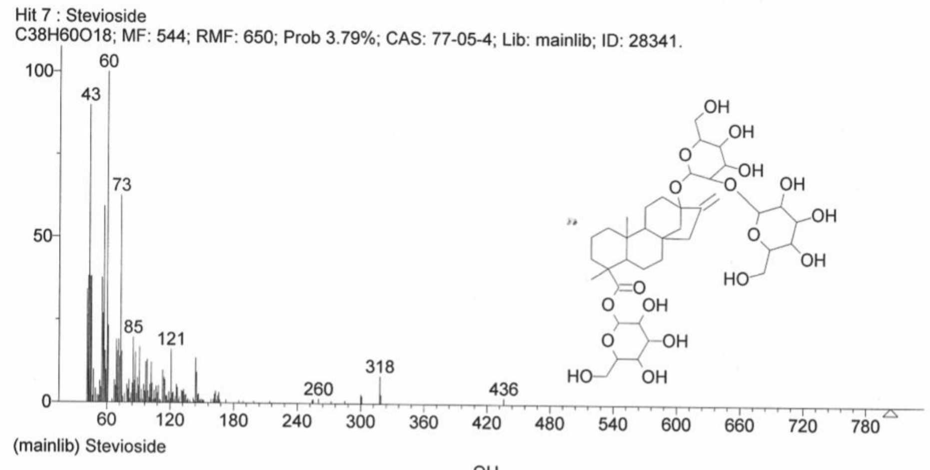

Hit 1 : 9-[2-Deoxy-β-d-ribohexopyranosyl]purin-6(1H)-one
C11H14N4O5; MF: 657; RMF: 676; Prob 25.0%; CAS: 107712-13-0; Lib: mainlib; ID: 37328.
HN
N
O
OH
HO
(mainlib) 9-[2-Deoxy-β-d-ribohexopyranosyl]purin-6(1H)-one

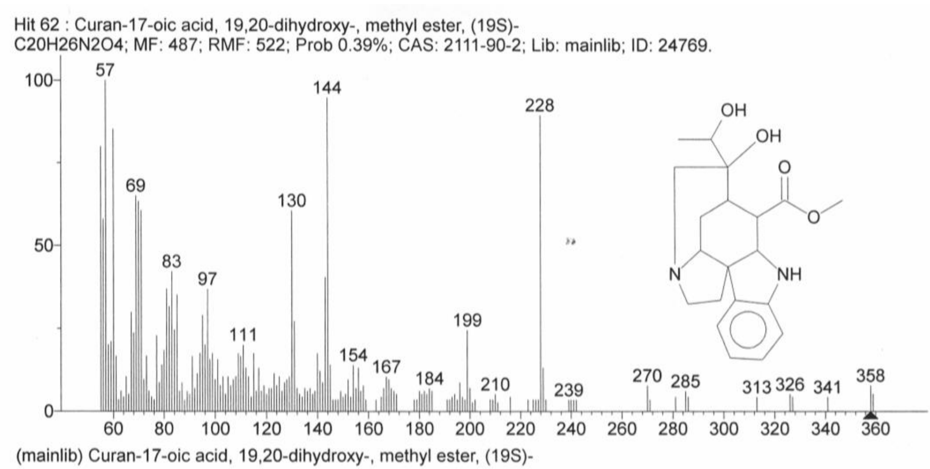

Hit 81 : Psicofuranine
C11H15N5O5; MF: 520; RMF: 546; Prob 0.11%; CAS: 1874-54-0; Lib: mainlib; ID: 107561.
OH
N
NH2
(mainlib) Psicofuranine

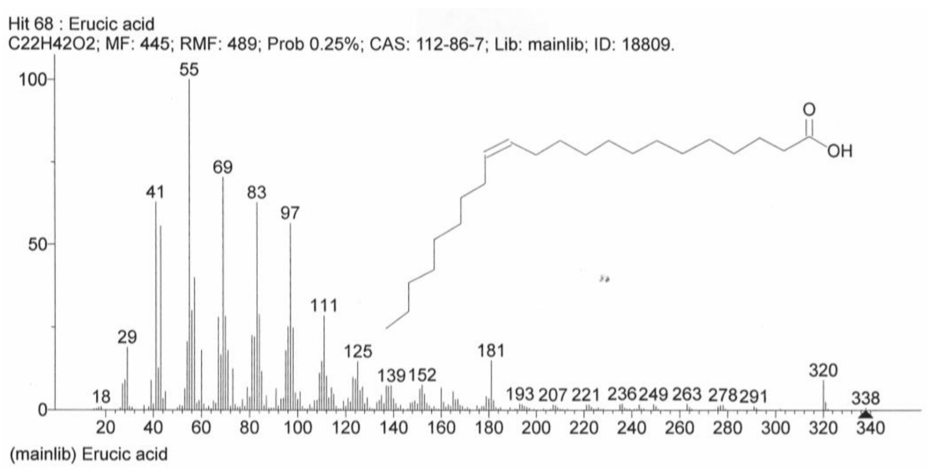

Hit 71 : Albuterol
C13H21NO3; MF: 522; RMF: 577; Prob 0.12%; CAS: 18559-94-9; Lib: replib; ID: 11642.
(replib) Albuterol


Hit 43 : Desulphosinigrin
C10H17NO6S; MF: 467; RMF: 501; Prob 0.63%; CAS: 5115-81-1; Lib: mainlib; ID: 28432.
(mainlib) Desulphosinigrin


Hit 39 : Solasonine
C45H73NO16; MF: 468; RMF: 552; Prob 0.66%; CAS: 19121-58-5; Lib: mainlib; ID: 7066.
(mainlib) Solasonine


Hit 7 : Stevioside
C38H60O18; MF: 544; RMF: 650; Prob 3.79%; CAS: 77-05-4; Lib: mainlib; ID: 28341.
100
50
0
43
60
73
85
121
260
318
436
60 120 180 240 300 360 420 480 540 600 660 720 780
(mainlib) Stevioside
OH
O
HO


Hit 62 : Curan-17-oic acid, 19,20-dihydroxy-, methyl ester, (19S)-
C20H26N2O4; MF: 487; RMF: 522; Prob 0.39%; CAS: 2111-90-2; Lib: mainlib; ID: 24769.
100
50
0
57
144
228
69
130
83
97
111
199
154
167
184
210
239
270
285
313
326
341
358
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360
(mainlib) Curan-17-oic acid, 19,20-dihydroxy-, methyl ester, (19S)-
OH
O
N
NH


Hit 68 : Erucic acid
C22H42O2; MF: 445; RMF: 489; Prob 0.25%; CAS: 112-86-7; Lib: mainlib; ID: 18809.
100
50
0
55
69
41
83
97
111
29
125
181
139
152
320
18
193 207 221 236 249 263 278 291
338
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
(mainlib) Erucic acid
O
OH

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