Cytotoxicity of the Chinese Herbal Remedy *Oldenlandia diffusa* and its anti-cancer effective constituents

Thesis

Submitted for the degree of Doctor of Philosophy

Munkhchimeg Ganbold

Faculty of Science

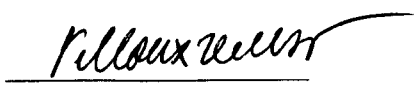
School of Pharmacy and Chemistry

Kingston University London, UK

March 2010

DECLARATION

This thesis entitled "**Cytotoxicity of the Chinese Herbal Remedy *Oldenlandia diffusa* and its anti-cancer effective constituents**" is based upon work conducted by the author in the School of Pharmacy and Chemistry at Kingston University London between May 2007 and March 2010. All of the work described herein is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other universities.



Munkhchimeg Ganbold

To my parents

Acknowledgements

I wish to thank to my supervisors Dr. James Barker, Dr. Mark Carew (2008-2010), Dr. Lucy Jones and Dr. Elizabeth Opara (2007-2008) for their advice, guidance and encouragement.

I would like to thank to Professor John Brown for the provision of the research facilities.

Also, I am grateful to Dr. Asoka Bandara and Richard Gurner for the warm reception and kind help on instruments.

I thank to all the technical staffs, foremost, Dr. Julian Swinden, Bert Bunby and Dr. Jean-Marie Peron for their advice and friendship.

Thankfull to Carole Brosseau (St. George's Hospital Medical School, UK) and Dr. Maggie Liu (Cancer Research Institute, UK) for their kind help and advice on Western blotting and NMR spectroscopic instruments.

I am also grateful to my all colleagues in EM 213 and my friends for their honest and kind friendship.

In addition, I would truly like to take this opportunity to thank my mother Mrs. Shijee Sanjid, my husband Dr. Chuluunzorigt Baz, my twin daughters Miss Tsogzolboot Chuluunzorigt, Miss Tsogjavkhlant Chuluunzorigt and my relatives. Without their love, support, encouragement and endless patience, I would not have done all this work.

In conclusion, I kindly acknowledge the fellowship from my Mongolian Government and the financial support from my husband for giving me the opportunity to do this research work in this country.

Abstract

The main objective of the study was to investigate the bioactive components, their effect on HL60 leukaemic and Caco-2 colon carcinoma cancer cell lines and their bioavailability from a decoction of a Chinese Herbal Remedy *Oldenlandia diffusa*, using a Caco-2 monolayer as a mimic for intestinal absorption.

The HPLC separation method was set up by investigating parameters such as column type, mobile phase, isocratic/gradient elution, flow rate, pH of buffer and detection wavelength. Using this method eleven fractions (F1-F11) were collected. Results from a cytotoxicity investigation using the CyQUANT NF, trypan blue and neutral red uptake assays showed that the decoction has a cytotoxic effect on HL60 (V=13.5±4.3%, n=3) and Caco-2 (V=50.0±1.4%, n=3) cancer cell lines. The most cytotoxic active fraction was F9 (V=62.2±7.3% (HL60) and V=32.1±7.9%, n=3 (Caco-2)). DAPI staining and Western blotting (detection of cleaved-PARP) studies on the decoction and F9 fraction indicated that mode of cell death was apoptosis, which was mediated by a caspase cascade. Fraction F9 was separated into eight further fractions (compound-fraction-1 to 8) by optimisation of the HPLC method. By using liquid-liquid extraction with ethyl acetate, eleven subfractions (compound-fraction-1 to 8 and ethyl acetate fractions (FEA-1 to 3) were collected purely. All collected fractions were analysed by high resolution-MS and their MWs were determined as 268.07, 238.07, 242.24, 280.38, 256.24, 282.25, 284.27, 456.36, 550.17, 328.22 and 330.24.

In the most cytotoxic fraction F9, oleanolic acid and ursolic acid were isolated and identified in concentrations of 0.068 mg/g and 0.166 mg/g, respectively. FEA-1 to 3 also showed cytotoxic effects on these cancer cells (V=53.3±2.2%, 55.4±4.4% and 50.6±11.3% (HL60) and V=63.4±13.5%, 45.8±5.9%, and 59.5±9.7%, respectively (Caco-2)). Fraction FEA-1 was identified as *E*-6-*O*-*p*-coumaroyl scandoside methyl ester (MW 550.17). Cytotoxicity assessment results showed that it has a growth inhibition effect on both cancer cell lines.

Bioavailability after ingestion of the decoction was studied using 21-day grown Caco-2 monolayers. The post absorption sample (PAS) of the decoction and fraction F9 were shown to have a good permeability (P_{app}=3.575x10^{-6} cm/s). The PAS also has a cytotoxic effect on cancer cells (V=67.0±7.5%, n=3). When analysed by LC-MS, the most of the compounds that had previously been seen in the original fractions were again observed.

Contents

List of Figures

examined by confocal fluorescence microscopy after DAPI staining. Examination on HL60 cells identifying the characteristic morphological features, (a)-Untreated control cells have remained intact, (b)-Actinomycin-D-treated cells, (c)-*Oldenlandia diffusa* decoction treated cells and (d)-F9 treated cells show characteristic blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (magnification, x400).

Figure 42. Image of confocal fluorescence microscopy of HL60 cells identifying the characteristic morphological features, (a)-control (untreated) cells (48 hr incubation) have remained intact, (b)-fraction F9 treated cells (48 hr incubation) show characteristic of cell shrinkage, blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (changed) (magnification, x400).

Figure 43. Morphological changes of apoptotic cell death of the Caco-2 cancer cells treated with *Oldenlandia diffusa* decoction, fraction F9 and actonimycin-D for 24 hr incubation was examined by confocal fluorescence microscopy after DAPI staining. Examination on HL60 cells identifying the characteristic morphological features, (a)-Untreated control cells have remained intact, (b)-Actinomycin-D-treated cells, (c)-*Oldenlandia diffusa* decoction treated cells and (d)-F9 treated cells show characteristic blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (magnification, x400).

Figure 44. Confocal fluorescence microscopy of HL60 cells identifying the characteristic morphological features, (a)-Untreated control cells (48 hr incubation) have remained intact, (b)-fraction F9 treated cells (48 hr incubation) show characteristic of cell shrinkage, blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (changed) (magnification, x400).

Figure 45. Effect of *Oldenlandia diffusa* decoction on HL60 cell line for investigation of apoptosis cell death by Western blotting. The HL60 cancer cells were treated with *Oldenlandia diffusa* decoction under the culturing condition for 24 and 48 h. Lane 1-Negative control (medium)-24 h, lane 2-*Oldenlandia diffusa* decoction-24 h, lane 3-Negative control (medium)- 48 h, lane 4-*Oldenlandia diffusa* decoction-48 h. Equal amounts of the total proteins (20 μg/lane) were subjected to 10% SDS-PAGE. The expression of

List of Tables

List of abbreviation

AAB	Ammonium acetate buffer
ACN	Acetonitrile
AD	Actinomycin-D
ADMET	The absorption, distribution, metabolism, excretion and toxicity
ATP	Adenosine triphosphate
ATP	Adenosine triphosphate
BrdU	Bromodeoxyuridine
Caco-2	Colon adenocarcenoma cancer cells
CAM	Alternative medicine or complementary medicine
CAPS	*N*-cyclohexyl-3-aminopropanesulfonic acid
Cdk	Cyclin-dependent kinases
CHES	*N*-Cyclohexyl-2-aminoethanesulfonic acid
CHM	Chinese Herbal Medicine
CV-PARP	Cleaved PARP
DAPI	4,6'-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
D-PBS	Dulbecco's Phosphate-Buffered Saline
EA	Ethyl actate extract
ECACC	The European Collection of Cell Cultures
ECL	Enhanced Chemiluminescence
ESI	Electrospray ionization
FBS	Fetal bovine serum
HBSS	Hank's Balanced Salt Solution,
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid
HEPPS	3-[4-(2-Hydroxyethyl)-1-piperazinyl]propanesulfonic acid
HL60	Leukaemic cell line
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography- Mass Spectrometry
M1	Moon 1 method
M2	Moon 2 method
M3	Moon 3 method

M4	Moon 4 method
M5	Moon 5 method
M6	Moon 6 method
M7	Moon 7 method
Ms	Moon separation method
Msp	Moon separation-preparative method
MALDI	Matrix-assisted laser desorption/ionization
MCP	Micropannel plate detectors
MOPS	3-(N-morpholino)propanesulfonic acid
MES	2-(4-morpholino)-ethane sulfonic acid
MS-MS	Tandem Mass Spectrometry
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or tetrazole
NaOH	Sodium hydroxide
NEAAs	Non-essential amino acids
NMR	Nuclear Magnetic Resonance
NP	Normal Phase
NRU	Assay Neutral Red Uptake Assay
O.diffusa	*Oldenlandia diffusa*
OA	Oleanolic acid
PAGE	PolyAcrylamide Gel Electrophoresis
PARP	(poly-ADP ribose polymerase)
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PFP(2)	Pentafluorophenylpropyl(2)
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
PTEN	Phosphatase and Tensin homolog
PVDF	Polyvinylidene difluoride
Q-TOF	Quadrupole-time of flight
Rb protein	Retinoblastoma protein
RIPA	Radioimmunoprecipitation assay (RIPA)
RP	Reverse Phase
SDS	Sodium dodecyl sulphate
SPE	Solid Phase Extraction
TCM	Traditional Chinese Medicine

TEER	Transepithelial electrical resistance
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TM	Traditional medicine
TOF	Time of flight
UV	Ultraviolet
UA	Ursolic acid
V%	Viability percent
WB	Western blotting

Chapter 1. Introduction

Chapter 1. Introduction

1.1. Traditional medicine

Traditional medicine (TM) has a 5000 year history. During this period, traditional medicine included the knowledge and practices of medicine which were based on the theories, skills, experiences and beliefs. TM was used in the prevention, diagnosis, and treatment of illness [WHO, 2000].

According to the World Health Organization (WHO), traditional medicine is defined as:

> "...the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being..." [WHO, 2003].

Despite its common use in Asia, Africa and Latin America, TM is not integrated into the main controlling health care system [WHO, 2000]. Since the 1990s, usage of TM has become more popular in many countries. In some countries, it is used with names such as "alternative medicine " or "complementary medicine" medicine (CAM).

Traditional medicines include practices such as herbal medicine, Ayurvedic medicine, Unani medicine, acupuncture, spinal manipulation, Siddha medicine, Traditional Chinese medicine, South African Muti, Yoruba Ifa, as well as other medical knowledge and practices. In this project, one plant, *Oldenlandia diffusa*, used in traditional Chinese herbal medicine was studied. Thus, in this chapter, herbal medicine and traditional Chinese herbal medicine practice will be discussed.

1.2. Herbal Medicine

Herbal medicine is known as herbalism, botanical medicine, medical herbalism, herbology, and phytotherapy. Herbal medicine is a traditional medicine (folk medicine) that is based on the use of herbs and herbal medicines [Deepak & Anshu, 2008]. There are many medicines that originate from plants. For example, vinca alkaloids derived from Madagscan periwinkle plant *Catharantus roseus*, aspirin from the bark of willow trees, the painkiller morphine from poppies, taxanes derived from the Pacific yew *Taxus brevifolia*,

camptothecins from the Chinese tree *Camptotheca acuminata*, and digoxin from foxgloves [Dennis *et al.*, 2000], [Bupa's health information, 2009].

Generally, herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products. Herbs involve whole or fragmented or powdered plant materials such as flowers, fruit, seed, leaves, stems, wood, bark, roots, rhizomes or other parts of plant. They are the basis for finished herbal products. It can include powdered herbal materials, extracts, decoctions, tinctures and fatty oils of herbal materials, which are produced by extraction, boiling, fractionation, purification, concentration or by other processes. In herbal preparations, one or more herbs may be used and this is called a 'finished herbal product' [Lai *et al.*, 2005], [WHO, 2010].

In addition, if there are more than one herb used, it is generally named as a 'herbal mixture product'. These finished herbal products and mixture herbal products can additionally contain excipients to the active ingredients, but the exact amount of the active ingredients should be standardized, if appropriate, by analytical methods available.

1.3. History and theory of Traditional Chinese Medicine

The full theoretical system of Traditional Chinese Medicine (TCM) was established 2,300 years ago, however, it has several thousand years' history. TCM was formed to diagnose and cure illness. TCM theory is very complex, and the main theories of TCM include those of Yin-yang, the Five-element theories, the human body's canal system, six confirmations and four layers. The clinical diagnoses and treatments in TCM mainly based on the Yin-yang and Five-element theories. In TCM, these theories concern the physiological activities and pathological changes of the human body and its interactions.

1.3.1. The theory of Yin-yang

Yin and Yang are the two basic terms of the traditional Chinese philosophy, and are also used in the theory of the traditional Chinese medical science. Yin and Yang express a negative and positive influence. According to the traditional Chinese philosophy, all things of the world are based on and are defined by Yin or Yang. For example, water belongs to Yin, while fire belongs to Yang. All things that relate to cold, inhibition and condensation belong to Yin, whereas all things that relate to warmth, brightness and evaporation belong to Yang.

Furthermore, Yin and Yang belong to Qi (chi) which is the smallest substance or the most basic material to form a body. So, Qi includes Yang and Yin, and is called Yang-Qi and Yin-Qi. In the body, if Yang-Qi and Yin-Qi are in imbalance, then people become ill. For example, in the case of excessive Yin-Qi, the body will feel cold, the tongue fur will be white and thick and faeces watery. If Yin-Qi is deficient, the body will feel cold and the tongue fur will be less. Therefore, the treatment is based on the weakening or increasing of the deficiency or excessivity of Yang-Qi and Yin-Qi by traditional Chinese medicines [Wang, 2006], [Ergil, 2002].

In the Book of History by Zuozhuan, Chapter 'Duke Shao', Yin and Yang are first defined as two of six Qi, and it follows:

> "There are six heavenly influences [*qi*] which descend and produce the five tastes, go forth in the five colours, and are verified in the five notes; but when they are in excess, they produce the six diseases. Those six influences are denominated the *yin*, the *yang*, wind, rain, obscurity, and brightness. In their separation, they form the four seasons; in their order, they form the five (elementary) terms. When any of them is in excess, they ensure calamity. An excess of the *yin* leads to diseases of cold; of the *yang*, to diseases of heat..." [Legge, 1994], [Internet Encyclopedia of Philosophy, 2009].

1.3.2. Five-Element theory

Yin-yang theory was extended to the Five-element theory. In the Era of Warring States (476 BCE-221 BCE), Zou Yan, who was the representative of the School of Yin-Yang, developed the theory of the Five-Element and connected it to the theory of Yin-yang [Yao, 2010]. According to this theory, there are basic five elements such as wood, fire, earth, metal, water, and they were formed by Qi. Each element corresponds to organs of the body such as wood corresponds to liver, fire is to heart, earth is to spleen/pancreas, metal is to lung and water is to kidney [Bellavite *et al.*, 1998]. These five elements are connected to each other by circular influences (Figure 1).

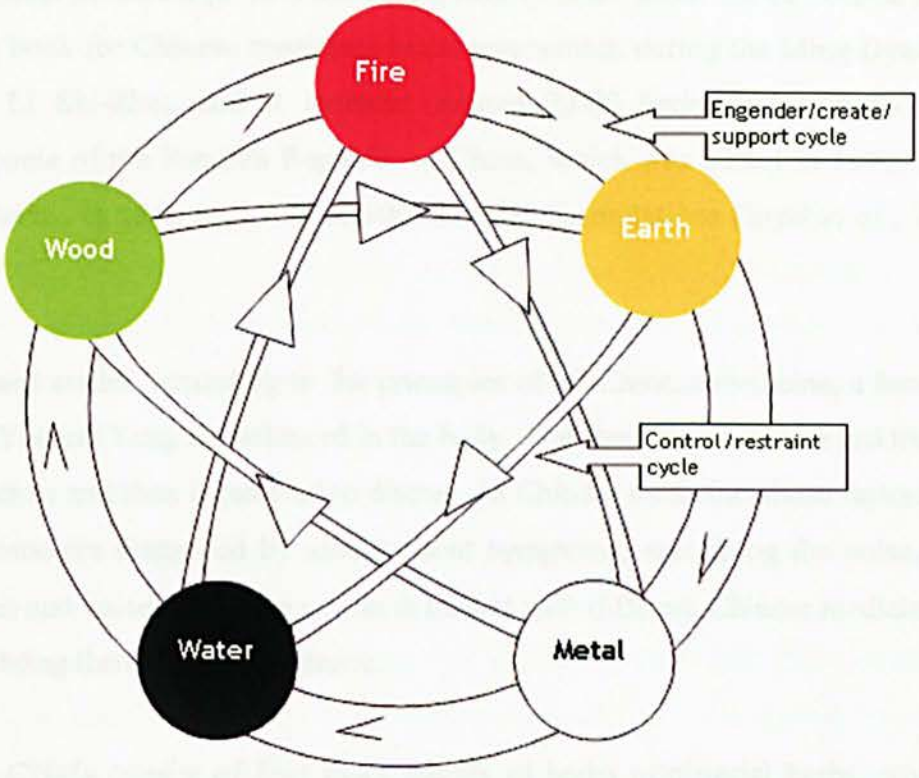


Figure 1. Representation scheme of Five-element Theory. The five elemental energies play a role in the Chinese herbal system. The creation and control cycles interact constantly to maintain an overall balance in the system.
[Taken from http://www.surfacingwellness.com/images/cycle.PNG]

The relations of the five elements interact in a cycle. Wood promotes fire, fire promotes earth, earth promotes metal, and metal promotes water. In addition, they control each other: water controls fire, fire controls metal, metal controls wood, wood controls earth while earth controls water. If the balance between these five elements are lost within the body, this can result in illness [Teng *et al.*, 2006].

Generally, TCM involves methods such as Chinese Herbal Medicine, Chinese food therapy, Cupping, Gua Sha, Moxibustion, Physical Qigong exercises such as tai chi chuan, Standing Meditation, Yoga, Brocade Ba Duan Jin exercises and esoteric methods, which are based on personal beliefs or specializations such as Fengshui and Bazi [Jahnke, 1991].

1.4. Chinese Herbal Medicine

Chinese Herbal Medicine (CHM) has a long history since about the 12th century A.D. until today. The book for Chinese medicinal herbs was written during the Ming Dynasty (1152-1578) by Li Shi-Zhen and it includes around 2,000 herbs and extracts. Later, the Pharmacopoeia of the People's Republic of China, which was edited in 1990, listed more than 500 herbs, extracts and around 300 complex formulations [Ergil *et al.*, 2002], [Ko, 1998].

As mentioned earlier, according to the principles of all Chinese medicine, a human will be healthy, if Yin and Yang are balanced in the body. But this balance can be lost by internal or external factors and then it can lead to disease. In Chinese medicine, those factors which are out of balance are diagnosed by asking about symptoms, examining the pulse, skin, hair, tongue, eyes and voice. Then, the patient is treated with different Chinese medicinal methods in order to bring them back into balance.

Generally, CHMs consist of four main groups of herbs ministerial herbs, which are the main effective herbs for the disease. Deputy herbs assist the ministerial herb or act for coexisting conditions. Assistant herbs are there to reduce the side effects of the ministerial and deputy herbs, and envoy herbs direct the therapy to a particular part of the body. The dosage forms of TCMs are mainly pills, powders, chopped slices and tablets [Ko, 1998]. For example, Chinese Pharmacopoeia (2005 edition) contains a list of 223 pills [Zhang *et al.*, 2008].

Chinese medicine is successfully used for a wide range of conditions or diseases such as skin disease (including eczema, acne, rosacea, urticaria), gastro-intestinal disorders (e.g. irritable bowel syndrome, chronic constipation), in gynaecology (including pre-menstrual syndrome and dysmenorrhoea, endometriosis, infertility), hepatitis and chronic fatigue syndromes, respiratory diseases (e.g. asthma, bronchitis and chronic coughs, allergic and perennial rhinitis, sinusitis), rheumatology (e.g. osteoarthritis and rheumatoid arthritis), as well as chronic cystitis, psychological problems (e.g. depression, anxiety) and children's diseases.

There are some individual herbs or extracts, but usually combinations of herbs are used for the prevention and treatment of cancer, however, more research is needed to determine the effectiveness of these herbs and their substances.

1.5. Cancer

Cancer is the Latin word for *crab* and the meaning of the word is a malignancy. Sometimes cancer is referred to as a malignancy, a malignant tumour or a neoplasm. Cancer is the uncontrolled proliferation of abnormal cells and in some cases, the metastasis of cells from one area of the body to another. There are more than 100 different groups of cancers [Cancer, 2004]. Worldwide cancer is a most common and serious disease. There were 7.6 million people in the world and 155,484 people in the UK who died of cancer in 2007 [Cancer Research, 2009].

Cancer is caused by abnormalities in the genome of the cells and their growth becomes uncontrolled and their metabolism is dysfunctional as the main two genes, namely tumour-suppressor genes that control the cell proliferation, and caretaker genes that control cell mutation [Kinzler & Vogelstein, 2002].

Carcinogens such as tobacco smoke, radiation, chemicals or other infectious agents can increase the risk of getting cancer by changing the cellular metabolism or damaging DNA in cells and leading to uncontrolled malignant cell division and formation of tumours. Typically, serious damage of DNA leads to programmed cell death, but if the cell cannot repair itself or the programmed cell death pathway is damaged, then it becomes a cancerous cell [Kerr *et al.*, 1972], [Roos & Kaina, 2006].

In cancer, there are two general classes of genes, oncogenes and tumour-suppressor genes that affect genetic abnormality of cells. In cancer cells, cancer-promoting oncogenes are activated and they give the cell new properties such as rapid growth and rapid cell division activities, protection against apoptosis, as well as the loss of boundaries to normal cells. Because of these activities tumor-suppressor genes, e.g. Rb protein (retinoblastoma protein), p53 (protein 53), PTEN (phosphatase and tensin homolog) and PARP 1 (poly-ADP ribose polymerase 1) are inactivated in cells [Polinsky, 2007]. It causes the loss of normal functions in normal cells, including accurate DNA replication, orientation and adhesion within tissues and control of the cell cycle.

1.5.1. Cell cycle

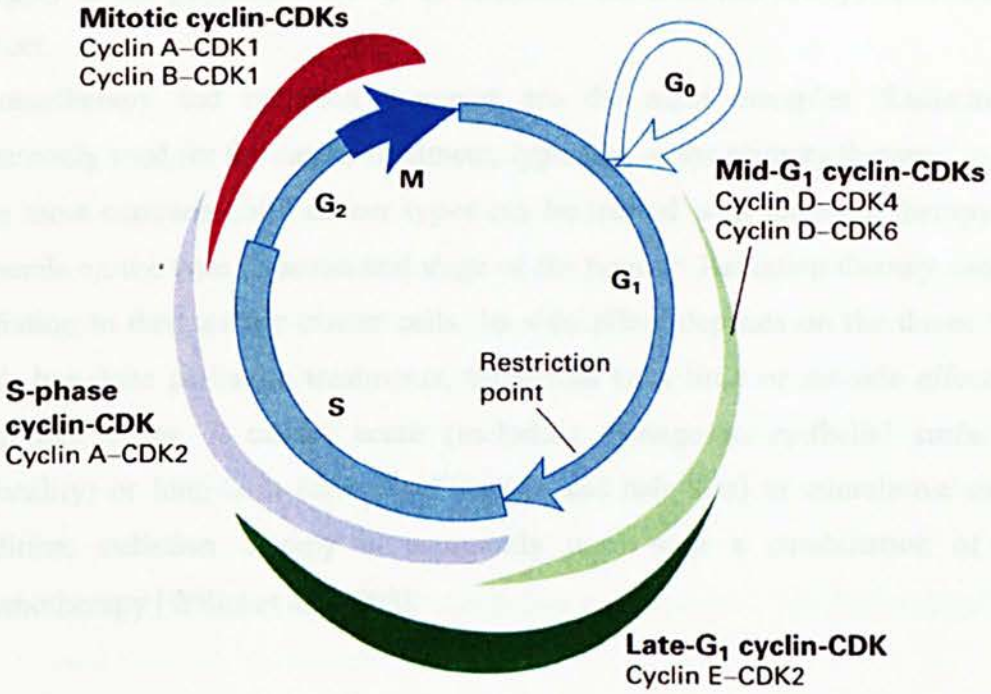
Cell cycling (cell division cycle) is the highly regulated series of events of eukaryotic cell production. Cancer cells have mutations in the genes that control the cell division cycle. Therefore, the DNA replicating and cell dividing processes can be described as a cell cycling process.

The cell division cycle is generally divided into four phases which are G_1 phase (growth), S phase (synthesis), G_2 phase (second growth) and M phase (mitotic). In addition, there is a G_0 (quiescent state) phase when the cell is not dividing. In cell division, cells move from the G_0 phase into the G_1 phase. In this stage, more proteins are produced in the cell. After that, when the cell transfers to the S phase, it replicates the DNA and creates two identical copies for daughter cells. Then, in the G_2 phase, cells continue to grow and synthesize all the proteins which it will need after the division. In the M phase, DNA is separated and then the cell divides into two [Arellano & Moreno, 1997] (Figure 2).

The cell cycle is controlled by proteins in the cytoplasm and are divided into two families:

1. Cyclins. There are four classes of cyclins that are each defined by a stage, but three of them participate in eukaryotic cells:
   - G_1/S cyclins (activate cyclin-dependent kinases (CDKs) in the late G_1)
   - S cyclins (stimulate the chromosome duplication)
   - M cyclins (activate Cdks that stimulate entry into mitosis at the G_2/M checkpoint)
2. Cyclin-dependent kinases (Cdks). There are four classes of Cdks:
   - G_1 Cdk (Cdk-4 and Cdk-6)
   - S Cdk (Cdk-2)
   - M-Cdk (Cdk-1)

In the cell cycle, Cdks promote other proteins to perform their functions by phosphorylating key amino acid residues, while cyclins bind to cyclin-dependent kinases to control their ability to phosphorylate those target proteins [Morgan, 2007], [Bruce *et al.*, 2008].



[Taken from Lodish *et al.*, 2008]

Figure 2. Schematic of the cell cycle and the action of the mammalian Cdk-cyclin complexes during the cell cycle. There are four phases of the cell cycle (G1-M). G1 is between M phase and S phase, while G2 is the gap between S phase and M phase. The width of colored bands is approximately proportional to the protein kinase activity of the indicated complexes.

During these process, each step is controlled by checkpoints in order to produce corect normal cells by preventing or repairing the damage of the cells. If cell division is not in correct regulation, then it turns to cancer. For example, the function of Rb protein is to prevent excessive cell growth by inhibiting cell cycle progression until a cell is ready to divide. It regulates the starting point of cell division by stopping the transcription of genes required for cell division, as well as sequestering the proteins that regulate DNA replication. Lack of or absence of the Rb gene in the regulation of cell division, causes many common types of cancer, such as lung, breast, and bladder cancer [Murphree & Benedict, 1987].

1.5.2. Conventional cancer therapies

Cancers can be treated by surgery, chemotherapy, radiation therapy, immunotherapy or other methods, but it depends on the cancer type, location and stage.

Surgery is the physical removal of tumours, but it is not always successful to remove cancer.

Chemotherapy and radiation therapies are the main therapies. Radiation therapy is commonly used for the cancer treatment, typically, as the primary therapy.

The most common solid cancer types can be treated with radiation therapy, but again it depends on the type, location and stage of the tumour. Radiation therapy uses the ionizing radiation to damage the cancer cells. Its side effect depends on the doses. For example, with low-dose palliative treatments, there will be a little or no side effects. For higher treatment doses, it causes acute (including damage to epithelial surfaces, swelling, infertility) or long-term (including fibrosis and hair loss) or cumulative side effects. In addition, radiation therapy is commonly used with a combination of surgery and chemotherapy [Willet *et al.*, 2003].

Chemotherapy is the use of chemical substances like cytotoxic drugs to treat the cancer. Generally, chemotherapeutic drugs involve the following groups of drugs: alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, monoclonal antibodies and other antitumour agents which affect cell division or DNA synthesis and the cell function. During chemotherapy, the normal cells within the body which are dividing rapidly, e.g. immune cells, bone marrow cells and hair follicle cells are typically affected by those treating chemotherapeutic drugs and it leads to adverse side effects for the patient [Verweij & de Jonge, 2000]. Common side-effects include: nausea, vomiting, diarrhea or constipation, anemia, malnutrition, memory loss, depression of the immune system, hence haemorrhage, infections and sepsis, secondary neoplasms, cardiotoxicity, hepatotoxicity, nephrotoxicity and death.

In addition, CHM treatments are part of medical and health care systems in cancer treatment, however, they are not part of conventional medicine.

1.6. Chinese Herbal Medicines for the treatment of cancer

Some experiments *in vitro* and *in vivo* demonstrated that Chinese Herbal Medicines (CHM) play a role in cancer treatment by inducing apoptosis and differentiation, enhancing the immune system and inhibiting angiogenesis, etc. Many herbal remedies play their anti-cancer roles through multiple mechanisms, however, the mechanism of their anti-cancer effects has not yet been fully elucidated [Ikezoe *et al.*, 2003 a], [Ikezoe *et al.*, 2003 b],

[Cheng *et al.*, 2003], [Hong-Fen *et al.*, 2001], [Hsieh *et al.*, 2002], [Wartenberg *et al.*, 2003].

Compared to synthetic chemical drugs, herbal medicines are more natural and have much fewer side effects, are gentler in action and give good effects. CHM can control and minimize the side effects of chemical drugs and enhance their therapeutic effects. They can also improve appetite, reduce nausea and vomiting, and alleviate stress, and also strengthen immune-system functions depressed by radiation [Cheng *et al.*, 2003].

The Chinese herbs used to treat cancer and other immune-deficient conditions are divided into three categories:

1. Tonic herbs that increase the number and activity of immunologically active cells and proteins in the body.
2. Toxin clearing herbs that clear the blood and waste products from the destruction of tumors.
3. Blood activating herbs that reduce the coagulation and inflammatory reactions associated with immune response.

So, the Chinese herbal preparations are complex and contain more than one herb. For example, there are common anti-cancer base formulae that contain *Oldenlandia diffusa* and *Scutellaria baicalensis* as main ingredients for cancers of the specified areas that are mentioned in the book 'Anticancer Medicinal Herbs'. These base formulae had been applied for cancer treatment at the hospital where it was being used. For example,

> "...for stomach cancer: combination of oldenlandia (90 g) and imperata (60 g) or combination of scutellaria (30 g) and imperata (30 g); for esophagus, rectum, and stomach cancer: combination of oldenlandia (70 g) and coix (30 g) and additional other herbs with small quantities; for esophagus cancer: combination of oldenlandia (60 g), scutellaria (60 g), cycas leaf (60 g), imperata (60 g) and cotton root (60 g); for rectum cancer: combination of oldenlandia (60 g), scutellaria (15 g), solanum (60 g), lonicera stem (60 g) and viola (15 g); for gastrointestinal tract cancers and lymphosarcoma: combination of oldenlandia (50 g), imperata (50 g), coix (20 g) and brown sugar (60 g); for ovary cancer: combination of oldenlandia (30 g), scutellaria (50 g), solanum (50 g S. Nigri and 30 g S. lyrati) and turtle shell (30 g); for pleura (metastasize to): combination of scutellaria (120 g), taraxacum (30 g);

and for liver, rectum, lung cancer: combination of oldenlandia (60 g) and scutellaria (60 g)..." [Subhuti, 2004].

In addition, Tang kuei (Angelica sinensis), has been used clinically in China to treat cancer of the oesophagus and liver and showed good results. There was success using this herb itself alone and in combination with other medicinal agents for treatment of cervical cancer and breast cancer in women [Cheng *et al.*, 2003]. It can be used in either infusion or douche form. In Japan, classical Chinese herbal formulae are prepackaged and standardized. Kampo, the Japanese version of Chinese herbalism, has reported many successes for treatment of cancer [Teng, 2006].

Generally, the pharmacological effects of CHM are still not clear even though it has proved to be effective against disease. This is the major reason for CHM in not being accepted over the world.

Some studies were conducted on the characteristics of some effective anti-cancer herbal remedies *in vitro*. For instance, the synergistic effects of various components in *Scutellaria baicalensis* extract (baicalin (80%), wogonoside (16%), baicalein (2%), wogonin (1%) and other compounds in small amounts) were confirmed. The research revealed that both the extract and its pure compound baicalein inhibited cancer cell growth. Also, the extract inhibited prostaglandin E2 (PGE2) production, but its pure compound baicalein could not inhibit it. Another pure compound wogonin showed significant inhibition on cyclooxygenase 2 (COX-2) activity in lipopolysaccharide (LPS)-stimulated macrophages. The various components in the herb act on different anti-cancer pathways such as COX-dependent and COX-independent pathways [Zhang *et al.*, 2003].

Furthermore, some experiments revealed that some herbs have selective effects on cancer cells, but not for normal cells. For instance, it was reported that the butanol fraction of *Mylabris phalerlata* was cytotoxic on human monocytic leukaemic U937 cells, but not on peripheral blood mononuclear lymphocytes [Huh *et al.*, 2003].

It was reported that magnolol at concentrations of 3-10 μmol/L inhibited DNA synthesis and decreased the COLO-205 and Hep-G2 cell numbers in a dose-dependent manner, but not in non-transformed human cells such as keratinocytes, fibroblasts, and human

umbilical vein endothelial cells. When the concentration of magnolol increased to 100 μmol/L, apoptosis was observed in COLO-205 and Hep-G2 cells, whereas in cultured human fibroblasts and human umbilical vein endothelial cells did not [Lin *et al.*, 2002b]. But it is still not clear how this anti-cancer specific killing occurs.

1.7. Introduction to Chinese herbal remedies *Oldenlandia diffusa*

TCM has become increasingly popular for the treatment of a variety of physiological disorders such as cancer [Cheng *et al.*, 2000], [Gordon & Weng, 1992], [Islam *et al.*, 2009]. Several Chinese herbs were reported to have been used to treat cancer patients or used successfully to prevent the cancer [Gu & Weng, 2001]. One of these CHRs is *Oldenlandia diffusa* (Henrik Bernard Oldenland). It is one of the most extensively used CHR in modern Chinese practice for treatment of viral infections (especially hepatitis) and cancers, as well as for some other diseases [Shan *et al.*, 2001], [Liang *et al.*, 2008], [Chung *et al.*, 2002]. In the Chinese Pharmacopoeia, *Oldenlandia diffusa* was officially listed as an ingredient of Chinese patent medicine [Pharmacopoeia of China, 2005].

Taxanomic classification of *Oldenlandia diffusa* are listed below:

***Oldenlandia diffusa* (Willd.) Roxb.**

Family name:	**Rubicaceae**
Pharmaceutical name:	**Herba hedyotidis Diffusae**
Common name:	**Snake-needle grass, Spreading hedyotis**
Synonyms:	***Hedyotis diffusa* (Willd.)**
Other names:	**Bai hua she she cao** (Chinese), **Byakkajazetsuso** (Japenese), **Paekhwasasolcho** (Korean)

1.7.1. Physical characteristics

Figure 3. Typical image of *Oldenlandia diffusa*.
[Taken from http://www.dkimages.com/discover/previews/825/25012996.JPG]

Oldenlandia diffusa has a pleasant cooling taste. It is a slender, spreading or ascending, smooth, annual herb reaching a length of 20-30 cm and grows ubiquitously as a weed. The leaves are linear and 1 to 3 cm long. The white and 3 mm long flowers occur singly in the axils of the leaves, and the pedicels are long as the calyx. The capsules are ovoid, about 4 mm long and not protruded beyond the calyx-segments. It is in flower from August to September. The flowers are hermaphrodite (have both male and female organs) [Ulasiman, 2007], [Plants for a Future, 2007]. It is collected in the summer or autumn, cleaned, cut into pieces, dried in sunlight, and used unprocessed or fresh [Bown, 1995], [Jiangsu, 1977].

1.7.2. Habitats and possible locations

The plant grows throughout south East Asia and southern China. It prefers light-sandy, medium-loamy and heavy-clay soils. The herb can grow in all soil pH's. It can grow in semi-shade and requires moist soil [Jiangsu, 1977].

1.7.3. Medicinal use of *Oldenlandia diffusa* for cancer

The whole plant is used as a medicine. *Oldenlandia diffusa* has been shown to lower fever and has anodyne, antibacterial, anti-inflammatory, cardiotonic, depurative, diuretic, febrifuge, sedative and anti-tumour properties. It has an effect mainly on the liver and also stimulates the immune system [Yoshida *et al.*, 1997], [Shan *et al.*, 2001], [Chung *et al.*, 2002], [Lin *et al.*, 2002],[Liang *et al.*, 2008]. It is taken internally in the treatment of fevers, coughs, asthma, jaundice, septicaemia, urinary tract infections, acute appendicitis, biliousness and cancers of the digestive tract. Externally, it is used in the treatment of snake bites, boils, abscesses and severe bruising. It is commonly used with other herbs in the preparation of anti-cancer formulations. For example, *Oldenlandia diffusa* (60 g) is the main ingredient for liver cancer treatments that are mentioned in the book of 'Anticancer medicinal herbs' along with a combination of scutellaria (60 g), cycis (18 g) and phragmites (30 g) [Subhuti, 2004], [Ninghon *et al.*, 1986].

Oldenlandia diffusa is used as a pill, powder, extract, infusion and in decoction forms [Ergil *et al.*, 2002]. In CHMs of Hong Kong, it is noted that *Oldenlandia diffusa* is used for treatment of "early stage of cancer of lungs, liver, and rectum", and the recommended dose is 60 g with 30 g of *Scutellaria barbarata* as a decoction, taken once a day [Ninghon *et al.*, 1986].

1.7.4. Chemical constituents of *Oldenlandia diffusa*

Oldenlandia diffusa has been studied by a number of researchers and they have demonstrated that *Oldenlandia diffusa* contains a majority of iridoid glucosides, triterpenoids, flavonoids and polysaccharides. For example, iridoid glycosides including E-6-O-p-coumaroyl scandoside methyl ester, E-6-O-p-methoxycinnamoyl scandoside methyl ester, 6-O-feruloyl scandoside methyl ester, oldenlandoside I, II, III, asperuloside [Nishihama *et al.*, 1981], [Takagi *et al.*, 1982], 10-O-benzoylscandoside methyl ester [Gu & Weng, 2001], asperulosidic acid [Shan *et al.*, 2001] and deacetylasperulosidic acid [Bown, 1995], as well as an anthraquinone [Ho *et al.*, 1986], oleanolic acid, trans p-coumaric acid (p-hydroxy cinnamic acid), ursolic acid, sitosterol, β-sitosterol, stigmasterol, sitosterol-d-glucoside, flavones [Chung *et al.*, 1998] have been isolated from *Oldenlandia diffusa* [Dong, 2005]. For example, the main five compounds of the *Oldenlandia diffusa* are shown in Figure 4.

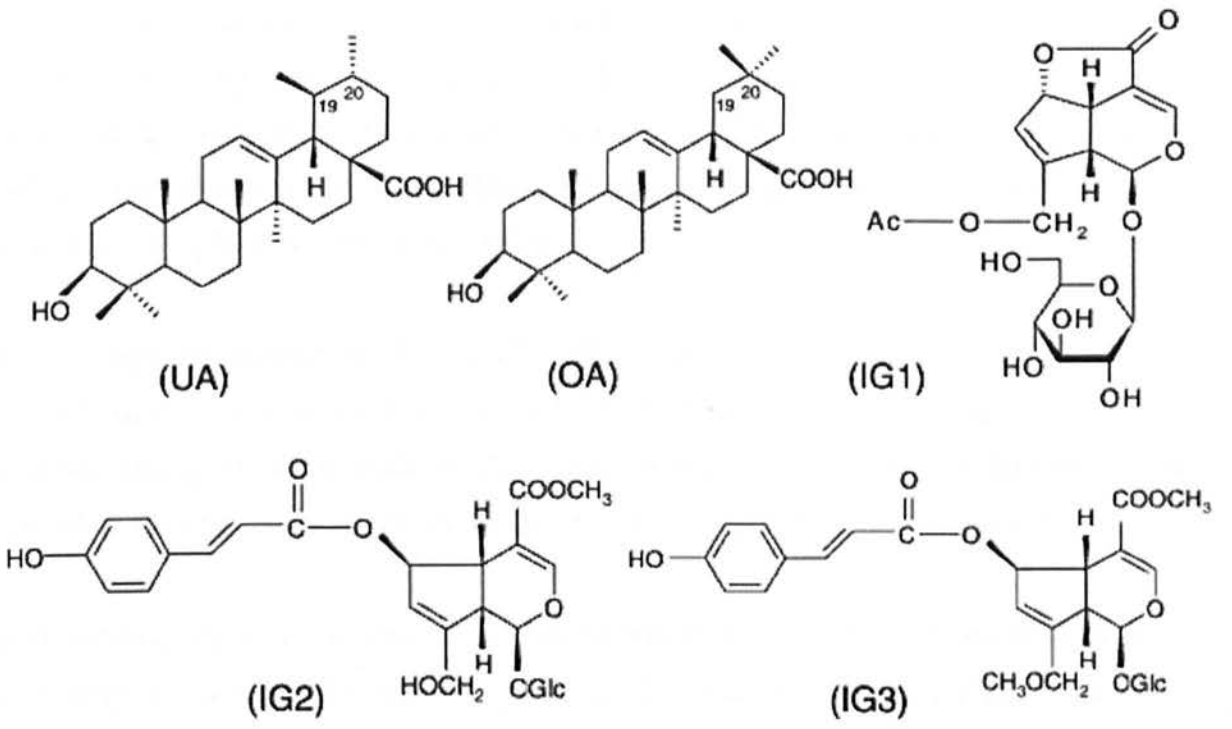


Figure 4. Stucture of the main five compounds of the *Oldenlandia diffusa* are. UA-ursolic acid, OA-oleanolic acid, IG1-asperuloside, IG2-E-6-O-p-coumaroyl scandoside methyl ester and IG3-E-6-O-p-coumaroyl scandoside methyl ester-10-methyl ether.

1.7.5. The cytotoxic potential of *Oldenlandia diffusa*

The *in vitro* anti-cancer effects of *Oldenlandia diffusa* is established, however the mechanisms are not yet deeply studied.

Some research work was conducted on ursolic acid, which was isolated from the methanol extract of *Oldenlandia diffusa*. Its cytotoxic effect was investigated on human lung, ovary, skin, brain, colon, stomach, murine leukaemia and murine melanoma and results revealed that ursolic acid has a significant inhibition on the proliferation of tumour cells. In addition, further work on ursolic acid suggested that ursolic acid had an apoptotic effect on HL60 cells [Shan *et al.*, 2001].

Moreover, the effect of aqueous extracts of *Oldenlandia diffusa* was investigated on the lung cancer cell line (H69), a multi-drug resistant small cell lung cancer cell line (H69VP) and a normal lung epithelium cell line (BEAS-2) [Sadava *et al.*, 2002]. The aqueous exract significantly inhibited the cell proliferation of these cells and apoptosis was observed in both the H69 and H69VP cell lines.

Some *in vivo* research revealed that oral administration of *Oldenlandia diffusa* aqueous extract on mice has a significant growth inhibition on murine renal carcinoma cells, and it enhanced macrophage function *in vitro* [Wong *et al.*,1996]. In a separate study, isolated polysaccharides from *Oldenlandia diffusa* showed anti-tumor activities against transplanted sarcoma-180 cells in mice [Li *et al.*, 2002].

On the aqueous extract of *Oldenlandia diffusa* some research work was undertaken to investigate the anti-cancer effects on the HL60 leukaemic cell line. It was shown that the aqueous extract of *Oldenlandia diffusa* is directly toxic to the HL60 cell line by inducing apoptosis via some form of activity specific to these cells [Willimott *et al.*, 2007].

Furthermore, *Oldenlandia diffusa* enhanced the induction of allo-antigen specific cytotoxic T lymphocytes and stimulated macrophages to produce interleukin-6 and tumor necrosis factor and the results suggested that it has an immunomodulating activity *in vitro* [Yoshida *et al.*, 1998].

There were some studies done on immunomodulatory activity and anti-tumour activity of *Oldenlandia diffusa in vitro* and this proved that *Oldenlandia diffusa* has immuno-modulating activity and anti-tumour activity *in vitro* through stimulating the immune system to kill or engulf tumour cells, which could be used clinically for immune function modulation and to treat tumour and other diseases [Shan *et al.*, 2001]. Therefore, anti-tumor activity has been identified as the main pharmacological effect of *Oldenlandia diffusa*.

1.8. Aims of the study

In traditional Chinese human medicine, herbs have been used as immunostimulants for thousands of years [Yoshida *et al.*, 1998]. Chinese Medicinal Remedies have been traditionally used to prevent and treat many kinds of diseases, especially autoimmune diseases and cancers [Cheng *et al.*, 2000], [Li *et al.*, 1986], and they became commonly used for the treatment of cancer. However, their anti-cancer effect and biological actions are not fully elucidated. One of the most popular used Chinese medicinal herbs is *Oldenlandia diffusa*. In order to study its anti-cancer effects, the most responsible constituent(s) to have a cytotoxic effect and the compounds' bioavailability will be investigated.

The first objective of this study will be to set up the separation method for *Oldenlandia diffusa* decoction using HPLC. To create the separation method, different types of chromatographic columns will be investigated with the following different parameters such as flow rates, absorption wavelengths, isocratic and gradient elutions of different types of mobile phases at different pHs. The decoction will be prepared by conventional method as used for treatment by boiling (refluxing) in water.

The second objective of this investigation will be to collect the fractions in order to narrow down and specify the study area. For this reason, preparative column scale will be examined, and fraction collecting points will be determined using HPLC and preparative column.

The third objective will be to assess the direct cytotoxic potential of the *Oldenlandia diffusa* decoction against cancer cells using an *in vitro* system. In addition, the cytotoxic effect of those separated and collected fractions will be investigated on cancer cells in order to select the most cytotoxic fraction.

The fourth objective of this investigation will be to characterize the chemotherapeutic potential of *Oldenlandia diffusa* decoction that shows cytotoxic action against the cancer cells. To characterize the mode of cell death, the decoction and chosen fraction's ability to induce the apoptosis in cancer cells will be examined.

The fifth objective of this investigation will be to set up the separation method for the chosen most cytotoxic fraction using HPLC. For this, different parameters namely, different types of chromatographic columns, flow rates, different wavelengths, isocratic and gradient elutions of different mixture of mobile phases will be examined.

The sixth objective of this investigation will be to isolate separated compounds in the chosen fraction and to identify them. To collect these separated compounds as pure and to identify them, the LC-MS, Tandem MS and NMR will be performed.

The seventh objective of this investigation will be to create a Caco-2 monolayer for studying of the bioavailability (post-absorption samples) of the *Oldenlandia diffusa*

decoction. For this reason, the growth rate of Caco-2 colon adenocarcinoma cells will be investigated and a gut mimicking wall (monolayer) will be created using Caco-2 cells.

The eighth objective of this investigation will be then to set up the post-absorption sample collection method. For this, the effect of the decoction on the Caco-2 cell monolayers, the post-absorption sample collection concentration and time interval on the created monolayers will be investigated. Also, cytotoxic effect of post-absorption sample will be assessed on cancer cell line.

The final objective of this investigation will be to assess the participation of determined cytotoxic compounds in the post-absorption sample whether they are in original or in metabolised form.

Chapter 2. Setting up the separation method to be used for *Oldenlandia diffusa* decoction.

Chapter 2. Setting up the separation method to be used for *Oldenlandia diffusa* decoction.

2.1. Introduction

In order to study the CHR *Oldenlandia diffusa*, the initial aim was to set up the separation method for the decoction. For this reason, the technique of high performance liquid chromatography was used and its parameters optimised.

2.1.1. High performance liquid chromatography

High performance liquid chromatography (HPLC) is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds which are soluble in a liquid. HPLC has been applied to many samples, such as pharmaceuticals, environmental matrices, forensic materials, food, cosmetics and industrial chemicals [Yuri & LoBrutto, 2007], [Skoog *et al.*, 2004].

Equipment: A HPLC system consists of the following main six parts (Figure 5):

1- Solvent Reservoir
2- Pump/s
3- Injection Port
4- Column
5- Detector
6- Data Acquisition System

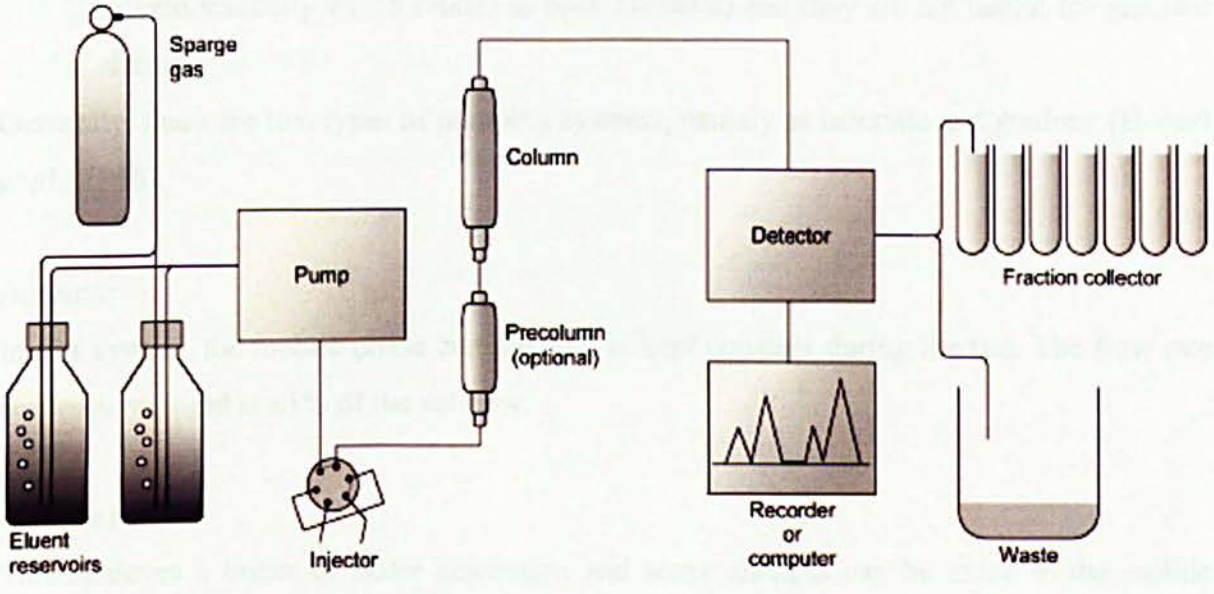


Figure 5. Schematic of typical HPLC system.

[Taken from http://www.medicinescomplete.com/mc/clarke/2009/images/clk9030f001.gif]

1. Solvent Reservoir

Solvent reservoirs are used to store the mobile phase. The solvent reservoir must be made of an inert material such as transparent or amber-coloured glass. It must be smooth so to avoid growth of microorganisms on its walls.

2. Pump

The conventional HPLC pump is a very important component of the system. The pumps deliver a constant flow of the mobile phase so that separation of the components in the mixture occurs at a reasonable time. The requirements for pumps include the ability to generate pressures up to 50,000 psi (lb/in^2), pulse-free output, with flow rates ranging from 0.1 to 10 mL/min. They must also be resistant to corrosion by a variety of solvents and have flow reproducibilities of 0.5% or better [Gooding & Regnier, 2002].

There are three types of pumps used in HPLC.

- A screw-driven syringe type called a displacement pump. This is pulse-free, with a small capacity (250 mL) and no gradient elution possibility with only one syringe.
- A reciprocating pump. It is the most widely used pump. It has a small internal volume (35-400 μL), high pressure (10^5 psi), gradient elution and pulse-free flow.
- A pneumatic or constant-pressure pump. This is the simplest, inexpensive and pulse free pump. It has limited capacity and pressure output (pumping rates depend on solvent viscosity which relates to back pressure) and they are not useful for gradient elution.

Generally, there are two types of pumping systems, namely as isocratic and gradient [Hobart *et al.*, 1998].

Isocratic

In this system, the mobile phase composition is kept constant during the run. The flow rate accuracy required is ±1% of the set flow.

Gradient

This achieves a better or faster separation and some changes can be made to the mobile phase composition during the particular sample run.

In addition, there are two types of gradient systems low pressure mixing and high pressure mixing two types of gradient systems. Each system has its own advantage. Low pressure mixing can work for three or four mobile phases. In high pressure mixing, generally two pumps are used so thus the choice is of two different mobile phases only.

The dwell volume is of practical importance only for gradient applications. This is the volume from the point that the mobile phase solvents are mixed until the head of the column. In low-pressure mixing systems, the dwell volume includes the internal piston cavity, the pulse dampener, the priming valve and the mixer, in addition to the fluidic volumes in the autosampler and all connection tubing before the column. The typical dwell volme in low-pressure mixing system is about 2-3 mL, but can be as high as 5-7 mL. High-pressure mixing system has inherently lower dwell volumes (1-2 mL) because the point of mixing is external to the pumps [Ahuja & Dong, 2005], [Dong, 2006].

3. Injection Port

The sample introduction device such as the injector introduces the sample into a flow of mobile phase at high pressure. The valve injection through a fixed or variable loop introduces the sample with sizes ranging from 5 to 500 μL. There are some loops for injection volume up to 2000 μL [Shimadzu, 2007], [Fifield & Kaelay, 2000].

4. HPLC Column

The HPLC column holds the stationary phase for separating the components of the sample. The columns are usually made from stainless steel tubing and glass or Tygon tubing (for lower pressure application (<600 psi).

In general, if the sample size increases, the size of the HPLC column need to become larger and the pump will need higher volume (flow rate) capacity. Determination of the capacity of a HPLC system is called selecting the HPLC scale. Table 1 shows a list of variables of scales for chromatographic columns [Fifield & Kaelay, 2000].

Table 1. HPLC column types, internal diameters and recommended particle sizes.

Scale	**Chromatographic objective**	**Column diameter, mm**	**Particle size, µm**
Analytical	Information (compound identification and concentration)	1-8	1.7-10
Semi-preparative	Data and small amount of purified compound (<0.5 g)	10-40	3-15
Preparative	Larger amounts of purified compound (>0.5g)	50-100	15-100
Process (Industrial)	Manufacturing quantities (grams to kilograms)	>100	100+

[Janson & Pettersson, 1992], [Ahuda, 2000], [Williams *et al.*, 2002].

From the above mentioned method scales, analytical and a semi-preparative columns were used for *Oldenlandia diffusa* decoction separation as their capacity (column volume) is the most appropriate.

In general, the range of HPLC columns is from 20 mm to 500 mm in length and 1 mm to 100 mm internal diameter. The analytical column is straight, 50-250 mm long with 3-5 mm internal diameter, 3-5 µm of particle size and 40 k-100 k plates/m. For semi-preparative work, we use a column with an internal diameter of 10-40 mm containing 3-15 µm particles. As the scale of chromatography increases, the column dimensions, especially the cross-sectional area increases as well. For optimization, mobile phase flow rates must increase in proportion to cross-sectional area. When a smaller particle size is needed for better separation, then pumps should work on higher mobile phase volume flow rates at high backpressure [Chen & Horvath, 1995], [Halasz *et al.*, 1975].

All columns were used with guard columns as it is necessary to remove the particulate matter and contamination from the analyte samples in order to protect the column. So, this increases the life-time of column. The composition of guard column should be similar to that of the column. The particle size is usually larger than the column's particle size.

5- HPLC detectors

Detectors detect various compounds as they elute from the column. The detector gives response in terms of a millivolt signal. Then, it will be processed by the computer (integrator) to give a chromatogram.

There are several detectors used for detection, for example, UV-Visible detector (UV), diode array detector, fluorescence detector, refractive index detector, conductivity detector and mass detector (MS). The detector used can be chosen on the nature of the sample. The most widely used detectors for liquid chromatography are based on absorption of ultraviolet or visible radiation [Willoughby *et al.*, 1998], [Niessen, 1999].

In this study, an ultraviolet (UV) detector and photo diode array detector were used. Ultraviolet (UV) detector detects absorbance of UV light by chromophores in the analyte compound [George, 1976], [Rouessac & Rouessac, 2007]. The photo diode array detector is the most used detector in liquid chromatography today. This detector gives a three dimensional view of chromatogram (intensity versus time) and spectra (intensity versus wavelength), and can be called a spectro-chromatogram [Hobart *et al.*, 1998], [Loconto, 2006].

6- Data Acquisition System

Data systems process the detector output and integrate it into a chromatogram. Modern integration systems do the processing of the chromatogram, calculations, statistical analysis and data storage.

2.1.2. Types of HPLC modes

There are several types of HPLC modes and these are classified by the nature of the stationary phase as follows:

1- Reversed phase chromatography (RP chromatography)

2- Normal phase chromatography (NR chromatography)

3- Ion-Exchange chromatography (IE chromatography)

4- Size-Exclusion chromatography (SEC chromatography) [Yuri & Rosario, 2007].

RP-HPLC was performed in the study of *Oldenlandia diffusa* decoction.

2.1.3. Reversed phase chromatography

RP-HPLC is useful in both analytical and preparative applications in the area of biochemical separation and purification.

The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, for example, the stationary phase [Elena *et al.*, 1998]. Reversed phase chromatography is an adsorptive process and is based on a partitioning mechanism by dispersive forces to effect separation. The solute molecules are partitioned between the mobile and stationary phases. The distribution of the solute between the two phases depends on the following characters:

- the binding properties of the medium,
- the hydrophobicity of the solute,
- the composition of the mobile phase.

Initially, experimental conditions dictate the adsorption of the solute from the mobile phase to the stationary phase. Then, the mobile phase composition is set to desorption of the solute from the stationary phase back into the mobile phase.

Generally, a reversed phase separation is performed in several basic steps: 1-Starting conditions-equilibration of the colum, 2-Adsorption of sample substances, 3-Start of desorption, 4-End of desorption and 5-Regeneration.

- In the first step of the gradient elution, equilibration of the column packed with the reversed phase medium under the initial mobile phase conditions, including pH, ionic strength and mobile phase hydrophobicity is performed. The polarity of the mobile phase is obtained by the addition of organic modifiers such as acetonitrile and methanol. The polarity of the initial mobile phase must be low enough to dissolve the hydrophobic solute and high enough to bind the solute to the reversed phase chromatographic medium.
- The second step is an adsorption step. In this step, the sample containing the solutes to be separated (dissolved in the same mobile phase used to equilibrate the

chromatographic bed) is applied. The sample is applied to the column at a flow rate where optimum binding will occur.

- In the next step or in starting of the desorption process, bound solutes are desorbed from the reversed phase column by adjusting the polarity of the mobile phase in order that, the bound solute molecules will desorb by order and elute from the column. In reversed phase HPLC, this usually involves decreasing the polarity of the mobile phase by increasing the percentage of organic modifier in the mobile phase. Generally, the pH of the initial and final mobile phase solutions remains the same. The gradual decrease of mobile phase polarity (increasing mobile phase hydrophobicity) is achieved by an increasing linear gradient from initial mobile phase containing no or very little organic modifier to 100% (or less) mobile phase containing a higher concentration of organic modifier. The bound solutes desorb from the reversed phase medium according to their hydrophobicities.
- In the fourth step of chromatographic process (end of desorption), previously non desorbed substances are removed from the column. This is generally achieved by changing the mobile phase to 100% or close to 100% organic modifier.
- The fifth step is re-equilibration of the chromatographic medium. In this stage, the final 100% mobile phase is put back to the initial mobile phase conditions for next run [Skoog *et al.*, 2007], [Skoog *et al.*, 2004].

So, separations in reversed phase chromatography depend on the adsorption and desorption of solute molecules with different degrees of hydrophobicity to a hydrophobic stationary phase.

2.1.3.1. Column for RP-HPLC

In RP-HPLC, the stationary phase is non-polar and the mobile phase is polar. Generally, this causes the elution of polar compounds earlier than non-polar compounds.

The stationary phase is generally made up from hydrophobic alkyl chains ($-CH_2-CH_2-CH_2-CH_3$) ($(O-Si-C_{18}H_{37})$) that interact with the analyte [IUPAC, 1987], [Phenomenex, 2007]. In general, longer alkyl chains, higher phase loading, and higher carbon loads provide higher retention of non-polar analytes. Commonly used reversed phase columns and their uses are given below.

Propyl (C3), Butyl (C4), Pentyl (C5), Octadecyl (C18) phases are widely used for ion-pairing chromatography. C3-C5 columns are generally retain non-polar solutes less when compared to C8 or C18 phases.

Octyl (C8, MOS), with the functionality-$Si(CH_3)_2C_8H_{17}$. Octyl phases are less retentive than the C18 phases, but they are quite useful for pharmaceuticals, nucleosides, and steroids.

Octadecyl (C18, ODS) columns are the most widely used. Their functionality is -$Si(CH_3)_2C_{18}H_{37}$. It is most retentive for non-polar analytes and has wide applicability (similar to C8 in addition to vitamins, fatty acids, environmental compounds).

Phenylpropyl (Ph, -$Si(CH_3)_2(CH_2)_3C_6H_5$) columns are used for their different selectivity from alkyl phases. They are generally less retentive than C8 or C18 phases. Phenyl columns are commonly used to resolve aromatic compounds.

Nitrile (CN or cyano, - $Si(CH_3)_2(CH_2)_3CN$) columns are more polar. They can be used in both reversed and normal phase applications and are often used to increase the retention of polar analytes.

Pentafluorophenyl propyl (PFP(2) -$Si(CH_3)_2(CH_2)_3C_6F_5$) is a new high performance stationary phase. Phenomenex introduced the columns of Luna PFP(2) in 2008. Luna PFP(2) columns are good for pharmaceutical and natural products research. The column uses multiple selectivity mechanisms, including hydrogen bonding, dipole-dipole, aromatic, hydrophobic, and these columns separate challenging compounds that are highly polar, halogenated, isomeric or aromatic [Phenomenex, 2008], [Joel, 2001].

The latter five types of RP-HPLC columns were examined for separation of *Oldenlandia diffusa* decoction. Column compatibility with various mobile phases is determined by the stationary phase.

2.1.3.2. Mobile phase for RP-HPLC

The mobile phase is the fluid that percolates in a definite direction, through the length of the stationary phase. In HPLC, the mobile phase is a liquid and is called the *eluent*, whilst the

portion of the mobile phase that has passed through the stationary phase and contains the compounds of interest in solution is called the *eluate*. In RP-HPLC, the mobile phase is composed from an aqueous buffer and a water miscible organic solvent (modifier) which usually has no or very little absorption above 200 nm, e.g. methanol.

Organic solvent

A large variety of organic solvents can be used in RP-HPLC. The common organic solvents are methanol, acetonitrile, isopropanol and tetrahydrofuran. Isopropanol (2-propanol) can be used because of its strong eluting properties, but it has a limited use, because it has a high viscosity, which results in lower column efficiencies and higher back-pressures. Both acetonitrile and methanol have less viscosity than isopropanol. All solvents are UV transparent. This is a crucial property for reversed phase chromatography since column elution is typically monitored using UV detectors.

The organic solvent is added to lower the polarity of the aqueous mobile phase. In reversed phase chromatography, the lower the polarity of the mobile phase the greater eluting strength [Weng, 2003].

Gradient elution

Gradient elution is the preferable method, when performing the preparative reversed phase chromatography of biomolecules. The main gradient elutions for the preparative reversed phase chromatography are linear and binary, for example, involving two mobile phases. The concentration of organic solvent is lower in the initial mobile phase (mobile phase A) than it is in the final mobile phase (mobile phase B). The gradient, always proceeds from high polarity (high aqueous content, low concentration of organic modifier) to low polarity (lower aqueous content, higher concentration of organic modifier) [Snyder & Dolan, 2006].

Gradient shape (combinations of linear gradient and isocratic conditions), gradient slope and gradient volume are all important considerations in reversed phase chromatography [Jandera, 1999]. In order to determine the optimum gradient shape for performing a reversed phase separation of a complex sample, a broad gradient is used for initial screening. After the initial screening, the gradient shape can be adjusted to optimize the separation of the desired components.

The choice of gradient slope will depend on how close the eluted components are to each other. Generally, decreasing its gradient slope increases resolution. However, peak volume

and retention time increase with decreasing gradient slope. Also, total gradient volume (gradient time x flow rate) affects resolution. To begin, the gradient volume can be approximately ten to twenty times the column volume. The slope can then be increased or decreased in order to optimize the resolution [John *et al.*, 1999].

Furthermore, there are some more critical parameters in RP-HPLC to take into count for compound separation, e.g. column length, flow rate and temperature.

Column length

The resolution of high molecular weight biomolecules in reversed phase separations is less sensitive than small organic molecules to column length. The partition coefficients of high molecular weight compounds are very sensitive to small changes in mobile phase composition. Therefore, large molecules desorb in a very narrow concentration range of organic modifier [Skoog *et al.*, 2007].

In reversed phase chromatography, the use of gradient elution further reduces the significance of column length for the resolution of large biomolecules.

Flow rate

In reversed phase separations, flow rate is an important factor for resolution of small molecules, while larger biomolecules are insensitive to flow rate. Generally, very low flow rate can decrease the resolution because of the increased longitudinal diffusion of compounds as they pass through the length of the column. The flow rate used during the loading of the sample solution is significant in large scale preparative reversed phase chromatography, but it is not critical during analytical experiments [Gooding & Regnier, 2002].

Temperature

Temperature can have an effect on reversed phase chromatography, especially for low molecular weight compounds. The viscosity of the mobile phase used in reversed phase HPLC decreases with increasing column temperature. Generally, the decreasing viscosity leads to more efficient mass transfer, therefore higher resolution is achieved. Increasing the temperature of a reversed phase column is effective for low molecular weight solutes, if they are stable at these temperatures.

2.2. Materials and methods

2.2.1. Materials

CHR *Oldenlandia diffusa* was verified and obtained locally from a TCM practitioner Ms Qiuyan Liu, Dr. TMC - Traditional Chinese Medicine (Mitcham, UK). A high performance liquid chromatography system was used for chromatographic separation, containing a L7150 2 pump system, injection port and UV detector (VARIAN ProStar, Model 210), with a Waters 600 controller, Waters 600 pump and Waters M 996 photodiode array detector, using Merck HSM software to control the data acquisition. Freeze-drier (TELSTAR Croydos, model 50, USA), UV-Visible spectrophotometer (Varian, UK) and 0.45 μm sterile filter (Nalgene, Hereford, UK) were used. Columns used were:

- C18 (LichroCART 250 x 10, LiChrospher 100 RP-18e 10 μm, Merck, Darmstadt, Germany),
- C18 (Luna 250 x 4.60 mm, 5 μm C18, Phenomenex, Germany),
- Phenyl column (Prodigy 5 μm Phenyl-3, 150 x 4.60 mm 5 μm, Phenomenex, UK),
- Amino column (Sphereclone NH_2, 250 x 4.60 mm 5 μm, Phenomenex, UK),
- Cyano column (Hypersil 3 CN, 150 x 4.60 mm 3 μ micron, Phenomenex, UK),
- PFP(2) (Luna 250 x 4.60 mm, 5 μm, Phenomenex, Germany),
- PFP(2) (Luna 20 cm x 15.1 mm, 5 μm, Phenomenex, Germany).

The following chemicals NaCl, KCl, $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, ammonium acetate (HPLC grade) were supplied by Sigma-Aldrich Inc, Poole, UK, and methanol, acetonitrile, tetrahydrofuran, hexane (HPLC grade) and purchased from by Fisher Scientific Inc, UK.

2.2.2. Sample preparation

Decoction preparation

5 g of aerial parts of CHR *Oldenlandia diffusa* were cut into 1-1.5 mm pieces and crushed using a mortar and pestle. Then, it was boiled with 50 mL of deionised water by refluxing for 45 minutes. After cooling the decoction was centrifuged at 3000 rpm for 20 minutes and then filtered through 0.45 μm filter.

Preparation of Phosphate Buffered Saline

PBS was prepared by dissolving:

- 8 g NaCl,
- 0.2 g KCl,

- 1.44 g $Na_2HPO_4 \cdot 12H_2O$
- 0.24 g KH_2PO_4 in 0.8 L of distilled water, and making up to 1 L.

The pH was 7.4. When making buffer solutions, its pH was measured directly using a pH meter and adjusted using sodium hydroxide.

Preparation of ammonium acetate buffer

Ammonium acetate buffer was prepared by dissolving 0.77 g ammonium acetate in 0.8 L deionised water and making up to 1 L. Its pH was measured using a pH meter and adjusted using 1 M ammonium hydroxide.

Before undertaking the HPLC separation, each mobile phase was degassed using a sonicator.

2.2.3. Determination of the concentration of prepared decoction

Decoction of *Oldenlandia diffusa* was prepared three times using the above mentioned method. Then the decoction was transferred to a round bottom flask and frozen. After that, it was connected to freeze-drier until dry. Each time the residue left was weighed and the average value was calculated.

2.2.4. Setting up the separation method for the decoction using HPLC

To set up the optimal method for separation of *Oldenlandia diffusa* decoction, the following parameters such as column used, flow rates, absorption wavelengths and mobile phases were examined.

2.2.4.1. Choice of HPLC column

Different reversed phase and normal phase chromatographic columns such as C18, amino, phenyl, cyano and PFP(2) columns were tested for the separation of the decoction into fractions.

These columns were examined with the following mobile phases: deionised water, methanol, phosphate buffered saline (PBS), ammonium acetate buffer and the mixtures water/methanol, water/methanol with 0.1% TFA, water/acetonitrile, water/acetonitrile with 0.1% TFA, ammonium acetate buffer/methanol, ammonium acetate buffer/acetonitrile, PBS/methanol, ammonium acetate buffer/methanol/tetrahydrofuran (THF), and methanol/THF/water. Also, on the amino column hexane and hexane/isopropanol mixtures were used.

2.2.4.2. Choice of absorption wavelength

In order to choose the most sensitive absorption wavelength for the decoction, the following wavelengths: 198-254 nm were checked on UV spectrophotometer.

2.2.4.3. Choice of mobile phase

To obtain the optimal mobile phase for the separation of the *Oldenlandia diffusa* decoction, both isocratic and gradient elution modes with deionised water, methanol, phosphate buffered saline, ammonium acetate buffer and their mixtures such as:

- water/methanol (25:75, 50:50, 75:25, v/v),
- water/methanol/0.1% TFA (25:75, 50:50, 75:25, v/v),
- water/acetonitrile (30:70, 50:50, 70:30, v/v),
- water/acetonitrile/0.1% TFA, (30:70, 50:50, 70:30, v/v),
- ammonium acetate buffer/methanol (20:80, 50:50, 80:20, v/v),
- ammonium acetate buffer/acetonitrile (20:80, 50:50, 80:20, v/v),
- PBS/methanol (20:80, 50:50, 80:20, v/v),
- water/methanol/tetrahydrofuran (50:40:10, 70:25:5 v/v);
- ammonium acetate buffer /methanol/tetrahydrofuran (THF) (50:40:10, 60:15:25, 70:25:5 v/v) were tested on different types of reversed phase high performance column.

While, on the amino HPLC column hexane and hexane/isopropanol (95:5, 90:10 v/v) were examined.

For gradient elution of mobile phase the following gradient shape and slopes were investigated such as:

Solvent A=organic modifier B=water or buffer

- Gradient elution-1:

  0-10 min 90% B

  10-40 min 100% A

  40-50 min 100% A

  50-60 min 90% B

- Gradient elution-2:

  0-5 min 100% B

5-15 min 30% A

15-30 min 50% A

30-40 min 100% A

40-50 min 100% B

- Gradient elution-3:

0-10 min 100% B

10-40 min 50% A

40-50 min 100% A

50-60 min 100% B

- Gradient elution-4:

0-10 min 100% B

10-40 min 100% A

40-50 min 100% A

52-60 min 100% B

Once the mobile phase, gradient shape and slope were chosen, it was necessary to choose the concentration of buffer and the pH of mobile phase. Different concentrations and pHs of buffers were examined under the chosen conditions.

2.2.4.4. Choice of buffer concentration

In order to find the best concentration of buffer solution for the separation of the decoction, 0.25 mM, 5 mM, 8 mM, 10 mM, 20 mM, 50 mM and 100 mM concentrations of PBS and ammonium acetate buffers were examined with methanol under the optimized conditions.

2.2.4.5. Choice of the flow rate of mobile phase

To obtain the optimal resolution, 1 and 2 mL/min for analytical columns, 3, 4, 10, 15 and 20 mL/min flow rates of mobile phase for semi-preparative column were examined.

2.2.4.6. Choice of the pH of mobile phase

The pHs of the ammonium acetate buffer within 3, 4.5, 5, 6.5, 7.4 and 8 were checked for the separation of the *Oldenlandia diffusa* decoction by HPLC, using 1 M ammonium hydroxide and 1 M acetic acid to get pH=3-8.

2.3. Results

2.3.1. The mass of prepared decoction

To determine the mass of *Oldenlandia diffusa* decoction, 5 g aerial sample was prepared three times by the method mentioned above using the same conditions but on different days. After filtration through the 0.45 μm filter, the decoction was placed in a previously weighed round bottom flask and it was connected to freeze-drier until dry. After drying, it was weighed.

As results, the weights of dry residue of those prepared decoctions are shown in Table 2.

Table 2. Amount of decoction for three experiments.

Decoctions	Dry residue, g
I-Decoction	0.520
II-Decoction	0.507
III-Decoction	0.510
Mean	**0.512**
STDEV	**0.007**

So, according to the above mentioned method, the mass of prepared *Oldenlandia diffusa* decoction is 0.512±0.007 g which means 10.24% (w/w) extracted dry mass.

2.3.2. Results for setting the separation method up for *Oldenlandia diffusa* decoction using HPLC

To set up the optimal separation method of *Oldenlandia diffusa* decoction using HPLC was an initial aim of the study. To achieve it, the best parameters for separation of decoction, e.g. column, flow rates, absorption wavelengths and mobile phase to be used were examined.

C18, amino, phenyl, cyano and PFP(2) normal phase and reversed phase chromatographic columns were investigated with different ratio of mixtures of different mobile phases as optimised in 2.2.4.3. at 198, 206, 216, 220, 226 and 254 nm wavelengths.

Separation results from using the amino, phenyl and cyano columns at different conditions showed low resolutions and only one to four major peaks (see Figures 6, 7, 10, 11).
Better separations were obtained on reversed phase chromatography using a C18 column, however, the PFP(2) column achieved the best peak (see Figures 8 and 12). Mobile phase of ammonium acetate buffer (pH=6.5)/methanol with gradient elutions at 1 mL/min flow rate were performable.

The most suitable concentration of ammonium acetate was 10 mM. The most responsive wavelength of the decoction was 226 nm as checked on UV detector.

Some representative chromatograms of a better separation of the decoction using the above mentioned chromatographic conditions are shown below:

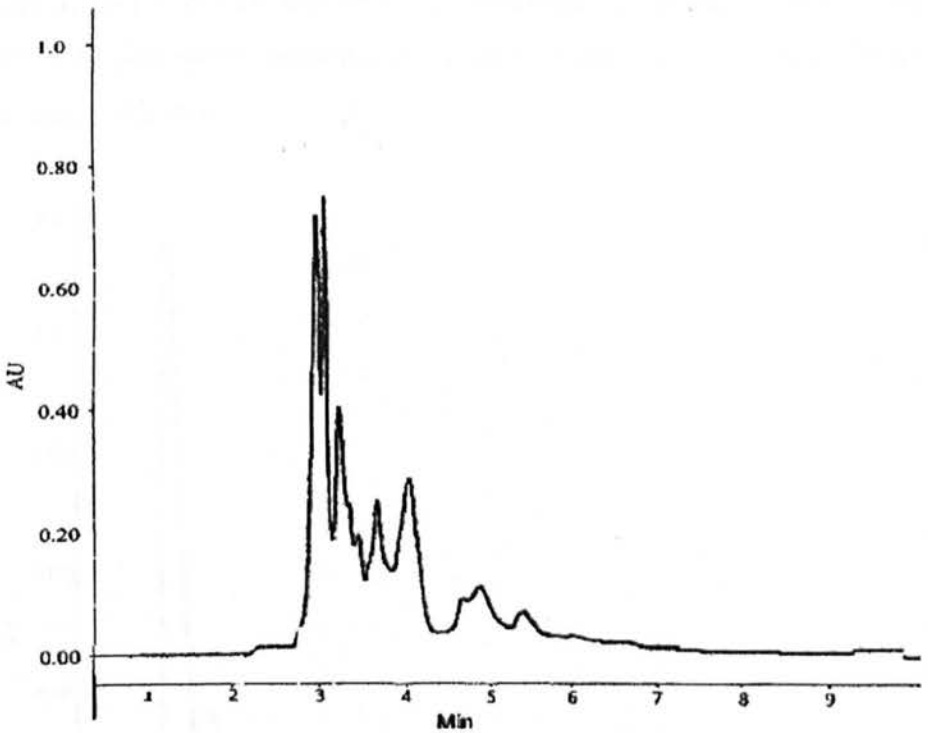


Figure 6. HPLC chromatogram for *Oldenlandia diffusa* decoction, hexane used as a mobile phase on analytical (250 x 4.60 mm 5 μm) amino column, UV detector, wavelength-220 nm, 20 μL injection volume, flow rate 1 mL/min.

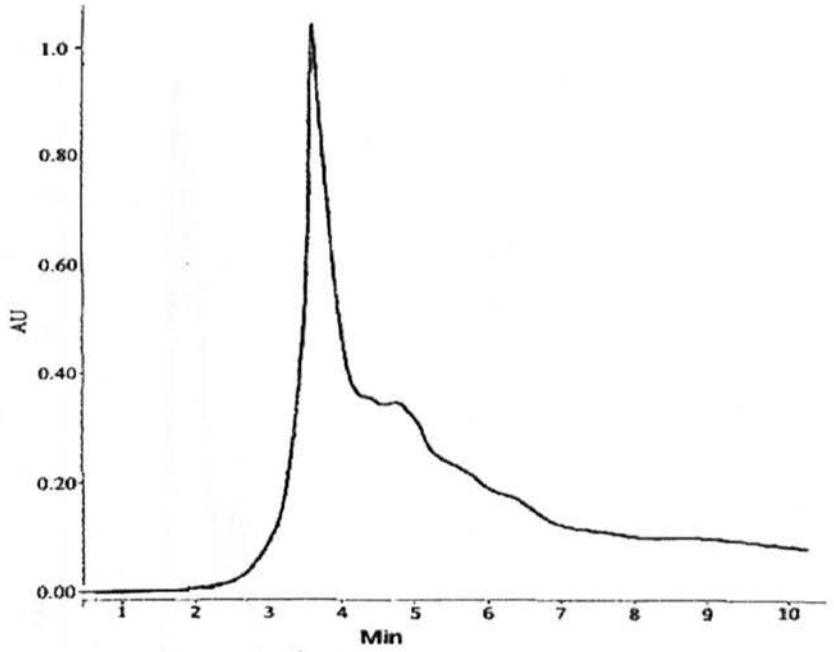


Figure 7. HPLC chromatogram for *Oldenlandia diffusa* decoction, water as a mobile phase on RP C18 semi-prep column, UV detector, wavelength-220 nm, 500 µL injection volume, flow rate 3 mL/min.

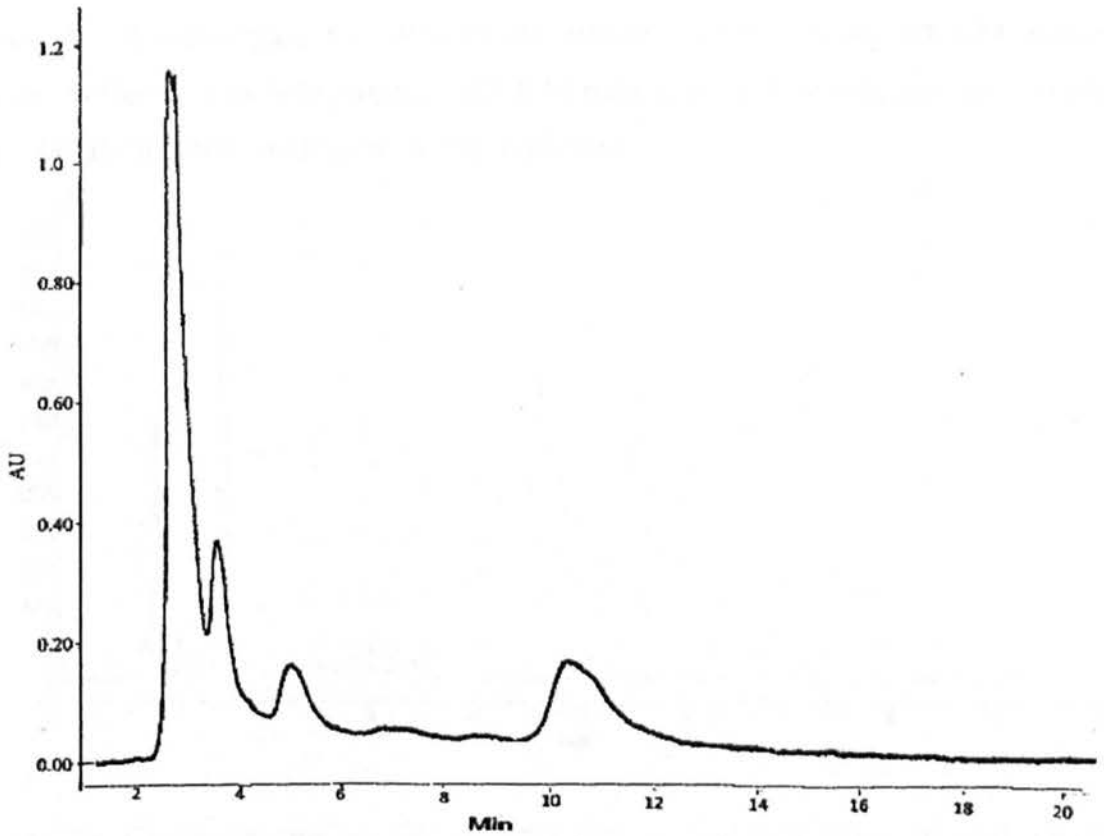


Figure 8. HPLC chromatogram for *Oldenlandia diffusa* decoction using 10 mM PBS (pH=7.2) on C18 semi-prep column, at 220 nm, 500 µL injection volume, flow rate 3 mL/min.

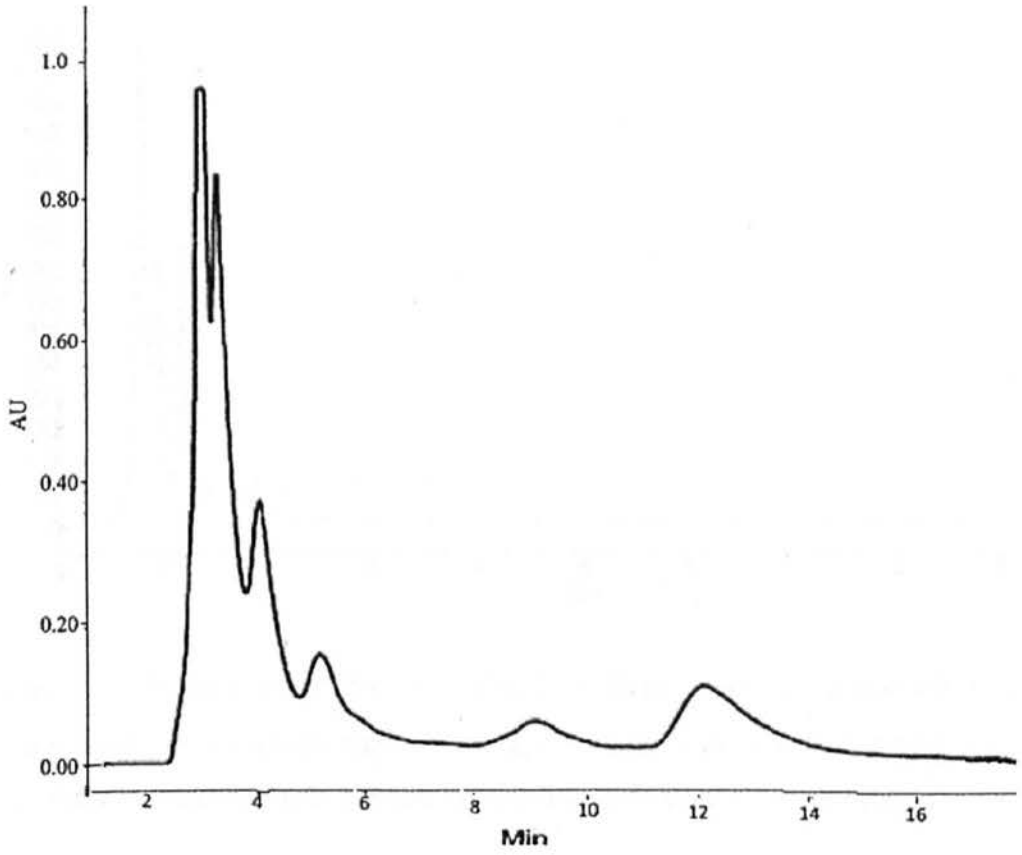


Figure 9. Chromatogram for *Oldenlandia diffusa* decoction using 10 mM ammonium acetate buffer as a mobile phase on RP C18 prep column, UV detector, wavelength-220 nm, 500 µL injection volume, flow rate 3 mL/min.

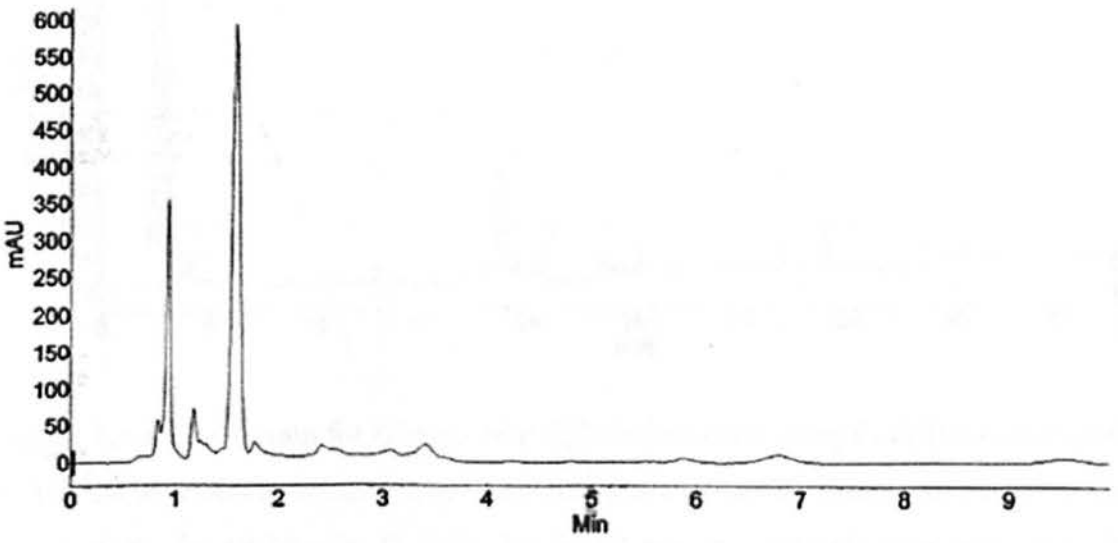


Figure 10. Chromatogram for *Oldenlandia diffusa* decoction using 10 mM ammonium acetate buffer as a mobile phase on analytical (250 x 4.60 mm 5 µm) phenyl column, diode array detector, wavelength-238 nm, 20 µL injection volume, flow rate 1 mL/min.

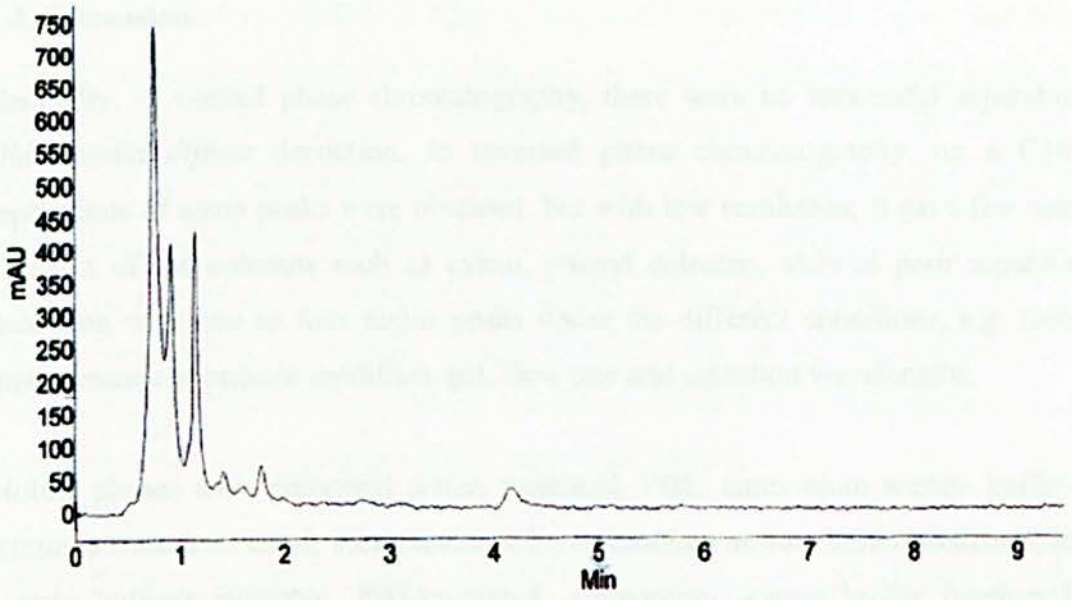


Figure 11. Chromatogram for *Oldenlandia diffusa* decoction using 10 mM ammonium acetate buffer as a mobile phase on analytical (250 x 4.60 mm 5 μm) CN column, diode array detector, wavelength-206 nm, 20 μL injection volume, flow rate 1 mL/min.

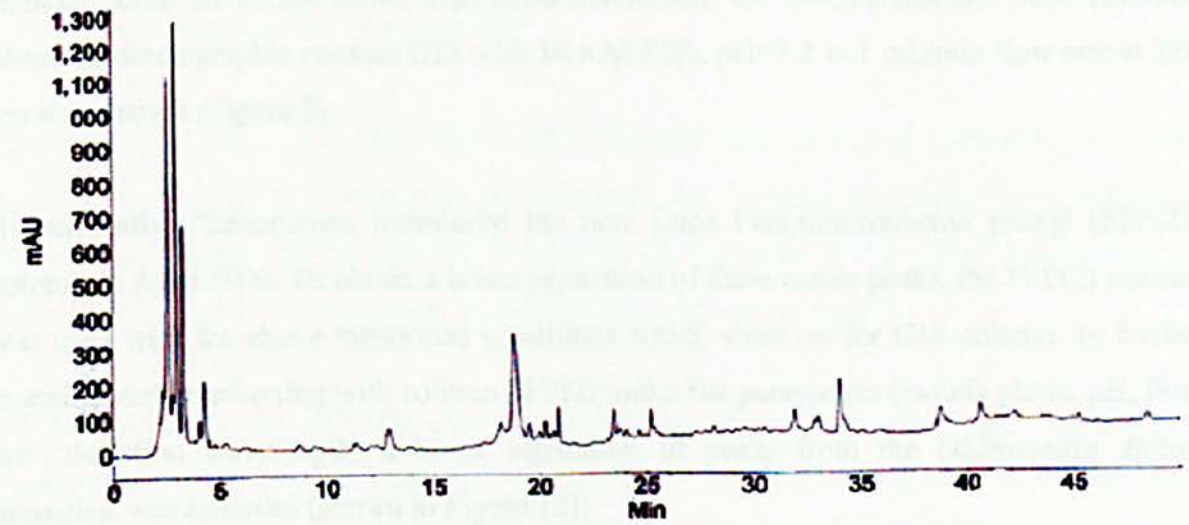


Figure 12. Chromatogram for *Oldenlandia diffusa* decoction using PFP(2) column, methanol (A)/10 mM ammonium acetate buffer (pH=6.5) (B) as a mobile phase with B 100% for 0-10 min, A 100% for 40-50 min, B 100% for 52-60 min at 1 mL/min flow rate and 226 nm wavelength.

2.4. Discussion

Generally, in normal phase chromatography, there were no successful separations of the *Oldenlandia diffusa* decoction. In reversed phase chromatography, on a C18 column, separations of some peaks were obtained, but with low resolution; it gave few major peaks. The rest of the columns such as cyano, phenyl columns, showed poor separation of the decoction with one to four major peaks under the different conditions, e.g. mobile phase (maintenance of organic modifier), pH, flow rate and detection wavelengths.

Mobile phases were deionised water, methanol, PBS, ammonium acetate buffer and their mixtures water/methanol, water/acetonitrile, ammonium acetate buffer/methanol, ammonium acetate buffer/acetonitrile, PBS/methanol, ammonium acetate buffer /methanol/THF for reversed phase-HPLC columns and on amino column were hexane and hexane/isopropanol with different ratio of mixtures and isocratic and gradient shape and slopes as mentioned in Section 2.2.4.3. at 198, 206, 216, 220, 226 and 254 nm wavelengths and pHs. There were no successes on examinations of water with different organic modifiers.

Initially, from all of the above mentioned conditions, the best parameters were: reversed phase chromatographic column C18 with 10 mM PBS, pH=7.2 at 1 mL/min flow rate at 226 nm wavelength (Figure 8).

Subsequently, Phenomenex introduced the new Luna Pentafluorophenyl propyl (PFP(2)) column in April 2008. To obtain a better separation of these major peaks, the PFP(2) column was used with the above mentioned conditions which were set for C18 column. In further examinations, performing with column PFP(2) under the parameters (mobile phase, pH, flow rate, detection wavelength) a better separation of peaks from the *Oldenlandia diffusa* decoction was achieved (shown in Figure 12).

Since gradient elutions are more preferable for biomolecules, the gradient slope and gradient shape were optimised for gradient elution.

It was necessary to consider not only from the side of cellular biology, but also for the chemistry analysis. For the further studies, after the separation of the *Oldenlandia diffusa* decoction, fractions will be collected and examined on cancer cell lines to choose the more

cytotoxic fraction(s). After collection of the fractions for cell biology investigations, their solvent must be appropriate for the cells to be living in. Methanol can be removed easily as it is an organic volatile solvent. Therefore, PBS was chosen as a buffer for the decoction separation. But for the chemical analysis, it was complicated to remove the PBS from the contaminated decoction (fraction(s)) to determine the cytotoxic content(s). Therefore, another easily removable, simple but adaptable buffer was needed to be used as a mobile phase. There are some suitable buffers for cells such as MOPS, HEPES, MES, CAPS, CHES, HEPPS, Tris, Tricine, PIPES, cacodylate and KH_2PO_4. Unfortunately, the PBS was the simplest buffer within these above mentioned buffers.

Furthermore, a removable buffer was the best choice for both cell biological and chemistry analysis. The most suitable simple and easily removable salt which can give similar separation to PBS was ammonium acetate. Its optimal concentration and pH were investigated using the PFP(2) column at a 1 mL/min flow rate with methanol with the same gradient slope of mobile phase. In order to remove the salt, it was necessary to use as low as possible amount of salt in the mobile phase. Results showed that less than 5 mM and above 10 mM was not suitable. The resolution of peaks with 5 mM ammonium acetate buffer was lower than the 10 mM buffer.

Its pH was 6.5, but before with PBS at pH=7.2 good separation was achieved. The buffer does an excellent job in keeping the pH between 6 and 8, there was almost no difference of separation between pH=6.5 and 7.2. Therefore, 10 mM ammonium acetate buffer with pH 6.5 were chosen for the separation of *Oldenlandia diffusa* decoction.

At the beginning, for HPLC method evaluation, a UV detector was used. Therefore, to determine the most absorbable wavelength for decoction its UV absorbances were measured on the UV spectrometer, and the following wavelengths 198, 206, 216, 220, 226 and 254 nm were checked for each run. The best wavelength to use was 226 nm.

2.5. Conclusion

The dry mass of *Oldenlandia diffusa* decoction is 10.24% (w/w), prepared as 5 g of dried aerial parts of herb boiled with 50 mL of deionised water by refluxing for 45 min, and after cooling and filtration, make up to 50 mL with deionised water.

The method that was set up for the separation of *Oldenlandia diffusa* decoction is:- in HPLC, using PFP(2) (250 x 4.60 mm, 5 μm) column, methanol (A)/10 mM ammonium acetate buffer (pH=6.5) (B) as a mobile phase with B 100% for 0-10 min, A 100% for 40-50 min, B 100% for 52-60 min gradient elution at 1 mL/min flow rate and 226 nm with 20 μL injection volume (Ms method).

Chapter 3. Fraction collection

Chapter 3. Fraction collection

3.1. Introduction

The fractions were collected from the *Oldenlandia diffusa* decoction, in order to determine the most cytotoxic one. It was necessary to remove the ammonium acetate salt from the fractions for the cell biological (cytotoxicity) assessments. The removal of the salt from the decoction was investigated using freeze-drying and solid phase extraction methods, and was performed on collected fractions before the cell biological investigations.

3.1.1. Solid phase extraction

Solid phase extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate one or one type of analyte from a solution. It is also a useful sample preparation technique. SPE is more efficient than liquid-liquid extraction. It is rapid and can be automated. Solid phase extraction is usually used to clean up a sample before using other analytical methods. They are available in a wide variety of types and sizes [Nijel *et al.*, 2000]. For example: reversed phase, normal phase, ion exchange, and adsorption [Eaton *et al.*, 2005]. Selecting the most suitable cartridge for the sample is important. In our study the RP-SPE was used.

3.1.2. RP-SPE

RP-SPE is a non-polar stationary phase. It includes several SPE materials, such as alkyl- or aryl-bonded silicas. Thus, LC-18, ENVI-18 (octadecyl bonded, endcapped silica), LC-8, ENVI-8 (octyl bonded, endcapped silica), LC-4 (butyldimethyl bonded, endcapped silica) and LC-Ph (phenyl bonded silica) tubes are involved in the reversed phase type.

The LC-18 SPE cartridge was used in our study. It is a monomerically bonded silica. RP-SPE uses a polar or fairly polar mobile phase and the analyte of interest is typically from mid to nonpolar [Loconto *et al.*, 2006], [Somenath, 2003]. Organic analytes are retained from polar solutions (e.g. water) onto these SPE by the nonpolar-nonpolar attractive forces (van der Waals forces) between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. Then, to disrupt this force that binds the compound to the silica surface or to elute the adsorbed compound from a RP-SPE tube, a nonpolar solvent can be used [Leo, 2004]. The general procedure for SPE involves cartridge

conditioning, sample loading on to the cartridge, then cartridge rinsing and at the end analyte elution [Lisk *et al.*, 1989], [Takayuki *et al.*, 1998].

3.1.3. Fraction collection

Usually, the purification of natural and biological compounds with any quantity requires the use of preparative HPLC. It is used to achieve the purified compound(s) of interest [Satyajit *et al.*, 2006]. However, there are several ways to purify compounds, including crystallization, SPE, distillation and filtration. Preparative HPLC was the most powerful, robust and versatile tool for purification [Mann, 1998], [Satyajit *et al.*, 2006].

In this Chapter, manual fraction collection of *Oldenlandia diffusa* decoction using preparative HPLC will be discussed.

3.1.4. Fraction collection using preparative HPLC

Fractions can be collected from HPLC by:

1. Peak gradient
2. Peak threshold
3. Time

1- Fraction collecting by peak gradient

Fraction collection by peak gradient can be performed using a fraction collector. In this fraction collecting method, the fraction collector measures the upslope of peaks. When the upslope is high enough the fraction collector begins the collection. When the peak has been reached, the fraction collector measures the down slope and stops collecting when the gradient of the peak becomes low. Broad, low peaks may be not detected by this method.

2- Fraction collecting by peak threshold

If it is necessary to collect the major peaks and lose the minor peaks, it could be done by collecting above the 'peak threshold'. It involves the collection of peaks over a set threshold (e.g. 10% of total detector response). Collecting by peak threshold or by peak gradient is also dependent on achieving baseline separation of the peaks.

3- Fraction collecting by Time

The fraction can be collected by a set time period. Sometimes the collected fractions are not pure, but this method helps narrow the field down to a particular region of the active

mixture [Satyajit *et al.*, 2006]. In addition, this method can be used for manual fraction collecting. Firstly, this method is used to create many fractions, especially when studying the unknowns, and secondly, because of the instrumental condition, the fractions were manually collected by this method from the *Oldenlandia diffusa* decoction.

It was necessary to find out some of the following questions:

- What is the delay from the detector to the fraction collecting point?
- Is the collection point correct or not?

There are many factors that affect the ability to collect the fractions. As mentioned above, the fractions were collected from preparative HPLC using the method which was set up for the separation of the decoction manually by time. Therefore, the first focusing basic parameters were delay time and detector settings.

3.1.5. Delay Time

The delay time is the time that an analyte molecule migrates from the detector cell to the fraction collecting point [Joan *et al.*, & Craig *et al.*, 2003], [Stavros, 2006]. This delay time can be converted to the flow rate delay volume.

3.1.6. Delay Volume

There is a fixed amount of tubing volume between the detector flow cell and the fraction collector point or waste output. To collect the fractions, it is important to determine the delay volume. The time-delay between the signal diverter valve and fraction delay sensor is the delay time. Depending on the flow rate used for the calibration procedure, the exact delay volume is automatically calculated by the system and stored in the fraction collector memory. The precise delay time can be calculated by the system for every flow rate. The delay volume, V is:

$$V = h \times (d/2)^2 \times \pi = h \times (d^2/4) \times 3.14 \qquad (1)$$

Hence, V is delay volume

h is length of tubing

d is internal diameter of tubing [Giddings *et al.*, 1983], [Agilent Application Note, 2002].

3.1.7. Detector delay

Most detectors have a detector noise filter that smoothes the data output signal. This detector smoothing process takes time. A delay of 2 or 3 sec can result in the loss of 50% of the sample, but with fast flow rates (20-200 mL/min), narrow peaks (5–60 sec), and long time constants, the sample can reach the fraction collector before the software receives the signal from the detector. Most modern detectors should have the option to set a time constant at or near zero to minimize this problem.

3.2. Materials and methods

3.2.1. Materials

The following equipments and materials were used for the fraction collecting experiments: The HPLC system was the same as mentioned in Chapter-2. In addition, a freeze-drier (TELSTAR Croydos, model 50, USA), UV-Visible spectrophotometer (Varian, UK), C18 SPE column (Fisher Scientific, UK), PFP(2) analytical column (Luna 250 x 4.60 mm, 5 µm, Phenomenex, Germany) and PFP(2) preparative column (Luna 20 cm x 15.1 mm, 5 µm, Phenomenex, Germany) were used. Chemicals used were ammonium acetate (HPLC grade) (Sigma- Aldrich Inc, Poole, UK) and methanol, acetonitrile (HPLC grade) (Fisher Scientific Inc, UK).

3.2.2. Methods

3.2.2.1. Sample preparation

Decoction of CHR *Oldenlandia diffusa* and ammonium acetate buffers were prepared as mentioned in Chapter 2.

In order to collect the fractions from the *Oldenlandia diffusa* decoction, the scale of column was changed from analytical to preparative. As the cytotoxicity of collected fractions needed to be investigated, the removal of ammonium acetate buffer was performed using C18 SPE.

3.2.2.2. Removal of salt by SPE

The general procedure for SPE is to load a solution onto the SPE cartridge, wash away undesired components and then wash off the compounds of interest with another solvent into a collection tube.

The following five steps were performed for sample cleaning by SPE:

1. Select the proper SPE cartridge
2. Condition the SPE cartridge
3. Add the sample
4. Wash the packing
5. Elute the compounds of interest

Step 1. Selecting the SPE tube

To remove the ammonium acetate salt and other inorganic impurities, it was necessary to select a SPE sorbent that will bind the compounds of interest and the sample impurities. As our sample sizes were approximately 50-200 mL in ammonium acetate buffer (pH=6.5) and separated by reversed phase chromatography column, C18 SPE with 6 mL capacity was used.

Step 2. Conditioning the SPE tube

Before extracting the sample, the tubes were washed with 10-12 mL of acetonitrile, followed by deionised water and resultant equilibration. Reversed phase type silicas and nonpolar adsorption media are usually conditioned with a water-miscible organic solvent such as acetonitrile and methanol. The SPE tubes were washed two times with twice their volume of acetonitrile. Acetonitrile wets the surface of the sorbent and penetrates bonded alkyl phases. After that the silica surface were washed twice with water to let it get wet.

This pre-conditioning is used to remove any impurities on the SPE cartridge and aids wetting the surface area to ensure a maximum contact with analytes [Lisk *et al.*, 1989], [Takayuki *et al.*, 1994].

Step 3. Loading a solution on SPE tube

The sample solution was accurately transferred to a SPE C18 tube, using a micropipette. Total sample volume for each tube did not exceed 6 mL volume.

The sample solution slowly passed through the extraction tube, using vacuum. Generally, the flow rate should not exceed 5 mL/min for SPE tubes. As the flow rate can affect the retention of certain compounds, the sample solution passed with 2.5 mL/min drop wise flow rate. When the sample was passed through the SPE tube, the selected components were retained. The effluent contains the sample without the adsorbed components.

Step 4. Washing away undesired components

As the compounds of interest were retained on the packing, unwanted materials and impurities were washed off using the water.

Step 5. Washing the compounds of interest with another solvent into a collection tube

The SPE tubes were rinsed with 10-12 mL volume of acetonitrile (a solution that removes compounds of interest) with 2.5 mL/min wise flow. The eluates were collected for further analysis.

Acetonitrile of collected eluate was evaporated on rotary-evaporator at 40 °C under vacuum. Then, the dry residue was dissolved back in water in order to bring it back to the same condition as the decoction and investigated by the Ms method which was set up for the decoction separation on HPLC.

3.2.2.3. Removal of ammonium acetate salt by freeze-drying

5 mL of prepared 10 mM ammonium acetate buffer was once, double and triple freeze-dried and 5 mL of deionised water was added back to round bottom flask and the absorbances of samples were measured by UV spectrometer.

3.2.2.4. Setting up the fraction collection method

Delay time for the preparative column was calculated using equation (1 and 2).

In addition, it was checked by injection of naphthalene to RP-HPLC, using the separation method which set up for the decoction on preparative column, but at 20 mL/min flow rate. Then, 1-5 fractions were collected at 5 seconds intervals, including before the naphthalene peak elution, on the naphthalene peak elution and after the naphthalene peak elution points. Their UV absorbances were measured with a UV spectrophotometer (Figures17-21).

3.2.2.5. Fraction collection

Fraction collection from the *Oldenlandia diffusa* decoction was performed on HPLC, on PFP(2) (Luna 20 cm x 15.1 mm, 5 µm) preparative column using Ms method at 20 mL/min flow rate (Msp method) by the time intervals of 2.0-4.0, 4.0-7.0, 7.0-17.0, 17.0-20.9, 20.0-24.0, 24.0-29.0, 29.0-33.5, 33.5-36.5, 36.5-41.5, 41.5-50.0 and 50.0-60.0 min.

3.2.2.6. UV absorbance of each fraction

UV absorption measurements were taken from each collected fraction using a UV spectrophotometer (at 200-700 nm) to ascertain the best absorbing wavelength for future studies.

3.2.2.6. Preparation of collected fractions for cell cytotoxicity investigation

All collected 11 fractions were evaporated on rotary-evaporator at 40 °C under vacuum. Then, each fraction was passed through the C18 SPE. Solvents were evaporated on a rotary-evaporator at 40 °C under vacuum and then connected to a freeze-drier to get a dry residue for each fraction.

3.3. Results

The chromatographic trace of the decoction on analytical and preparative scale columns remained the same.

3.3.1. Removal of salt by SPE

To remove the ammonium acetate salt from the sample, C18 SPE tube was used. The decoction was passed through the SPE and post-extraction sample was investigated back on HPLC using the same method as used for the separation (Ms method).

Results are shown below:

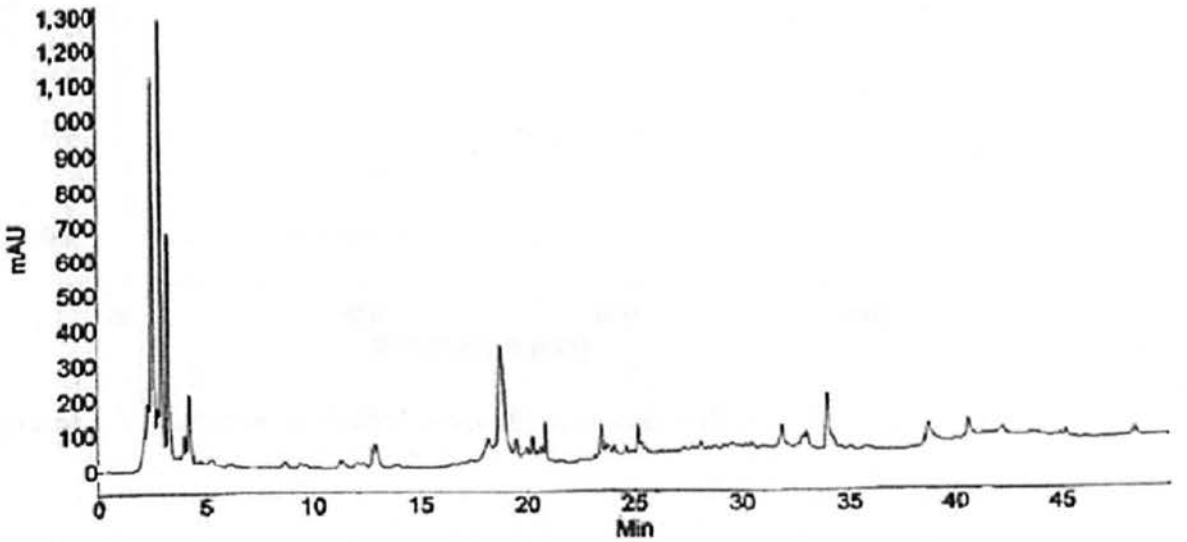


Figure 13. Chromatogram for *Oldenlandia diffusa* decoction (before SPE) using Ms method.

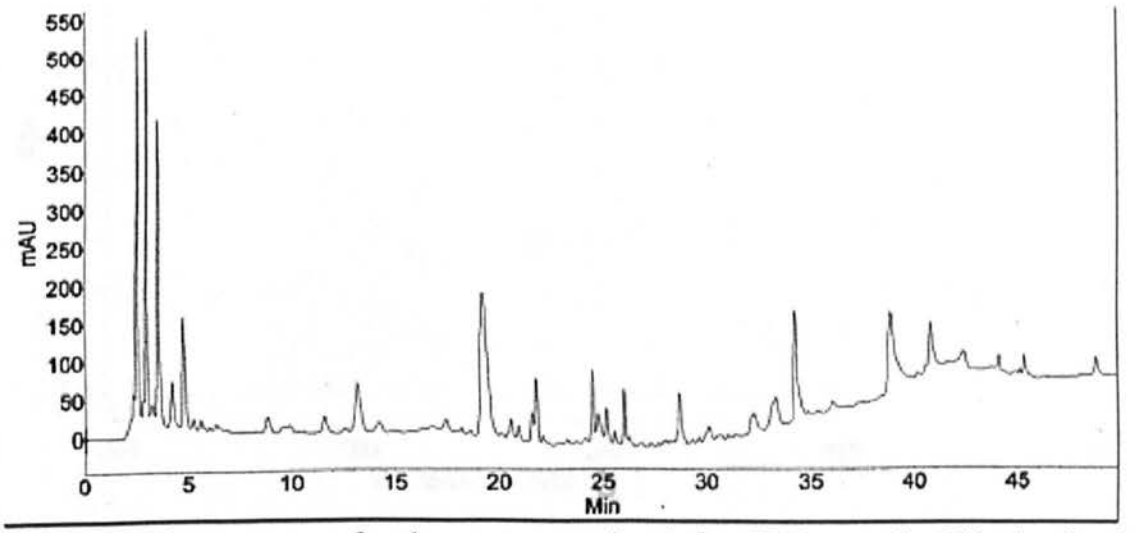


Figure 14. Chromatogram for the post-extraction (after SPE passed) *Oldenlandia diffusa* decoction using Ms method.

3.3.2. Removal of ammonium acetate salt by freeze-drying

Results for the ammonium acetate salt removal by freeze-drying are shown in Figures 15-18.

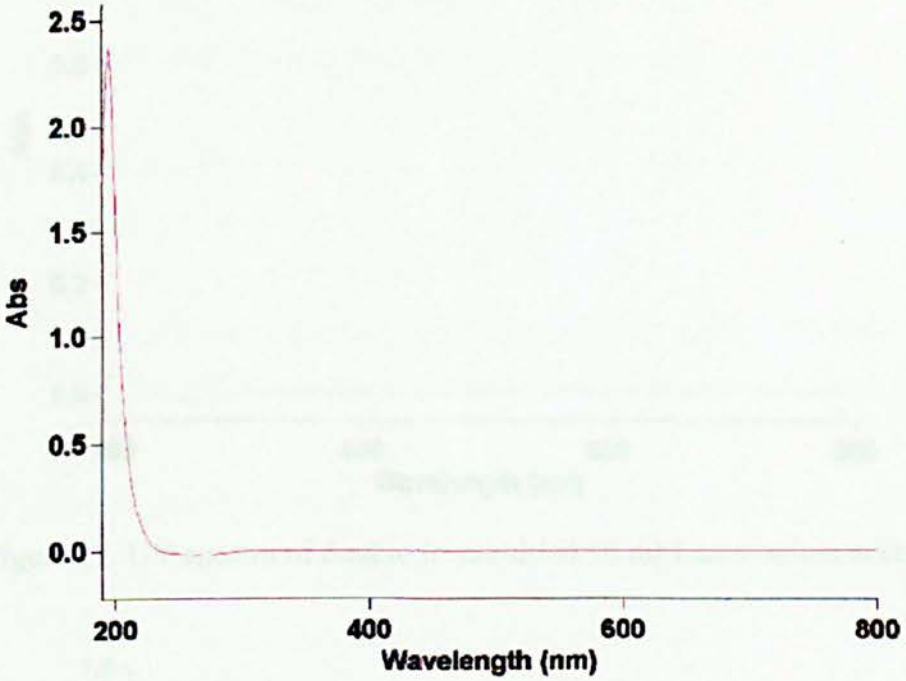


Figure 15. UV spectra of 10 mM ammonium acetate buffer.

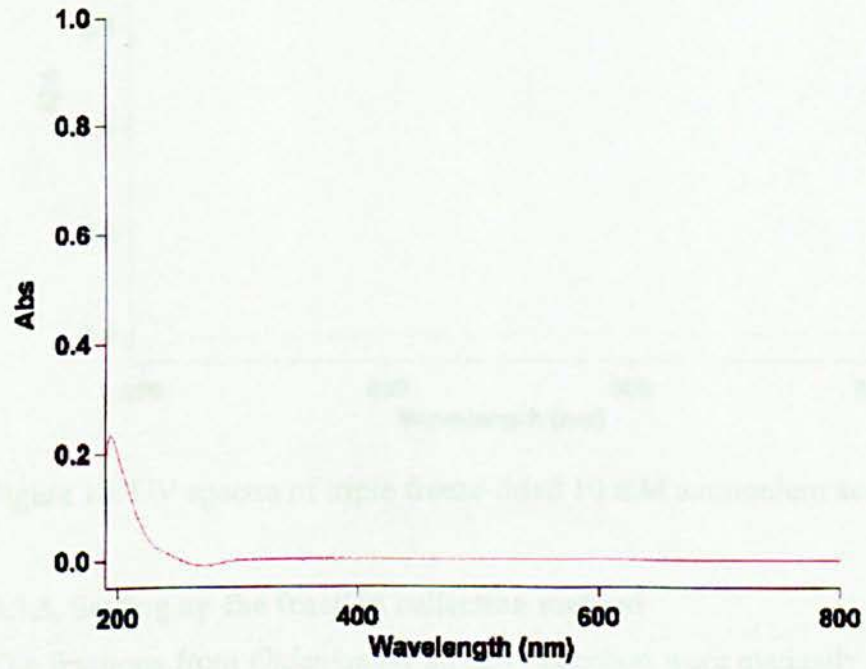


Figure 16. UV spectra of one time freeze-dried 10 mM ammonium acetate buffer.

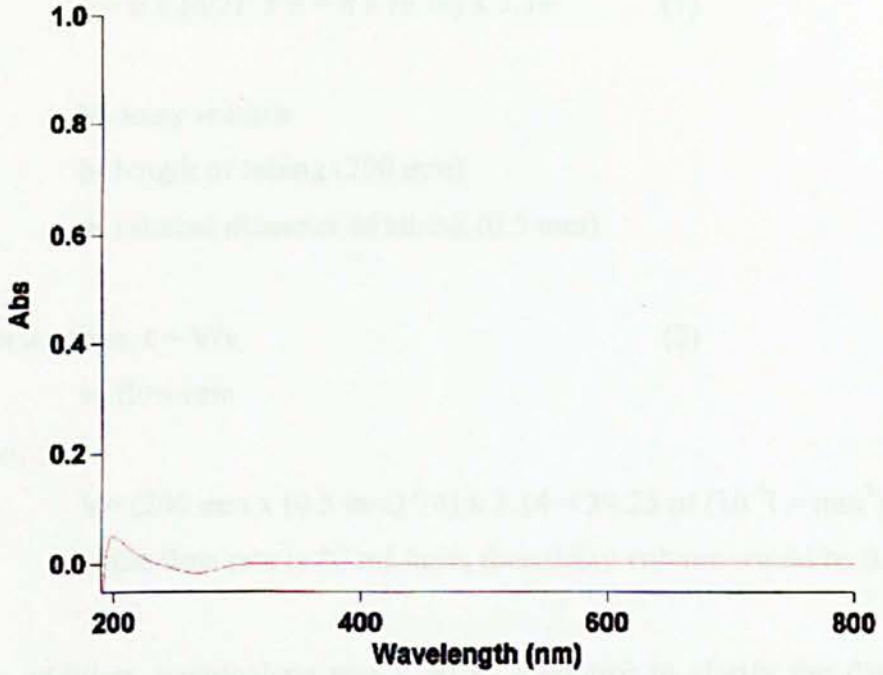


Figure 17. UV spectra of double freeze-dried 10 mM ammonium acetate buffer.

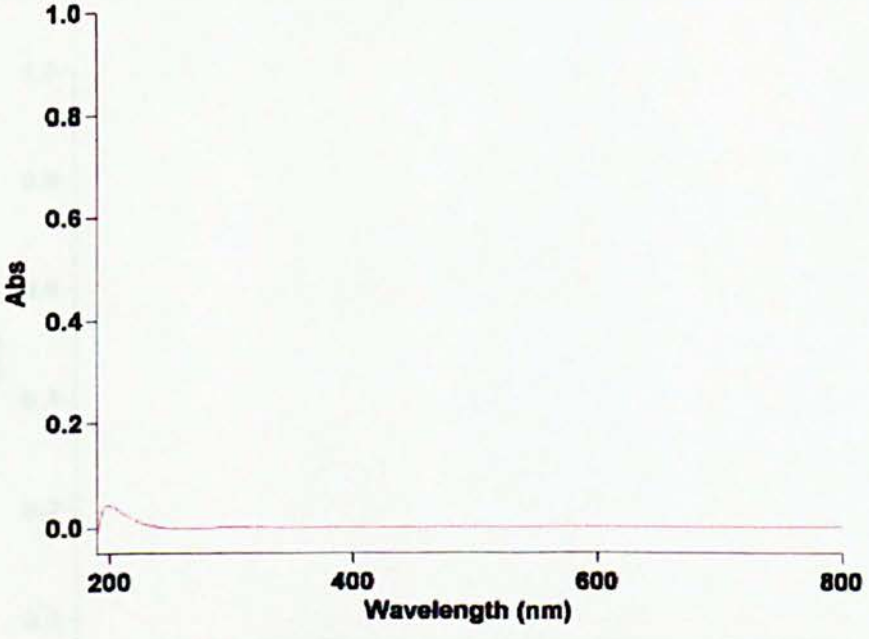


Figure 18. UV spectra of triple freeze-dried 10 mM ammonium acetate buffer.

3.3.3. Setting up the fraction collection method

The fractions from *Oldenlandia diffusa* decoction were manually collected from HPLC by time interval. For that, it was necessary to determine the delay time. The delay time for the preparative column was calculated as follows:

$$V = h \times (d/2)^2 \times \pi = h \times (d^2/4) \times 3.14 \qquad (1)$$

V-delay volume

h- length of tubing (200 mm)

d- internal diameter of tubing (0.5 mm)

Delay time, $t = V/v$ (2)

v- flow rate

So,

$V = (200\ mm \times (0.5\ mm)^2/4) \times 3.14 = 39.25\ \mu l\ (10^{-6}L = mm^3)$

If the flow rate is 20 mL/min, then delay volume would be 0.118 seconds.

In addition, naphthalene was used as a control to clarify the delay time, and collected fractions' (1-5) UV absorbances were measured using UV spectrometer and the results follow.

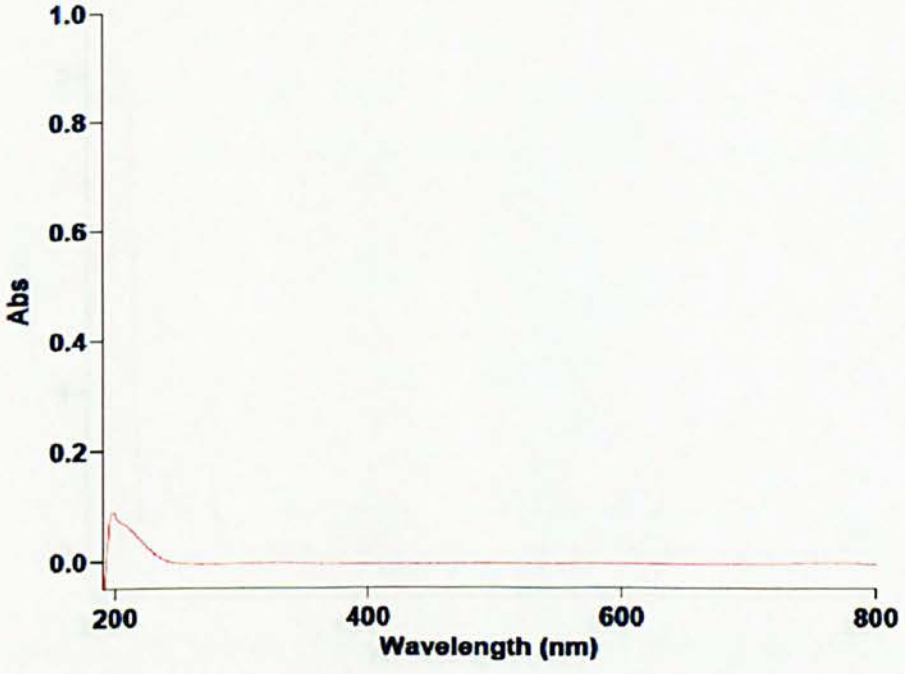


Figure 19. UV spectra of fraction-1, 10 seconds before the naphthalene peak collected from the HPLC on prep PFP(2) column using Msp method.

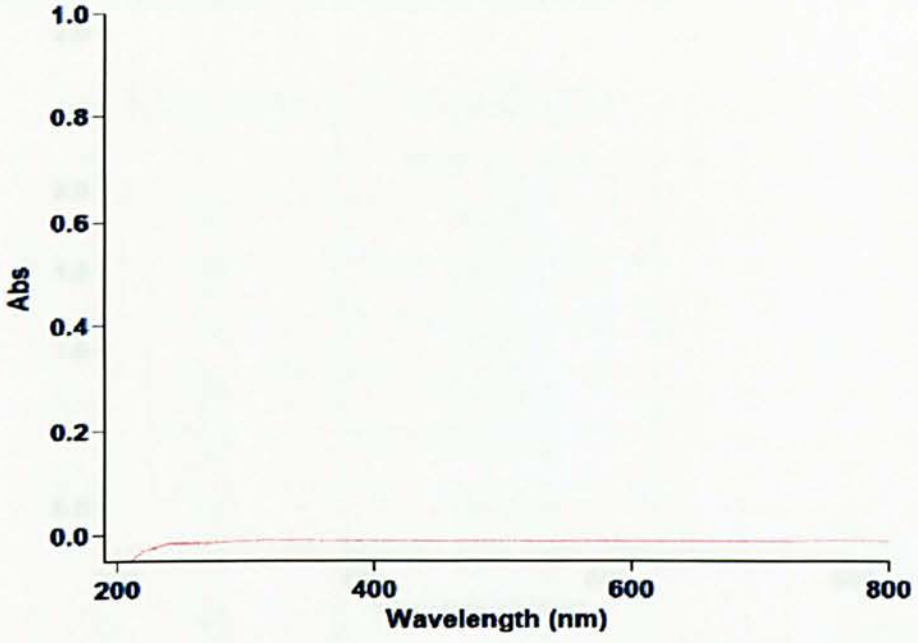


Figure 20. UV spectra of fraction-2, 5 seconds before the naphthalene peak collected from the HPLC on prep PFP(2) column using Msp method.

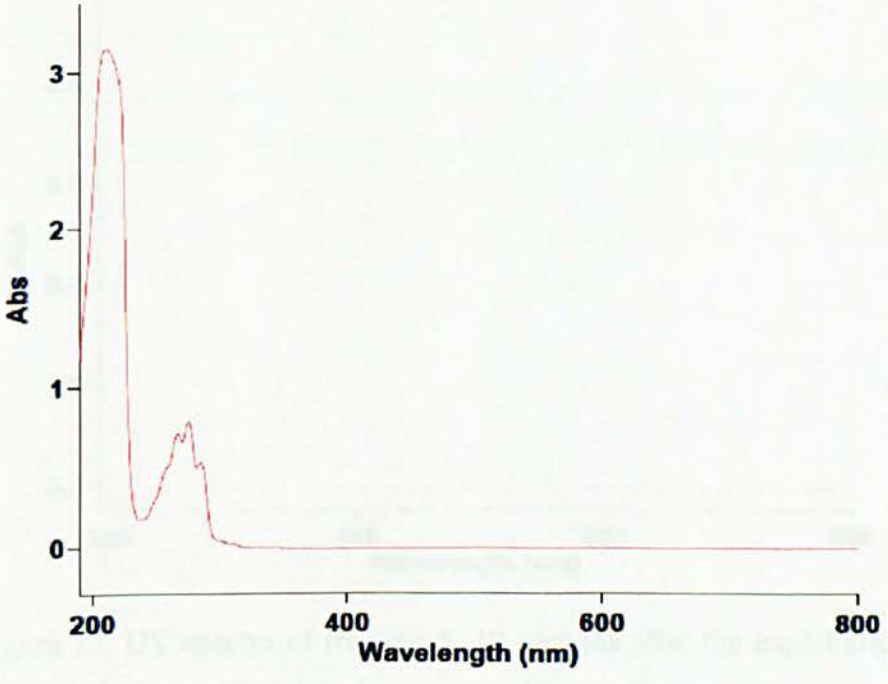


Figure 21. UV spectra (at 275 nm) of fraction-3 which is the naphthalene peak collected from the HPLC on prep PFP(2) column using Msp method.

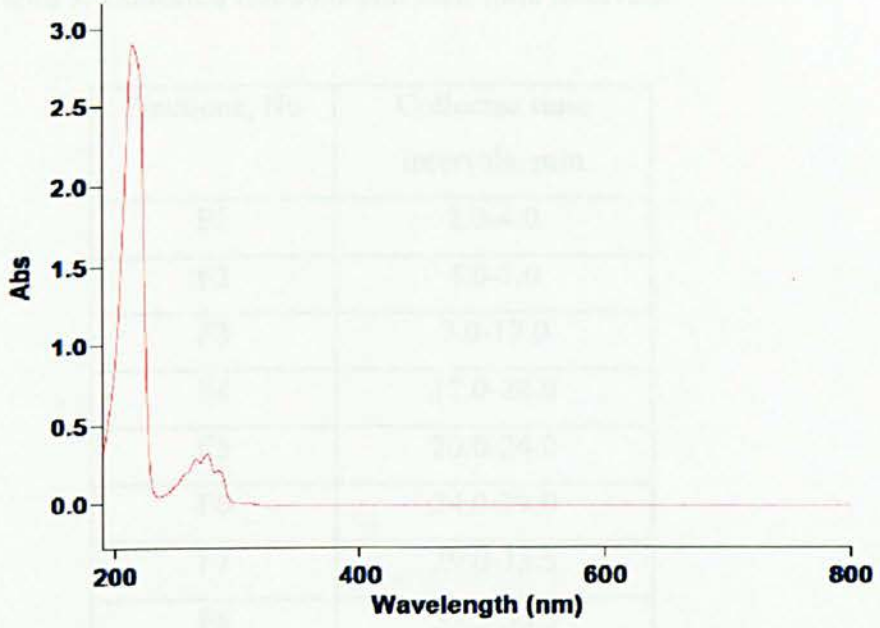


Figure 22. UV spectra (at 275 nm) of fraction-4, 5 seconds after the naphthalene peak collected from the HPLC on prep PFP(2) column using Msp method.

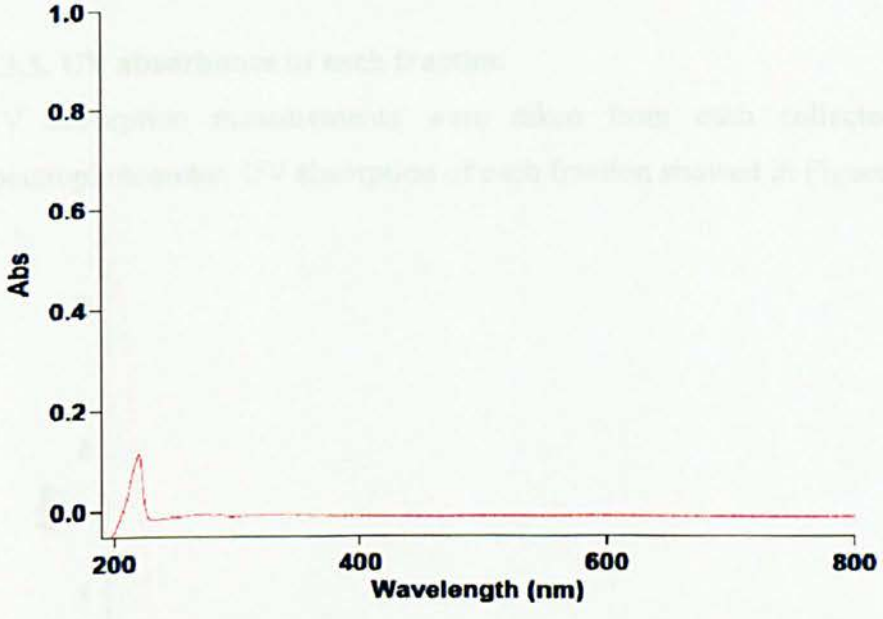


Figure 23. UV spectra of fraction-5, 10 seconds after the naphthalene peak collected from the HPLC on prep PFP(2) column using Msp method.

3.3.4. Fraction collection

There were 11 fractions (F1-F11) collected from the *Oldenlandia diffusa* decoction using the separation method on preparative column.

Table 3. Collected fractions and their time intervals.

Fractions, No	Collected time intervals, min
F1	2.0-4.0
F2	4.0-7.0
F3	7.0-17.0
F4	17.0-20.0
F5	20.0-24.0
F6	24.0-29.0
F7	29.0-33.5
F8	33.5-36.5
F9	36.5-41.5
F10	41.5-50.0
F11	50.0-60.0

3.3.5. UV absorbance of each fraction

UV absorption measurements were taken from each collected fraction using UV spectrophotometer. UV absorption of each fraction showed in Figures 24-34.

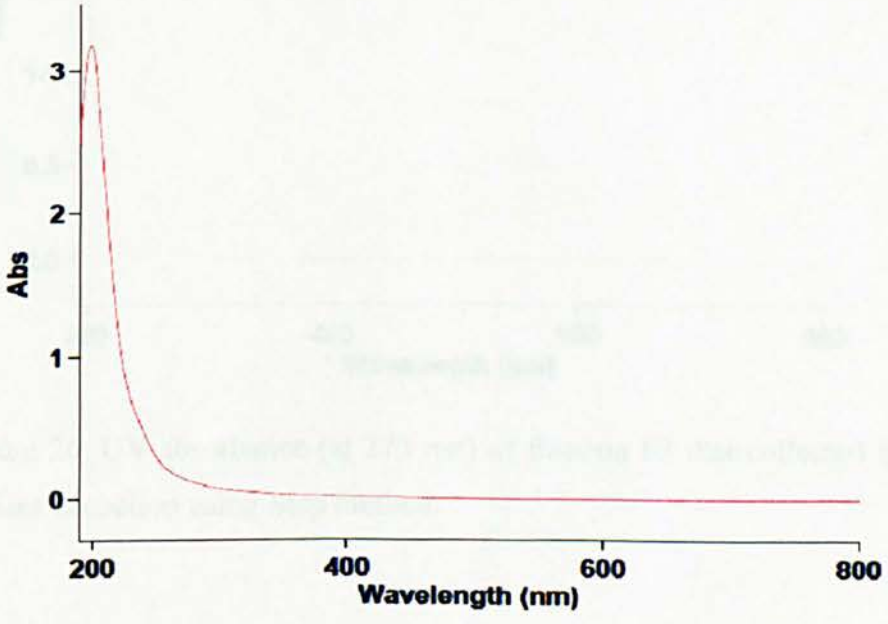


Figure 24. UV absorbance (at 245 nm) of fraction F1 that collected from the *Oldenlandia diffusa* decoction using Msp method.

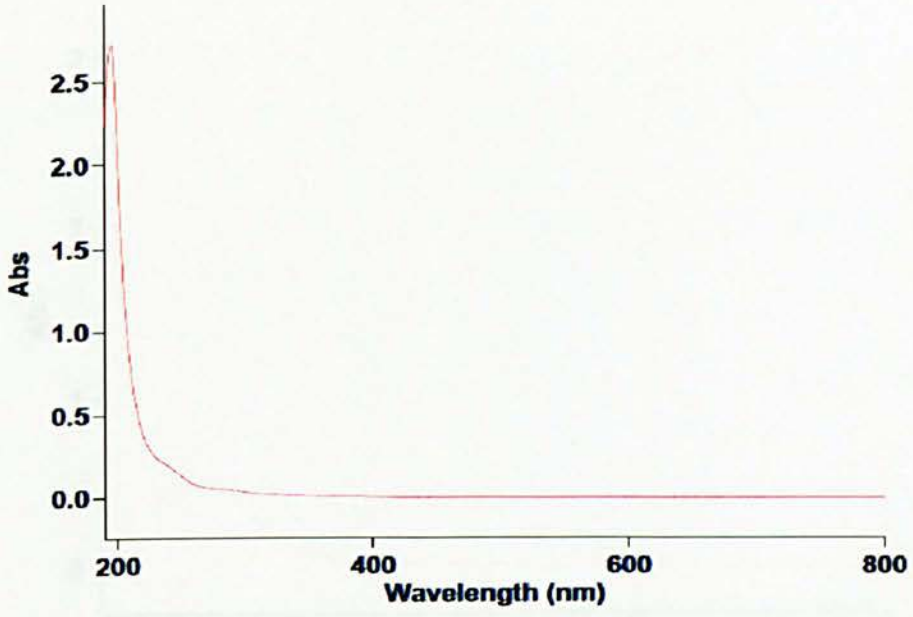


Figure 25. UV absorbance (at 244 and 281 nm) of fraction F2 that collected from the *Oldenlandia diffusa* decoction using Msp method.

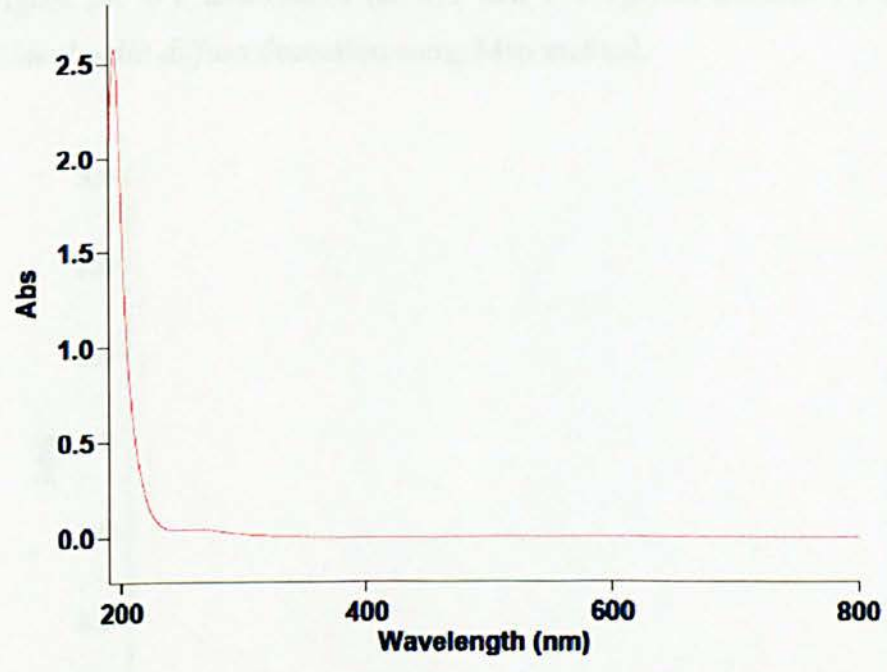


Figure 26. UV absorbance (at 273 nm) of fraction F3 that collected from the *Oldenlandia diffusa* decoction using Msp method.

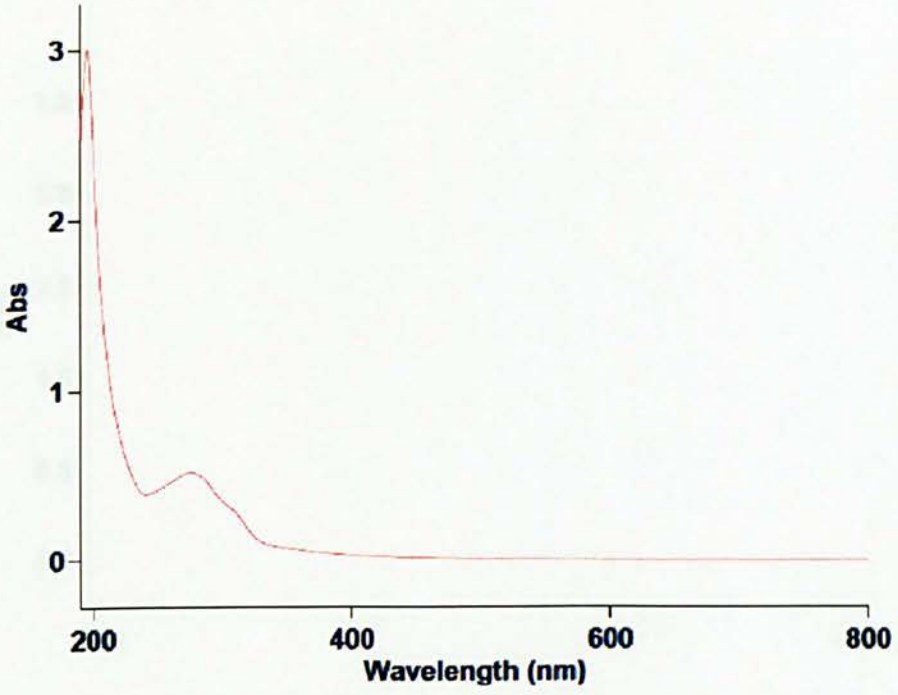


Figure 27. UV absorbance (at 273 and 314 nm) of fraction F4 that collected from the *Oldenlandia diffusa* decoction using Msp method.

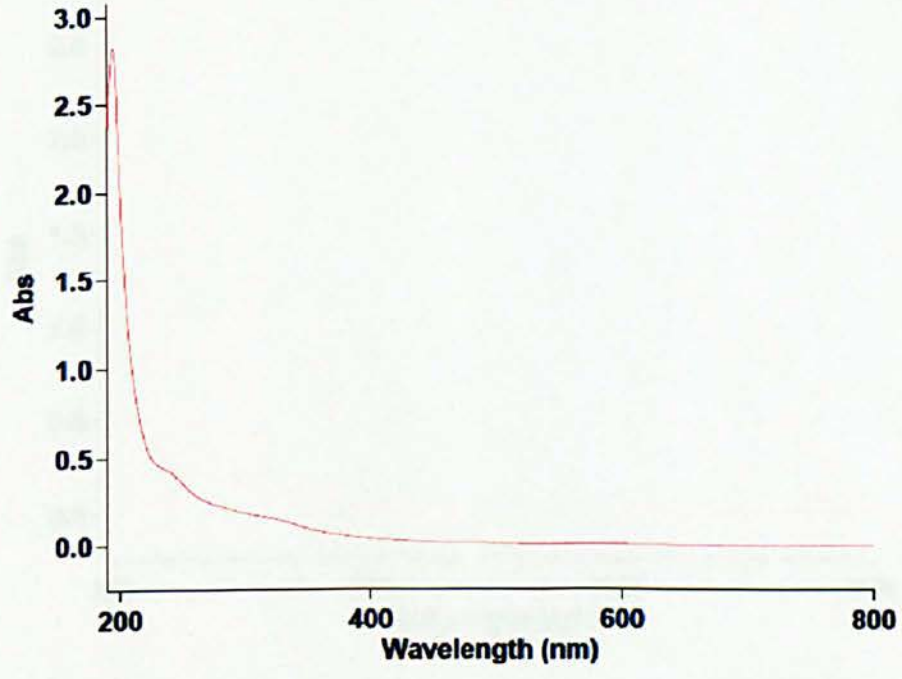


Figure 28. UV absorbance (at 239, 278 and 320 nm) of fraction F5 that collected from the *Oldenlandia diffusa* decoction using Msp method.

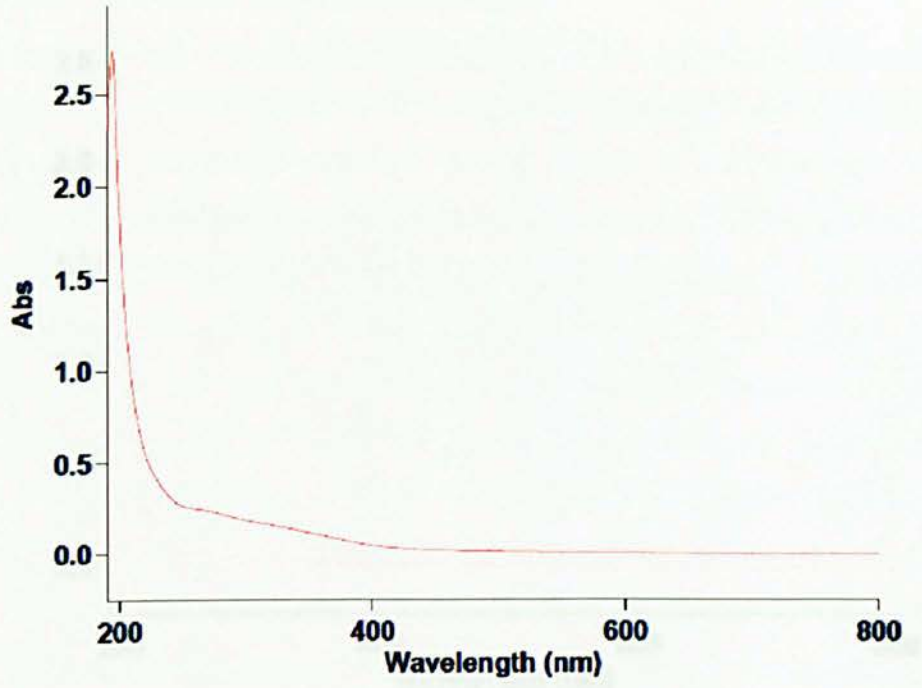


Figure 29. UV absorbance (at 236 and 315 nm) of fraction F6 that collected from the *Oldenlandia diffusa* decoction using Msp method.

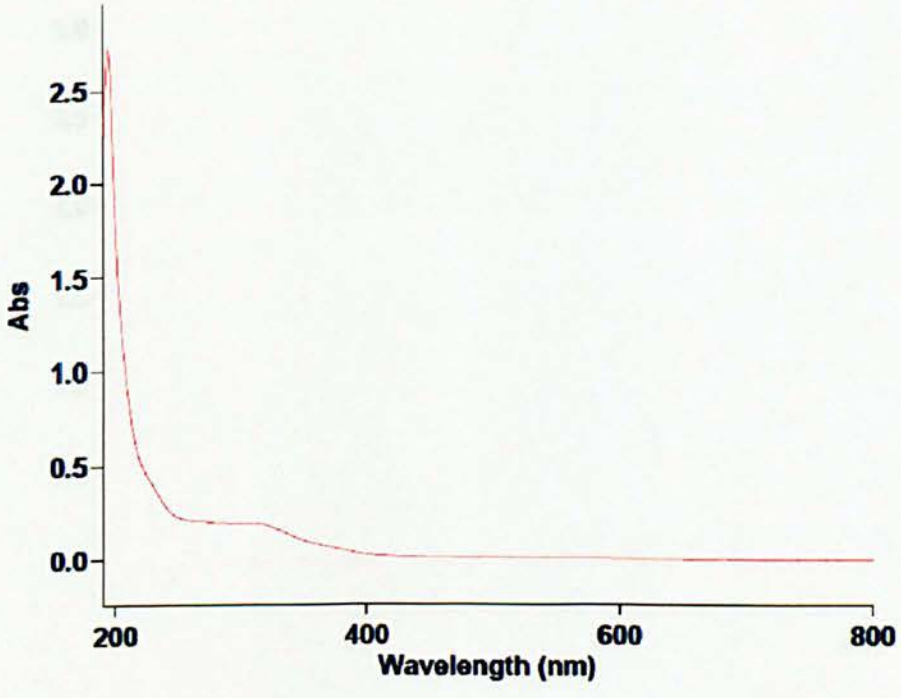


Figure 30. UV absorbance (at 236 and 315 nm) of fraction F7 that collected from the *Oldenlandia diffusa* decoction using Msp method.

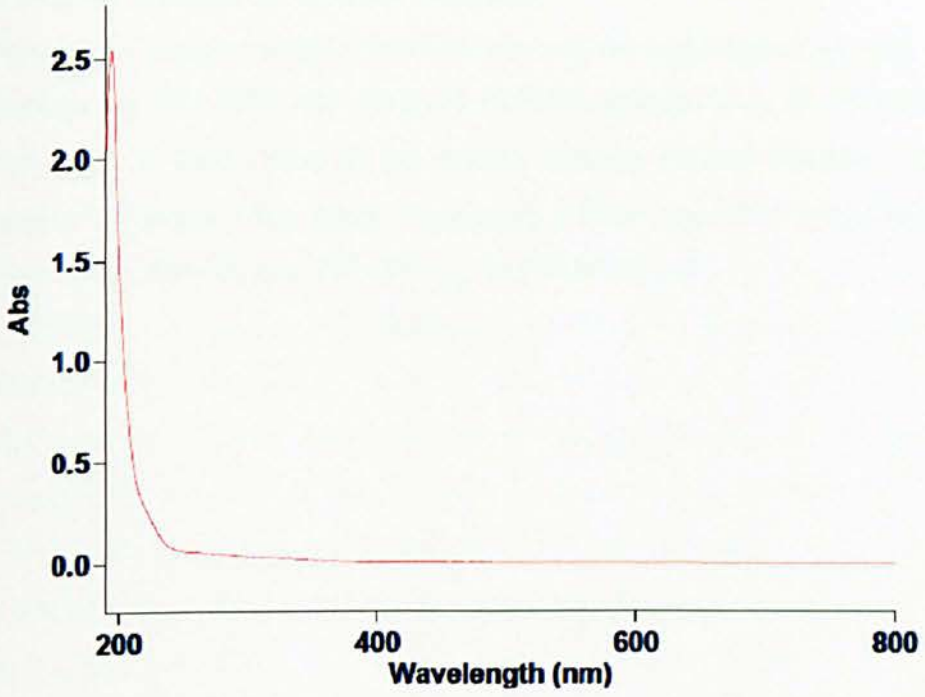


Figure 31. UV absorbance (at 224 and 269 nm) of fraction F8 that collected from the *Oldenlandia diffusa* decoction using Msp method.

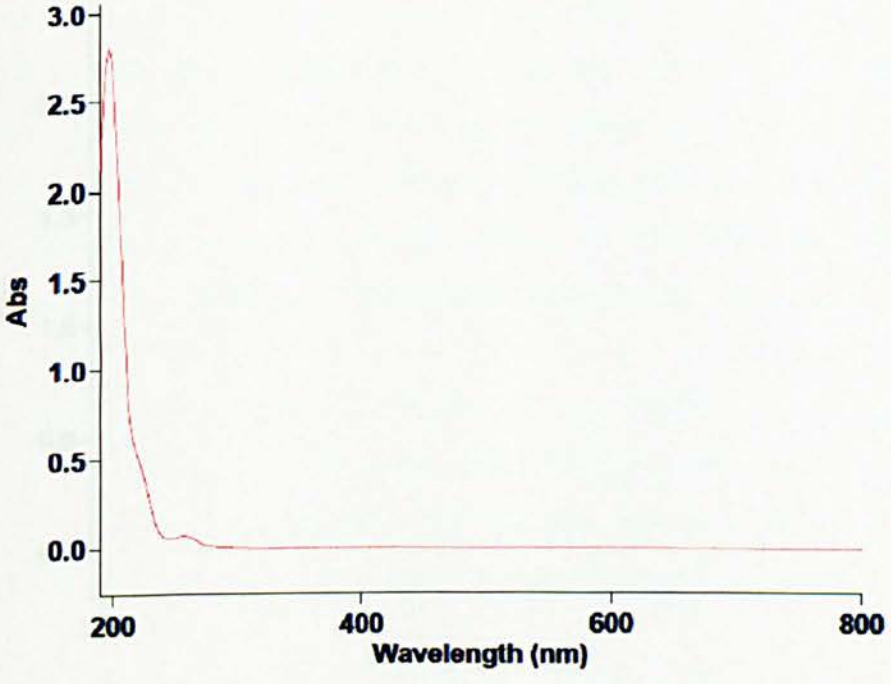


Figure 32. UV absorbance (at 221 and 255 nm) of fraction F9 that collected from the *Oldenlandia diffusa* decoction using Msp method.

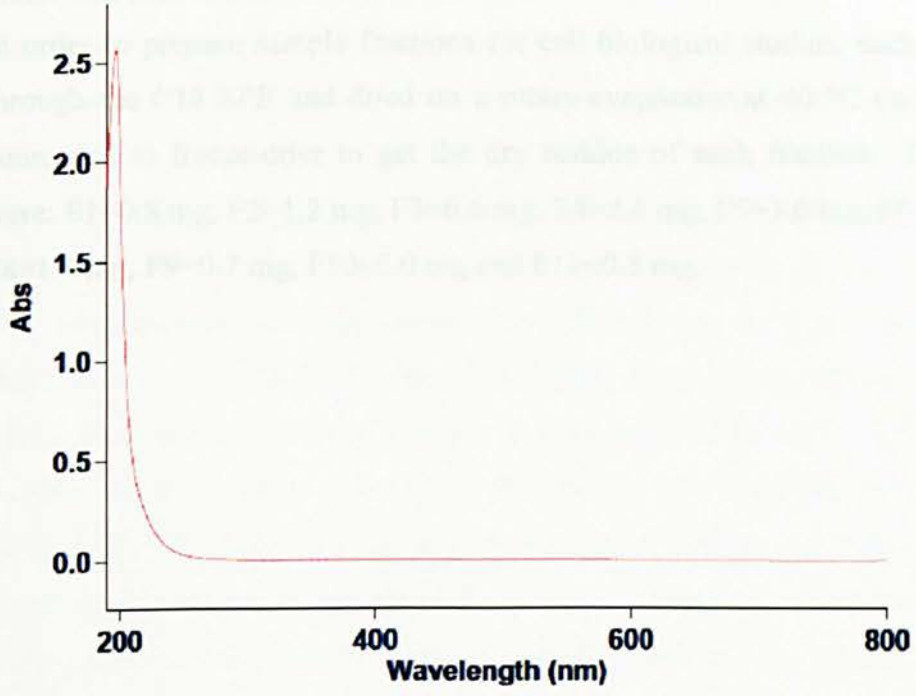


Figure 33. UV absorbance of fraction F10 that collected from the *Oldenlandia diffusa* decoction using Msp method.

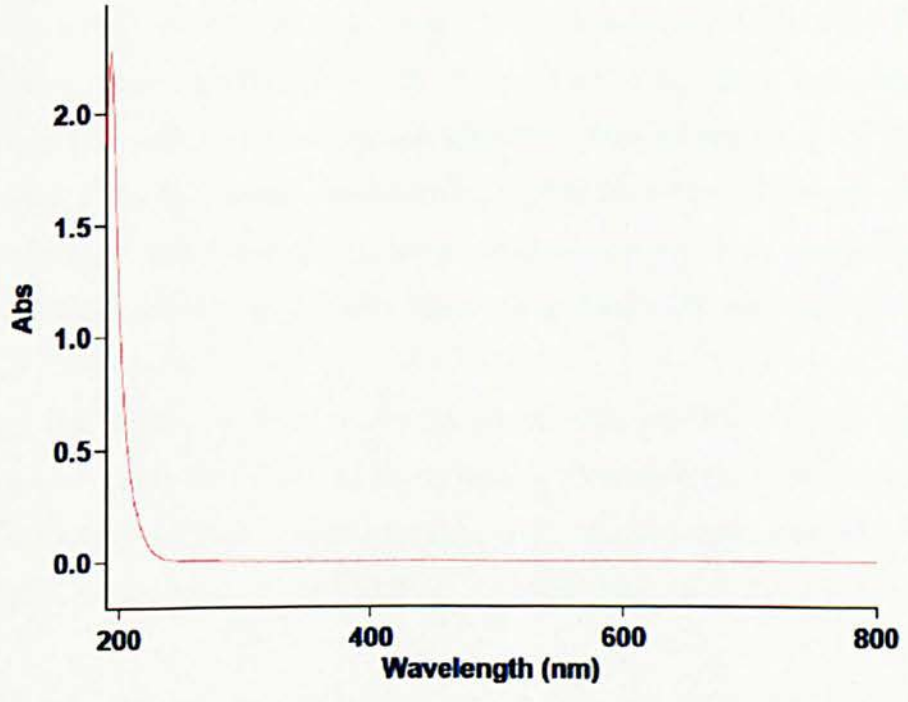


Figure 34. UV absorbance of fraction F11 that collected from the *Oldenlandia diffusa* decoction using Msp method.

3.3.6. The amount of collected fractions

In order to prepare sample fractions for cell biological studies, each fraction was passed through the C18 SPE and dried on a rotary-evaporator at 40 °C under vacuum and then connected to freeze-drier to get the dry residue of each fraction. Dry residues weights were: F1=0.8 mg, F2=1.2 mg, F3=6.6 mg, F4=2.9 mg, F5=3.0 mg, F6=1.6 mg, F7=3.5 mg, F8=1.6 mg, F9=0.7 mg, F10=5.0 mg and F11=0.8 mg.

3.4. Discussion

The aim of this part of the study was to collect the fractions from the *Oldenlandia diffusa* decoction and prepare the correct sample for a cell biology study.

At first, the chromatographic trace remained the same in both analytical and preparative scale columns. For the preparative PFP(2) (Luna 20 cm x 15.1 mm, 5 µm) column, the flow rate and injection volumes used were 20 mL/min and 2 mL, respectively. Therefore, the fractions can be collected from this column using the separation method which was set up for the decoction. Prior to this, it was necessary to find out the best way to get the good samples for cell biology studies. For this reason, the ammonium acetate salt was removed using C18 SPE tubes. To make sure that the contents remained the same in the post-extraction decoction, the sample was investigated using HPLC under the same conditions and by same method used in the decoction. As a result, it was clearly shown that the traces remained the same in both *Oldenlandia diffusa* decoction and post-extraction decoction, however, the quantity was lowered in the latter one (shown in Figures 13 and 14).

In addition, in some publications it was shown that the ammonium acetate salt can be removed by double or triple freeze-drying [Anthony *et al.*, 1977], [Berne & Alan, 1983], [Rainer *et al.*, 1990]. Therefore, the removal of the salt was investigated using a freeze-dryer and subsequently by measuring the absorbances on a UV spectrometer. The UV cutoff of 10 mM ammonium acetate is <200 nm [Yuri & Rosario 2007]. The ammonium acetate salt was reduced or removed after double and triple freeze-drying as there were no (or little) absorbances that could be measured on a UV spectrometer (see Figures 15-18).

For the fraction collection, the length of tube was 200 mm, because the fractions were manually collected from the waste tubing. Therefore, the delay time was 0.118 second and flow rate used was 20 mL/min; thus fractions were collected when the detector response signal is initiated and stopped when the peak drops back down to baseline.

Naphthalene was used to verify that the collection point was correct. In the fraction 4 of naphthalene, which was collected after 5 seconds elution of the naphthalene peak (refer to Figures 19-23), contained approximately 28% of the sample amount. So, the collected fractions will contain the certain amount of the previous peak (cross over).

Furthermore, there were 11 fractions (F1-F11) collected from the *Oldenlandia diffusa* decoction using the separation method which was set up for the decoction separation, and their UV maxima were 245 nm (F1), 244 and 281 nm (F2), 227 and 273 nm (F3), 273 and 314 nm (F4), 239, 278 and 320 nm (F4), 269 nm (F6), 236 and 315 nm (F7), 224 and 269 nm (F8), 221 and 255 nm (F9), and only at 200 nm for F10 and F11.

In order to remove ammonium acetate salt, each fraction was passed through the C18 SPE, and then solvents were removed on the rotary-evaporator at 40 °C under vacuum. Then, fractions were dried on freeze-dryer. A final weight of each fraction was calculated and were between 0.7-6.6 mg.

3.5. Conclusion

Eleven fractions (F1-F11) were collected from *Oldenlandia diffusa* decoction from HPLC, using prep PFP(2) (Luna 20 cm x 15.1 mm, 5 μm) preparative column, methanol (A)/10 mM ammonium acetate buffer (pH=6.5) (B) as a mobile phase with B 100% for 0-10 min, A 100% for 40-50 min, B 100% for 52-60 min gradient elution at 20 mL/min flow rate and 226 nm wavelength at time interval (Msp method).

Mobile phase modifier ammonium acetate salt was successfully removed from fractions using a C18 SPE tube and freeze-drying.

The amount of collected fractions from one run were F1=0.8 mg, F2=1.2 mg, F3=6.6 mg, F4=2.9 mg, F5=3.0 mg, F6=1.6 mg, F7=3.5 mg, F8=1.6 mg, F9=0.7 mg, F10=5.0 mg and F11=0.8 mg.

Chapter 4. Inhibition of growth of cancer cell lines *in vitro* by *Oldenlandia diffusa* decoction

Chapter 4. Inhibition of growth of cancer cell lines *in vitro* by *Oldenlandia diffusa* decoction

4.1. Introduction

CMRs have been traditionally used to prevent and treat many kinds of diseases, especially autoimmune diseases and cancers [Cheng, 2000], [Li, 1986].

In order to determine the possible cytotoxic compound(s) presenting in the decoction of *Oldenlandia diffusa* and the cytotoxic action of ammonium acetate buffer and collected fractions (F1-F11) from HPLC were investigated on HL60 and Caco-2 cancer cell lines *in vitro*. However, ammonium acetate salt can be removed by SPE; its toxic effect was examined on the HL60 cell line. In addition, in some sources, it was confirmed that the ammonium acetate salt can be removed by freeze-drying (2-3 times) [Demsey *et al.*, 1977], [Berne & Alan, 1983], [Rainer *et al.*, 1990].

4.1.1. The cytotoxic potential of CHR *Oldenlandia diffusa*

CHR *Oldenlandia diffusa* is traditionally used for the treatment of malignant tumours of lung, liver and stomach [Joe, 2006], [Shan *et al.*, 2001]. The anti-cancer effect of CHR *Oldenlandia diffusa* is well established. For example, ursolic acid which was isolated from a methanol extract of *Oldenlandia diffusa* demonstrated a significant inhibition of the proliferation of cultured tumour cells: A549 (human lung), SK-OV-3 (ovary), SK-MEL-2 (skin), XF498 (brain), HCT-15 (colon), SNU-1 (stomach), L1210 (murine leukaemia) and B16-F-0 (murine melanoma). A study suggested that UA had an apoptotic effect on HL60 cells [Shan *et al.*, 2001]. The effect of water extract of *Oldenlandia diffusa* was investigated on a cell lung cancer cell line (H69), a multi-drug resistant small cell lung cancer cell line (H69VP) and a normal lung epithelium cell line (BEAS-2) [Sadava *et al.*, 2002]. IC50 values showing the concentration of *Oldenlandia diffusa* needed to inhibit the growth of each cell line by 50% over 4 days revealed *Oldenlandia diffusa* to be equally toxic to the H69 and H69VP cell lines. Also, it showed a not significantly reduced effect on the normal BEAS-2 cell line at the same concentration. Apoptosis was observed in both the H69 and H69VP cell lines after exposure to *Oldenlandia diffusa* [Gupta *et al.*, 2004].

In addition, *Oldenlandia diffusa* enhanced the induction of allo-antigen specific cytotoxic T lymphocytes and stimulated macrophages to produce interleukin-6 and tumour necrosis

factor. The results suggest that *Oldenlandia diffusa* has immunomodulating activity *in vitro* and this activity could be used clinically for the modulation of immune responses [Chung *et al.*, 1998]. Some researchers studied immunomodulatory activity and anti-tumour activity of *Oldenlandia diffusa in vitro*; they proved that *Oldenlandia diffusa* has immunomodulating activity and anti-tumour activity *in vitro* through stimulating the immune system to kill or engulf tumour cells, which could be used clinically for immune function modulation and to treat tumour and other diseases [Shan *et al.*, 2001].

Furthermore, some researchers studied the anti-cancer activity of the water extract of *Oldenlandia diffusa* on the HL60 leukaemic cell line, and compared this effect to that of water extracts of *Oldenlandia diffusa* on PBLs (primary human blood lymphocytes), in order to look for evidence of *Oldenlandia diffusa* inducing cancer cell-specific cytotoxic activity. It was shown that the aqueous extract of *Oldenlandia diffusa* was found to be directly toxic to the HL60 cell line, inducing apoptosis via some form of activity specific to these cells [Willimott *et al.*, 2007].

Cytotoxicity study may involve the use of an *in vivo* model or an *in vitro* model [Davila *et al*, 1998]. Cytotoxicity can be studied with greater reproducibility, less expense, fewer ethical and safety concerns, and with greater detail and considerably faster *in vitro* model [Davila *et al*, 1998]. However, sometimes results found *in vitro* studies do not translate well to real life. The cytotoxic potential of decoction can be assessed *in vitro* using cancer cell lines by examining one of the characteristics related with toxicity.

4.1.2. Cancer cell

Cancer cells can be obtained by removing the malignant tumour by dissociating the tissue into its separate cells. Then, culturing the cells *in vitro* in the laboratory. There are many lines of cultured cells that have been collected from human tumours. The behaviour of cancer cells can be easily studied when the cells are growing in the culture [Karp, 2008]. Cancer cells can usually grow on much simpler culture medium than the normal cells' [Cancer cells in culture, 2004].

In this study, HL60 and Caco-2 cell lines were used for investigation of the *Oldenlandia diffusa* decoction and its fractions. This was because *Oldenlandia diffusa* showed cytotoxic

effect on HL60 cell line [Willimott *et al.*, 2007], and Caco-2 cell line is a colon carcinoma cell line.

4.1.3. HL60 cell line

The HL60 cell line was established in 1977 from with acute promyelocytic leukaemia. It provides a unique *in vitro* model system for studying the cellular events involving the cell proliferation [Collins *et al.*, 1977], [Collins *et al.*, 1987]. HL60 cells spontaneously differentiate and differentiation can be stimulated by butyrate, hypoxanthine, phorbol myristic acid (PMA, TPA), dimethylsulfoxide (DMSO, 1% to 1.5%), actinomycin-D and retinoic acid [Collins *et al.*, 1978]. The cells exhibit phagocytic activity and responsiveness to chemotactic stimuli [Gallagher *et al.*, 1979]. Some details of these cells are following:

- Morphology is lymphoblast-like
- Species is human, Caucasian female 36 years old
- Tissue: peripheral blood
- Growth properties: Suspension
- Tumor is leukaemia, promyelocytic
- Properties: phagocytosis; antitumour testing; differentiation; pharmacodynamics; cloning; Susceptible to: HIV-1, HTLV-1.

Complete growth medium for HL60 cell line is RPMI 1640 with 2 mM glutamine and 10-20% Foetal Bovine Serum (FBS). Growth atmosphere and temperature are 5% CO_2 and 37°C, respectively. Its freezing conditions are: RPMI 1640 medium with 20% FBS and 10% DMSO [ECCAC, 2008].

4.1.4. Caco-2 cell line

This is a human colonic carcinoma cell. When cells reach confluence, they show characteristics of enterocytic differentiation [Jumarie & Malo, 1991]. Caco-2 cells express retinoic acid binding protein I and retinol binding protein II [Wang *et al.*, 2007]. Some important details of these cells are:

- Morphology is epithelial
- Species is human, Caucasian female 72 years old
- Tissue is colon
- Growth properties are adherent
- Tumor is colorectal adenocarcinoma.

Caco-2 cells' complete growth medium is Dulbecco's Modified Eagle Medium (DMEM) with 2 mM glutamine, 1% Non essential amino acids (NEAA) and 10% FBS. Growth atmosphere and temperature are 5% CO_2 and 37 °C, respectively. During routine subculture, the cells should always be subcultured before they achieve confluence. Freezing conditions are: FBS with 10% DMSO [ECCAC, 2007].

The cytotoxicity can be examined by one of a number of characteristics associated with toxicity which includes inhibition of cell proliferation, decreased metabolic activity and decreased cell viability [NIH, 2001].

4.1.5. Cytotoxicity Assays

Cytotoxicity is the quality of toxicity to living cells. Treating the cells with a cytotoxic compound can result in a variety of cell changes [Riss & Moravec, 2004], [Promega, 2007].
Generally, there are three groups of cytotoxicity assays which are based on their measurement parameters.

At first, the often tested assay type is the measurement of cellular metabolic activity. The reduction in metabolic activity is an early sign of cellular damage. Therefore, tests which can measure metabolic function measure cellular ATP levels or mitochondrial activity, e.g. MTT Assay [Todd *et al.*, 1999].

The second group of assays are based on the measurement of membrane integrity. When cells are damaged or lose their membrane integrity they become leaky, so membrane integrity can be determined by measuring lactate dehyrogenase (LDH) in the extracellular medium. Some assays measure the uptake of fluorescent dye that is normally excluded from intact cells. These effects on membrane integrity are indication of more serious injury and could lead to cell death [Decker & Lohmann-Matthes, 1988].

In the third, there are some assays which measure cell number, since dead cells normally detach from a culture plate and are washed away in the medium. Cell numbers can be measured by direct cell counting or by the measurement of total cell protein or DNA, which is proportional to the number of cells, for example CyQUANT NF assay and neutral red uptake assay [Todd *et al.*, 1999].

4.1.6. Cytotoxicity Assays performed in the present assessment

4.1.6.1. Trypan blue

Trypan blue is a blue acidic dye that contains two azochromophores [Green, 1990]. Trypan blue has been used in vital staining of various tissues [Lillie *et al.*, 1977]. The reaction of this vital dye is based on the fact that the chromopore is negatively charged and does not interact with the cell, if the membrane is not damaged. Therefore, all dead cells are coloured as blue and the cells which are alive are excluded from the dye. Therefore, this staining method is named a dye exclusion method [Freshney, 1987]. The trypan blue exclusion method cannot distinguish whether there is necrotic or apoptotic cell death.

4.1.6.2. CyQUANT NF assay

Invitrogen's CyQUANT NF cell proliferation kit was used to measure cytotoxicity of the decoction and its separated and collected fractions. Manual cell counting on a haemocytometer using trypan blue was too time consuming for the numbers of assays and replicates, so the CyQUANT NF assay was performed. This assay kit was very good for counting large numbers of cell samples.

The CyQUANT NF assay is based on measurement of cellular DNA content via fluorescent dye binding. Because cellular DNA content is highly regulated, it is proportional to cell number. The degree of proliferation is determined by comparing the treated cell numbers (treated with compounds of interest) to untreated cell numbers (controls). The CyQUANT NF cell proliferation assay kit provides a fast (can be completed in one hour) and sensitive method for counting cells. This method is more sensitive than MTT and AlamarBlue assays. It has a linear detection range from 100 to 20,000 cells/well. The CyQUANT NF assay protocol has few steps such as aspiration of growth medium (for adherent cells), replacement with dye binding solution, incubation for 30–60 minutes, and then measurement of fluorescence in a microplate reader [Molecular Probes, 2006], [Lansing *et al.*, 2006]

4.1.6.3. Neutral red uptake assay

The neutral red method was developed by Borenfreund and Puerner [Borenfreund & Puerner, 1985]. It is simple, accurate method and gives reproducible results [Jones *et al.*, 1999].

The neutral red uptake (NRU) assay system measures the living cells via the uptake of the vital dye neutral red (basic red 5, toluylene red). Viable cells take up the dye by active transport and incorporate the dye into lysosomes, whereas non-viable cells will not take up the dye. After the incorporation of the dye, cells are washed with an acidified ethanol solution, thus the incorporated dye is then liberated from the cells. NRU assay kit includes the following components: neutral red solution. 0.33% in D-PBS 20 mL, NR assay. Fixative, 0.1% $CaCl_2$ in 0.5% formaldehyde 125 mL and NR assay solubilization solution, 1% acetic acid in 50% ethanol 125 mL [Triglia *et al.*, 1989].

4.2. Materials and methods

4.2.1. Materials

The HL60 cell line and Caco-2 cell line were obtained from The European Collection of Cell Cultures (ECACC, Sailsbury, UK). DMEM, L-glutamine, NEAAs, RPMI 1640 were purchased from Gibco, Paisley, UK.

Actinomycin-D (cell culture tested), ammonium acetate (HPLC grade), CyQUANT NF assay kit, trypan blue and NRU Kit were purchased from Sigma, Poole, UK. Haemocytometer, 0.20 μm and 0.45 μm sterile filter were obtained from Nalgene, Hereford, UK. All used consumables such as 96 well plates, 24 well plates, sterile pipettes, eppendorf tubes, universals were Nunc and Corning (Gibco, Paisley, UK).

4.2.2. Methods

4.2.2.1. Cell culture

The HL60 cancer cell line was grown in complete growth RPMI 1640 medium containing 2 mM glutamine, 10% FBS. The Caco-2 cancer cell line was grown in complete growth medium DMEM containing 10% v/v FBS, 2 mM L-glutamine and 2 mM NEAAs. All cell lines were grown and incubated at 37 °C in an atmosphere containing 5% CO_2. Cells were subcultured when the concentrations reached to 10^6 cells/mL, and diluted to 10^4-10^5 cells/mL, thereby maintaining a stock solution containing cells at concentrations ranging from 10^5 to 10^6 cells/mL.

4.2.2.2. Preparation of samples

Preparation of decoction

Oldenlandia diffusa decoction was prepared as mentioned in Chapter 2.

Preparation of the collected fractions

All collected and prepared fractions (F1-F11) from the decoction (see Chapter 3) were dissolved back in cell growth complete medium in DMEM and RPMI with 0.35 mg/mL concentration.

Preparation of ammonium acetate buffer

10 mM ammonium acetate buffer was prepared as mentioned in Chapter 2. There were three different concentrations of ammonium acetate buffers (10 mM, 1 mM and 2 times freeze-dried) investigated on cancer cell lines.

Preparation of CyQUANT NF assay kit

1. To prepare 11 mL of 20% Hank's balanced salt solution (HBSS) buffer, 2.2 mL of stock HBSS buffer was diluted with 8.8 mL of deionised water.
2. To prepare 50% dye binding solution, added 22 µL of CyQUANT NF dye reagent to 5.5 mL of 20% HBSS buffer.

Preparation of NRU assay kit

Neutral red solutions were freshly prepared before the experiment

1- Neutral red solution was prepared with 40 µg/mL concentration in phenol red free DMEM.

2- Neutral red assay fixative was prepared with 0.1% concentration of $CaCl_2$ in 125 mL 0.5% concentration of formaldehyde.

3- Neutral red assay solubilization solution was prepared with 1% concentration of acetic acid in 125 mL 50% concentration of ethanol.

4.2.2.3. Cytotoxic effect of the ammonium acetate buffers on HL60 cell line

The cytotoxic effect of ammonium acetate buffer (1.0 mM, 10 mM and double freeze-dried) was investigated on HL60 cancer cell line by the trypan blue exclusion method. HL60 cells were seeded on 24 well plates with $1x10^5$ cells/mL density and treated with ammonium acetate samples for 48 hours at 37 °C in an atmosphere containing 5% CO_2. Cell viability was determined by the trypan blue exclusion method.

4.2.2.4. The determination of the cytotoxic effect of *Oldenlandia diffusa* decoction and its collected fractions (F1-F11) on HL60 cell line using trypan blue and CyQUANT NF assay.

HL60 cells were grown in RPMI 1640 medium and the cells with 18-19 passage numbers were seeded to 96 well plates with $1x10^4$ cells/well density. The *Oldenlandia diffusa* decoction (0.35 mg/mL and 2.5 mg/mL), fractions (F1-F11) (0.35 mg/mL), negative

control (medium) positive control actinomycin-D (10 μg/mL) were added to HL60 cells and incubated for 48 hours at 37 °C in an atmosphere containing 5% CO_2.

After the incubation period, 20 μL of treated cell suspension were taken from each well and placed in an eppendorf tube and 20 μL of 0.4% of trypan blue were added to each tube and mixed gently. Stained cells (10 μL) were placed in a both chambers of haemocytometer, and the numbers of viable cells were counted. The average number of cells in each quadrant were calculated and multiplied by $2x10^4$ to find cells/mL.

The rest of the treated cells were transferred to eppendorf tubes and sedimented by centrifugation at 150 x g for 5 minutes. Then, the supernatant was removed and the cells resuspended in 100 μL of CyQUANT NF assay. The 96 well microplate was covered and incubated at 37 °C for 45 minutes as this incubation period is required for equilibration of dye-DNA binding. After that, the fluorescence intensity of each sample was measured using a fluorescence microplate reader with excitation at 485 nm and emission detection at 530 nm. Fluorescence measurements were performed at ambient temperature.

4.2.2.5. The determination of the cytotoxic effect of *Oldenlandia diffusa* decoction and collected fractions on Caco-2 cell line using NRU assay.

Caco-2 cells were grown in DMEM under the same conditions as above. The cells with passage number 58-59 were seeded to 96 well plates with $2x10^4$ cells/well density. *Oldenlandia diffusa* decoction (0.35 mg/mL and 2.5 mg/mL), fraction F4 and F9 (0.35 mg/mL), negative control (medium) and positive control actinomycin-D (10 μg/mL) were added to Caco-2 cells and were incubated for 48 hours at 37 °C in an atmosphere containing 5% CO_2.

After the incubation period, the routine culture medium (with test chemical) was carefully removed and 200 μL of NRU (40 μg/mL) was added to all wells including the blanks. The 96 well microplate was incubated at 37±0.5 °C, 90±5% humidity, and 5.0±0.5% CO_2/air for 2 and 1/2 hours, and then NRU was removed. The cells were carefully rinsed with 200 μL of pre-warmed D-PBS. After this, exactly 200 μL of neutral red desorb (ethanol/acetic acid) solution was added to all wells, including blanks. Microplate was protected from light by using a cover and agitated on a plate shaker for 30 minutes to extract neutral red from the cells and form a homogeneous solution.

After this, the absorption (within 60 minutes of adding neutral red desorb solution) of the resulting coloured solution were measured at 540 nm in a microplate reader (spectrophotometer).

4.2.2.6. Data presentation and statistical analysis

Data for the cytotoxicity study are expressed as mean ± standard deviation from the mean. The mean values of each fraction were compared to control values using ANOVA. One-way ANOVA with Dunnett's post test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

4.3. Results

4.3.1. Cytotoxic effect of the ammonium acetate buffer on HL60 cell line

Results from the assessment of the toxic effect of ammonium acetate buffer on HL60 are shown a Figure 33 as cell viability V=81.07±2.39% (n=3) for 10 mM ammonium acetate buffer, V=94.91±13.02% (n=3) for 1 mM ammonium acetate buffer and V=99.21±3.58% (n=3) for freeze-dried ammonium acetate buffer.

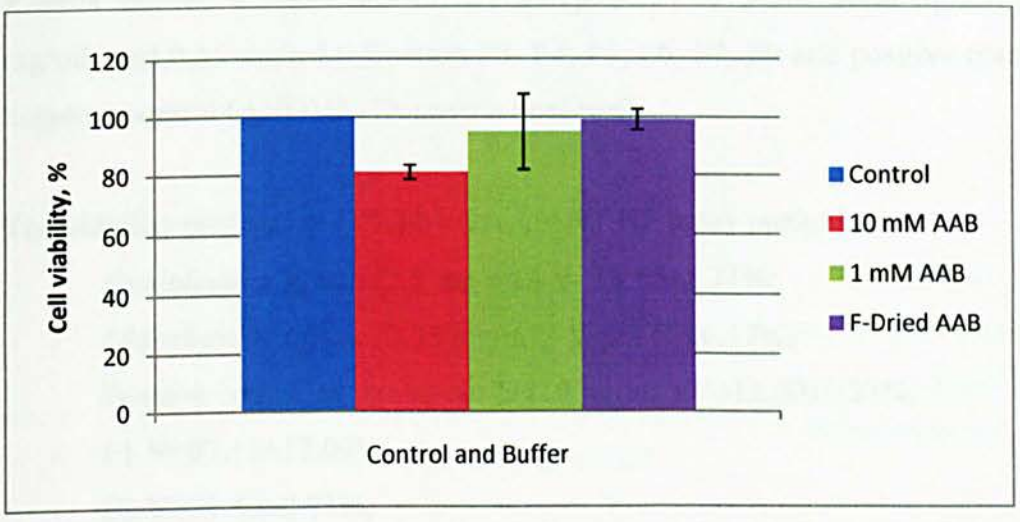


Figure 35. Cell viability, V% (n=3) of 10 mM, 1 mM and freeze-dried ammonium acetate buffers and negative control treated HL60 cells, assessed using trypan blue exclusion method.

4.3.2. The determination of the cytotoxic effect of *Oldenlandia diffusa* decoction and its collected fractions (F1-F11) on HL60 cell line using trypan blue and CyQUANT NF assay kit.

The cytotoxicity study showed that the decoction has a toxic effect on HL60 and Caco-2 cell lines. In addition, some collected fractions showed a cytotoxic activity on both cell lines. As a result, the viability percentage (V%) by trypan blue exclusion method were:

- *Oldenlandia diffusa* (2.5 mg/mL) V=0.48±0.41%,
- *Oldenlandia diffusa* (0.35 mg/mL) V=41.16±3.15%,
- Positive control-actinomycin-D (10 µg/mL) V=0.89±0.19%,
- F1 V=105.79±2.41%,
- F2 V=82.12±3.42%,
- F3 V=69.99±19.34%,
- F4 V=61.52±21.90%,
- F5 V=60.58±13.61%,

- F6 V=54.50±7.22%,
- F7 V=67.25±17.16%,
- F8 V=73.69±16.36%,
- F9 V=37.61±9.39%,
- F10 V=93.59±15.33%,
- F11 V=78.14±8.37% (0.35 mg/mL) (Figure 34).

Results showed a significant difference ($p<0.05$) of *Oldenlandia diffusa* decoction (2.5 mg/mL and 0.35 mg/mL), fraction F3, F4, F5, F6, F7, F9 and positive control against the negative control (ANOVA, Dunnett's Post-test).

The viability percentage (V%) by CyQUANT NF assay method were:

- *Oldenlandia diffusa* (2.5 mg/mL) V=13.65±2.71%;
- *Oldenlandia diffusa* (0.35 mg/mL) V=25.57±6.17%;
- Positive control-actinomycin-D (10 μg/mL) V=12.00±0.21%,
- F1 V=97.13±17.09%,
- F2 V=93.40±0.91%,
- F3 V=86.98±11.92%,
- F4 V=76.74±5.42%,
- F5 V=77.74±7.65%,
- F6 V=85.56±6.35%,
- F7 V=86.95±5.20%,
- F8 V=81.36±7.79%,
- F9 V=62.16±7.32%,
- F10 V=101.55±14.64%,
- F11 V=103.83 ±3.30% (0.35 mg/mL).

Results showed a significant difference ($p<0.05$) of *Oldenlandia diffusa* decoction (2.5 mg/mL and 0.35 mg/mL), fraction F4, F5, F9 and positive control against the negative control (ANOVA, Dunnett's Post-test) (Figure 37).

Figures for investigation of cytotoxicity activity of *Oldenlandia diffusa* decoction and its fractions (F1-F11) compared to negative control (medium) and positive control actinomycin-D.

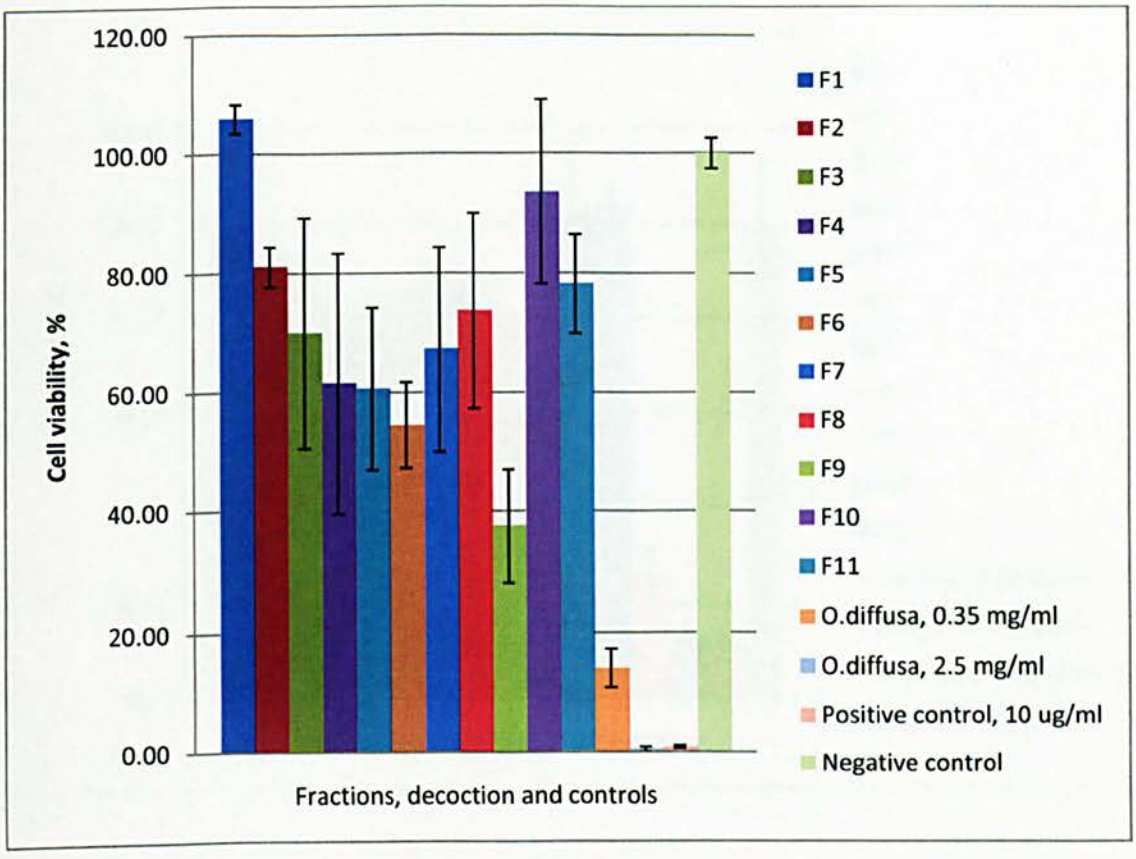


Figure 36. Cell viability, V% (n=3) of *Oldenlandia diffusa* decoction (0.35 mg/mL and 2.5 mg/mL), fractions (F1-F11) (0.35 mg/mL), negative control (medium) and positive control actinomycin-D (10 µg/mL) treated HL60 cells, using trypan blue method. The control was set to 100%.

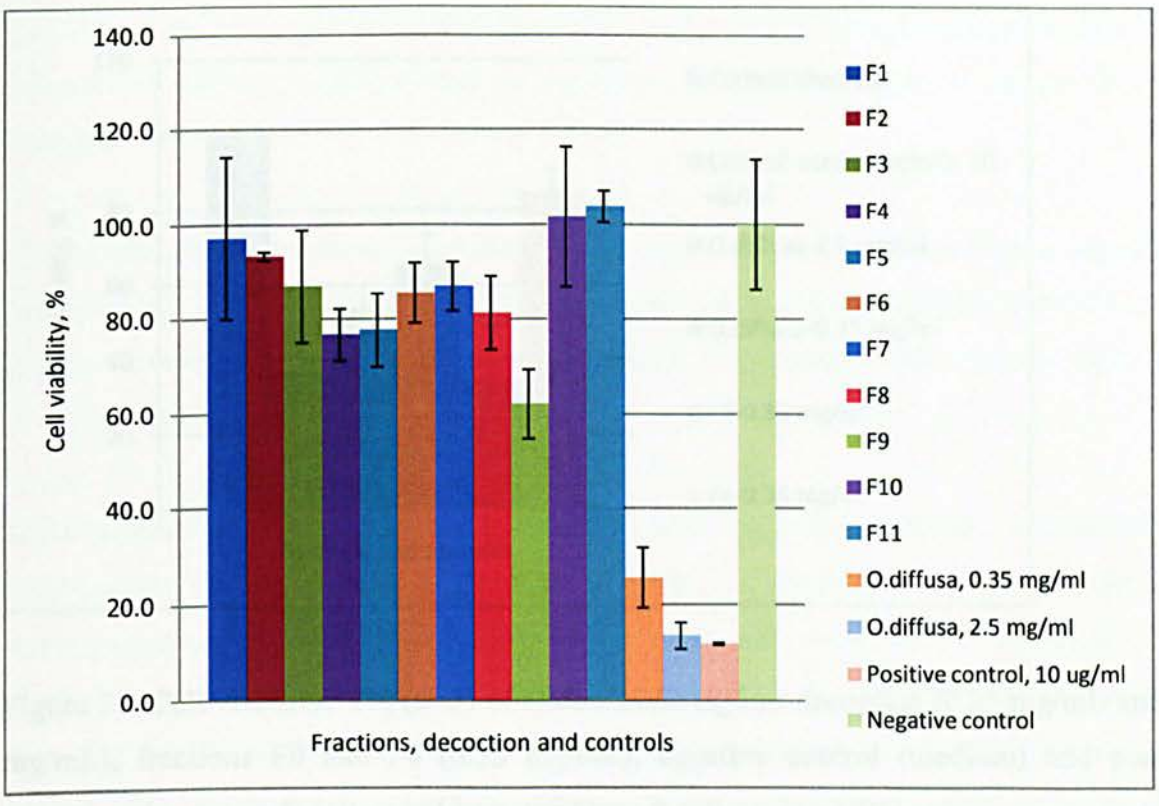


Figure 37. Cell viability, V% (n=3) of *Oldenlandia diffusa* decoction (0.35 mg/mL and 2.5 mg/mL), each fraction (F1-F11) (0.35 mg/mL), negative control (medium) and positive control actinomycin-D (10 µg/mL) treated HL60 cells, by CyQUANT NF assay.

4.3.3. The determination of the cytotoxic effect of *Oldenlandia diffusa* decoction and collected fractions on Caco-2 cell line using NRU assay.

This showed a greater toxic effect on the Caco-2 cell line, killing the cells within 48 hours of exposure with statistically significant ($p<0.05$) effect. Viability percentages (V%) were:

- *Oldenlandia diffusa* (2.5 mg/mL) V=50.04±1.4%;
- *Oldenlandia diffusa* (0.35 mg/mL) V=66.63±9.7%;
- Positive control-actinomycin-D (10 µg/mL) V=37.00±6.0%,
- F9 V=32.05±7.9%
- F4 V=86.26±2.4% (see Figure 36).

There were significant differences on all treatments (decoctions, fraction F4, F9 and positive control) against the negative control (ANOVA, Dunnett's Post-test).

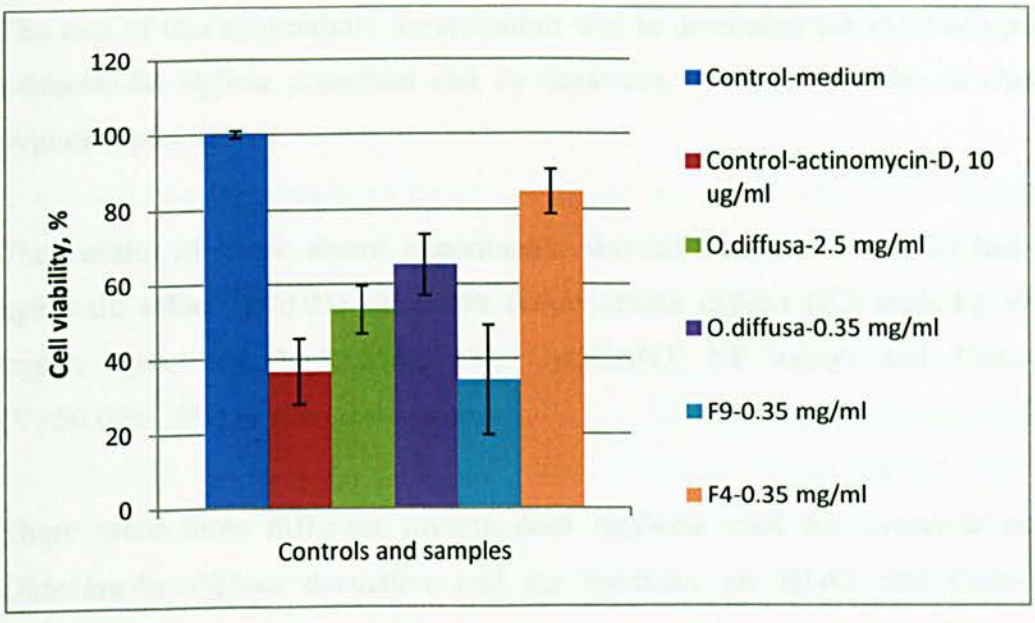


Figure 38. Cell viability, V% (n=3) of *Oldenlandia diffusa* decoction (0.35 mg/mL and 2.5 mg/mL), fractions F9 and F4 (0.35 mg/mL), negative control (medium) and positive control actinomycin-D (10 µg/mL) treated Caco-2 cells, using NRU assay.

4.4. Discussion

The aim of this cytotoxicity investigation was to determine the cytotoxic potential of the *Oldenlandia diffusa* decoction and its separated fractions in order to choose the most cytotoxic part of it.

The results of these above experiments showed that the decoction has a significant cytotoxic effect ($p<0.05$) on HL60 (*Oldenlandia diffusa* (2.5 mg/mL) V=0.48±0.41%, trypan blue, and V=13.65±2.71%, CyQUANT NF assay) and Caco-2 cell lines (V=50.04±1.4%) *in vitro* test system.

There were three different investigation methods used for cytotoxic activity of the *Oldenlandia diffusa* decoction and its fractions on HL60 and Caco-2 cells. The conventional trypan blue exclusion method was used as a control for this experiment. The trypan blue exclusion method was time consuming to do for replicate experiments. Therefore, the CyQUANT NF cell proliferation assay, which is based on the incorporation of thymidine analogs such as 3H thymidine or bromodeoxyuridine (BrdU) during DNA synthesis or on measurement of metabolic activity indices such as oxidoreductase activity or ATP levels was used [Lansing *et al.*, 2006]. Significant differences were indicated in this assessment (ANOVA, $p<0.05$).

As viability percentage of cells, the most cytotoxic effect occurred on the decoction (0.35 mg/mL) (by trypan blue: V=4.16±3.26%; CyQUANT NF assay: V=25.6±6.17%) and then F9 (0.35 mg/mL) (by trypan blue: V=37.61±9.39%; CyQUANT NF assay: V=62.2±7.32%), then F4 (0.35 mg/mL) (by trypan blue: V=61.52±21.90; CyQUANT NF assay: V=76.7±5.42%) from the all the collected fractions (F1-F11) which were separated and collected from the decoction on HPLC.

Overall the trypan blue assay returned lower values of viability than the CyQUANT NF assay. One reason for this may be that the CyQUANT dye detected DNA in unviable but still left cells, where as such cells would appear blue and be deemed non-viable in the trypan blue assay. Thus, the CyQUANT NF assay may overestimate the number of viable cells. However, the use of both assays was essential in confirming the relative effects of the decoction and fractions. The effects of the decoction at 0.35 mg/mL were not very much different to its effects at the higher concentration.

In addition, the decoction exhibited cytotoxic effects *in vitro* on Caco-2 colon cancer line. The cell viability was determined using NRU assay. In these experiments, the decoction, F9 and F4 were examined.

If the viability percentages (V%) are compared, the F9 fraction of the decoction had more growth inhibition effect on Caco-2 cell line than HL60. The viability percentages on HL60 cell line were F9 (0.35 mg/mL) V=62.2±7.32%), and F4 (0.35 mg/mL) V=76.7±5.42%, while for Caco-2 cell line were F9 (0.35 mg/mL) V=32.05±7.9% and F4 (0.35 mg/mL) V=86.26±2.4%. Our results confirmed that the *Oldenlandia diffusa* decoction has activity against HL60 and Caco-2 cells by a number of different assays, and the most active fraction was identified as F9. Also, we can use both cell lines for the further investigation of cytotoxic activity of *Oldenlandia diffusa* decoction and its constituent(s).

There were some concerns that maybe the ammonium acetate is giving an additional toxic effect on cells. In previous work, the ammonium acetate buffer was first removed by SPE, and then by freeze-drying from fractions for further cell biology studies. In addition, in some researchers' study it was shown that ammonium acetate salt can be removed by 2-3 times freeze-drying [Anthony *et al.*, 1977], [Berne & Alan, 1983], [Rainer *et al.*, 1990]. Therefore, toxic effect of 10 mM, 1 mM and freeze-dried ammonium acetate buffers were investigated on HL60 cell line, and results showed that the cell viability was high for 1 mM and freeze-dried ammonium acetate buffer V=94.91±13.02% and V=99.21±3.58%, respectively. So, the ammonium acetate did not effect to the cytotoxicity degree of decoction and fractions.

4.5. Conclusion

Oldenlandia diffusa decoction has a cytotoxic effect on HL60 (V=13.49±4.31%, n=3) and Caco-2 cancer cell lines (V=50.04±1.4, n=3).

The most cytotoxic active fraction which collected (F1-F11) from HPLC of *Oldenlandia diffusa* decoction is F9. Its viability percentages were V=62.2±7.32%, n=3, and V=32.05±7.9%, n=3, respectively on HL60 and Caco-2 cancer cell lines.

Chapter 5. Characterization of the mode of cell death induced by *Oldenlandia diffusa* decoction and its most cytotoxic fraction

Chapter 5. Characterization of the mode of cell death induced by *Oldenlandia diffusa* decoction and its most cytotoxic fraction

5.1. Introduction

In a previous study (Chapter 4), the cytotoxic activity of *Oldenlandia diffusa* decoction and its fractions were tested against cancer cells including a leukaemic cell line (HL60), colon adenocarcenoma cancer cells (Caco-2) in an *in vitro* test system to reveal their cytotoxic potential, but the data can only gave an indication of the degree of toxicity. Therefore, the mode of cell death (necrosis or apoptosis) of cancer cells exposed to *Oldenlandia diffusa* decoction and its fractions were investigated in this part of the assessment.

5.1.1. Mode of cell death

Cytotoxicity is the degree of toxicity to living cells. If cells treated with toxins, they can die in two different ways: necrosis (cells are killed by injuries) and apoptosis (cells are induced to commit suicide).

Necrosis is cell death by injury. Cells can be damaged by injury, such as by mechanical damage and exposure to toxic chemicals. In necrosis, a series of changes occur, for example, disruption of plasma membrane, swelling of organelles, followed by rupturing of the cell membrane and cell lysis [Majno & Joris, 1995], [Roos *et al.*, 2006].

In contrast, apoptosis is the process of programmed cell death. Apoptotic cells can be recognised by morphological, biochemical and molecular changes. The following changes occur in apoptosis: cell shrinkage, cell shape change and condensation of cytoplasm, nuclear envelope changes, nuclear fragmentation, loss of cell surface structures, blebbing, forming of apoptotic bodies, cell detachment and the removal by phagocytosis [Bruce *et al.*, 2008]. Morphologically, the membrane shows shrinkage and blebbing. These formulated blebs separate from the dying cell and form apoptotic bodies [Lorenzo & Susin, 2007].

Also, in the mitochondrial outer membrane some changes occur such as loss of its electrochemical gradient and the formation of pores. Thus, substances (e.g. cytochrome c)

leak from the mitochondrial outer membrane into the cytoplasm, and macrophages phagocytose apoptotic bodies and dead of cells [Kerr & Harmon, 1991]. Morphological processes in apoptosis necrosis are shown in Figure 39.

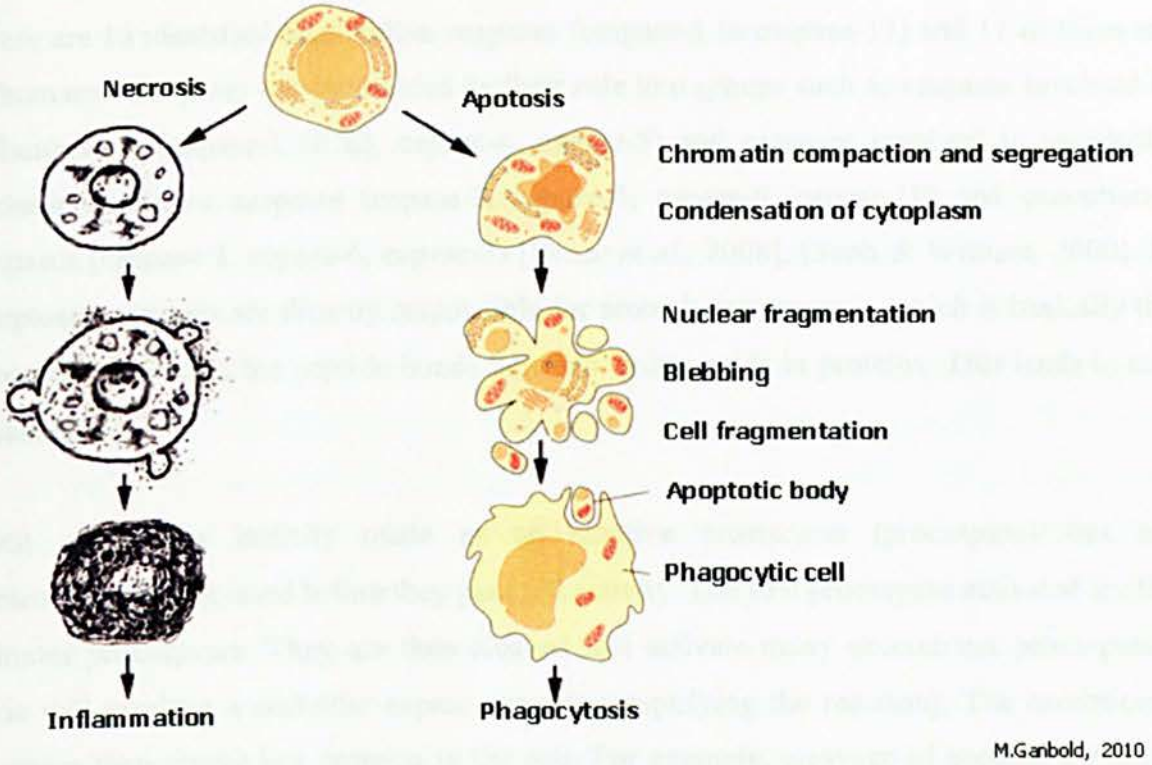


Figure 39. Morphological changes during necrosis and apoptosis and their difference. [Adopted from http://www.celldeath.de/encyclo/aporev/revfigs/rev_2.jpg]

In addition, apoptosis is a form of programmed cell death involving a biochemical cascade, including proteins such as Bcl-2, Bax, Apaf-1 or apoptotic protease activating factor-1, caspases such as caspase-9, caspase-3, caspase-7 and caspase-8, and proteins involved in digestion of proteins, degradation of DNA and phagocytosis.

5.1.2. Caspases

In apoptosis, cell suicide requires specialized machinery. The main compartment of this machinery is a proteolytic system, involving caspases (enzymes). A family of cysteine-dependent aspartate-directed proteases are called caspases (cysteine and aspartic acid). It depends on a family of proteases that have a cysteine at their activation site and cleave their target proteins at specific aspartic acids [Bruce *et al.*, 2008], [Liss, 1987]. They

participate in a cascade, which is activated in response to pro-apoptotic signals and lead to cleavage of proteins, resulting in disassembly of the cell [Cohen, 1997], [Nicholson & Thornberry, 1997], [Bump *et al.*, 1995], [Tewari *et al.*, 1995].

There are 13 identified mammalian caspases (caspase-1 to caspase-13) and 11 of them are in humans. Caspases can be divided by their role into groups such as caspases involved in inflammation (capase-1 (ICE), capase-4, capase-5) and caspases involved in apoptosis, including initiator caspases (capase-2, capase-8, capase-9, capase-10) and executioner caspases (caspase-3, capase-6, capase-7) [Bruce *et al.*, 2008], [Scott & William, 2000]. In apoptosis, caspases are directly responsible for proteolytic cleavages which is basically the process of breaking the peptide bonds between amino acids in proteins. This leads to cell disassembly.

Each caspase is initially made as an inactive proenzyme (procaspase) that are proteolytically processed before they gain full activity. The first procaspase activated are the initiator procaspases. They are then cleaved and activate many executioner procaspases. This will produce a protelitic capase cascade (amplifying the reaction). The executioner caspases then cleave key proteins in the cell. For example, cleavage of specific cytosolic proteins and nuclear lamins lead to the controlled death of the cell [Bruce *et al.*, 2008].

There are three pathways that caspases can proceed. Firstly, one pathway of caspase proceeds from death receptors through caspase-8 to caspase-3 and then to caspase-6 [Takahashi *et al.*, 1997], [Stennicke *et al.*, 1998]. A second pathway proceeds from the cytochrome *c*: Apaf-1 complex through caspase-9 to caspases-3 and caspases-7 and then to caspase-6 [Srinivasula *et al.*, 1998], [Kuida *et al.*, 1998], [Srinivasula *et al.*, 1996]. These pathways show that there are upstream and downstream events and interactions within caspases. For example, caspase-3 can activate procaspase-9, and caspase-6 can activate caspase-3 [Srinivasula *et al.*, 1996], [Liu *et al.*, 1996].

In addition, several protein substrates, including Poly (ADP-ribose) polymerase (PARP), lamin B and histone H1 are cleaved by caspases during the execution phase of apoptosis [Deveraux *et al.*, 1998]. Some caspases show overlapping specificities for some substrates, e.g. caspase-3 and caspase-7 can both cleave PARP, whereas other caspases have unique

substrate specificity, e.g. caspase-6 is the only known caspase that cleave lamins (see Figure A1).

For apoptosis examination of treated cells by *Oldenanldia diffusa* decoction and its fraction, PARP was used as its cleavage is one of the classical characteristics of apoptosis.

5.1.2.1. Poly (ADP-ribose) polymerase

Poly (ADP-ribose) polymerase (PARP) is a protein involved in a number of cellular processes involving mainly DNA repair and programmed cell death. DNA cleavage by enzymes involved in cell death. PARP may be the best characterized proteolytic substrate of caspases. It is cleaved in the apoptosis in many systems, including HL60 cells and breast cancer cell lines [Deveraux *et al.*, 1998], [Liu *et al.*, 1996]. Intact PARP (116 kDa) is cleaved to 24 kDa and 89 kDa fragments [Deveraux *et al.*, 1998]. ATP is required for PARP activity [Cohen, 1997]. So it can reduce the ATP of a cell. ATP depletion in a cell leads to lysis and cell death [Jones *et al.*, 2001]. Caspase-3 and caspase-7 are primarily responsible for PARP cleavage during apoptosis.

5.1.2.2. Caspase-3 (CPP32/YAMA/APOPAIN)

Caspase-3 is a member of the CED-3 subfamily of caspases. It is an important mediator of apoptosis in the immune system [Resnicoff *et al.*, 1998]. Caspase-3 is one of the key executioners of apoptosis. It is responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme PARP, which are cleaved in many different systems during apoptosis. During the apoptosis, caspase-3 is responsible in whole or in part for the proteolysis of a large number of substrates [Lazebnik, 1994]. Granzyme B can activate caspase-3 (or cleave another caspase which in turn activates caspase-3) as well as its ability to cleave PARP to its signature fragment [Quan *et al.*, 1996].

5.1.2.3. Caspase-7 (Mch3/ICE-LAP3/CMH-1)

Caspase-7 is a member of the CED-3 subfamily. It is a 303-amino-acid protein with high similarity to caspase-3. Caspase-7, like caspase-3, cleaves PARP and the peptide substrate Ac-DEVD-AMC. The competitive peptide aldehyde inhibitor Ac-DEVD-CHO is a potent inhibitor of both caspase-3 and caspase-7 enzymes. Caspase-3 and caspase-7 are functionally similar and have similar substrate specificities, so the cleavage of PARP

during apoptosis is a combination of the action of both these caspases. Active caspase-7 is made up of two subunits, similar to other caspases [Fernandes-Alnemri *et al.*, 1995]. Caspase-7 is activated to its catalytically active large subunit in intact cells when proceeding the apoptosis [Duan *et al.*, 1996], [Chandler *et al.*, 1997]. Following cleavage at Asp-198 and Asp-23, granzyme B activates pro-caspase-7 to a form that cleaves PARP to its fragment of 85 kDa [Chinnaiyan *et al.*, 1996].

5.1.2.4. Caspase-9 (ICE-LAP6/Mch6)

Caspase-9 is a member of the CED-3 subfamily and has high similarity to caspase-3. Procaspase-9 contains two processing sites between its large and small subunits, $P^{312}EPD\downarrow A^{316}$ and $D^{327}QLD\downarrow A^{331}$. The motif $D^{327}QLD\downarrow A^{331}$ is similar to the DEVD↓G site in PARP, so caspase-9 activated by caspase-3. $P^{312}EPD\downarrow A^{316}$ is a granzyme B cleavage site, as it contains an acidic residue in the P_3 position.

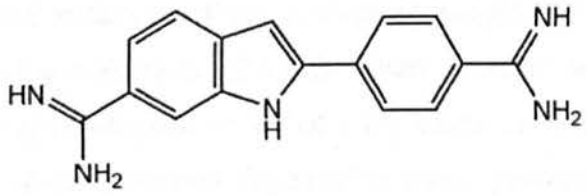
Both caspase-3 and granzyme B activate pro-caspase-9. Granzyme B cleaves procaspase-9 at both sites and generating an active enzyme capable of cleaving PARP to its fragment of 85 kDa. Caspases have different substrate specificity. A single activated caspase may not directly activate all other family members. For example, caspase-9 activates procaspase-3 and procaspase-7, but cannot activate procaspase-6 [Srinivasula *et al.*, 1998].

Caspases kill cells by cleaving proteins in different ways. For instance, caspases inactivate the inhibitors of proteins that promote apoptotic changes, or destroy cell structures, or deregulate proteins, resulting in loss or gain of function.

5.1.4. Deducing the mode of cell death induced by *Oldenlandia diffusa*

To distinguish between apoptotic and necrotic cell death or to investigate the morphological characteristics to apoptotic cell death, different experimental techniques and assays can be used such as fluorescence microscopy or flow cytometry (or fluorescence activated cell sorting (FACS)) by FITC-Annexin V and propidium iodide (PI) assays [Tuschl & Schwab, 2004]. In our experiments, confocal fluorescence microscopy was used with DAPI staining.

5.1.5. DAPI stain



DAPI is a blue nuclear counterstain for fluorescence microscopy. Its alternative names are 4,6'-diamidino-2-phenylindole, dihydrochloride and 4',6-diamidine-2-phenyl indole. It is soluble in dimethylformamide (DMF), water and various non-phosphate aqueous buffers. When DAPI passes through an intact cell membrane, it stains both living and fixed cells. It is widely used in fluorescence microscopy. On fluorescence microscopy, DAPI is excited with UV light. It is strongly bound to the minor groove of double stranded DNA and its fluorescence is approximately 20 times greater than in the nonbound state. Its absorption maximum is at 358 nm and its emission maximum is at 461 nm [Fuh *et al.*, 1998].

As DAPI is used for measuring the nuclear content [Hammarton *et al.*, 2003], assessing of apoptosis [Lai *et al.*, 2003], [Choi *et al.*, 1998] and detecting nuclei, it was used for investigation of apoptotic death of *Oldenlandia diffusa* decoction and its fraction treated cancer cells, namely Caco-2 and HL60.

However, the cell death mode of cancer cells exposed to analytes can be determined by examining the morphological characteristics unique to apoptosis in cancer cell lines *in vitro*, it cannot give information about their mode of biological action. To elucidate their mode of biological action or the activation of pro-apoptotic signaling caspase cascade in response to the cytotoxic effects of the decoction and its fraction, Western blotting was used.

5.1.6. Western blotting

The Western blot (protein immunoblot) is an analytical technique used to identify and locate proteins based on their ability to bind to specific antibodies or to detect the target proteins in a given sample.

There are four main steps in the Western blotting: electrophoresis, blotting, labeling and detection. In the electrophoresis stage, the proteins in a mixture (e.g. cell culture lysate) are separated by their molecular weight or isoelectric point, using polyacrylamide gel electrophoresis (PAGE). Then proteins are transferred from the gel to a membrane (nitrocellulose or PVDF) for easier handling. Next stage is the labeling. Choosing the labeling method depends on many parameters including level of sensitivity and ease of usage. There are four kinds of labeling systems that are generally used such as chemiluminescence, radioactivit, fluorescence and chemifluorescence. In the chemifluorescence method, an enzyme, alkaline phosphatase (AP), converts to a fluorescent precipitate that can be detected on a fluorescence scanner.

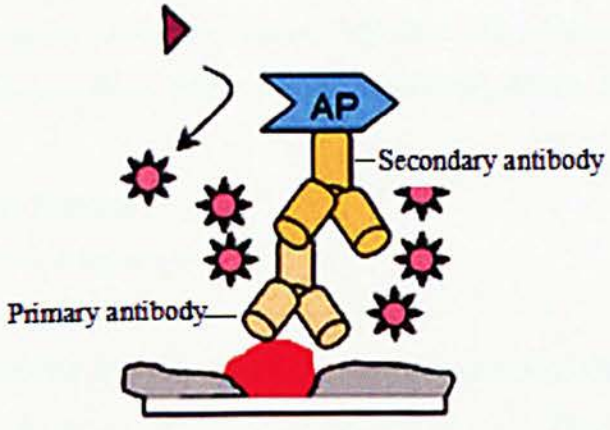


Figure 40. Schematic representation of Western blotting.

[Adopted from http://www.eurogentec.com/EGT/images/Eurogentec-proteomics-identification-western-blot-530x407.jpg]

Here the target protein is labeled using antibodies. The nitrocellulose membrane is incubated with a primary antibody. So, the protein is labeled with the primary antibody. The primary antibody is the specific antibody and it sticks to the protein and forms an antibody-protein complex with a certain protein. Then, the nitrocellulose membrane is incubated with a secondary antibody. This antibody should be an antibody-enzyme conjugate. The secondary antibody is an antibody against the primary antibody. Thus, the secondary antibody sticks to the primary antibody. Then it can be visualized using reagent that specific for the enzyme.

In the detection stage, target proteins are detected using the appropriate detection reagents to generate a signal that can be quantitated. For chromogenic methods, the signal is captured directly on the membrane. For radiolabeling, chemiluminescent, and chemifluorescent methods, the signal is captured using film or an imaging system. For example, the application of photo film on the gel to detect a flash of light, which is given by the enzyme [Ouyanga *et al.*, 1998], [Biji & Hal, 2006].

5.2. Materials and methods

5.2.1. Materials

CHR *Oldenlandia diffusa* and other chemicals, cells, content of complete medium are the same as mentioned in Chapter 2-4. Chemicals and consumables for morphological studies were DAPI (Fisher, UK), coverslip, glass slide, paraformaldehyde (Sigma, Poole, UK). confocal fluorescence microscopy was Leica TCS SP2 AOBS^{TM}, Milton Keynes, UK. For Western blotting experimental: PARP (Gibco, Paisley, UK), PARP, B-actin, CV-PARP (Cell Signaling Technology, UK), laemmli sample buffer (Bio-Rad, UK), skimmed milk powder (Fluka, UK), eppendorf tube, methanol (Fisher, UK), acrylamide, tris, SDS, ammonium persulfate, dual colour standard (Bio-rad, UK), membrane (Hybond-C Extra), sponge, ECL (GE Healthcare, UK), film (GE Health care, UK), protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), sodium deoxycholate, developer, fixer, phosphate buffered saline, TWEEN 20, Wattman paper, TEMED and NP 40 were purchased form Sigma Aldrich Company, Poole, UK.

5.2.2. Methods

5.2.2.1. Preparation of sample

Preparation of decoction and frcation

Decoction of *Oldenlandia diffusa* and fraction F9 were prepared as mentioned in Chapter 4.

Preparation of 4% paraformaldehyde (PFA)

2 g of PFA were put in the glassware and 50 mL of PBS was added on it and was heated up to 60 °C. It was adjusted to pH=7.2 using 10N NaOH. Then, the solution was cooled and filtered through 0.22 μm filter.

Preparation of RIPA buffer

RIPA buffer was prepared as content of 1% NP 40, 0.5% sodium deoxycholate, 0.1% SDS in PBS, and protease inhibitor cocktail, 1 tab/mL.

Preparation of transfer buffer

It was prepared mixing of 200 mL methanol, 100 mL (10x) PBS buffer and 700 mL of deionised water.

Preparation of milk for primary and secondary antibody

Solutions of primary and secondary antibody were prepared by adding 1 in 10000 antibody to 5 % of skimmed milk in PBS (10x) and 1% TWEEN 20.

Preparation of ECL

It was prepared by mixing, 125 µL solution B (these were the names given it patented reagent by GE Healthcare) was added to 5 mL solution A and mixed well.

5.2.3. Cell culture

HL60 cancer cell line was grown in complete growth medium RPMI 1640 containing 2 mM glutamine, 10% FBS. Caco-2 cell line was grown in complete growth medium DMEM containing 10% FBS, 2 mM L-glutamine and 2 mM NEAAs. All cell lines were grown and incubated at 37 °C in an atmosphere containing 5% CO_2. Cell lines were passaged when concentrations reached 10^6 cells/mL and diluted to 10^4-10^5cells/mL, thereby maintaining a stock solution containing cells at concentrations ranging from 10^5 to 10^6 cells/mL.

5.2.4. Experimental for DAPI staining

Cell seeding

Coverslips were previously sterilized in ethanol for 30-45 min and dried in sterile condition. In separate two 12 well plates these coverslips were placed one by one. Caco-2 and HL60 cells were seeded with $5x10^5$ cells/well density on it. Then, negative control (medium), positive control (actinomycin-D 10 µg/mL), *Oldenlandia diffusa* decoction (2.5 mg/mL) and fraction F9 (0.35 mg/mL) were added. Cells were treated for 24 and 48 hours under the above mentioned conditions.

5.2.4.1. Experimental protocol for DAPI staining

Counterstaining protocol for Caco-2 for fluorescence microscopy

After the incubation period, the samples were briefly equilibrated with PBS. Then, the cells fixed with 4% PFA for 15 min at room temperature. Fixed cells were permeabilized with methanol for 5 min. Cells were incubated with DAPI with a mounting medium and sample cells on coverslip was attached to glass slide and stabilized. The samples were analysed by a confocal fluorescence microscope.

Counterstaining protocol for HL60 cells

After exposure, the cells were centrifuged and the supernatants were discarded. Cells were washed with PBS at room temperature and pellets were transferred to coverslips and dried. Then, cells were fixed with 4% PFA solution for 15 min at room temperature and permeabilized with methanol for 5 min. Cells were incubated with DAPI counterstain and kept at -4 °C for further analysis.

5.2.5. Experimental for Western blotting

Stage-1 Treatment of cells (cell seeding)

Caco-2 and HL60 cells were seeded at $5x10^5$ cells/well density. Then, negative control (medium), positive control (actinomycin-D 10 µg/mL), *Oldenlandia diffusa* decoction (2.5 mg/mL) and fraction F9 (0.35 mg/mL) were added. Cells were treated for 24 and 48 hours under the above mentioned conditions.

Stage-2 Production of cell lysates

Production of Caco-2 cell lysates

Medium was removed from each well. Cold PBS was added and cell suspensions were transferred to eppendorf tubes. Freshly prepared RIPA with Protease inhibitor cocktail was added to the pellet and left for 15 minutes at 4 °C. Then it was sonicated for 10 seconds. Samples were centrifuged at 15,000 rpm for 20 min at 4 °C. Supernatents were taken and kept in a fridge (-4 °C) for further analysis.

Production of process for HL60 cell lysates

After incubation period, samples were transferred to eppendorf tubes and centrifuged at 1000 rpm for 10 min and supernatants removed. Pellets were washed with cold PBS and then freshly prepared RIPA with protease inhibitor cocktail was added on the samples. Samples were left for 15 minutes at 4 °C and sonicated for 10 seconds. After that, samples were centrifuged at 15,000 rpm for 20 min at 4 °C. Supernatents were collected and kept in a fridge for further analysis.

Stage-3 Bio-Rad Assay

Standard protein samples were prepared as mentioned by the instructions from the Bio-rad company. Seven different concentrations (0.2; 0.4; 0.5; 0.8; 1.0; 1.2; 1.4 mg/mL) of standard samples were prepared. All standard samples (5 µL) were placed in 96 well plates, then 25 µL and 200 µL of Reagent A (these were the names given it patented reagent by GE Healthcare) and B were added to each well, respectively. After 5 min shaking, absorbances were taken at 750 nm. A standard curve was used for determination of prepared sample concentration.

Stage-4 Western blotting

Firstly, on 20 µL of each sample was added to 10 µL of Laemli sample buffer and warmed up for 4-5 min by leaving it in boiling water. Then, 10 mL of resolving gel and 3 mL of stacking gels were prepared as followed:

a. For resolving gel, 4 mL deionised water, 3.3 mL 30% acrylamide mix, 2.5 mL 1.5 M tris (pH=8.8), 0.1 mL 10% SDS, 0.1 mL 10% ammonium persulfate and 0.004 mL TEMED were mixed and added to PAGE chambers.
b. Stacking gel was prepared adding by 0.5 mL 30% acrylamide mix, 0.38 mL 1M tris (pH=8.8), 0.03 ml 10% SDS, 0.03 ml 10% ammonium persulfate to 2.1 mL water and 0.003 mL TEMED. This was added to PAGE chambers after 10 min of loading of the resolving gel. After formulation of wells, samples were placed to each well and chambers were connected to electic, 80 W, 36 mA. It was run for 1 hour.

- Transfer stage:

To transfer the pattern from the gel to membrane, sandwiches were prepared using membrane (Hybond-C Extra), Wattman papers, sponges and transfer buffer. After that, sandwiches were placed in PAGE chambers (filled up with transfer buffer) and connected to electricity, 300 MA, 100 W and left for 1.5 hours.

- Labeling stage:

After blotting, membranes were left overnight in 5% skimmed milk with 1 in 10000 antibody PARP (rabbit), CV-PARP (cleaved PARP-rabbit) and β-actin (mouse)) on the shaker at 4 °C for labeling. Then, membranes were removed from the milk containing primary antibody and washed with 5% skimmed milk twice on shaker for 5 min. Secondary

antibodies (antirabbit and antimouse) were added to membranes and it was shaken for 1 hour. After that, the antibodies were removed and membranes were washed with 5% milk twice, PBS containing TWEEN (1%) 2 times and with PBS 2 times for 4-5 min on shaker.

- Detection stage:

When procedure of the membrane washing finished, it was transferred onto previously cut and prepared clean film. Then, the light on the antibody was turned on using reagent Enhanced Chemiluminescence (ECL) by leaving it at ambient temperature for 3-4 min. The signal was captured using film, developer (40% v/v) and fixer (30% v/v).

5.3. Results

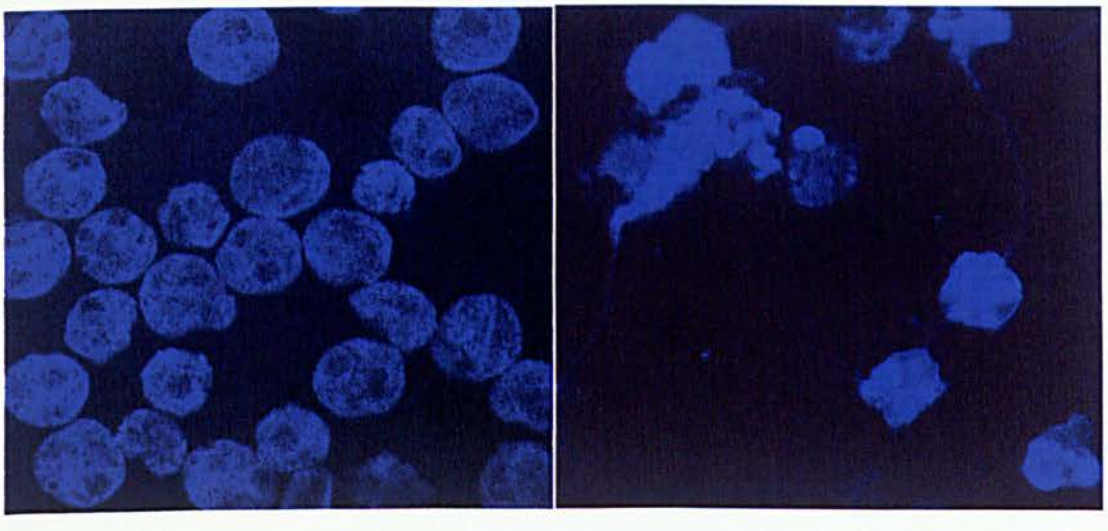
5.3.1. Mode of cell death induction by DAPI staining on confocal fluorescence microscopy

DAPI staining examination revealed apoptotic cell death induction in both HL60 and Caco-2 cell lines exposed to *Oldenlandia diffusa* decoction. In experiments, activities were compared to positive control actinomycin-D treated cells.

In *Oldenlandia diffusa* decoction and its fraction-F9 treated HL60 and Caco-2 cells (24 and 48 hours), apoptotic characteristics such as nuclei disintegration, blebbing followed by forming apoptotic bodies occurred, results are showed in Figures 41-44.

Results for DAPI staining on HL60 cell line (24h):

(a)- Control-(untreated cells) (b)-Cells treated with actinomycin-D



(c)- Cells treated with *Oldenlandia diffusa* decoction

(d)- Cells treated with fraction F9

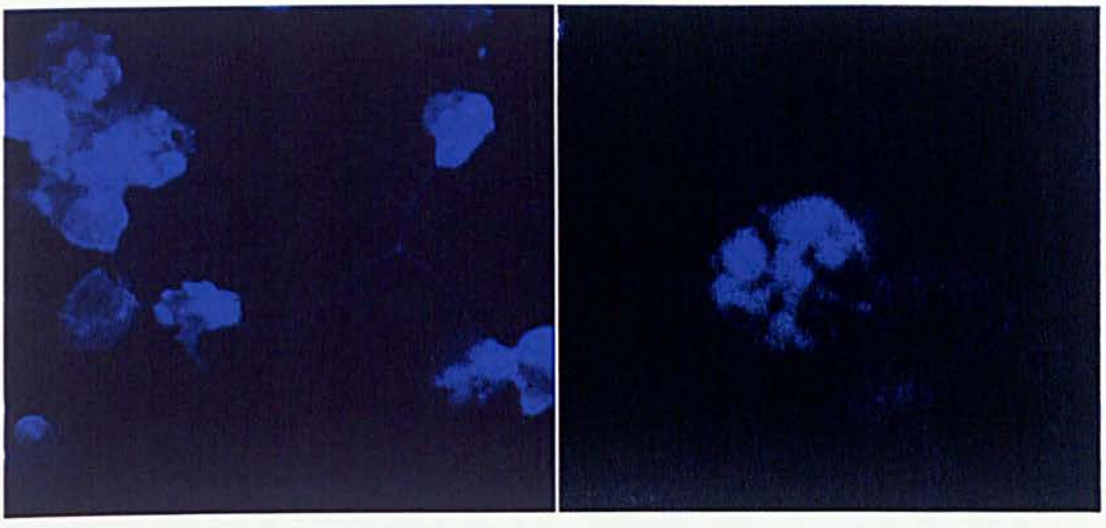


Figure 41. Morphological changes of apoptotic cell death of the HL60 cancer cells treated with *Oldenlandia diffusa* decoction, fraction F9 and actonimycin-D for 24 hr incubation examined by confocal fluorescence microscopy after DAPI staining. Examination on HL60 cells identifying the characteristic morphological features, (a)-Untreated control cells have remained intact, (b)-Actinomycin-D-treated cells, (c)-*Oldenlandia diffusa* decoction treated cells and (d)-F9 treated cells show characteristic blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (magnification, x400).

Results for DAPI staining on HL60 cell line (48h) for F9:

(a)- Control (untreated cells) (b)- Cells treated with fraction F9

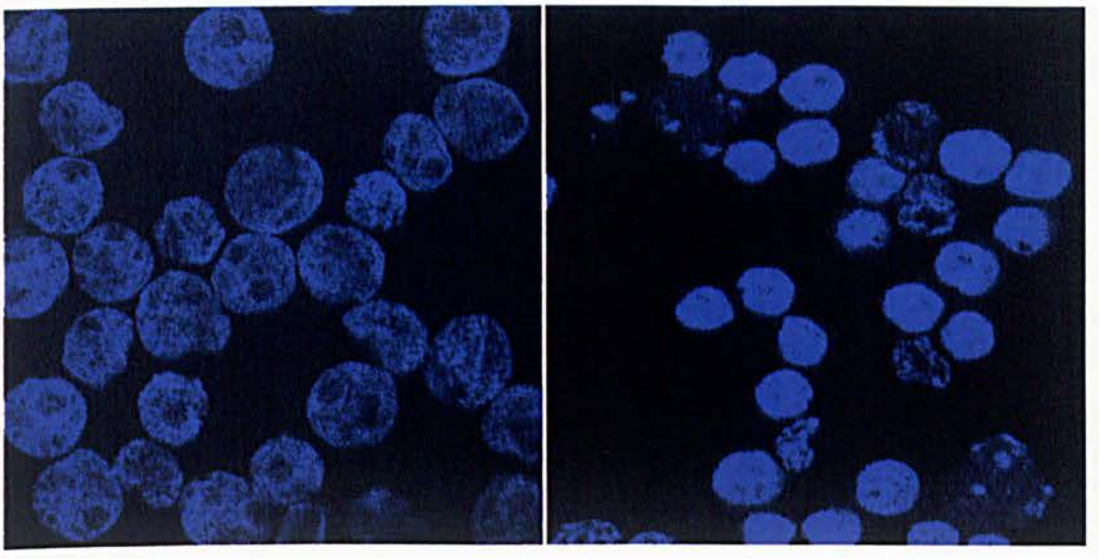
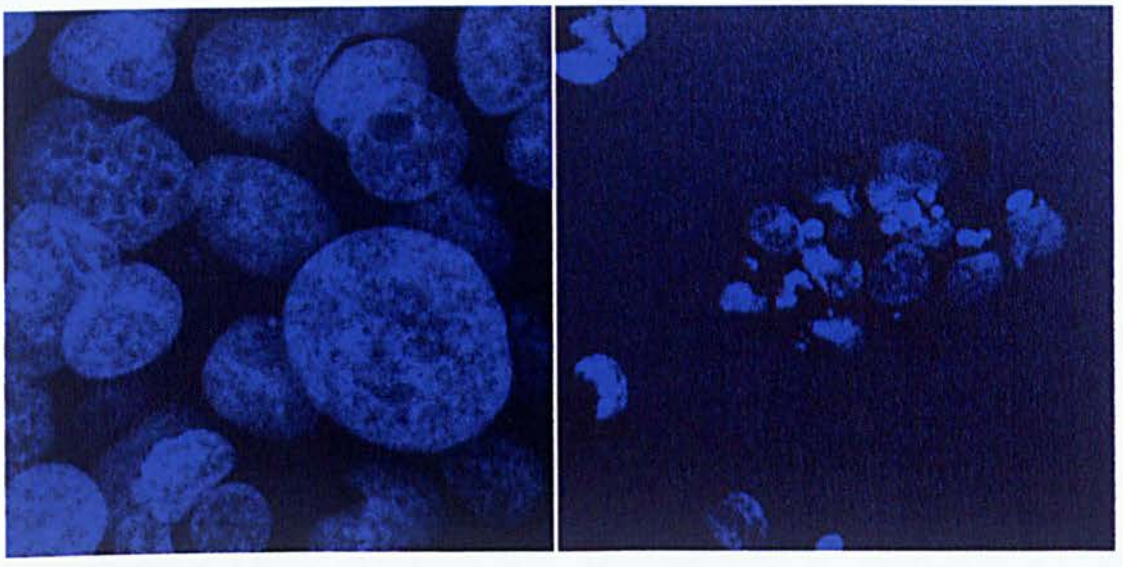


Figure 42. Image of confocal fluorescence microscopy of HL60 cells identifying the characteristic morphological features, (a)-untreated control cells (48 hr incubation) have remained intact, (b)-fraction F9 treated cells (48 hr incubation) show characteristics of cell shrinkage, blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (magnification, x400).

Results for DAPI staining on Caco-2 cell line (24h):

(a)- Control-untreated cells

(b)- Cells treated with actinomycin-D



(c)- Cells treated with *Oldenlandia diffusa* decoction

(d)- Cells treated with fraction F9

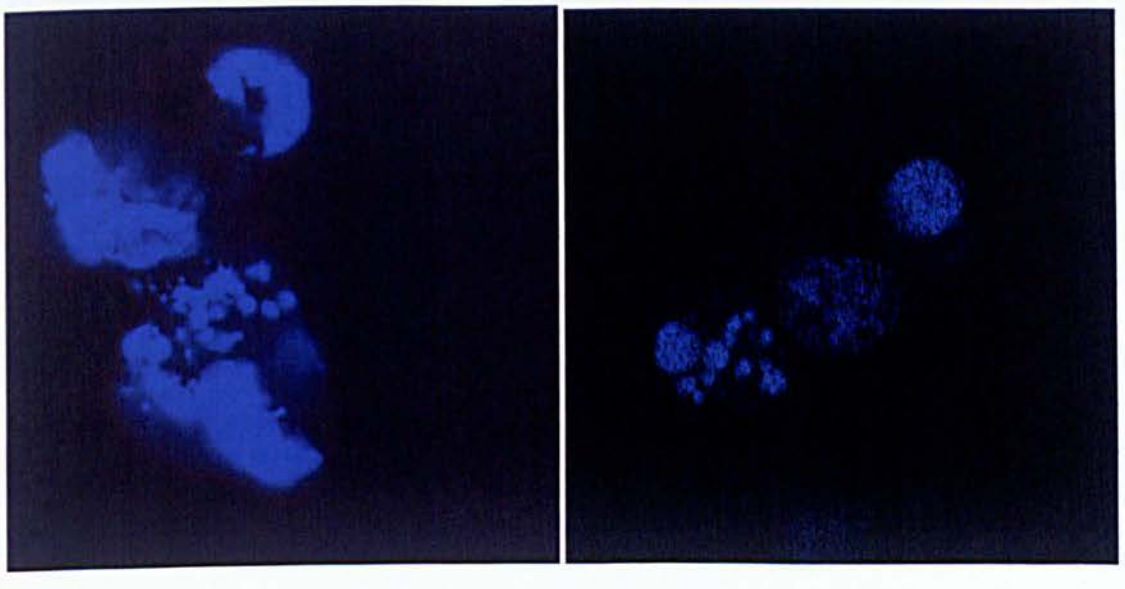


Figure 43. Morphological changes of apoptotic cell death of the Caco-2 cancer cells treated with *Oldenlandia diffusa* decoction, fraction F9 and actonimycin-D for 24 hr incubation examined by confocal fluorescence microscopy after DAPI staining. Examination on HL60 cells identifying the characteristic morphological features, (a)-Untreated control cells have remained intact, (b)-Actinomycin-D-treated cells, (c)-*Oldenlandia diffusa* decoction treated

cells and (d)-F9 treated cells show characteristic blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (magnification, x400).

Results for DAPI staining on Caco 2 cell line (48h) for F9:

(a)- Control-untreated cells (b)- Cells treated with fraction F9

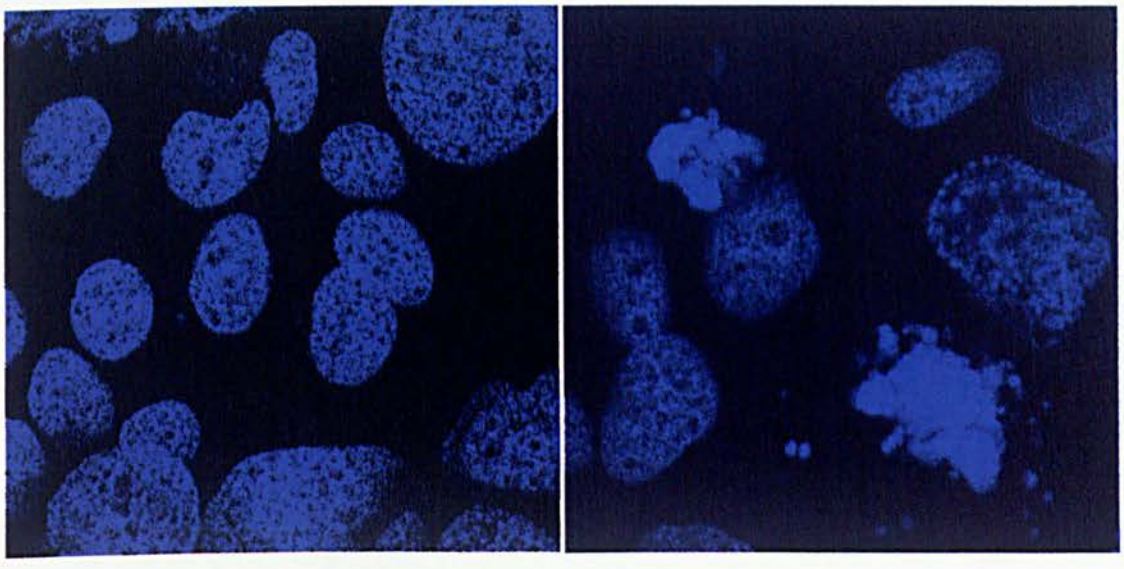


Figure 44. Confocal fluorescence microscopy of HL60 cells identifying the characteristic morphological features, (a)-Untreated control cells (48 hr incubation) have remained intact, (b)-fraction F9 treated cells (48 hr incubation) show characteristics of blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (magnification, x400).

5.3.2. Results for Western blotting on HL60 and Caco-2 cell line

Apoptotic cell death investigation was performed by Western blotting method. On untreated HL60 cancer cells, bands at 116 kDa for PARP were detected, whereas on *Oldenlandia diffusa* decoction (2.5 mg/mL) and its fraction F9 (0.35 mg/mL) treated cells, bands at 85 kDa for cleaved-PARP, and 47 kDa for β-actin for total protein were detected (Figures 45, 47).

On Caco-2 cells, initially, bands at 116 kDa for PARP were obtained for all untreated and treated cells, almost none of these bands were detected as cleaved-PARP (85 kDa), including cells treated with positive control actinomycin-D, see Figure 46. In further experiments, the antibodies (new) were changed and on the decoction, its fraction F9 and positive control actinomycin-D treated Caco-2 cells, the poly (ADP-ribose) polymerase cleavage was detected at 85 kDa. Results are shown in Figure 48.

Experimental of Western blotting for HL60 cancer cell line

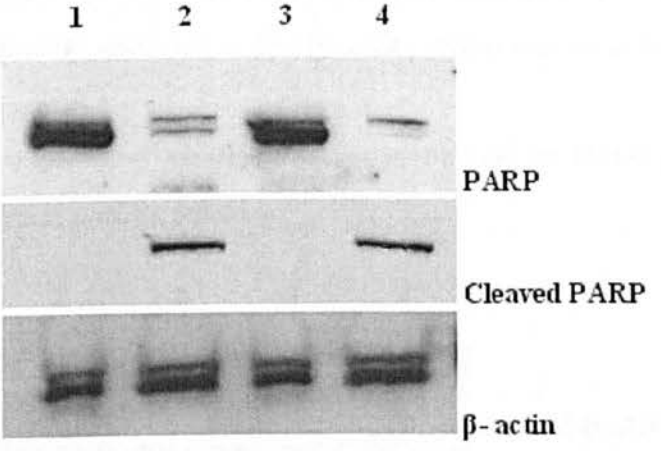


Figure 45. Effect of *Oldenlandia diffusa* decoction on HL60 cell line for the investigation of apoptosis cell death by Western blotting. The HL60 cancer cells were treated with *Oldenlandia diffusa* decoction under culturing condition for 24 and 48 h. Lane 1-Negative control (medium)-24 h, lane 2-*Oldenlandia diffusa* decoction-24 h, lane 3-Negative control (medium)-48 h, lane 4-*Oldenlandia diffusa* decoction-48 h. Equal amounts of the total proteins (20 µg/lane) were subjected to 10% SDS-PAGE. The expression of cleaved PARP, PARP and β-actin was detected by the specific antibodies at 85 kDa, 116 kDa and 47 kDa, respectively. The β-actin protein was used as the internal control.

Experimental of Western blotting for Caco-2 cancer cell line

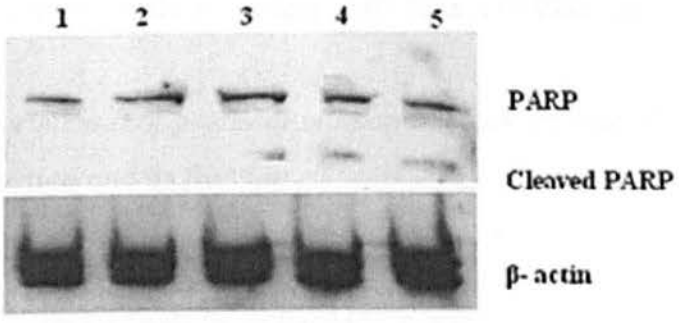


Figure 46. Examination of effect of *Oldenlandia diffusa* decoction on Caco-2 cell line for apoptosis cell death by Western blotting. The Caco-2 cancer cells were treated with *Oldenlandia diffusa* decoction under culturing condition for 24 and 48 h. Lane 1-Negative control (medium)-24 h, lane 2-*Oldenlandia diffusa* decoction-24 h, lane 3-Negative control (medium)-48 h, lane 4-*Oldenlandia diffusa* decoction-48 h, lane 5-Positive control actinomycin-D-48 h. Equal amounts of the total proteins (20 µg/lane) were checked on 10% SDS-PAGE. The expression of cleaved PARP, PARP and β-actin was detected using the specific antibodies. The β-actin protein was used as a loading control.

Experimental of potential of apoptotic cell on HL60 cells treated by Oldenlandia diffusa decoction and its fraction

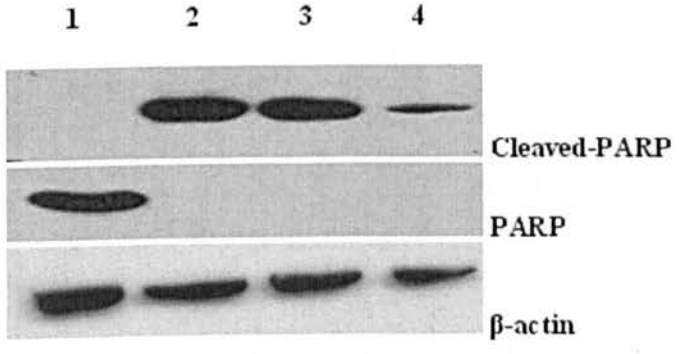


Figure 47. Effect of *Oldenlandia diffusa* decoction and its fraction-F9 on HL60 cell line for the investigation of apoptosis cell death by Western blotting. The HL60 cancer cells exposed to treating sample, negative and positive controls under culturing condition for 24 h. Lane 1-Negative control (medium), lane 2-*Oldenlandia diffusa* decoction, lane 3-Positive control (actinomycin-D), lane 4-Fraction-F9. 20 µg/lane proteins were subjected

to 10% SDS-PAGE. The expression of cleaved-PARP, PARP and β-actin was detected using the specific antibodies at 85 kDa, 116 kDa and 47 kDa, respectively.

Experimental of potential of apoptotic cell on Caco-2 cells treated by Oldenlandia diffusa decoction and its fraction

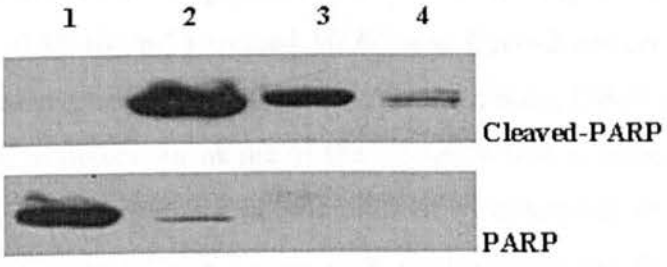


Figure 48. Effect of *Oldenlandia diffusa* decoction and its fraction- F9 on Caco-2 cell line for investigation of apoptosis cell death by Western blotting. The Caco-2 cancer cells were treated with sample, negative and positive controls under culturing condition for 24 h. Lane 1-Negative control (medium), lane 2-*Oldenlandia diffusa* decoction, lane 3-Fraction-F9, lane 4-Positive control (actinomycin-D). 20 µg/lane proteins were subjected to 10% SDS-PAGE. The expression of cleaved-PARP and PARP were detected by Western blotting using the specific antibodies at 85 kDa and 116 kDa, respectively.

5.4. Discussion

The apoptotic mode involves an active cascade self-destruction in the affected cells. This can involve DNA degradation via endonuclease activation, nuclear disintegration, and the formation of apoptotic bodies.

The induction of apoptosis in *Oldenlandia diffusa* decoction (2.5 mg/mL) and its fraction F9 (0.35 mg/mL) treated HL60 and Caco-2 cancer cells were investigated by confocal fluorescence microscopy after DAPI staining. DAPI specifically stains DNA and is widely used to detect shrinkage of the nuclei, which is indicative of apoptosis [Choi *et al.*, 1999]. Typical fluorescence photographs of shrunken nuclei and blebbing after the 24 h treatment of HL60 and Caco-2 cells with treatment by the decoction and fraction F9 compared to positive and negative control are shown in Figures 41-44. It strongly suggests that apoptosis was induced by the decoction and fraction F9 in HL60 and Caco-2 cells. After 24 hours exposure on fraction F9, the characteristic of apoptotic bodies forming was not clear on Caco-2 cells, therefore, treatment period was extended to 48 hours for both cells. Results are shown in Figures 42 and 44. Results showed that the fraction F9 induced apoptosis of those both cancer cell lines and maybe in a dose-dependent manner.

Experiments on Western blotting, molecules were separated using gel electrophoresis. Then, the separated molecules were blotted onto membrane. The transferred protein was complexed with an enzyme-labeled antibody. PARP, cleaved PARP and β-actin primary antibodies were used to detect an antigen. PARP cleavage is one of the classical characteristics of apoptosis. Cleaved-PARP antibody specifically recognizes the 85 kDa fragment of cleaved PARP.

Results on *Oldenlandia diffusa* decoction and fraction F9 showed that exposed HL60 and Caco-2 cancer cells, a 85 kDa band (cleaved-PARP) was detected; it gives the information that cells were apoptotic. ICE family members, such as caspase-3 and caspase-7 cleave PARP to give 85 kDa and a 25 kDa fragments [Nicholson *et al.*, 1995], [Tewari *et al.*, 1995]. Thus, it could be that the cell death was induced by capase-9, caspase-3 and caspase-7. According to results in both cell lines, capacity of detected band in PARP cleavage was less than the decoction. We could say that it was concentration dependant as the cells were exposed to much less amount of fraction F9 than the decoction.

Generally, as results of DAPI staining and Western blotting experiments showed, HL60 and Caco-2 cancer cells exposed to *Oldenlandia diffusa* decoction and its fraction F9 were apoptotic as showed the characteristics of blebbing, apoptotic body forming, nucleus degradation and cleavage of PARP from a 116 k Da to 85 and 24 kDa subunits, but in both cell lines, the size of detected band in cleaved-PARP and morphological changes were smaller than the decoction. So, the cell death process was concentration dependant as concentrations were 2.5 mg/mL for *Oldenlandia diffusa* decoction and 0.35 mg/mL for fraction F9.

5.5. Conclusion

DAPI staining for morphological examination and Western blotting analysis for detection of PARP cleavage on apoptotic cells were performed by investigating the *Oldenlandia diffusa* decoction and its most cytotoxic fraction F9 treated HL60 and Caco-2 cells. These results provide evidence that HL60 and Caco-2 cancer cells exposed to *Oldenlandia diffusa* decoction and its fraction F9 are apoptosis, which is mediated by a caspase cascade.

Chapter 6. Study of cytotoxic content(s)

Chapter 6. Study of cytotoxic content(s)

6.1. Introduction

In previous studies, *Oldenlandia diffusa* decoction was successfully separated using the method which was set up on HPLC and eleven fractions were collected. Cytotoxicity study results revealed that the decoction and the fraction F9 have significant cytotoxic effect on HL60 and Caco-2 cancer cell lines. Furthermore, it was shown that the cell death was induced by apoptosis. Therefore, in order to determine the content(s) of the most cytotoxic fraction F9, the initial aim of this part of assessment was to set up the separation method for this fraction. For that, HPLC and LC-MS analyses were performed.

In addition, the HPLC method for collecting fraction F9 was expensive and took lots of time. Therefore, liquid-liquid extraction was evaluated. Some additional fractions were collected and their cytotoxic activity was investigated.

6.1.2. Liquid Chromatography-Mass Spectrometry

Liquid Chromatography-Mass Spectrometry (LC-MS) is a powerful technique that is used for many applications. It has very high sensitivity and specificity. Compounds that are thermally labile, exhibit high polarity or have a high molecular mass are analysed using LC-MS. This technique provides both molecular weight and structural information for peak identification and confirmation. In addition, this technique has an application for the specific detection and identification of compounds in a complex mixture [Lee, 2002]. Specific applications include stability indicating assays and purity checking of LC peaks employing UV and visible wavelength monitoring with simultaneous mass spectrometry detection [Krstulovic *et al.*, 2002], [Simal-Gandara *et al.*, 2002], [Marquet, 2002], [Thomas, 2001], [Hogendoorn & Zoonen, 2000].

LC-MS includes a HPLC attached, via a suitable interface, to a mass spectrometer. The chromatographic scale of LC-MS is performed on a much smaller size of column to that of HPLC, so uses a low flow rate. Capillary columns of 75 μm to 1 mm are common for LC-MS work. In LC-MS as the analyte ions have to be nebulised in order to be charged at low flow rate (up to 200 μL/min or less), the MS gives improved sensitivity.

In LC-MS analysis, on HPLC column, compounds are separated according to their relative interaction with the stationary phase and the mobile phase eluting from the column. Then,

the eluting compounds from the chromatographic column are introduced to the mass spectrometer via a specialised interface. In the interface, all solvents must be evaporated and compounds in the peaks need to be ionized. They can then be analysed by a mass analyzer and ions are counted by suitable detector [Marvin, 2005], [Pavia *et al.*, 2009].

6.1.3. Interfaces

There are different types of interfaces namely, electron impact ionization, fast atom bombardment, MALDI, atmospheric pressure chemical ionization and electrospary ionization. The two most common interfaces used for LC-MS are the atmospheric pressure chemical ionisation and electrospray ionisation (ESI) interfaces [Aprino, 1989], [Murray, 1998], [Aprino, 2005]. The latter one was used for this study.

6.1.3.1. Electrospray ionization (ESI)

ESI is especially useful for non-volatile molecules to be analyzed directly from the liquid phase, small amounts of large (105 Da) or non-volatile compounds such as drugs, pesticides, carbohydrates, long chain fatty acids, analysis and sequencing of proteins and oligonucleotides and mass determination of biomolecules [Barker, 1998], [Patrick, 1989]. The ESI source operates at atmospheric pressure and produces neutrals, ions and clusters of ions.

Usually an analyte is prepared in a mixture of water and an organic, volatile solvent such as methanol or acetonitrile that can be evaporated easily for ion formation. When the analayte elutes from the column, it passes through the electrospray needle with 1 μL/min (typically) flow rate. There will be a high potential difference of 3-6 kV between the needle and the counter electrode to ESI. Then, the analyte solution is dispersed by a nebulizer to a fine aerosol into a strong electric field which is formed by a high potential difference in the presence of a warm nitrogen gas flow. As the droplets pass through the space between the needle tip and the cone, the solvent will evaporate. As the solvent evaporation occurred in a region of a vacuum of several torr (approx=760 torr), it causes the charge to increase on the droplets. Gas-phase ions are formed when the droplets from the Taylor cone evaporate. The ions carrying excess charge are released into the gas-phase. Then, the multiply charged ions enter the analyzer [Fenn *et al.*, 1990], [Bruins, 1998], [Kuo *et al.*, 2005].

Both positive and negative ion spectra can be obtained. For positive ion mode, 0.1% formic acid or acetic acid is usually added into the analyte solution to enhance protonation and increase sensitivity. For negative ion mode, 0.3% NH_4OH is usually added, however ammonium acetate was used in our study, into the analyte solution to help deprotonation and increase sensitivity. ESI ionisation produces low fragmentation and it is cheaper and compatible with various separation techniques. However, it shows limited transfer of ions through MS and has poor sensitivity and sources of instability, interference, background and competition effects [Cech & Enke, 2001], [Hewlett Packard, 1994].

6.1.4. Mass analyzer

Once the analyte is ionized, the beam of ions is accelerated by an electric field and then passes into the mass analyzer. In the mass analyzer, ions are separated according to their mass-to-charge (m/z) ratios. There are a several types of mass analyzers that can be used in LC-MS such as quadrupole, ion trap, time of flight (TOF) and quadrupole-time of flight (Q-TOF).

Quadrupole mass analyzer

The quadrupole mass analyzer is one type of mass analyzer used in mass spectrometry. It filters the sample ions according to their mass-to-charge (m/z) ratio [Hoffmann & Scroobant, 2003]. Quadrupole mass analyzers are often used for quantitative applications and can be used either to measure one or a few given ions in a mixture or to scan a range of m/z values [Hobart *et al.*, 1998]. The quadrupole mass analyzer consists of a parallel set of 4 circular rods. A direct current (DC) voltage and a radiofrequency (RF) are connected to the rods. When they are applied, an oscillation occurs of the electrostatic field produced in the region between the four rods. The ions acquire an oscillation in this electric field and pass through a quadrupole mass filter. All other ions do not have a stable path through the quadrupole mass analyzer and will collide with the quadrupole rods and thus not reach the detector [Hoffmann & Scroobant, 2003], [Rouessac & Rouessac, 2007].

Time-of-Flight mass analyzers

A time of flight (TOF) mass analyzer is based on the measurement of mass-dependent time. Ions with different masses to move from the ion source to the detector at a similar kinetic energy. The equation for the time of flight mass analyzer separation is:

$$\frac{m}{z} = \frac{2Vt^2}{L^2} \quad (3)$$

m/z is mass-to-charge ratio of the ion

V is kinetic energy of an ion accelerated through an electrical potential

L is the length of flight path

t is the measured time of the ion as it passes through the drift tube

When the ions pass along the field free drift zone, they are separated by their masses which mean the lighter ions will have a higher velocity. As the lighter ones are fastest, they will come to the detector first. Then, in the detector, ions are measured relating to their mass to charge ratio. A wide range of mass to charge ratios can be measured with good sensitivity and analysis is very rapid (40+ kHz). However, its major disadvantage is low resolution. In the TOF mass analyzer, the mass resolution is directly proportional to the time of flight of the ion. Therefore, a longer drift tube could increase the resolution [Duckworth *et al.*, 1990], [Hobart *et al.*, 1998].

6.2. Materials and methods

6.2.1. Materials

The HPLC equipment for the separation of fraction F9 were the same as mentioned in Chapter 3. Chemicals such as methanol, acetonitrile (HPLC grade), hexane, ethyl acetate, dichloromethane and n-butanol (analytical grade) were supplied by Fisher Scientific Inc, UK, and ammonium acetate (HPLC grade) was purchased from Sigma-Aldrich Inc, Poole, UK. LC-MS device was Waters, UK. Chemicals and equipments for cell biological studies were the same as mentioned in Chapters 4 and 5.

6.2.2. Methods

6.2.2.1. Sample preparation

Preparation of sample fraction F9

Fraction F9 was separated and prepared from *Oldenlandia diffusa* decoction by the Msp method that was set up on HPLC for the separation of the decoction (Chapter 3) between 36.5 to 41.5 min intervals. Then, it was passed through a C18 SPE tube and dried. Dry residue of fraction F9 was dissolved back in methanol or acetonitrile/water 50:50 or 60:40, depending on the mobile phase used.

Preparation of ammonium acetate

Ammonium acetate buffer was prepared as mentioned in Chapter 2.

Preparation of reagents and buffers

NRU assay kit, 4% PFA, RIPA buffer, transfer buffer and ECL were prepared in similar fashion to that described in Chapters 4 and 5.

6.2.2.2. Determination of the amount of fraction F9

Fraction F9 was collected from pooling 7 HPLC runs of *Oldenlandia diffusa* decoction using the method mentioned above. The solution dried on rotary-evaporator under vacuum at 40 °C and freeze-dried. The amount of dry residue was calculated. The experiment was repeated 3 times.

6.2.2.3. Setting up the separation method for fraction F9

In order to separate the fraction F9, the following columns including, C18, PFP(2) and amino chromatographic analytical columns were examined with different isocratic and gradient elutions using different combinations of buffer, water, methanol, acetonitrile, isopropanol, THF and hexane solvents as a mobile phase.

Using the isocratic mode, the investigation was started with 100% of methanol for a total 30 mins run, then transferred to water/methanol 50/50, 80/20, 40/60, 20/80 on C18 and PFP(2) analytical columns. Then, it was followed by utilising of different ratios of mixtures of ammonium acetate buffer/methanol, buffer/acetonitrile with 50/50, 80/20, 40/60, 20/80, ammonium acetate buffer/methanol/THF with 50/40/10, 65/10/25 v/v on reversed phase columns and with hexane on amino column.

Then, mobile phase mode was transferred to gradient elutions on PFP(2) (250 x 4.60 mm, 5 µm) column and optimised methods were:

Solvent A=MeOH/ACN B=water/buffer

- M1 method:
  - 0-5 min 80% B
  - 5-15 min 100% A
  - 15-25 min 100% A
  - 26-30 min 80% B gradient elution at 1 mL/min flow rate.
- M2 method:
  - 0-5 min 100% B
  - 5-15 min 100% A
  - 15-25 min 100% A
  - 26-30 min 100% B gradient elution at 1 mL/min flow rate
- M3 method:
  - 0-10 min 100% B
  - 10-40 min 100% A
  - 40-50 min 100% A
  - 50-60 min 100% B gradient elution at 1 mL/min flow rate.
- M4 method:
  - 0-5 min 70% B

5-15 min 95% A
15-16 min 95% A
16-25 min 100% A
26-30 min 70% B gradient elution at 1 mL/min flow rate.

- M5 method:

0-5 min 70% B
5-12 min 90% A
12-15 min 90% A
16-25 min 100% A
26-30 min 70% B gradient elution at 1 mL/min flow rate.

- M6 method:

0-5 min 70% B
5-10 min 90% A
10-14 min 90% A
15-25 min 100% A
26-30 min 70% B gradient elution at 1 mL/min flow rate at 210 nm.

- M7 method:

0-10 min 50% B
10-50 min 100% A
50-55 min 100% A
56-60 min 100% A gradient elution at 1 mL/min flow rate at 210 nm.

6.2.2.4. Optimising the method to achieve greater amount of fraction F9.

The collection of fraction F9 from HPLC was expensive and time consuming, therefore, it was necessary to set up another possible method which can improve the quantity of fraction F9 achieved.

Oldenlandia diffusa decoction was extracted with four different polarities of organic solvents hexane, ethyl acetate, dichloromethane and n-butanol, three to four times for each solvent extraction. Then, extracts were subjected to rotary-evaporator under vacuum at 40 °C to dryness. After that, each extract was dissolved in acetonitrile/water (50:50) solvent for further analysis.

All extracts were studied on HPLC, using Ms method on analytical PFP(2) (250 x 4.60 mm, 5 µm) column (see Section 2.5).

6.2.2.5. Cytotoxicity of ethyl acetate fraction and fractions FEA-1, FEA-2 and FEA-3.

Cell culture

HL60 and Caco-2 cancer cell lines were grown in complete growth medium RPMI and DMEM, respectively under the condition as mentioned in Chapter-2.

Cells were seeded to 96 well plates with $2x10^4$ cells/well density. *Oldenlandia diffusa* decoction (2.5 mg/mL), ethyl acetate fraction (0.5 mg/mL) and ethyl acetate fractions, including fraction ethyl acetate-1 (FEA-1) (0.5 mg/mL), FEA-2 (0.5 mg/mL), FEA-3 (0.5 mg/mL), negative control (medium) and positive control actinomycin-D (10 µg/mL) were added to both cell lines and were incubated for 48 hours at 37 °C in an atmosphere containing 5% CO_2. Cell viabilities were determined using NRU assay at 540 nm in a microplate reader (spectrophotometer).

6.2.2.6. Characterization of the mode of cell death by ethyl acetate fraction, using DAPI staining.

Counterstaining protocol for fluorescence microscopy

Counterstaining protocol for HL60 and Caco-2 cell were the same as mentioned in Chapter-5. The cells were treated with 0.5 mg/mL of ethyl acetate fraction sample and control for 48 hours. Counterstained cells were kept at -4 °C for further analysis.

6.2.2.7. Characterization of the mode of cell death and apoptosis induced by ethyl acetate fraction, using Western blotting.

Experimental protocol was the same as mentioned in Chapter-5. HL60 cells ($5x10^5$ cells/well) were treated with control (medium) and ethyl acetate fraction (0.5 mg/mL) 24 hours under the correct cell growth condition. Cells were lysed in RIPA buffer (1% NP 40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail. Briefly clarified protein lysates (20 µg) were resolved electrophoretically on 10% denaturing SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. After blocking in 5% skimmed milk, membranes were probed with the following primary antibodies specific for: PARP and CV-PARP. Proteins were visualized with antirabbit and antimouse secondary antibodies. Antigen–antibody complexes were detected using the ECL system on X-ray film.

6.3. Results

6.3.1. Determination of the amount of F9

The results from triplicate experiments for determination of the amount of the separated fraction F9 is showed in Table 4.

Table 4. The amount of fraction F9 in 7 times run collection.

Fraction F9	Dry residue, mg
I- collection from 7 runs	4.7
II- collection from 7 runs	5.7
III- collection from 7 runs	5.9
Mean	**5.43**
STDEV	0.64

The average amount of the fraction F9 for 7 run was 5.43±0.64 mg which means for 1 run can be obtained 0.77 mg of fraction F9.

6.3.2. Setting up the separation method for fraction F9

In order to set up the separation method for the chosen fraction F9, reversed phase chromatographic C18 and PFP(2) columns and normal phase amino column were performed with different modes of mobile phase. Mobile phase optimisation started with isocratic with different combinations of buffer, water, methanol, acetonitrile, isopropanol, THF and hexane solvents and then followed by the gradient elutions which were mentioned in section Method 6.2.2.3.

Some selected results are shown in Figures 49-56.

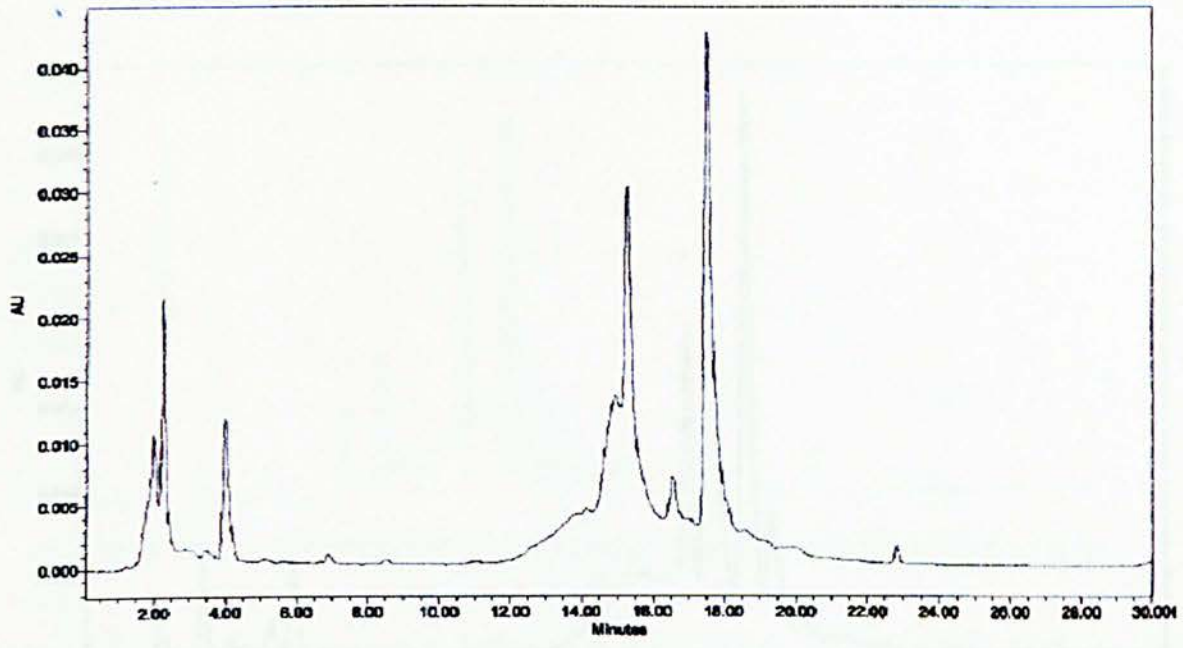


Figure 49. Chromatogram for separation of SPE tube passed fraction F9 on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using M1 method.

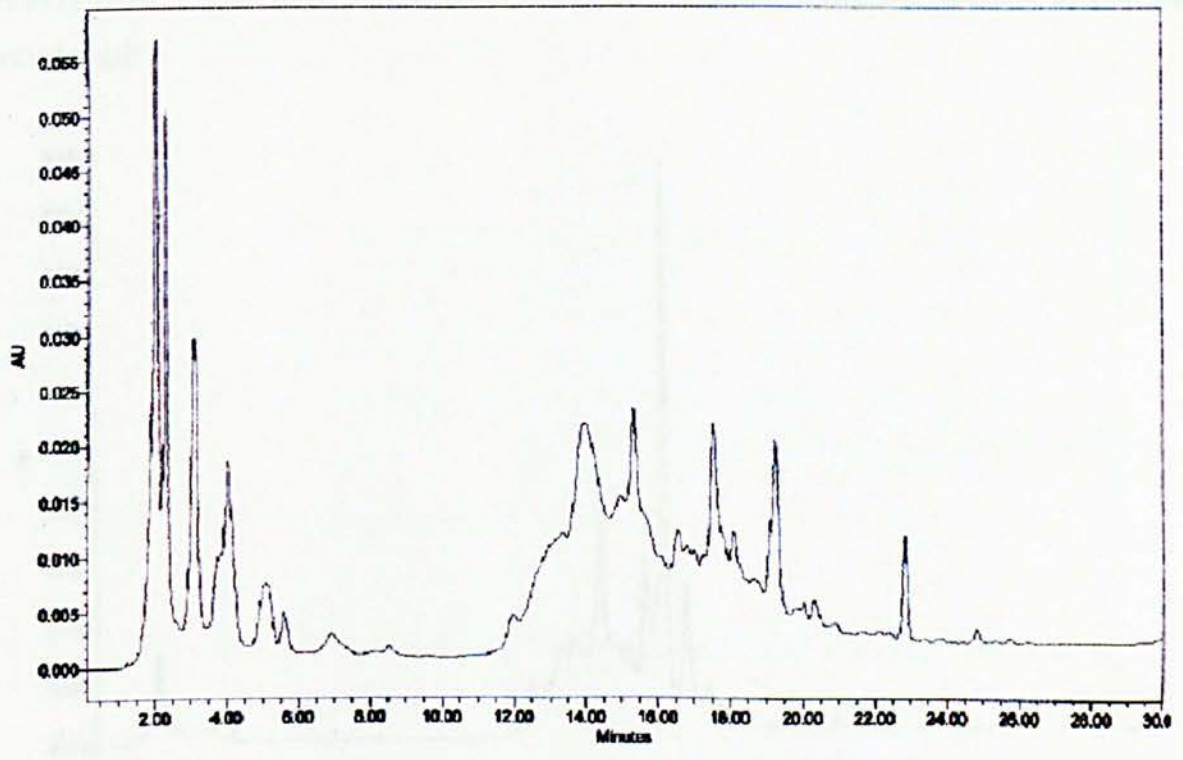


Figure 50. Chromatogram for separation of fraction of SPE tube passed F9 on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using M4 method at 226 nm.

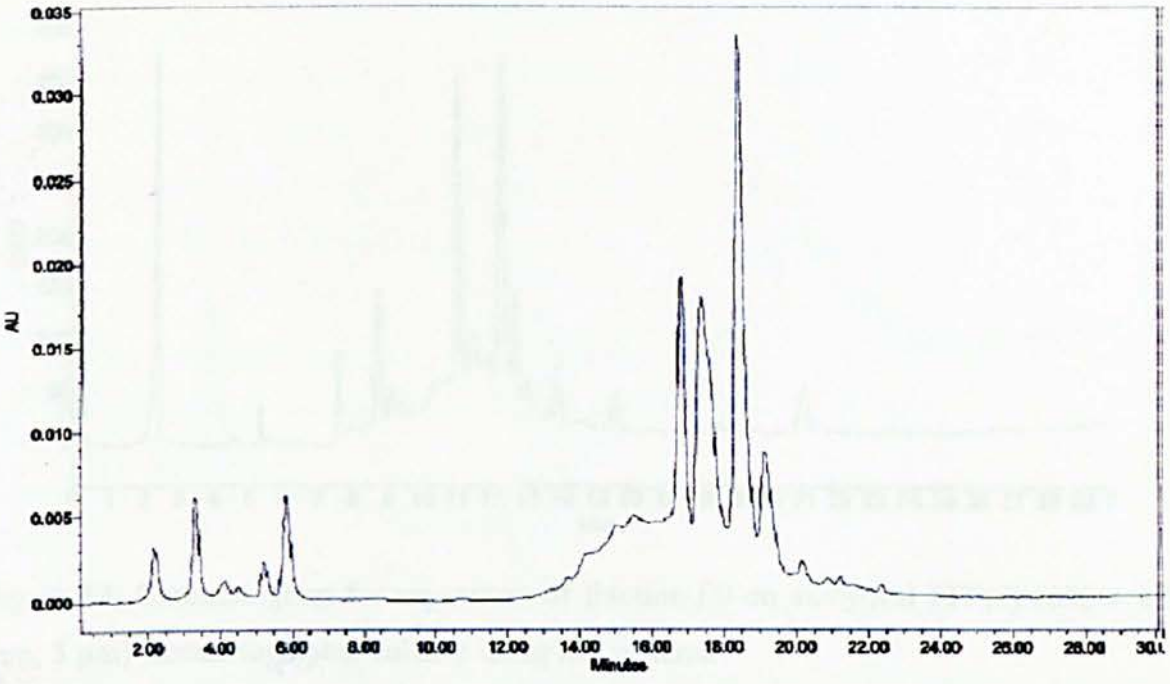


Figure 51. Chromatogram for separation of fraction of SPE tube passed F9 on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using M2 method at 238 nm wavelength.

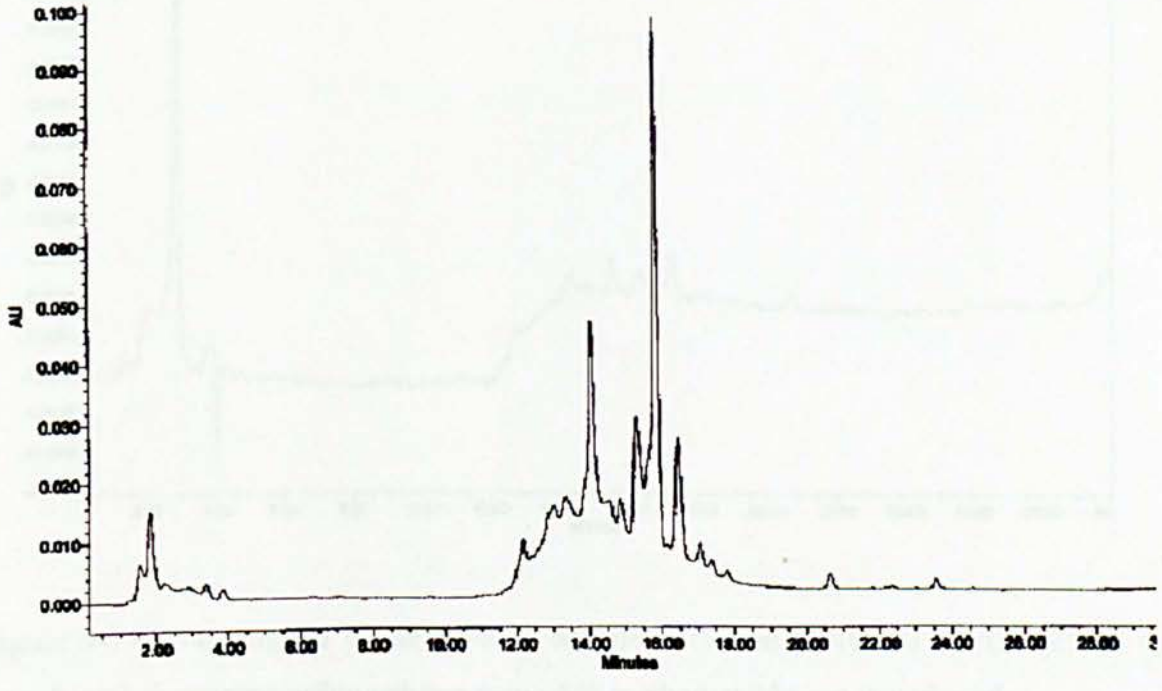


Figure 52. Chromatogram for separation of fraction of F9 on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using M5 method at 238 nm wavelength.

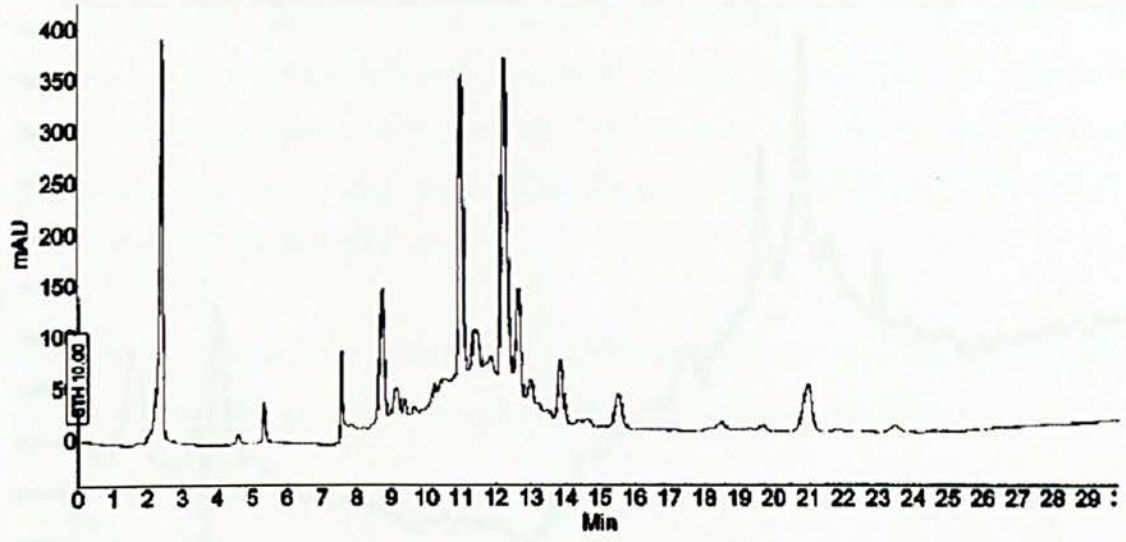


Figure 53. Chromatogram for separation of fraction F9 on analytical PFP(2) (250 x 4.60 mm, 5 μm) chromatographic column using M6 method.

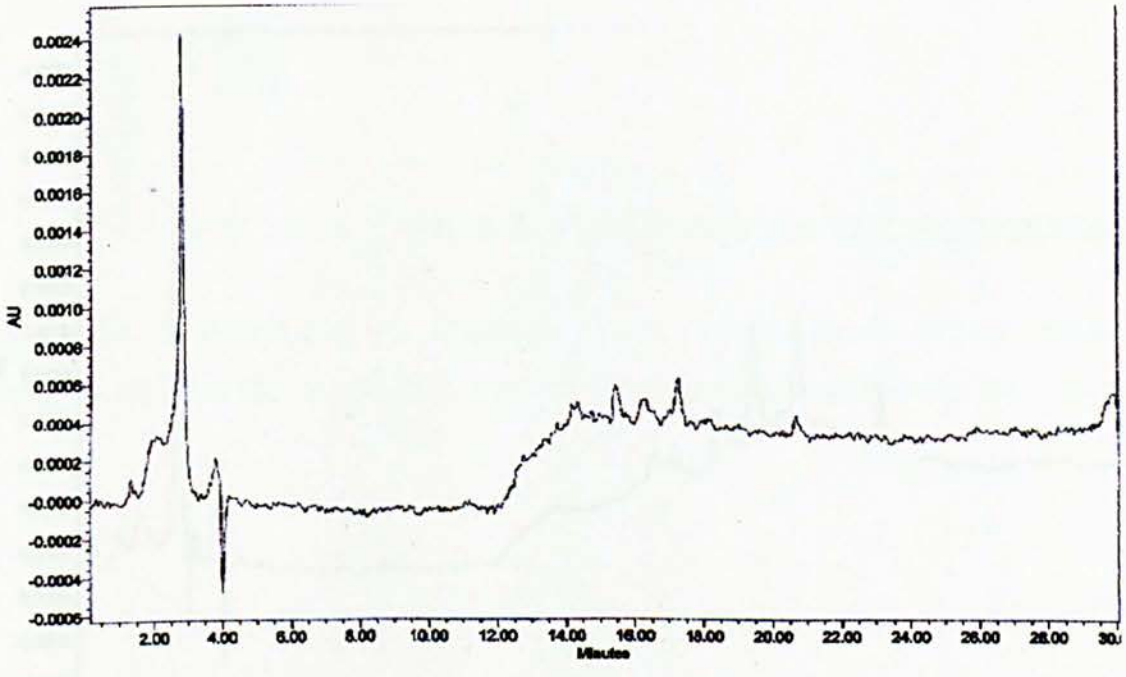


Figure 54. Chromatogram for ammonium acetate buffer on analytical PFP(2) (250 x 4.60 mm, 5 μm) chromatographic column using M5 method at 226 nm wavelength.

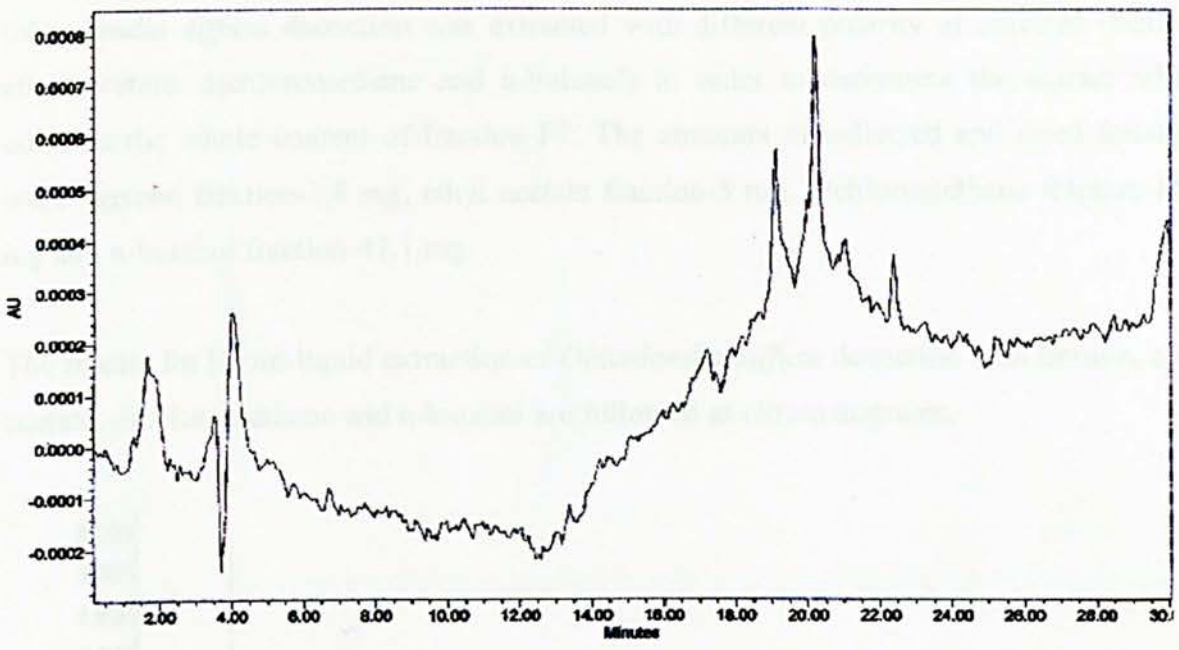


Figure 55. Typical chromatogram for solvent blank on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column M4 method at 226 nm wavelength.

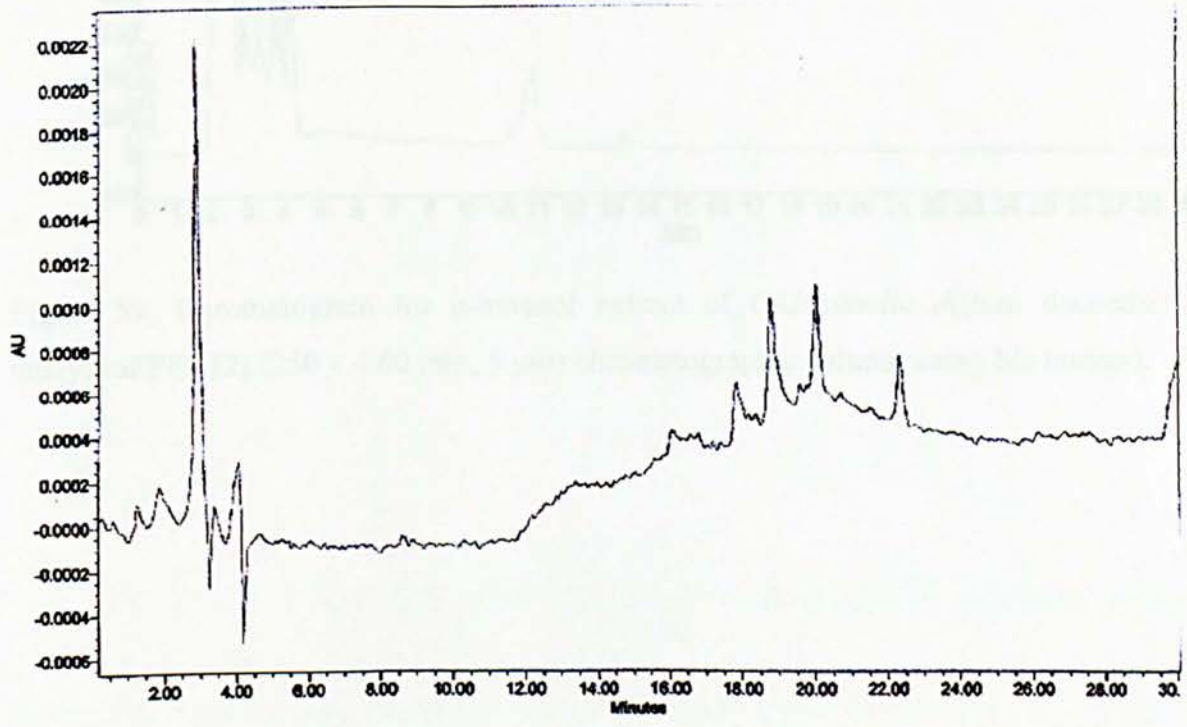


Figure 56. Typical chromatogram for water flush after fraction F9 passed through the SPE on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic columnusing M6 method.

6.3.3. Optimising the method to achieve greater amount of fraction F9.

Oldenlandia diffusa decoction was extracted with different polarity of solvents (hexane, ethyl acetate, dichloromethane and n-butanol) in order to determine the extract which contains the whole content of fraction F9. The amounts of collected and dried fractions were: hexane fraction-1.8 mg, ethyl acetate fraction-5 mg, dichloromethane fraction-15.7 mg and n-butanol fraction-47.1 mg.

The results for liquid-liquid extraction of *Oldenlandia diffusa* decoction with hexane, ethyl acetate, dichloromethane and n-butanol are followed as chromatograms.

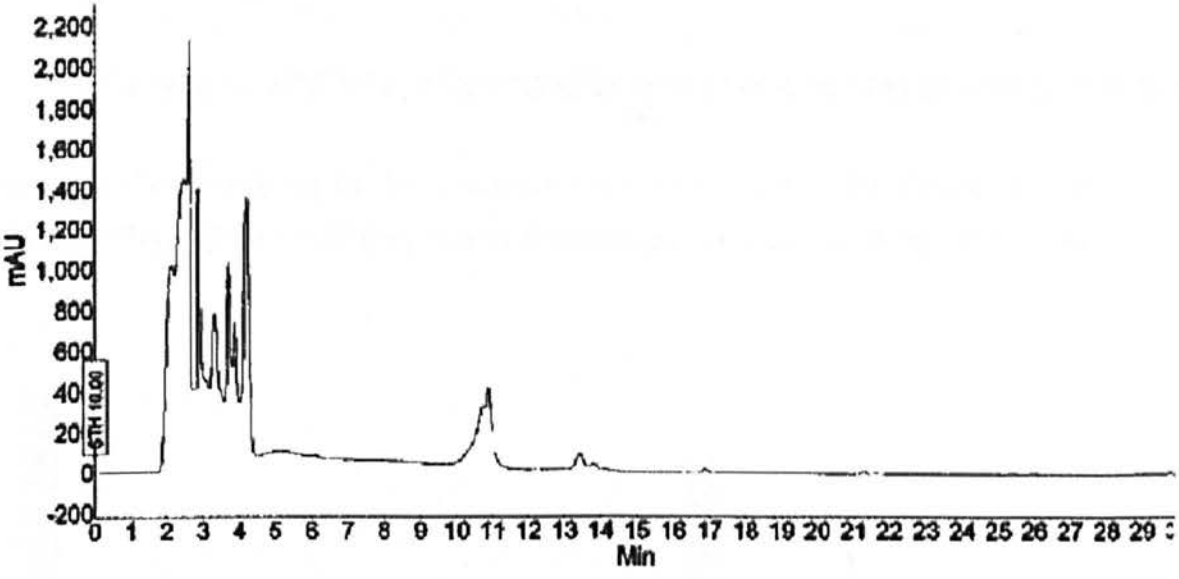


Figure 57. Chromatogram for n-butanol extract of *Oldenlandia diffusa* decoction on analytical PFP (2) (250 x 4.60 mm, 5 µm) chromatographic column using Ms method.

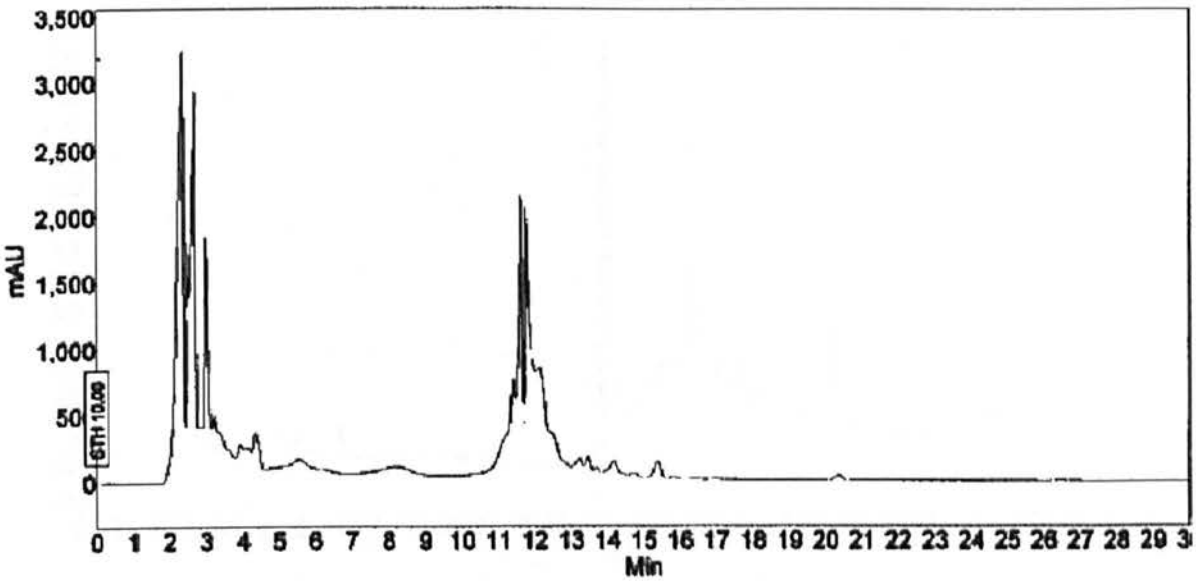


Figure 58. Chromatogram for ethyl acetate extract of *Oldenlandia diffusa* decoction on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using Ms method.

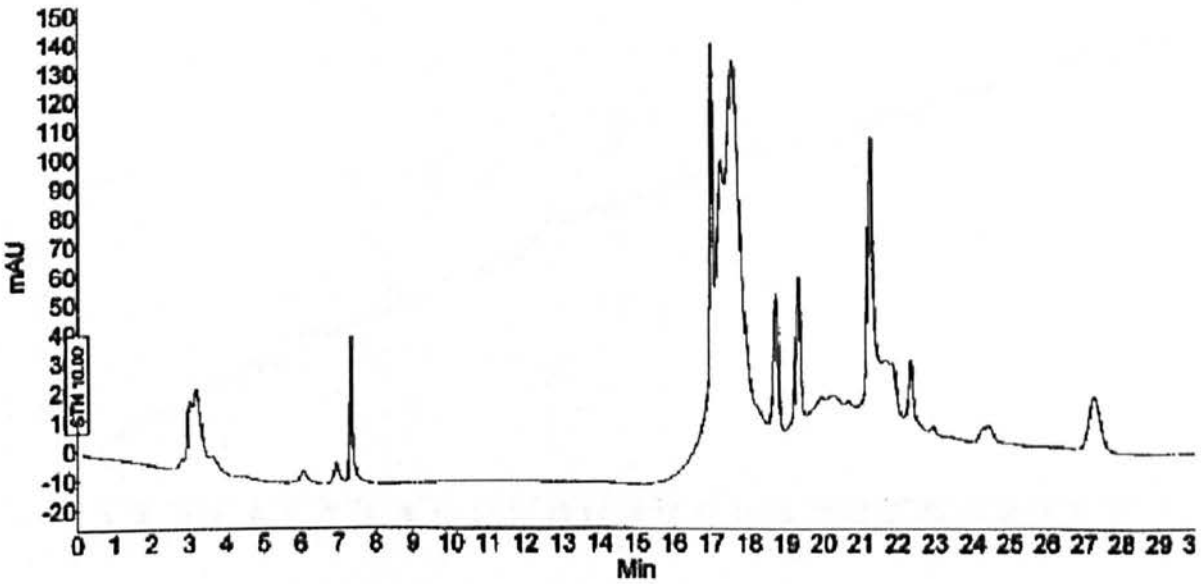


Figure 59. Chromatogram for dichloromethane extract of *Oldenlandia diffusa* decoction on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using Ms method.

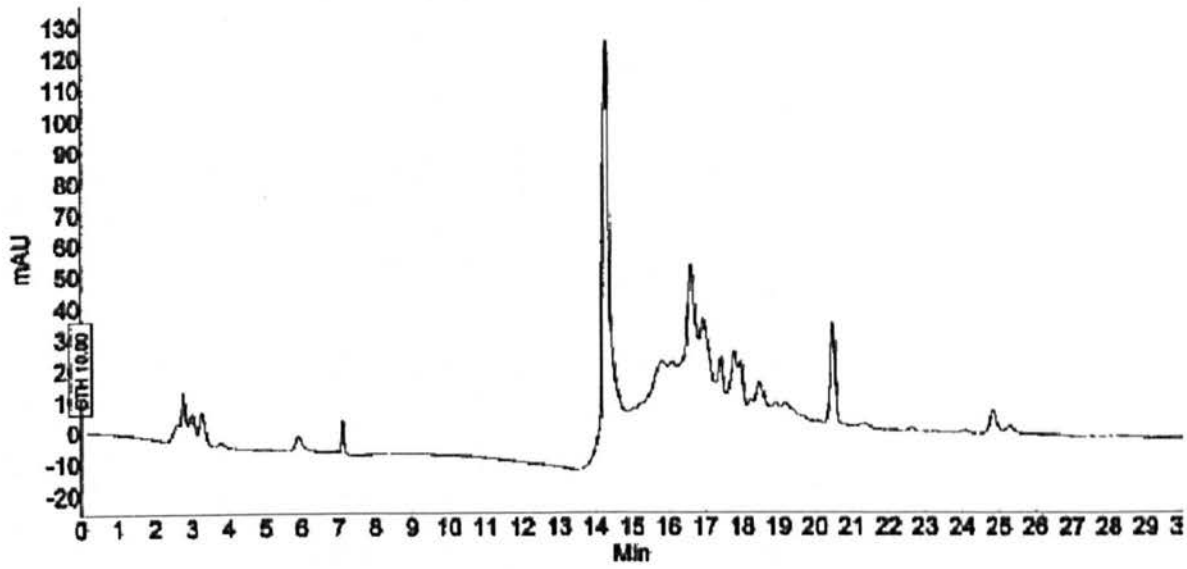


Figure 60. Chromatogram for hexane extract of *Oldenlandia diffusa* decoction on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using Ms method.

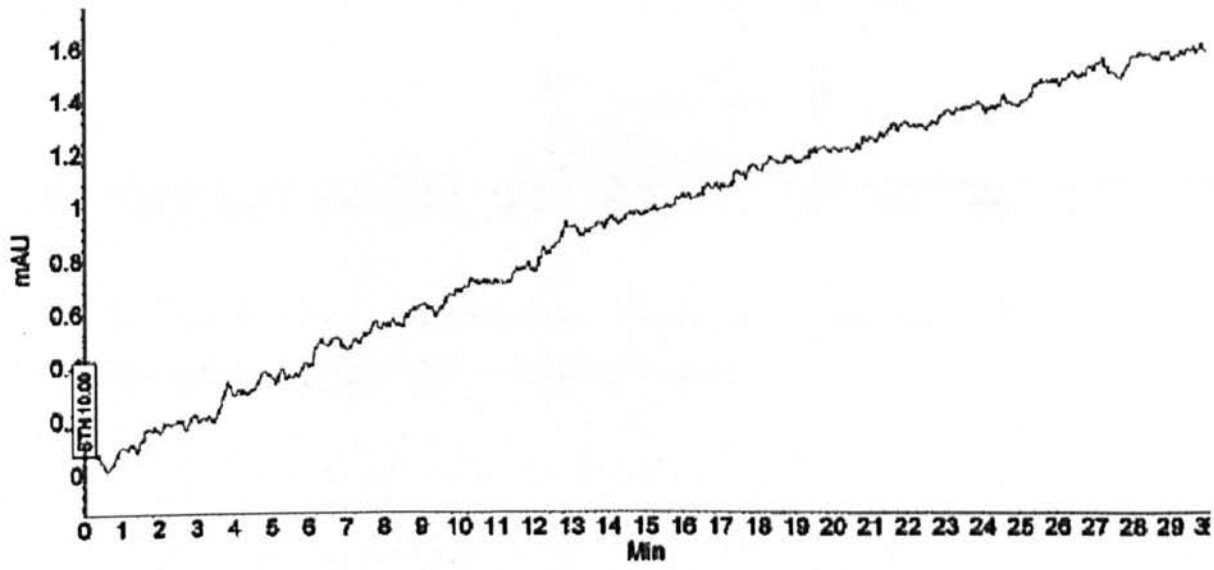


Figure 61. Chromatogram for solvent blank for above extractions on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using Ms method.

Results obtained for the fraction F9 and ethyl acetate fraction by optimized method (M7) on LC-MS are followed.

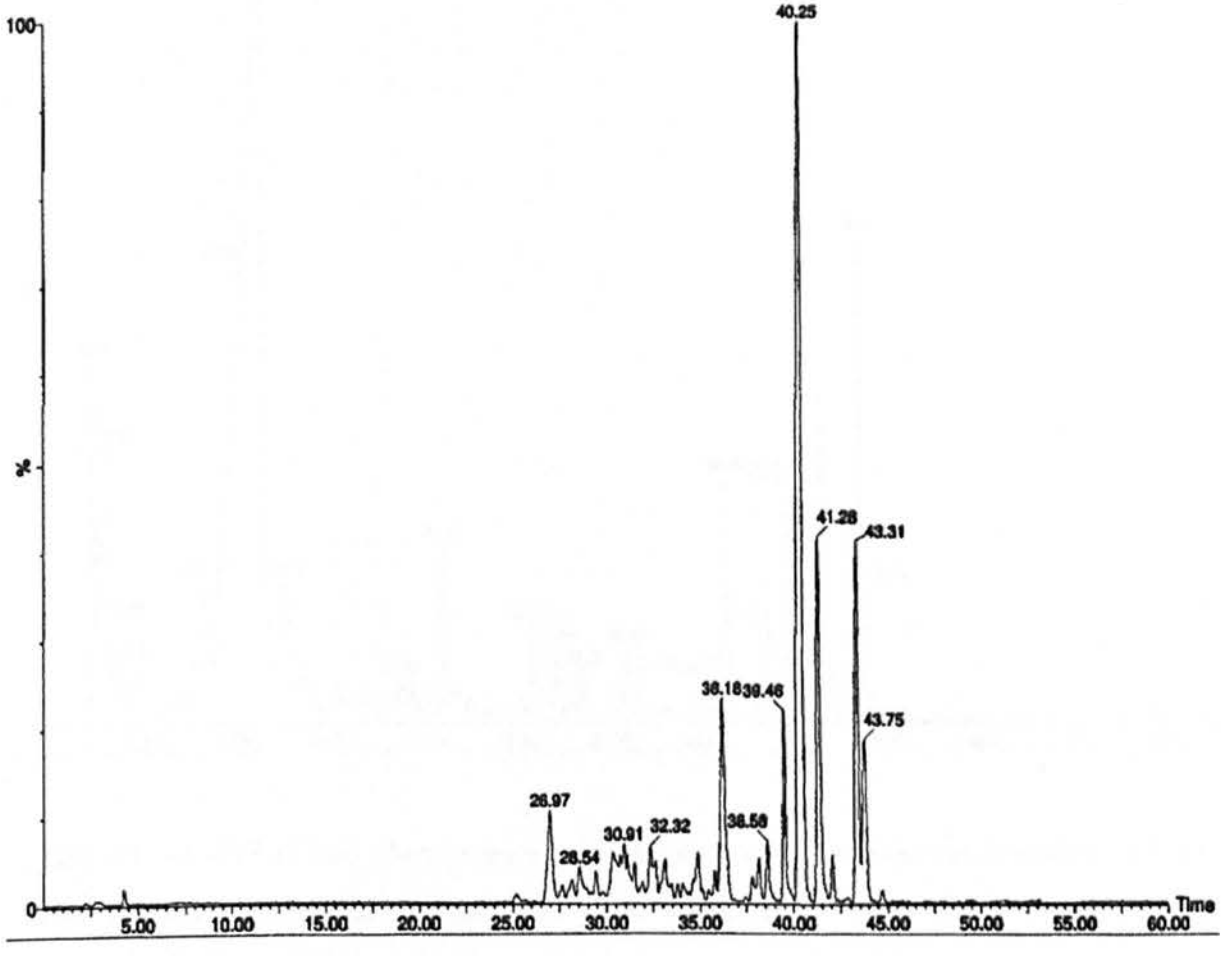


Figure 62. LC-MS (ESI-) chromatogram for fraction F9 on analytical PFP(2) (250 x 4.60 mm, 5 μm) chromatographic column using M7 method.

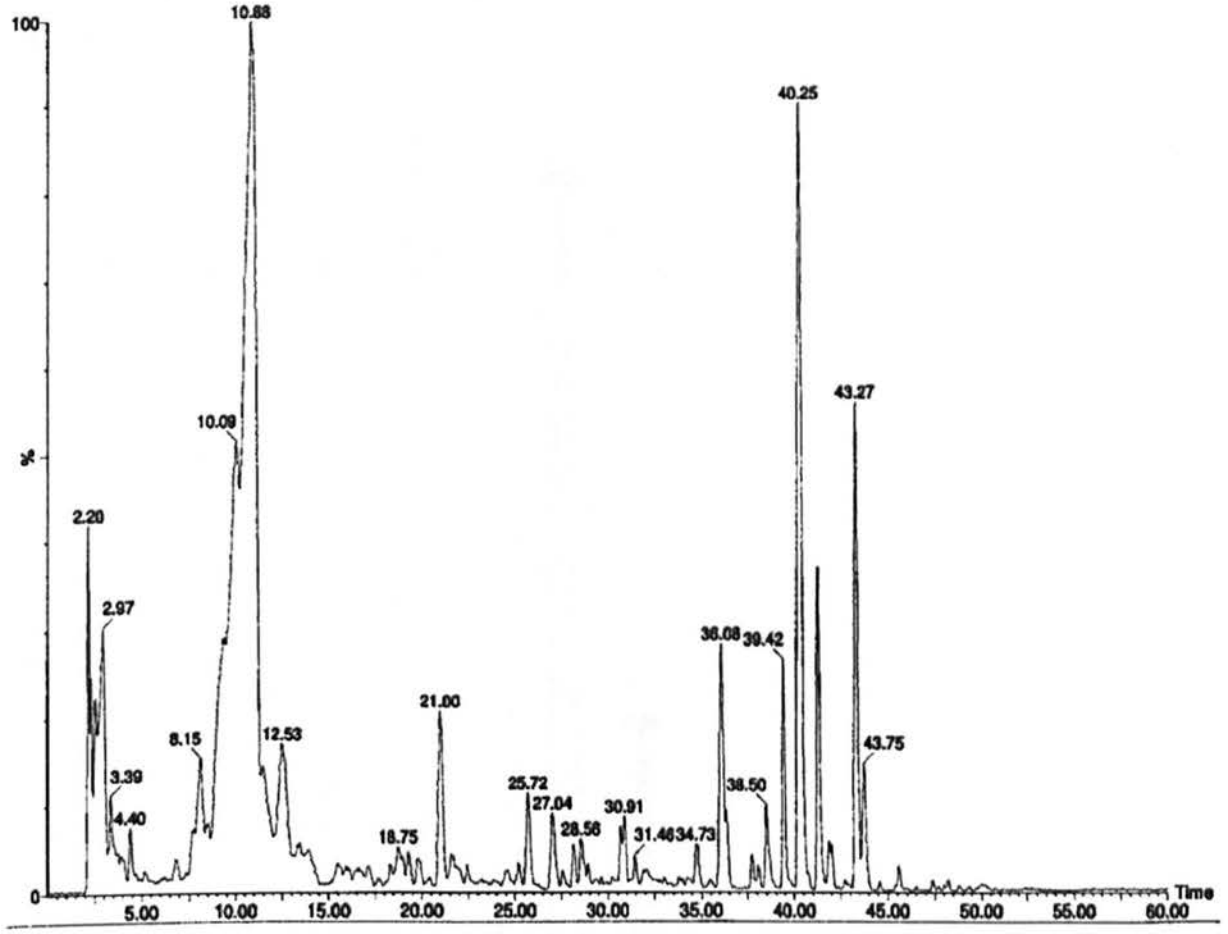


Figure 63. LC-MS (ESI-) chromatogram for ethyl acetate fraction on analytical PFP(2) (250 x 4.60 mm, 5 μm) chromatographic column using M7 method.

Comparison of LC-MS chromatograms for fraction F9 and same part of ethyl acetate fraction:

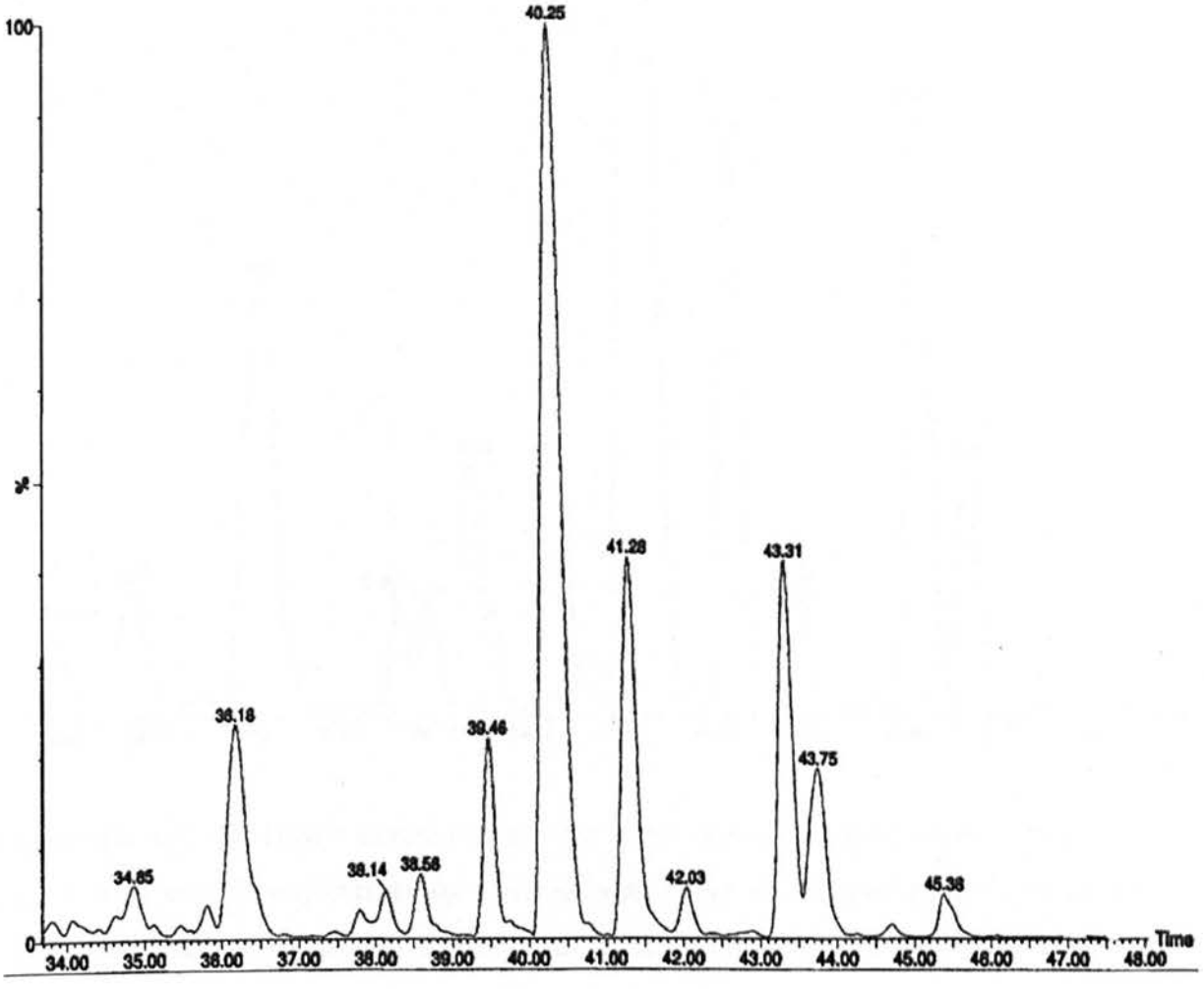


Figure 64. LC-MS (ESI-) chromatogram for fraction F9 on analytical PFP(2) (250 x 4.60 mm, 5 μm) chromatographic column using M7 method.

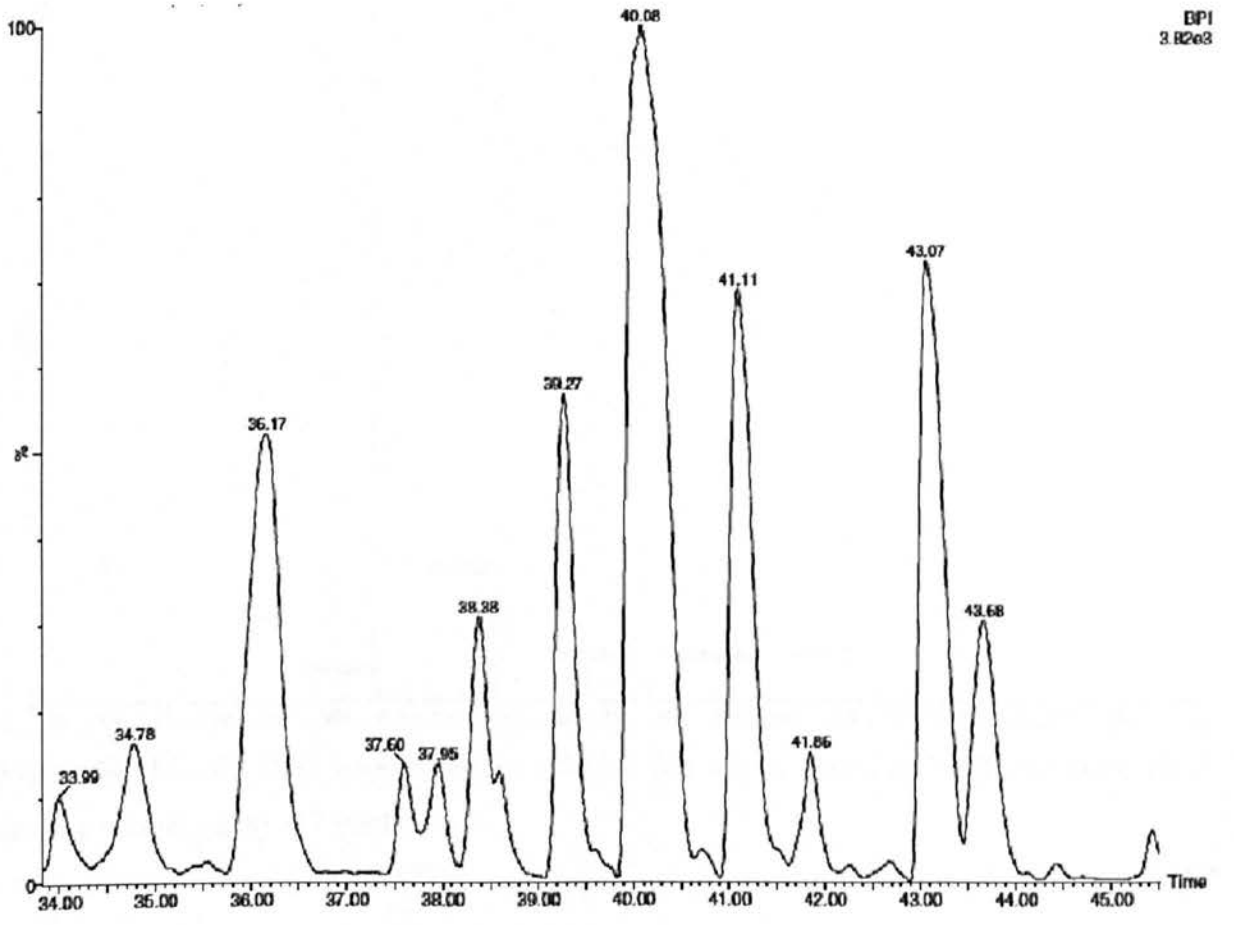


Figure 65. LC-MS (ESI-) chromatogram for ethyl acetate fraction on analytical PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using M7 method (the Figure of 3.82e3 refer to the TIC. Value>10^2 indicated a significant amount of ionisation).

6.3.4. Separated compounds of fraction F9 in ethyl acetate extract

The separated ions that related to fraction F9 and ethyl acetate extract were: m/z 267 at 34.78 min, m/z 237 at 36.03, m/z 241 at 38.52 min, m/z 279 at 39.40 min, m/z 255 at 40.29 min, m/z 281 at 41.25 min, m/z 283 at 43.27 min and m/z 455 at 43.75 min and are shown in the following Figures 66-73.

In addition, apart from fraction F9, there were some interesting compounds separated in the ethyl acetate fraction, they were m/z 549 at 10.92 min, m/z 327 at 7.98 min and m/z 329 at 12.28 min.

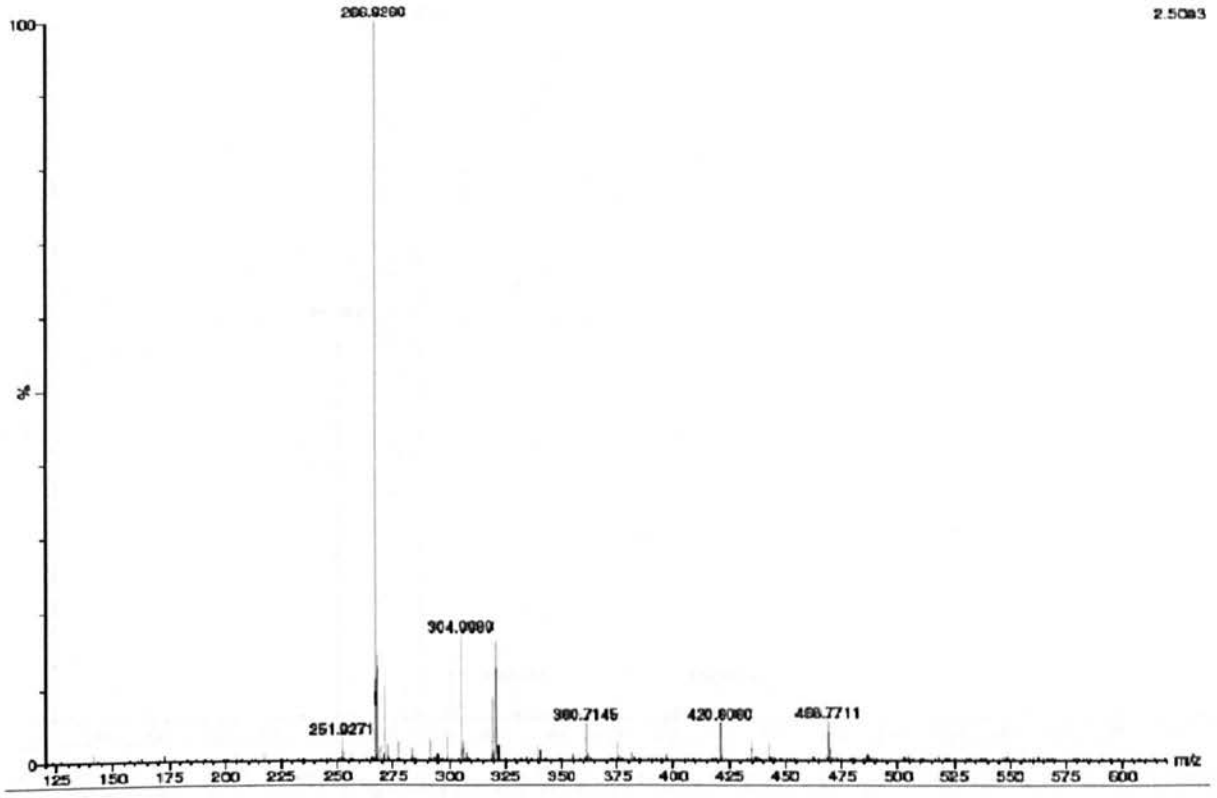


Figure 66. LC-MS (ESI-) spectrum for m/z 267 ion which eluted at 34.78 min from ethyl acetate extract using M7 method.

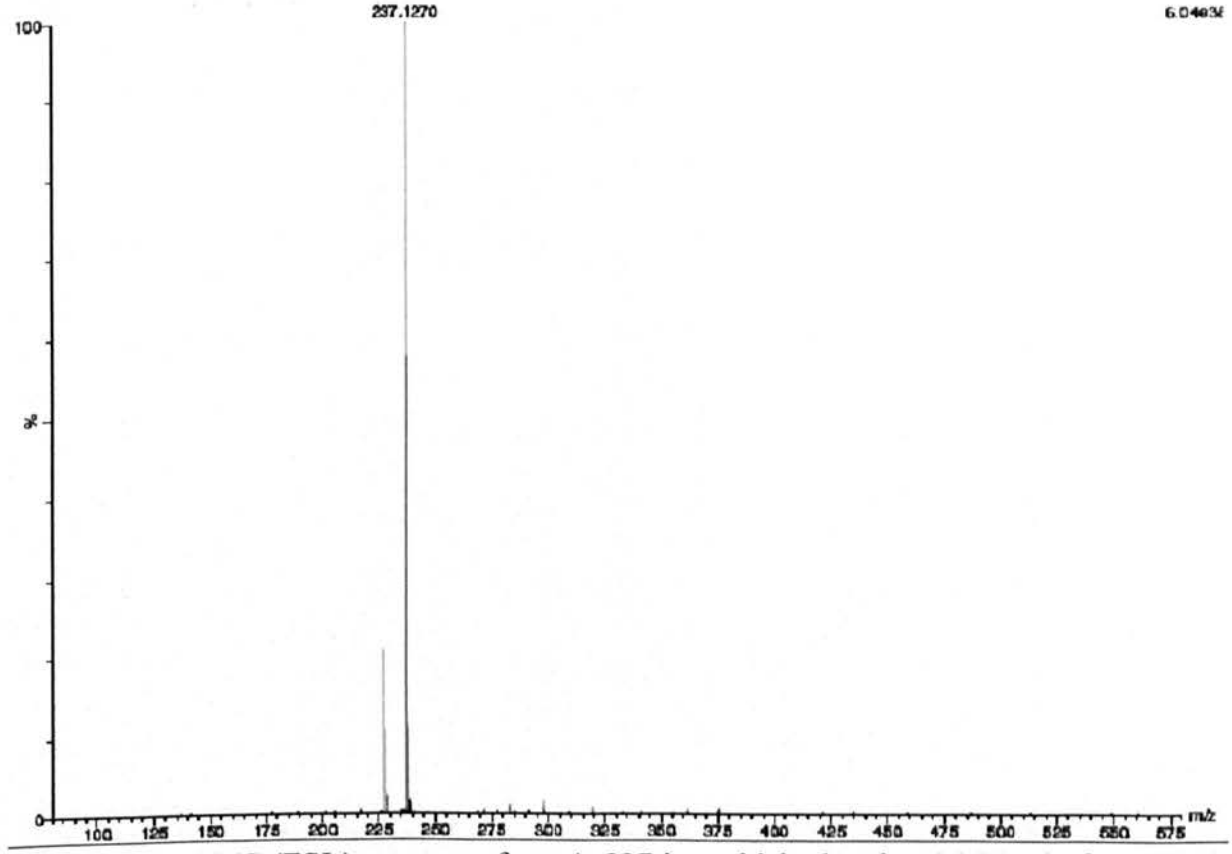


Figure 67. LC-MS (ESI-) spectrum for m/z 237 ion which eluted at 36.05 min from ethyl acetate extract using M7 method.

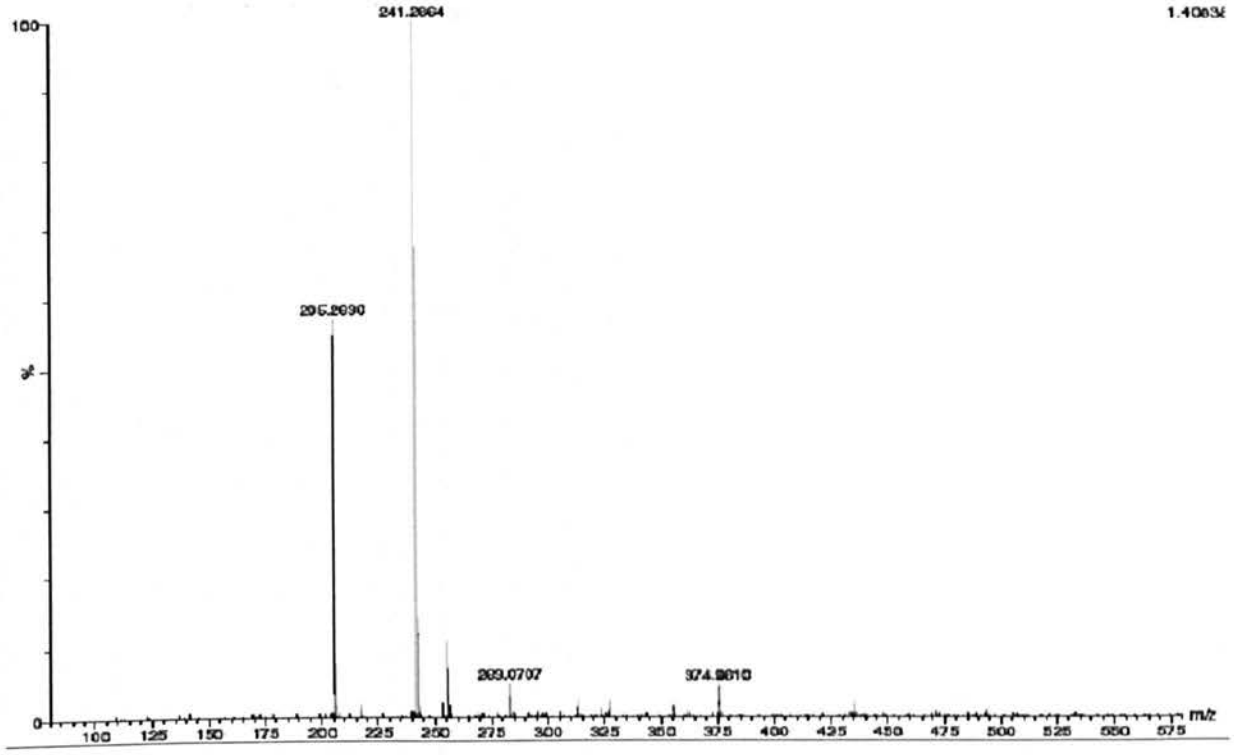


Figure 68. LC-MS (ESI-) spectrum for m/z 241 ion which eluted at 38.52 min from ethyl acetate extract using M7 method.

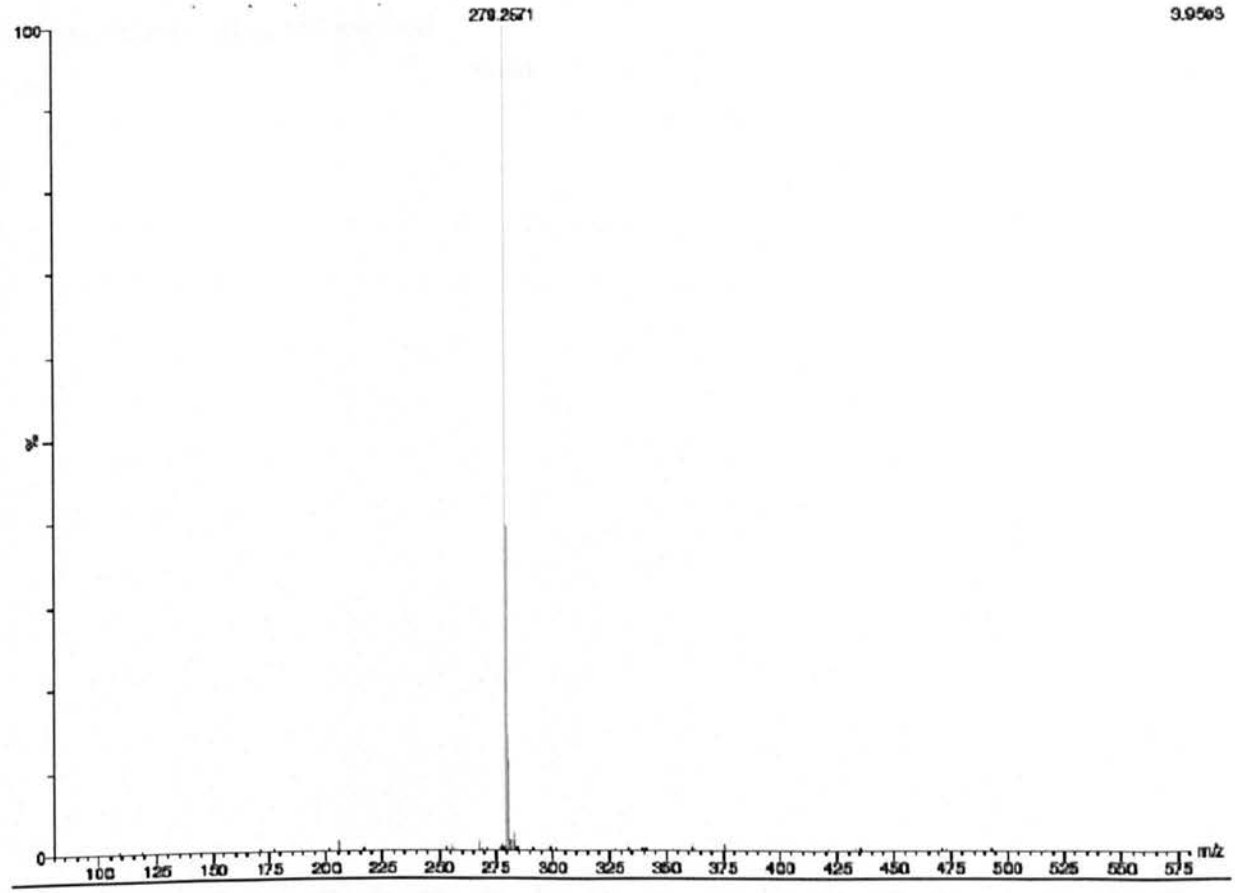


Figure 69. LC-MS (ESI-) spectrum for m/z 279 ion which eluted at 39.40 min from ethyl acetate extract using M7 method.

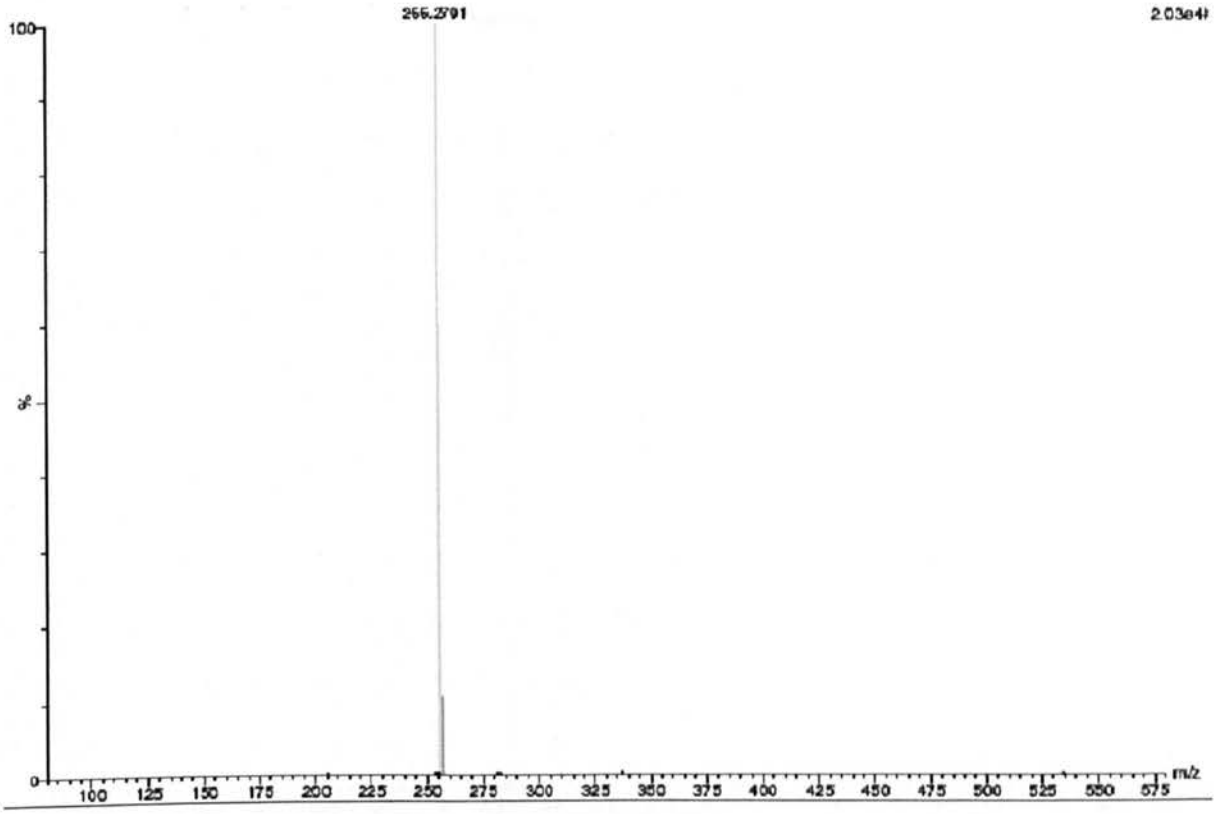


Figure 70. LC-MS (ESI-) spectrum for m/z 255 ion which eluted at 40.29 min from ethyl acetate extract using M7 method.

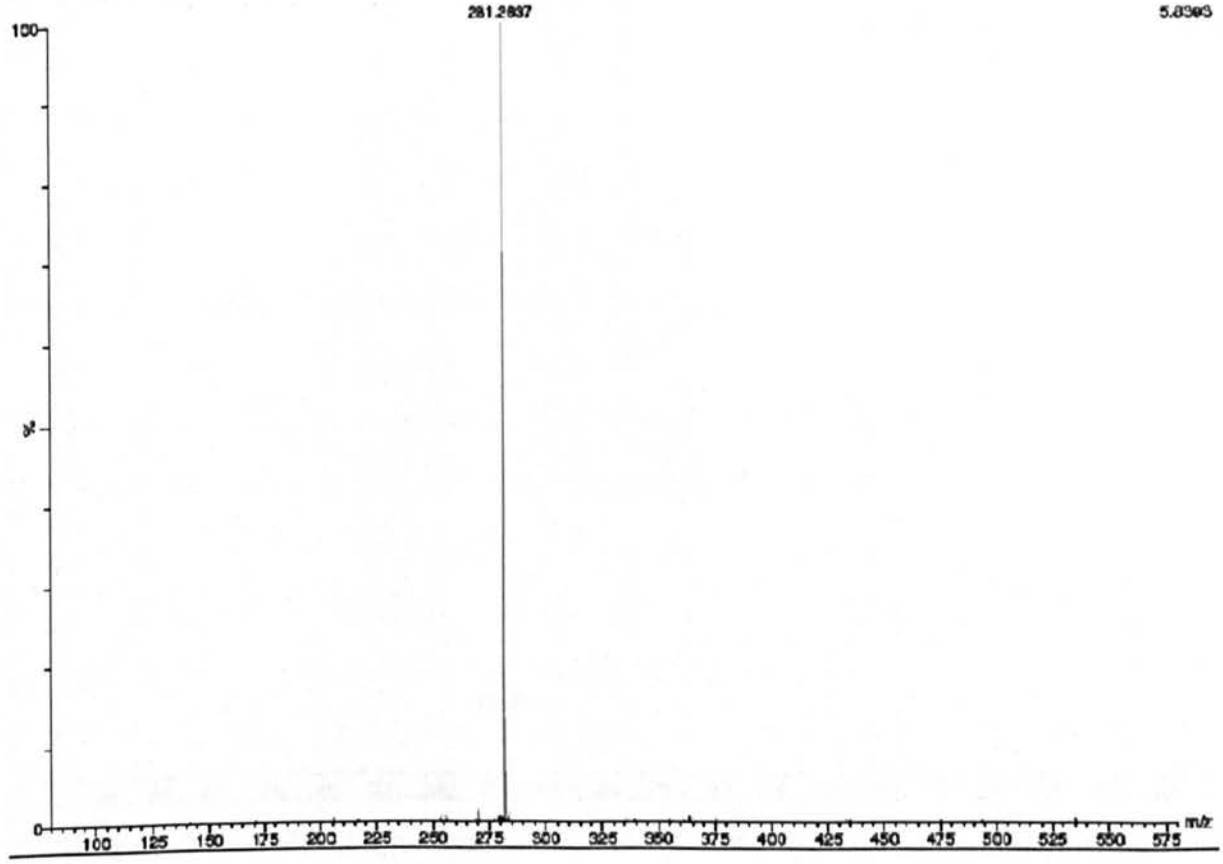


Figure 71. LC-MS (ESI-) spectrum for m/z 281 ion which eluted at 41.25 min from ethyl acetate extract using M7 method.

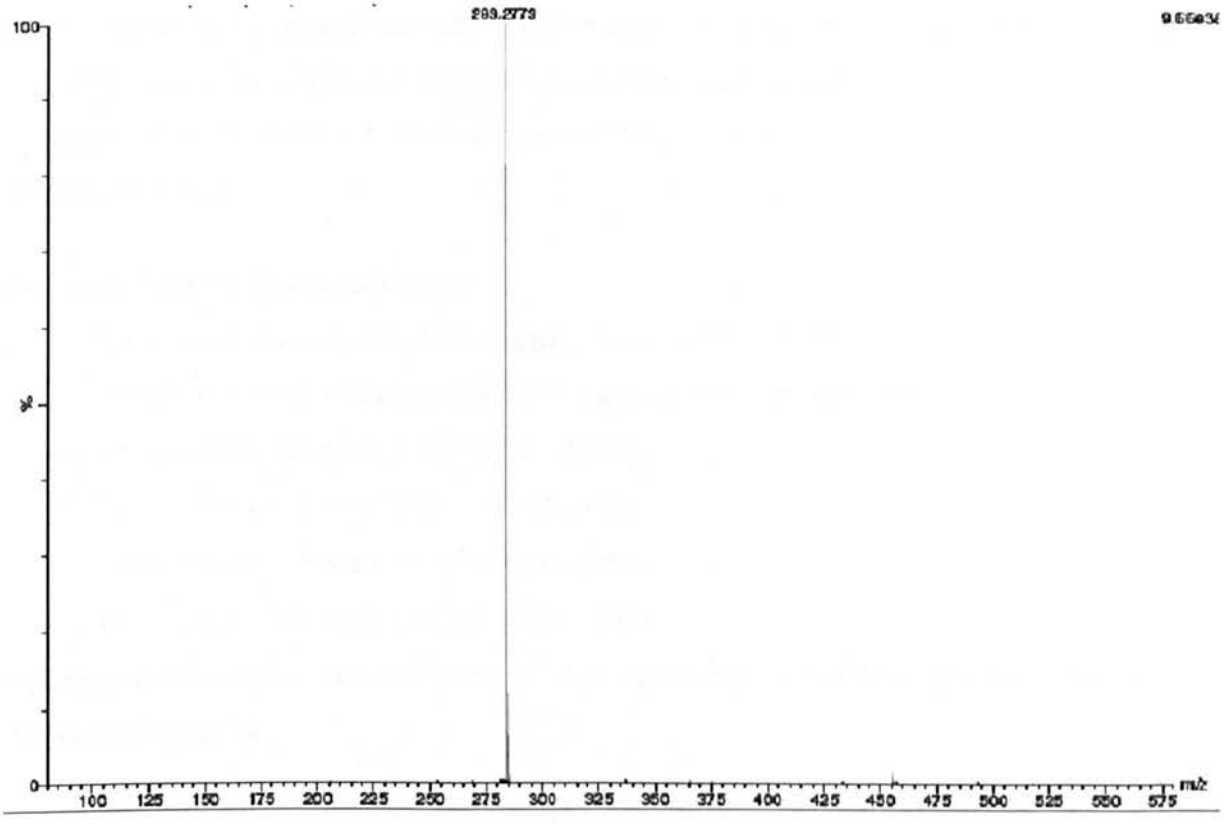


Figure 72. LC-MS (ESI-) spectrum for m/z 283 ion which eluted at 43.27 min from ethyl acetate extract using M7 method.

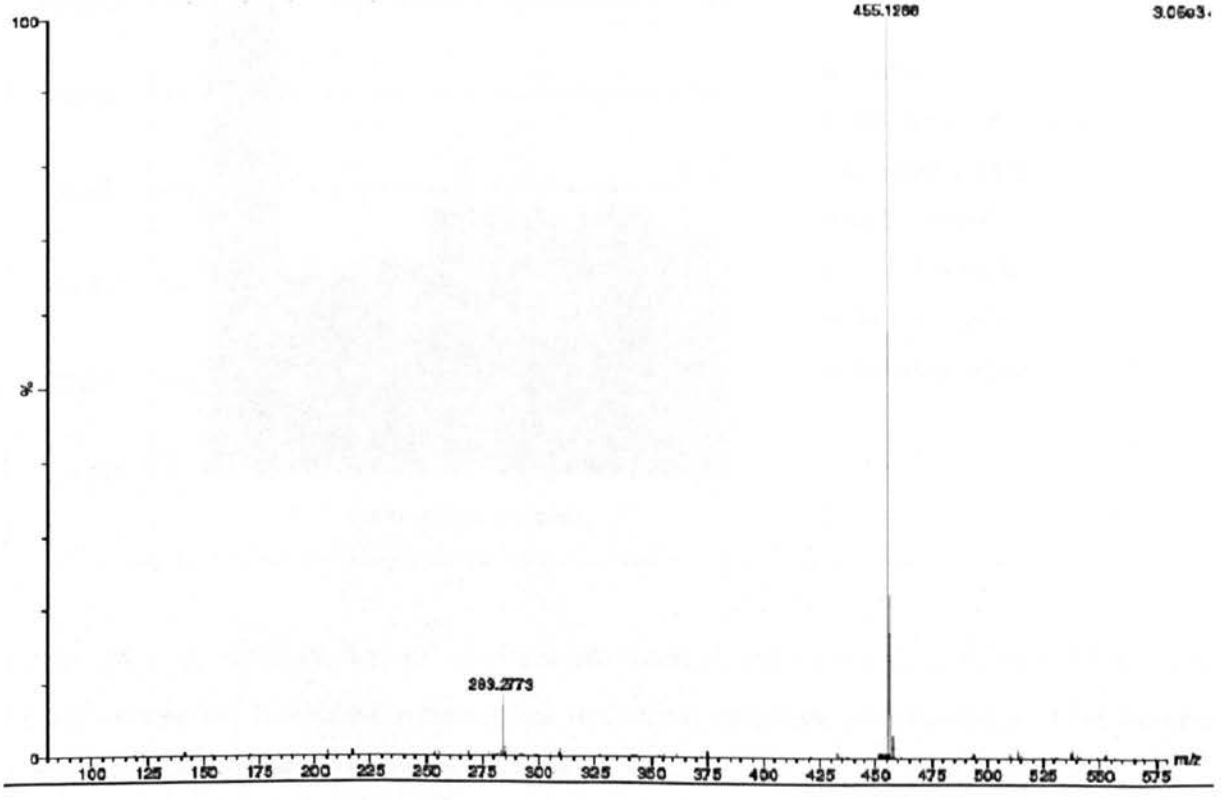


Figure 73. LC-MS (ESI-) spectrum for m/z 455 ion which eluted at 43.75 min from ethyl acetate extract using M7 method.

6.3.5. Cytotoxicity of ethyl acetate fraction and fractions FEA-1, FEA-2 and FEA-3.

The cytotoxic effects of ethyl acetate fraction and fractions FEA-1, FEA-2, FEA-3 were investigated on HL60 and Caco-2 cancer cell lines. Their cell viabilities were determined using NRU assay.

Cell viabilities on HL60 cells were:

- On *Oldenlandia diffusa* (2.5 mg/mL) V=45.071±0.767%,
- Positive control-actinomycin-D (10 µg/mL) V=41.804±3.149%,
- Ethyl acetate fraction V=56.377±9.954%,
- FEA-1 (m/z 549 ion) V=53.344±2.199%,
- FEA-2 (m/z 327 ion) V= 55.374±4.404%,
- FEA-3 (m/z 329 ion) V=50.576±11.281%.

Significant differences are indicated in this assessment (ANOVA, $p<0.05$). Results are shown in Figure 74.

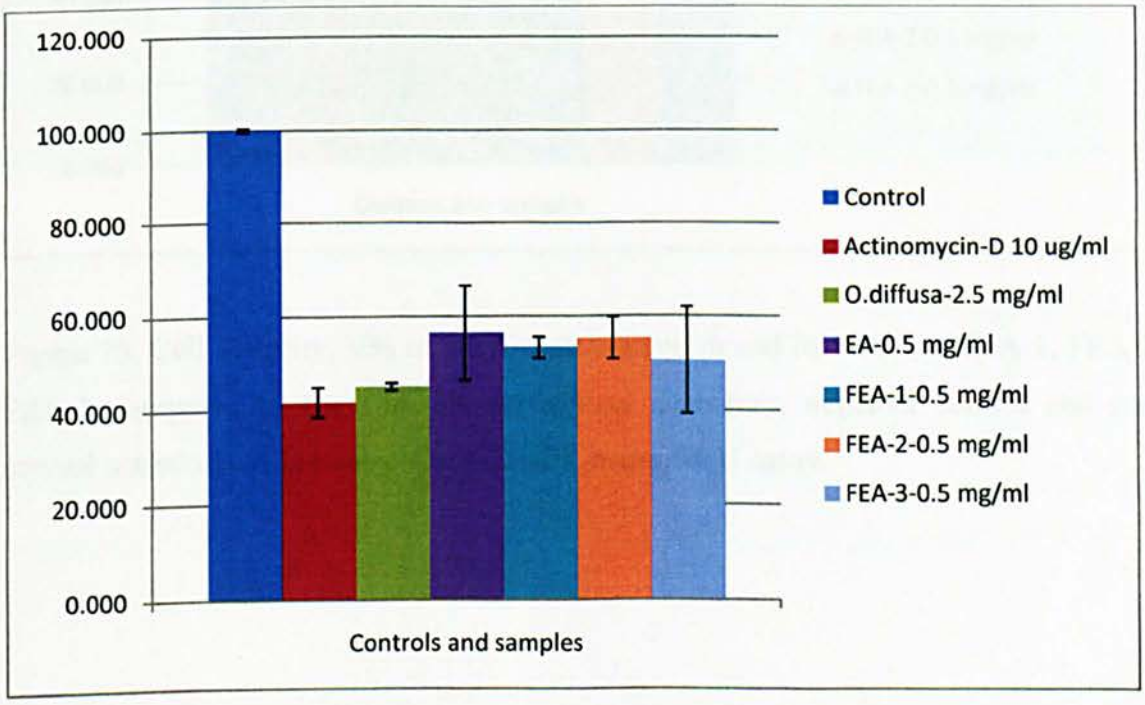


Figure 74. Cell viability, V% of ethyl acetate fraction and its fractions FEA-1, FEA-2 and FEA-3 compaired to *Oldenlandia diffusa* decoction, negative control and positive control actinomycin-D treated HL60 cells, using NRU assay.

Cell viabilities on Caco-2 cells were:

- *Oldenlandia diffusa* (2.5 mg/mL) V=41.755±1.709%,

- Positive control-actinomycin-D (10 µg/mL) V=44.366±5.333%,
- Ethyl acetate fraction (0.5 mg/mL) V=51.239±3.317%,
- FEA-1 V=63.429±13.477%,
- FEA-2 V=45.838±5.920%,
- FEA-3 V=59.452±9.699%

The significant differences are indicated in this assessment (ANOVA, $p<0.05$). Results are shown as a graph in Figure 75.

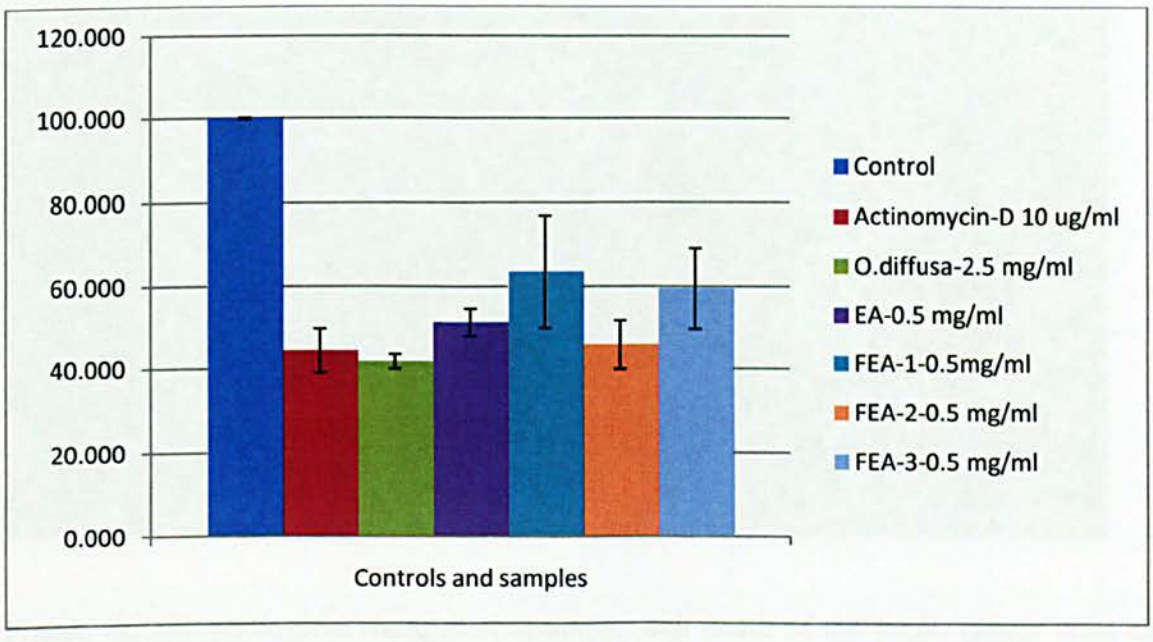


Figure 75. Cell viability, V% of ethyl acetate fraction and its fractions FEA-1, FEA-2 and FEA-3 compared to the *Oldenlandia diffusa* decoction, negative control and positive control actinomycin-D treated Caco-2 cells, using NRU assay.

6.3.6. Characterization of the mode of cell death induced by ethyl acetate fraction, using DAPI staining.

HL60 and Caco-2 cell were treated with ethyl acetate fraction sample (0.5 mg/mL) and control for 48 hours. Results follow:

Results for DAPI staining on HL60 cell line (48h):

(a)- Control-(untreated cells) (b)- Cells treated with ethyl acetate fraction

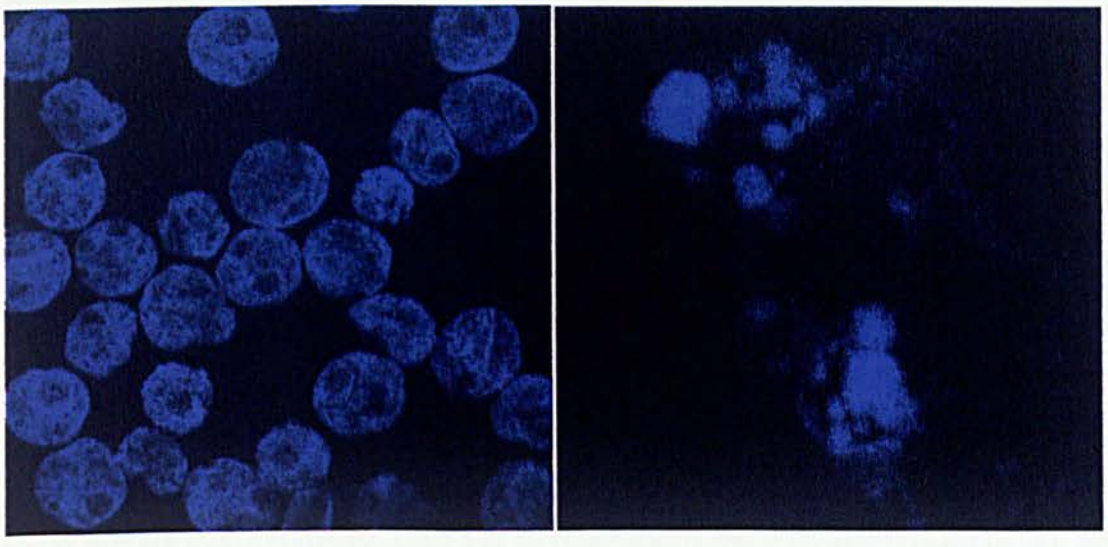


Figure 76. Morphological changes of apoptotic cell death of the HL60 cancer cells treated with ethyl acetate fraction and control (medium) for 48 hr incubation examined by confocal fluorescence microscopy after DAPI staining. Examination on HL60 cells identifying the characteristic morphological features, (a)-Untreated control cells have remained intact, (b)-Ethyl acetate fraction treated cells show characteristics blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (magnification, x400).

Results for DAPI staining on Caco-2 cell line (48h):

(a)-Control-(untreated cells) (b)-Cells treated with ethyl acetate fraction

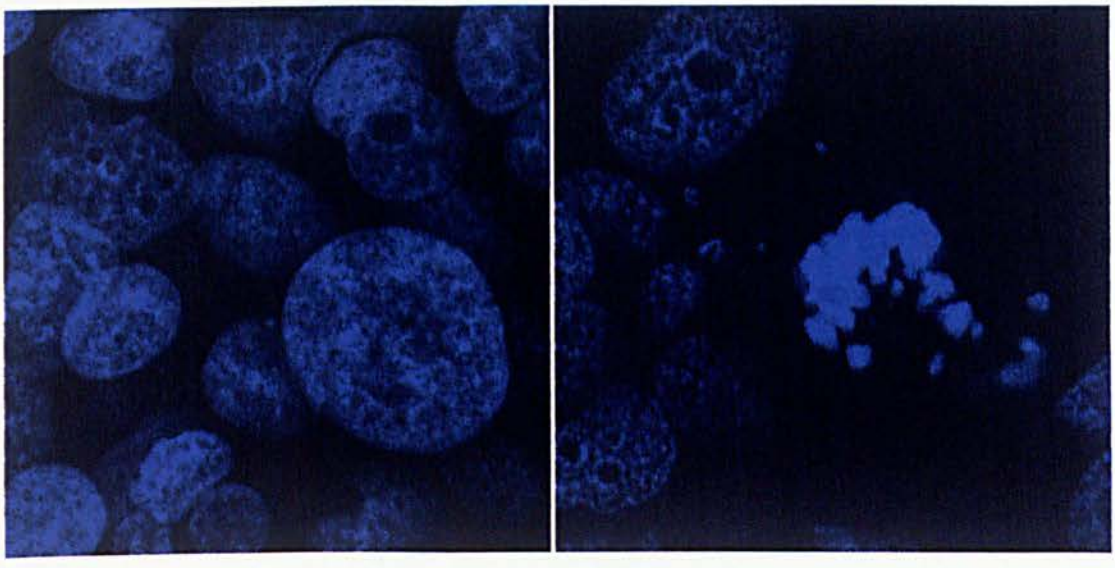


Figure 77. Images of confocal fluorescence microscopy of Caco-2 cells identifying the characteristic morphological features, (a)-Untreated control cells have remained intact, (b)-Ethyl acetate fraction treated cells (48 hr incubation) show characteristics of cell shrinkage, blebbing and apoptotic bodies as evidenced by the presence of condensed chromatin. The cell size is significantly decreased (changed) (magnification, x400).

6.3.7. Characterization of the mode of cell death and apoptosis induced by ethyl acetate fraction, using Western blotting.

The apoptotic cell death investigation of ethyl acetate fraction exposed HL60 cells was performed by Western blotting. On untreated HL60 cancer cells, the band at 116 kDa for PARP and on ethyl acetate fraction treated cells and band at 85 kDa for cleaved-PARP were detected (see Figure 78).

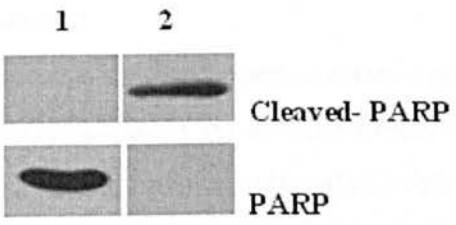


Figure 78. Effect of ethyl acetate fraction on HL60 cell line for the investigation of apoptosis cell death by Western blotting. The HL60 cancer cells were treated with ethyl acetate fraction negative control under culturing condition for 24 hrs. Lane 1-negative control (medium), lane 2-ethyl acetate fraction (0.5 mg/mL). 20 µg/lane proteins were subjected to 10% SDS-PAGE. The expression of cleaved PARP and PARP was detected at 85 kDa and 116 kDa, respectively.

6.4. Discussion

Fraction F9 was collected from 7 times run of *Oldenlandia diffusa* decoction by the created method (Msp method) on PFP(2) preparative chromatographic column within the same region 36.5-41.5 min time intervals (Table 4). Then, all samples were collected and dried on rotary-evaporator under vacuum at 40 °C and freeze-dried. Experiments were done in triplicate and the amount of dry residue was calculated in each time. Average amount was 5.43±0.64 mg which means that from each run of 2 mL of *Oldenlandia diffusa* decoction we can get 0.77 mg of fraction F9. This means that the content of fraction F9 in *Oldenlandia diffusa* decoction is 3.76% w/w.

The initial aim of this part of the study was to separate the compounds in fraction F9. For this reason, some chromatographic columns such as C18, PFP(2) and amino columns examined with different isocratic and gradient elutions of different combinations of buffer, water, methanol, acetonitrile, isopropanol, THF and hexane solvents as a mobile phase.
There were one or two major peaks obtained during isocratic elution. On the gradient elution, separations obtained on PFP(2) (250 x 4.60 mm, 5 µm) column, 80% B for 0-5 min, 100% A at 15 min, 100% A for 15-25 min, 80% B for 26-30 min gradient elution at 1 mL/min flow rate (M1 method) (see Figure 49). Then, the method was optimized by the following analysis by creating M2, M3, M4, M5, M6 methods, and at the end M7 method (see Figures 49-56).

The solvent blank, water flush after SPE extracted fraction F9 and UA analysis were studied with acetonitrile (A)/water (B) mobile phase with optimized M6 method.

The M7 method was optimized on LC-MS from the M6 method changing from water to 10 mM ammonium acetate buffer and acetonitrile to methanol. Gradient elution was optimised to 50% B for 0-10 min, 100% A at 50 min, 50% A 56-60 min. Therefore, the M7 method was set up for the separation and compound fraction collection.

As mentioned earlier, fraction F9 was collected from *Oldenlandia diffusa* decoction using the method which was set up for the separation of the decoction on HPLC, using PFP(2) (Luna 20 cm x 15.1 mm, 5 µm) preparative column, Msp method.

But the scale was too small and it was necessary to find a way to obtain more and more fraction F9 sample for further studies. To obtain the fraction F9 sample by such chromatographic method was expensive and time consuming. For instance, fraction F9 samples that were collected from 100 runs was used for setting up the separation method and for some other analysis, e.g. some cell biology experiments, and more sample was needed for compound fraction collection for further analysis.

Therefore, a liquid-liquid extraction method was performed for extraction of *Oldenlandia diffusa* decoction with different polarity of solvents, namely hexane, ethyl acetate dichloromethane and n-butanol in order to obtain the contents of fraction F9. After three to four times of extractions, collected extracts were dried on the rotary-evaporator under vacuum at 40 °C. Obtained amounts were for hexane fraction-1.8 mg, ethyl acetate fraction-5 mg, dichloromethane fraction-15.7 mg and n-butanol frcation-47.1 mg. Then, each fraction was dissolved in water/acetonitrile (50:50) and subjected into HPLC on the PFP(2) (250 x 4.60 mm, 5 µm) column using Ms method. Chromatograms are shown in Figures 57-61. Results showed that the chromatographic trace of ethyl acetate fraction was similar to the chromatographic trace of the fraction F9 (Figure 53).

As mentioned above, M7 method was optimized on LC-MS for the fraction F9 separation. Therefore, both fractions F9 and ethyl acetate were examined on LC-MS using the M7 method.

The LC-MS chromatograms are shown in Figures 62-65. They clearly show that the ethyl acetate fraction from *Oldenlandia diffusa* decoction extraction with ethyl acetate solvent can be used to get the fraction F9 contents.

The separated ions in fraction F9 and ethyl acetate extract were the same. In Figures 66-73, the representations of separated ions from ethyl acetate extract are shown. Separated and selected ions for the collection were m/z 267 at 34.78 min, m/z 237 at 36.03, m/z 241 at 38.52 min, m/z 279 at 39.40 min, m/z 255 at 40.29 min, m/z 281 at 41.25 min, m/z 283 at 43.27 min and m/z 455 at 43.27 min are shown in the figures. In addition, three more fractions (1 compound in each) (m/z 549 at 10.92 min, m/z 327 at 7.98 min and m/z 329 at 12.28 min) were chosen. These three fractions (FEA-1 (m/z 549 ion), FEA-2 (m/z 327 ion), FEA-3 (m/z 329 ion)) were investigated on HL60 and Caco-2 cell lines by NRU assay.

Generally, cell viabilities of both HL60 and Caco-2 cell lines were low in all samples, excluding the control (see Figures 74-75).

On the HL60 cell line, the ethyl acetate fraction (0.5 mg/mL) showed the cytotoxic effect ($p<0.05$) with V=56.377±9.954%, as well as the other three compounds as their cytotoxicity were FEA-1 (0.5 mg/mL) V=53.344±2.199%, FEA-2 (0.5 mg/mL) V= 55.374±4.404% and FEA-3 (0.5 mg/mL) V=50.576±11.281% and were close to *Oldenlandia diffusa* (2.5 mg/mL) V=45.071±0.767% and positive control-actinomycin-D (10 µg/mL) V=41.804±3.149%. However, all these three compounds showed cytotoxic effect, FEA-3 showed the most cell inhibition effect, then FEA-1 and FEA-2.

According to results on Caco-2 cells, all ethyl acetate fraction, FEA-2, FEA-3 and FEA-1 (0.5 mg/mL) have cytotoxic effect ($p<0.05$) as their viability percents were V=51.239±3.317%, V=45.838±5.920%, V=59.452±9.699% and V=63.429±13.477%, respectively. Generally, all those three compounds showed cytotoxic effect on Caco-2 cells, but FEA-2 has the most cell growth inhibition effect, then FEA-3 and FEA-1.

Characterization of cell death mode of cells exposed to ethyl acetate fraction was investigated on HL60 and Caco-2 cells using DAPI staining on confocal fluorescence microscopy. According to results, both HL60 and Caco-2 cells exposed to ethyl acetate fraction induced apoptosis as the blebbing and producing apoptotic bodies were evidenced by morphological investigation (see Figures 76-77).

Furthermore, the cytotoxic effects of ethyl acetate fraction treated cells that induced apoptosis were assessed by Western blotting by comparing it to untreated cells. On untreated cells, a band at 116 kDa for PARP was detected, while on the cells which were treated with ethyl acetate fraction, a 85 kDa band was detected for cleaved-PARP. This showed that the cells treated with ethyl acetate fraction were apoptotic (see Figure 78).

6.5. Conclusion

The separation method which was set up for fraction F9 contents is:- PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column, methanol (A)/10 mM ammonium acetate buffer (B) as a mobile phase with 50% B for 0-10 min, 100% A at 50 min, 50% A 56-60 min (M7 method) with flow rate 1 mL/min, at 210 nm wavelength.

For compound fraction collection, the same contents of fraction F9 can be obtained using liquid-liquid extraction method of *Oldenlandia diffusa* decoction with ethyl acetate solvent.

The selected ions for further compound fraction collection were m/z 267 at 34.78 min, m/z 237 at 36.03, m/z 241 at 38.52 min, m/z 279 at 39.40 min, m/z 255 at 40.29 min, m/z 281 at 41.25 min, m/z 283 at 43.27 min and m/z 455 at 43.27 min, and m/z 549 at 10.92 min, m/z 327 at 7.98 min and m/z 329 at 12.28 min.

In addition, collected fractions from ethyl acetate extract such as FEA-1, FEA-2 and FEA-3, and ethyl acetate fraction have a cytotoxic effect that induces apoptosis.

Chapter 7. Purification and determination of content(s) in the most cytotoxic fraction

Chapter 7. Purification and determination of content(s) in the most cytotoxic fraction

7.1. Introduction

The cytotoxicity-guided fractionation of the decoction of *Oldenlandia diffusa* led to the isolation of compounds in the most cytotoxic fraction and they were successfully isolated using the method which was set up for the separation.

The aim of this part of study was to determine the content(s) of the cytotoxic fraction in the decoction of *Oldenlandia diffusa.*

However, from previous other researchers' studies, it was suggested that the extracts of the *Oldenlandia diffusa* has significant cytotoxic effect on range of cancer cells *in vitro* [Sadava *et al.*, 2002], [Kim *et al.*, 1998], [Gupta *et al.*, 2004]. The compound(s) that are responsible for the action are not fully determined.

According to phytochemical studies, *Oldenlandia diffusa* contains some major compounds, including *E*-6-O-p-coumaroyl scandoside methyl ester, asperuloside [Nishihama *et al.*, 1981], [Takagi *et al.*, 1982], *E*-6-O-p-coumaroyl scandoside methyl ester-10-O-methyl ether [Gu & Weng, 2001], oleanolic acid and ursolic acid [Chung *et al.*, 1998].

One of the compounds giving the anti-cancer effects is the pentacyclic triterpenoid ursolic acid (UA) that is found in the methanol extract of *Oldenlandia diffusa.* UA showed a significant inhibition of the proliferation of A549 (human lung), SK-OV-3 (ovary), SK-MEL-2 (skin), XF498 (brain), HCT-15 (colon), SNU-1 (stomach), L1210 (murine leukaemia) and B16-F0 (murine melanoma) tumour cells. The nucleosomal DNA of HL60 cells pretreated with UA was cleaved into several oligomeric fragments due to apoptosis. But the cytotoxic effect of UA on tumour cells decreased in a dose dependent manner [Sung *et al.*, 1998].

In addition, other co-workers have suggested that OA may possess both cancer chemotherapeutic and chemopreventive properties. Results showed that OA can suppress the growth of human epithelial cancer cell line without toxicity to the normal cells [Pui *et al.*, 2009].

Moreover, 6-O-(*E*)-p-coumaroyl scandoside methyl ester that was isolated from 95% ethanol extract of *Oldenlandia diffusa* showed a moderate anti-proliferation effect on PC3 human androgen-independent prostate cancer cells while 10(S)-hydroxypheophytin *a.* showed moderate anti-proliferation effect on PC3 cells but strong anti-proliferation effects on LNCaP human androgen-sensitive prostate cancer cells [Li *et al.*, 2009].

However, no anti-cancer effective compounds in a decoction of *Oldenlandia diffusa* that has been prepared by the conventional method (boiling in water) which people use to drink in their treatment has yet been reported.

7.2. Materials and methods

7.2.1. Materials

HPLC and LC-MS equipments for the separation of fraction F9 were the same as mentioned in Chapters 3 and 6. C18 column (250 mm x 4.6 mm, 5 μm) column was Phenomenex, UK. Chemicals such as methanol, acetonitrile (HPLC grade) were supplied by Fisher Scientific Inc, UK, and ammonium acetate (HPLC grade), ursolic acid and oleanolic acid were purchased from Sigma-Aldrich Inc, Poole, UK. Mass spectrometry device was Thermo Electron Triple Quadrupole TSQ^{TM}, Thermo Scientific, UK.

7.2.2. Methods

7.2.2.1. Sample preparation

Preparation of fraction F9 sample

Dry residue of fraction F9 was dissolved back in methanol or acetonitrile/water 50:50 or 60:40, depending on the performing mobile phase.

Preparation of fraction Oldenlandia diffusa decoction and ammonium acetate buffer

Oldenlandia diffusa decoction and ammonium acetate buffer were prepared as mentioned in Chapter 2.

Preparation of Quercetin solution

Stock solution of 100 ppm quercetin was prepared in 50 mL HPLC grade methanol, and then necessary concentrations of reference solutions were prepared by dilution in methanol.

Preparation of ursolic and oleanolic acids solutions

Purchased OA and UA were used as an internal standard in this study. There were pure UA and OA solutions and UA and OA a mixture solution. These were prepared with 100 ppm concentration in HPLC grade methanol.

7.2.2.2. Subfraction collection from ethyl acetate extract

Eleven selected subfractions (compound-fraction-1 to 8 and ethyl acetate fraction FEA-1 to 3) were collected from ethyl acetate extract using a HPLC, PFP(2) analytical (Luna 250 x 4.6 mm, 5 μm) column and Ms method at the related time intervals.

It was important to set the correct collecting point to get the possible pure subfraction. In order to optimise the best tube length (from column to detector) for subfraction collection, a series of measurement of tubes (25-23 cm long) were made. After many attempts, subfractions were collected from exact midpoint of each peak elution. Eleven subfractions were collected, including m/z 267, 237, 241, 279, 255, 281, 283, 455, and m/z 549, 327 and 329 ions. The purity of each collected compound fraction was examined back on MS by direct injection.

7.2.2.3. Determination of molecular weights by high resolution-MS

The molecular weight of each collected subfraction (ion) was determined using high resolution-MS. Quercetin (with MW 302.24) solution in methanol was used as a reference. 10, 5, 1 and 0.5 ppm concentrations of quercetin solutions were subjected to mass spectrometry in order to determine the appropriate reference concentration. Samples were drawn by autosampler and directed (direct injection) to MS by tube which split to pumping system in order to bring the reference into the flow of sample analyte.

7.2.2.4. OA and UA study in determining fraction F9 contents

Previously prepared UA solution, OA solution and mixture of both OA and UA solutions were subjected to same PFP(2) column using M6 method. Their retention times were compared to the peak which was obtained in fraction F9 peak's retention time.

7.2.2.5. MS-MS assessment of subfractions, OA and UA

Mass spectrometric analyses were performed using MS-MS spectroscopy. Product ion scans were performed for OA, UA and compound-fraction-8 using direct infusion. The optimised MS (ESI-) conditions for the parent ion were: capillary temperature 400 °C, sheath gas pressure 10 mL/min and spray voltage 4500 V. The mass spectrometer was programmed to detect the product ions from the samples in the range of m/z 100-500. All data acquisition and processing were controlled by Xcalibur^{TM} software.

7.2.2.6. Comparison HPLC assessment of compound-fraction-8 and control OA and UA

In order to check content of compound-fraction-8 on HPLC, sample and control OA and UA and their mixture were investigated on C18 column (250 mm x 4.6 mm, 5 μm) with mobile phase methanol-water (83:17 containing 0.2% ammonium acetate, pH=6.7) at a flow-rate of

1.0 mL/min (room temperature). The detection wavelength was set at 210 nm [Liang *et al.*, 2009].

7.2.2.7. Comparison LC-MS analysis of compound-fraction-8

To check the ions of the separated two peaks in compound-fraction-8, the sample and the mixture of OA and UA were investigated on LC-MS (ESI-, at capillary pressure 3255 V, desolvation temperature 250 °C and source temperature 90 °C) using the above (see Section. 7.2.2.6) mentioned method.

7.2.2.8. Comparison NMR studies of fraction FEA-1

Fraction FEA-1 (3 mg) was dissolved in acetone D6 and a drop of DMSO. NMR spectra were recorded with an AV500 (500 MHz for ^{1}H; 100 MHz for ^{13}C) spectrometer.

7.3. Results

7.3.1. Subfraction collection from ethyl acetate extract

After setting of the collecting point of each compound, fractions were carefully collected to obtain possible pure compound from the LC-MS. The optimised tube length was found to be 32 cm long (from column to detector). The purity of each collected subfraction was examined back on MS by direct injection. Results are shown in Figures 79-89.

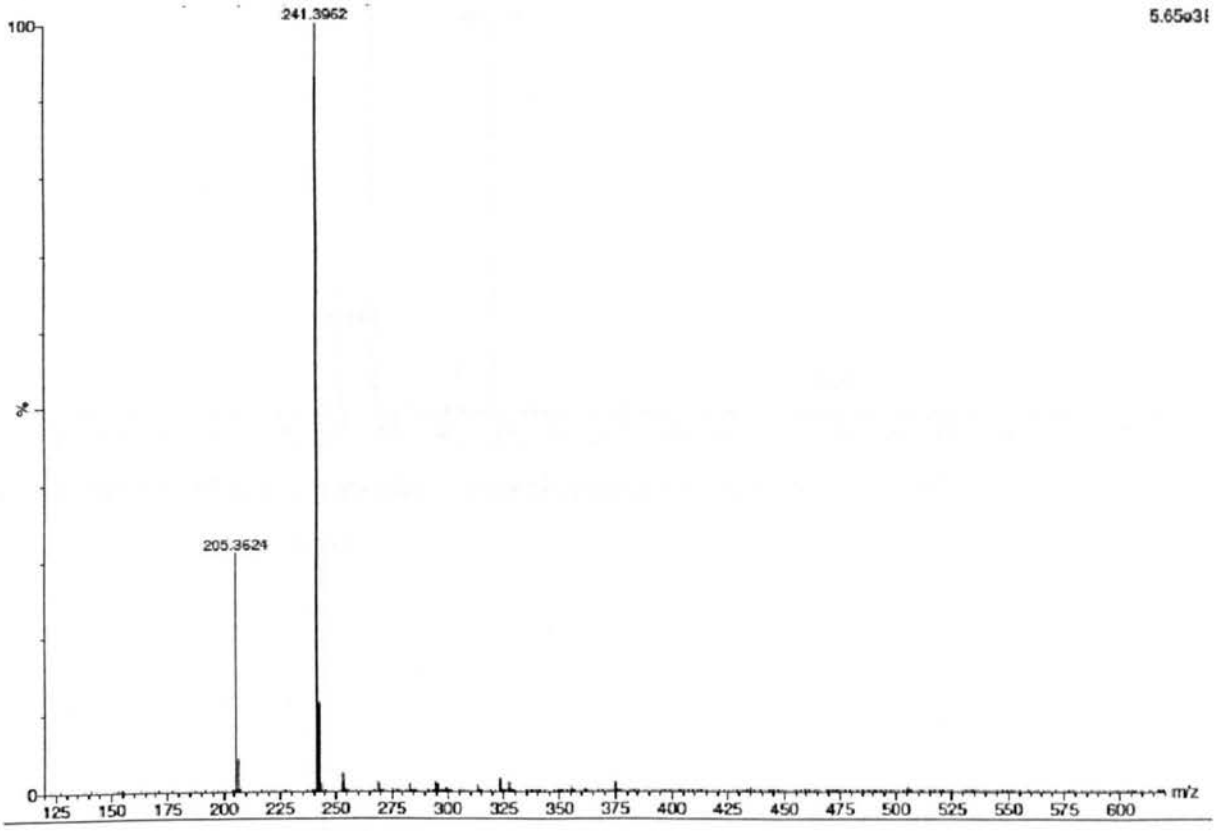


Figure 79. LC-MS (ESI-) spectra collected compound-fraction-1 (m/z 241 ion).

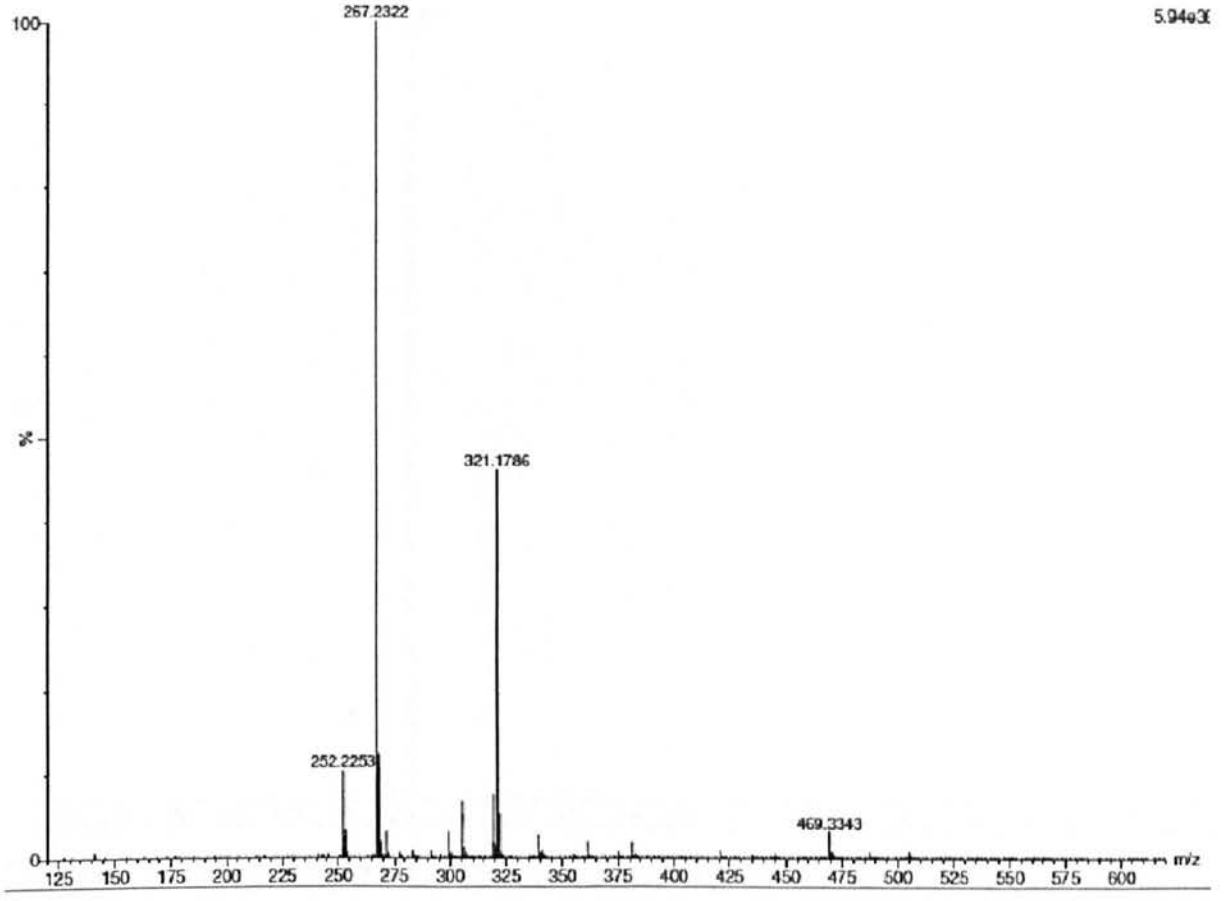


Figure 80. LC-MS (ESI-) spectra collected compound-fraction-2 (m/z 267 ion).

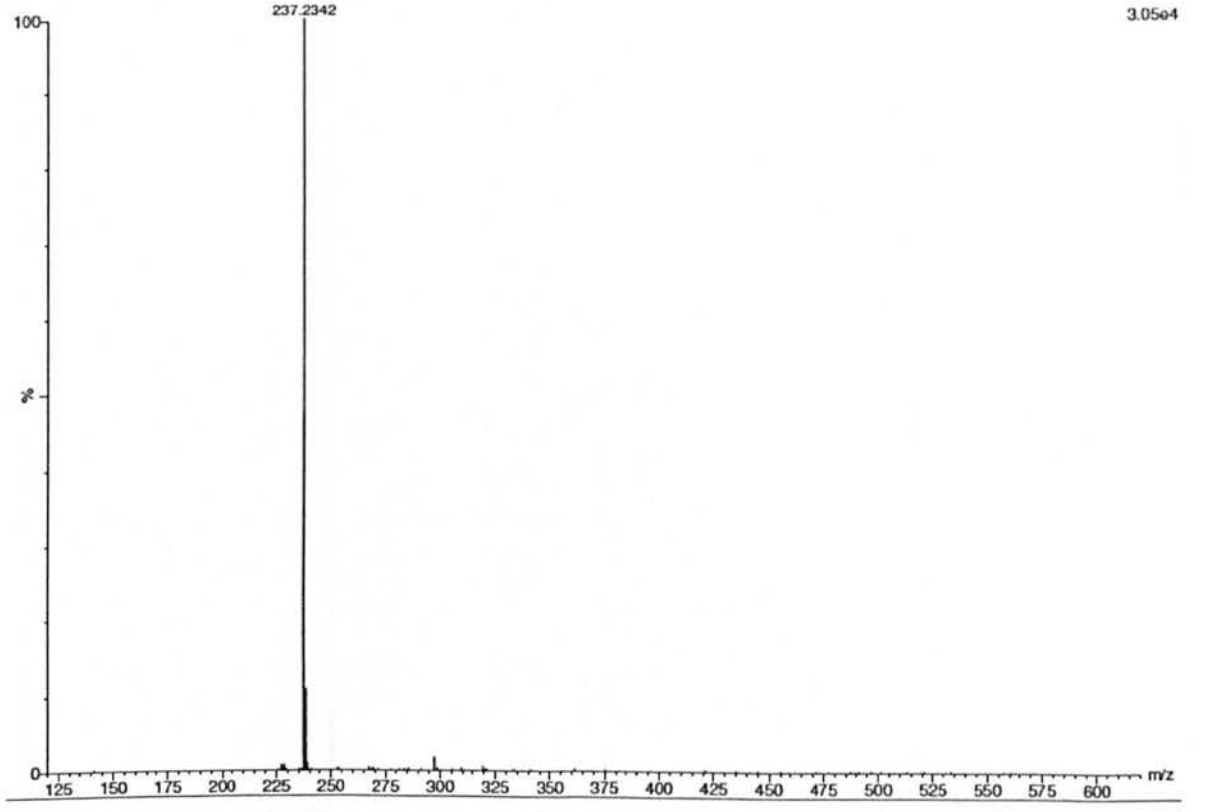


Figure 81. LC-MS (ESI-) spectra collected compound-fraction-3 (m/z 237 ion).

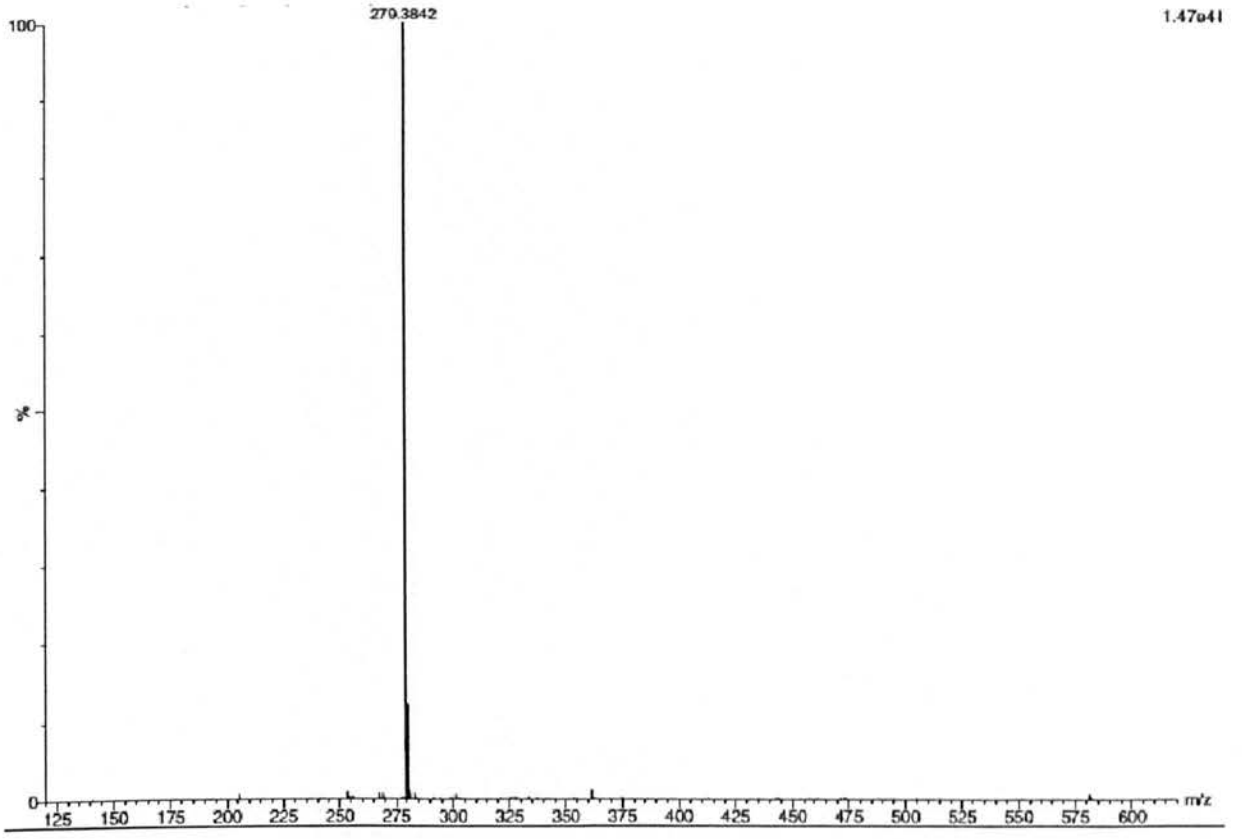


Figure 82. LC-MS (ESI-) spectra collected compound-fraction-4 (m/z 279 ion).

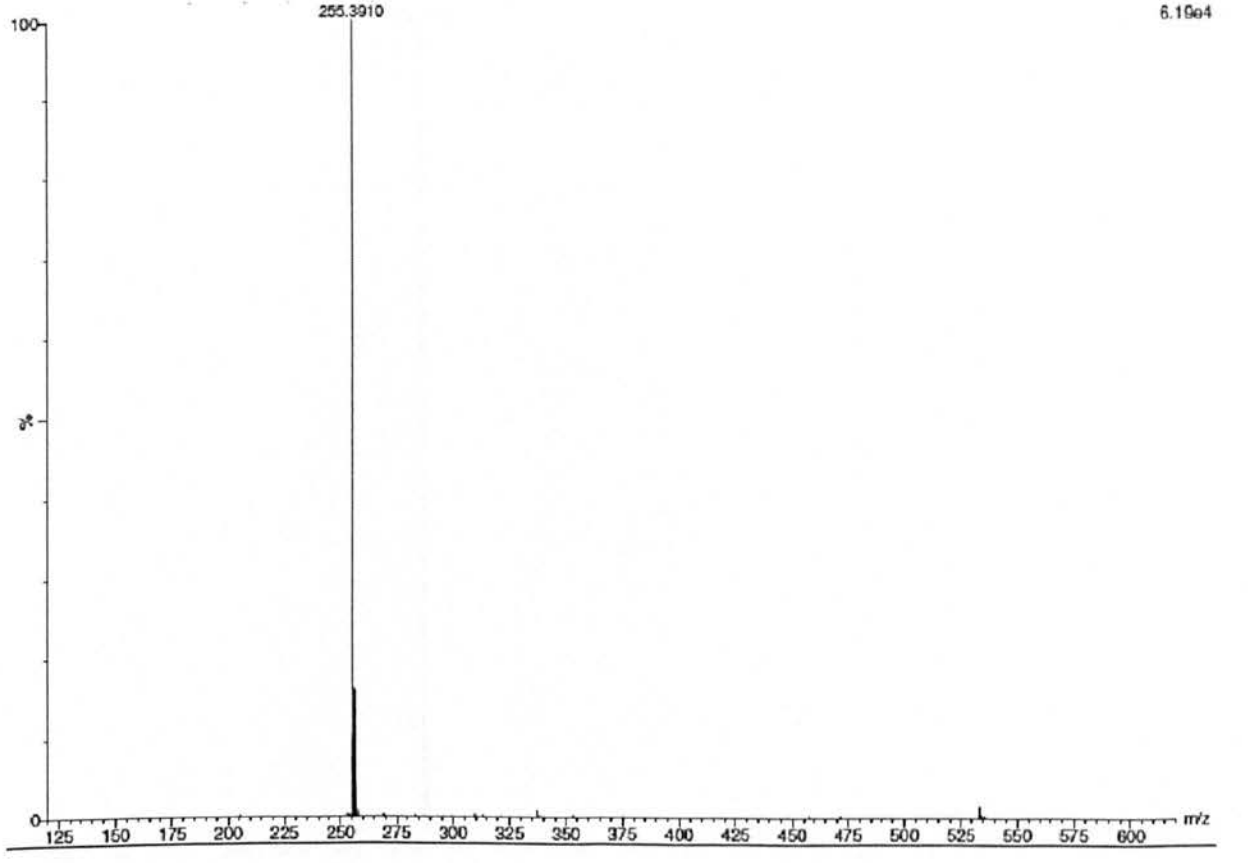


Figure 83. LC-MS (ESI-) spectra collected compound-fraction-5 (m/z 255 ion).

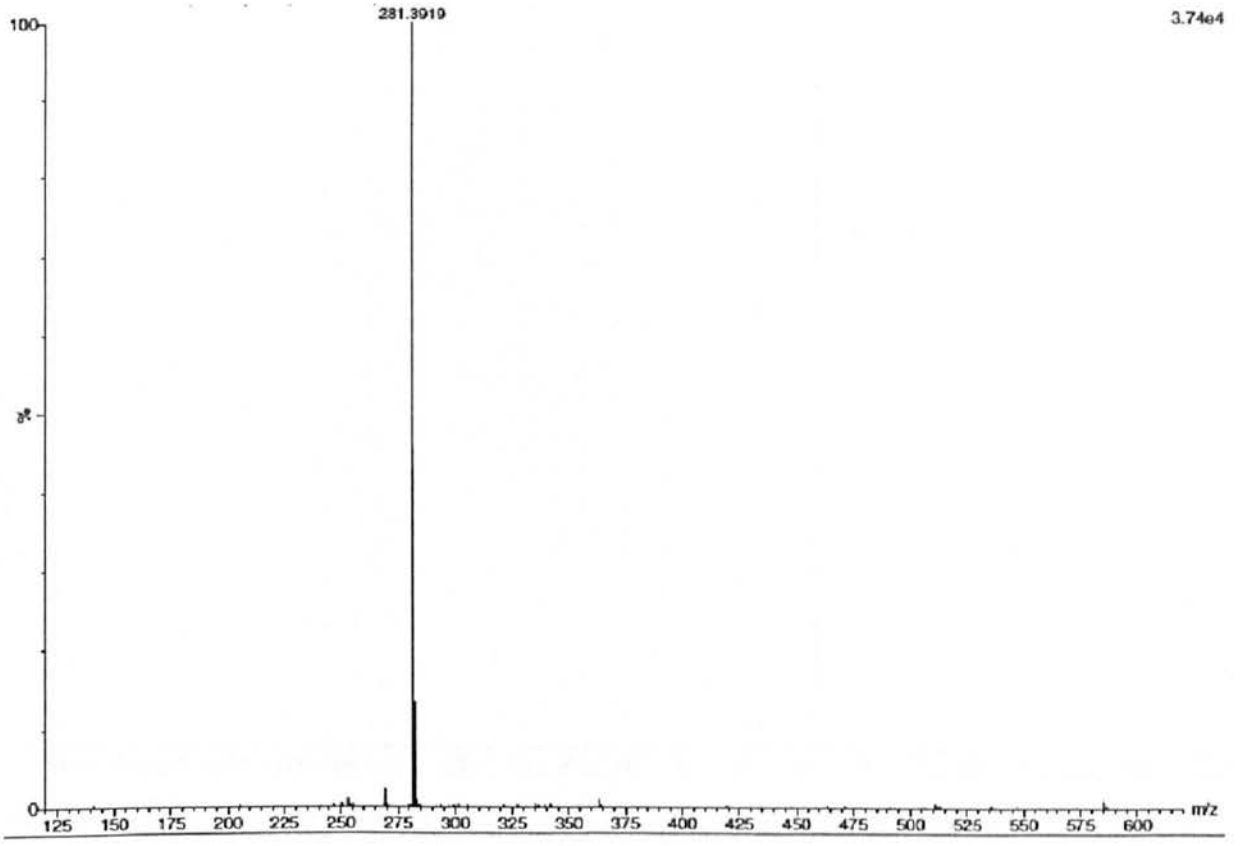


Figure 84. LC-MS (ESI-) spectra collected compound-fraction-6 (m/z 281 ion).

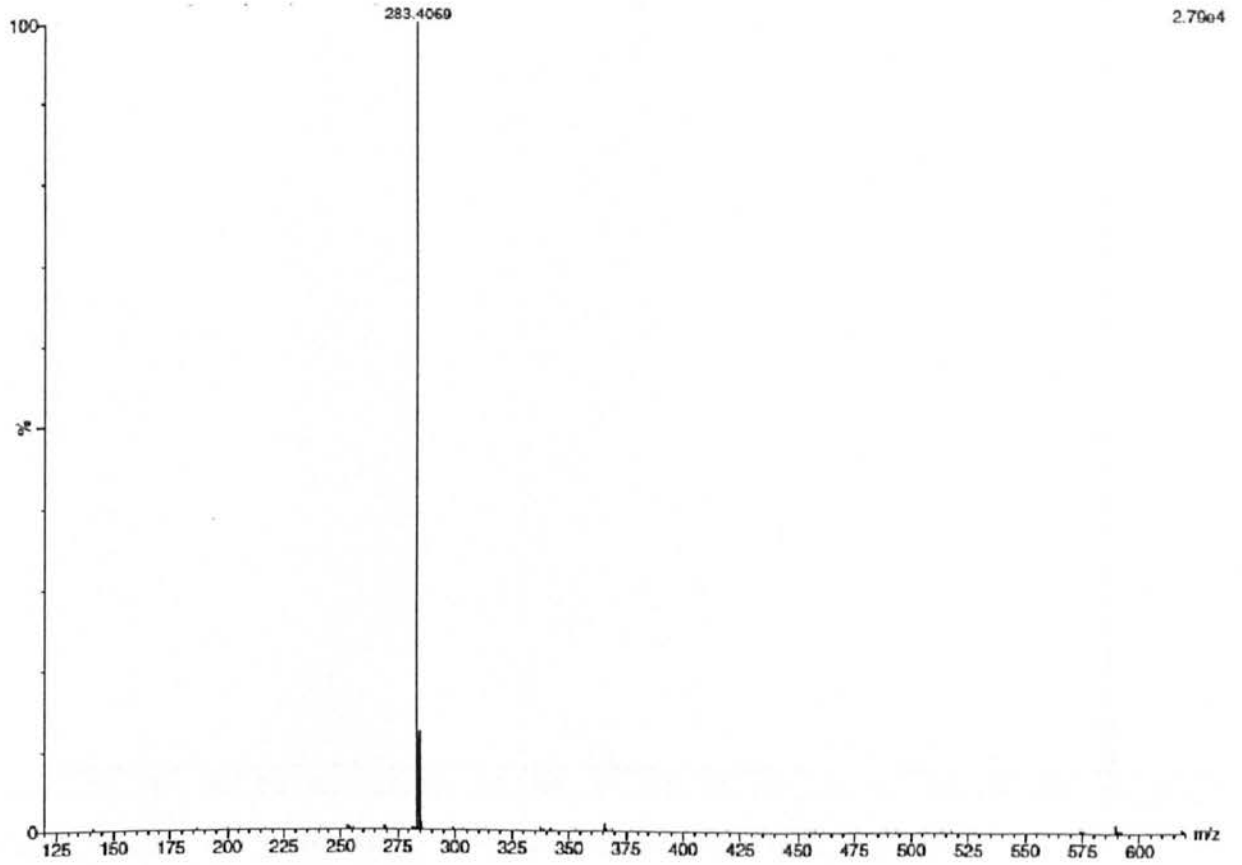


Figure 85. LC-MS (ESI-) spectra collected compound-fraction-7 (m/z 283 ion).

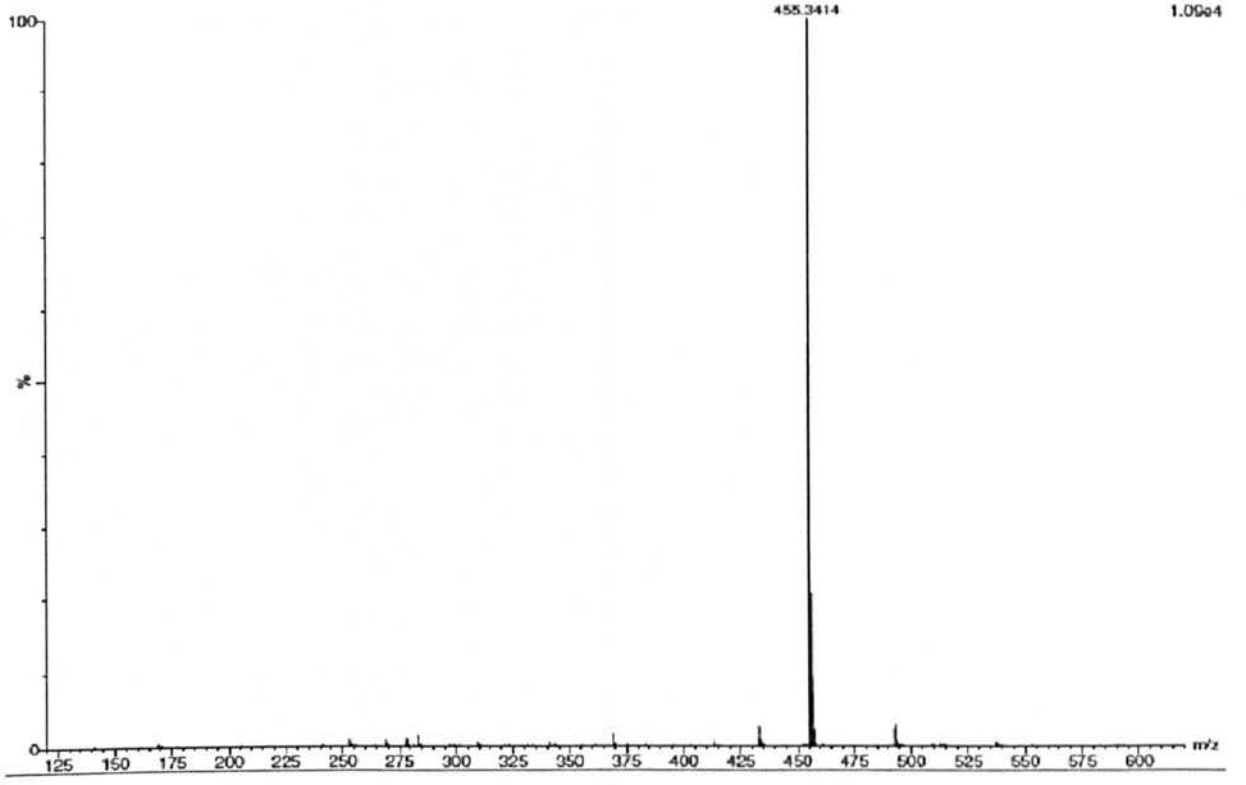


Figure 86. LC-MS (ESI-) spectra collected compound-fraction-8 (m/z 455 ion).

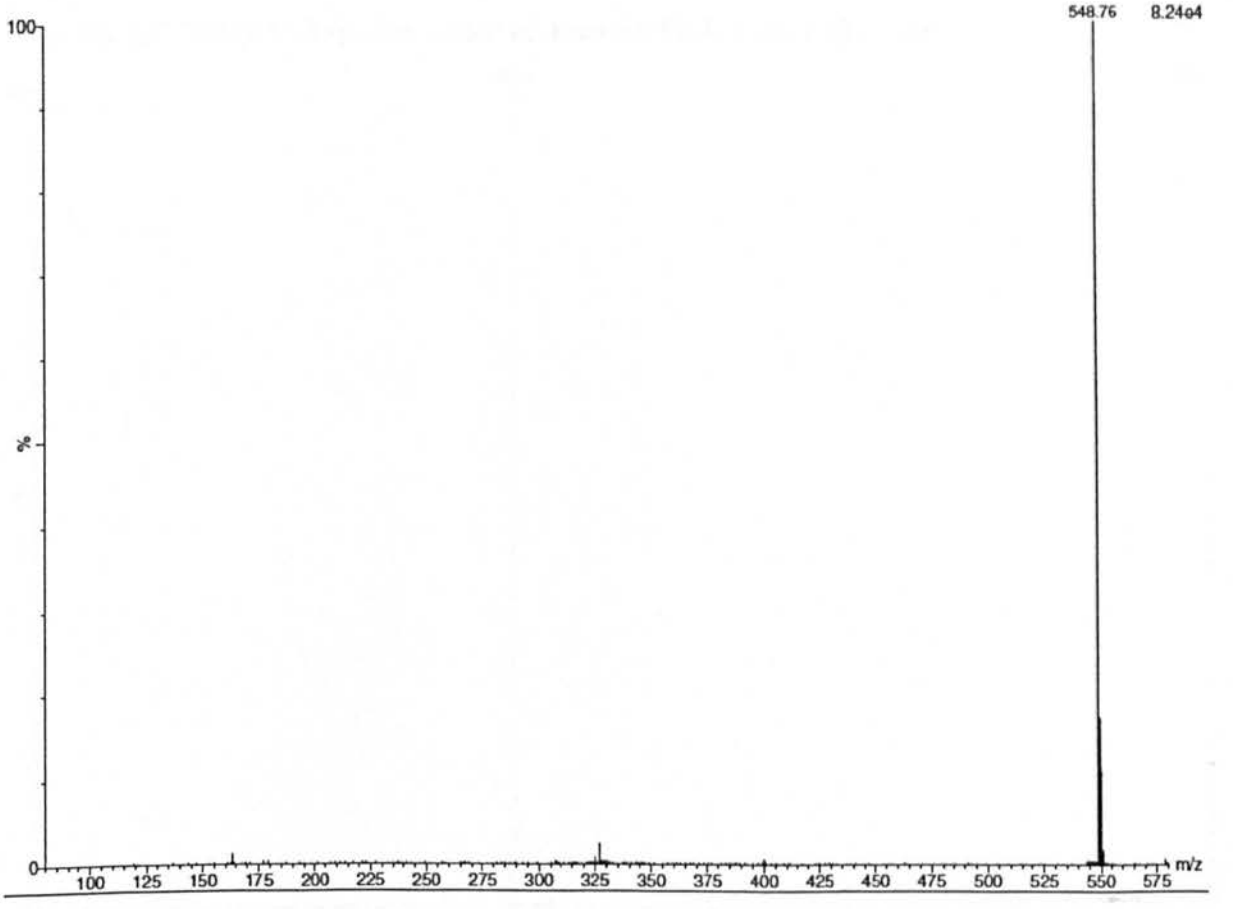


Figure 87. LC-MS (ESI-) spectra collected fraction FEA-1 (m/z 549 ion).

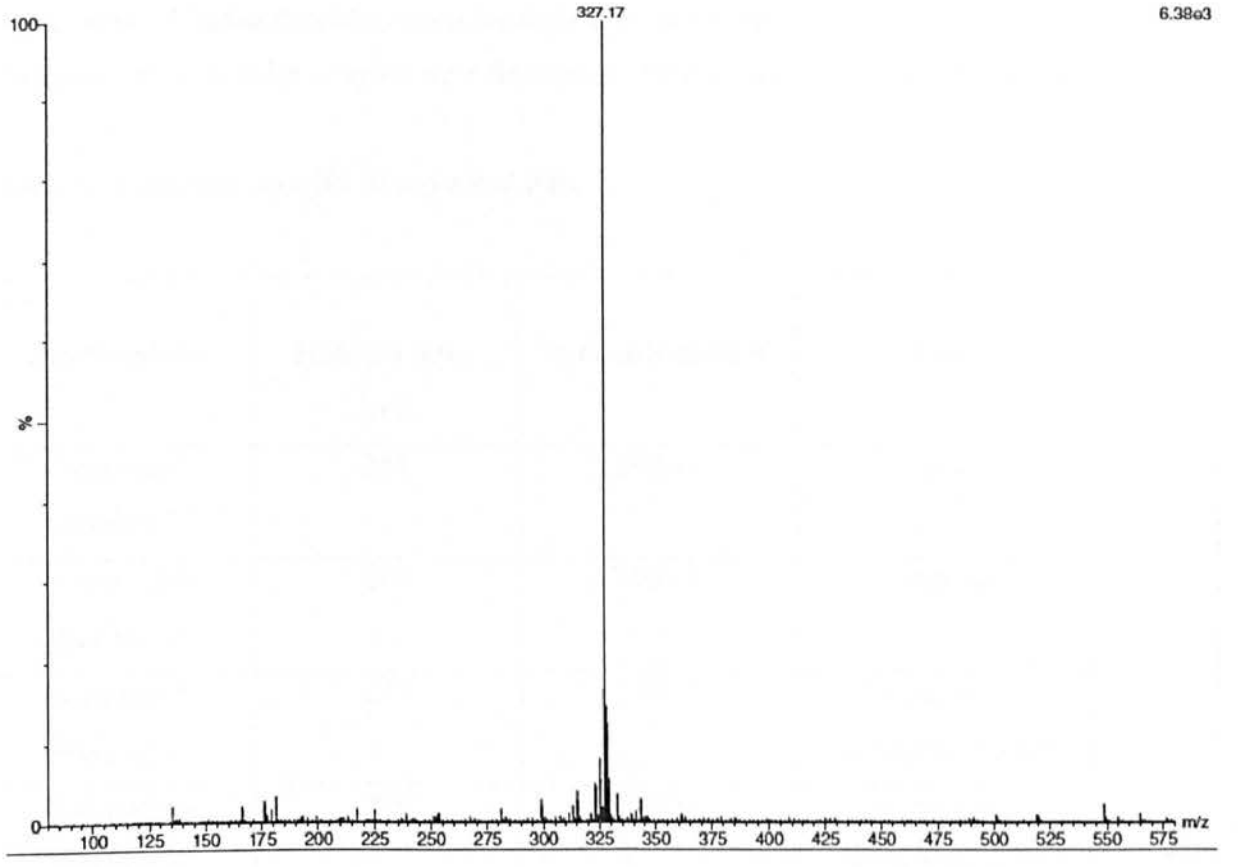


Figure 88. LC-MS (ESI-) spectra collected fraction FEA-2 (m/z 327 ion).

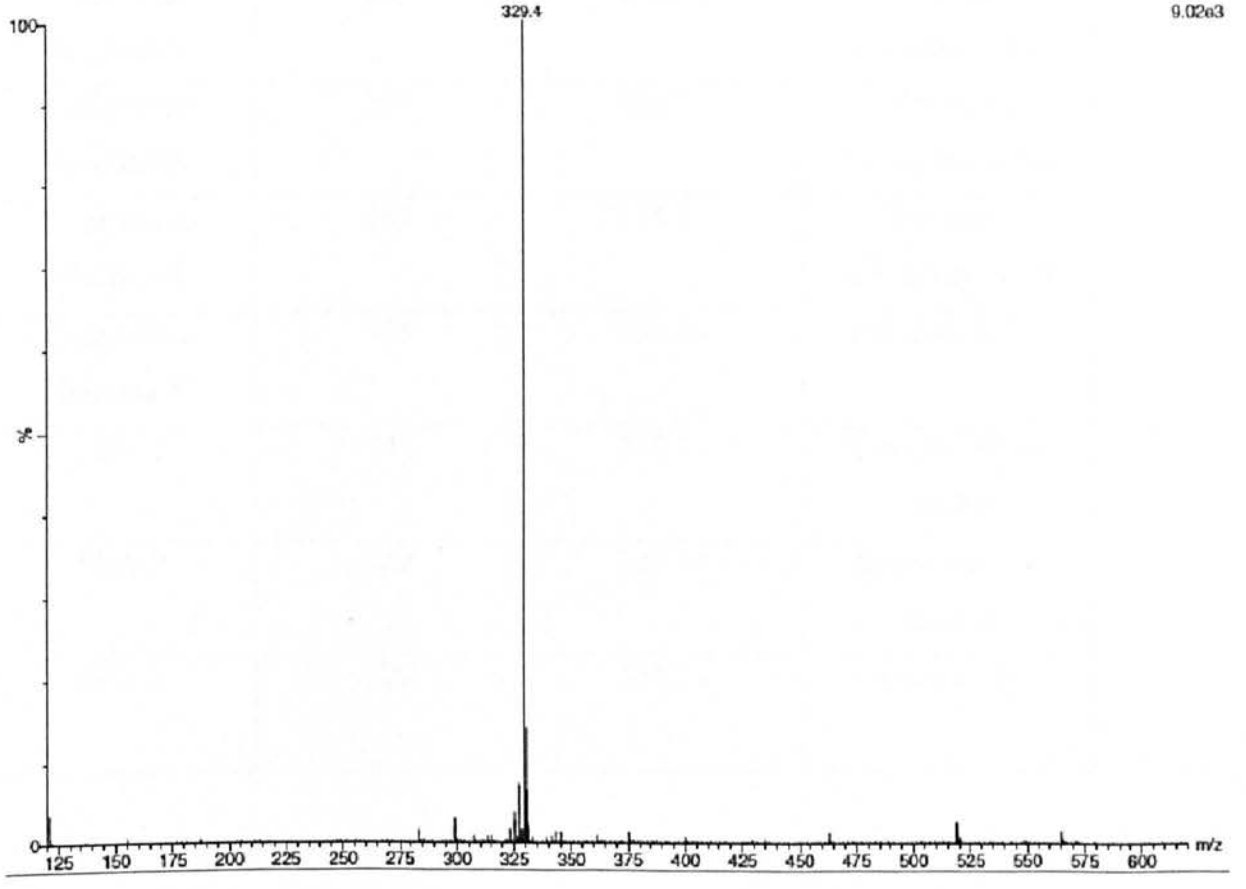


Figure 89. LC-MS (ESI-) spectra collected fraction FEA-3 (m/z 329 ion).

7.3.2. MW of isolated subfractions by high resolution-MS

Compounds' molecular weights were determined by high resolution-MS (see Table 5).

Table 5. Molecular weights of separated ions.

Subfractions	Isolated ions, m/z	Determined MW	Colour
Compound-fraction-1	241	242.24	impure
Compound-fraction-2	267	268.08	impure
Compound-fraction-3	237	238.07	Green-yellow amorphous solid
Compound-fraction-4	279	280.38	Whitish or colourless solid
Compound-fraction-5	255	256.24	Whitish or colourless solid
Compound-fraction-6	281	282.25	Whitish or colourless solid
Compound-fraction-7	283	284.27	Whitish or colourless solid
Compound-fraction-8	455	456.36	Whitish solid
FEA-1	549	550.17	Orange-yellow residue
FEA-2	327	328.22	Brown-yellow residue
FEA-3	329	330.24	Brown oily

7.3.3. OA and UA studies in fraction F9 contents

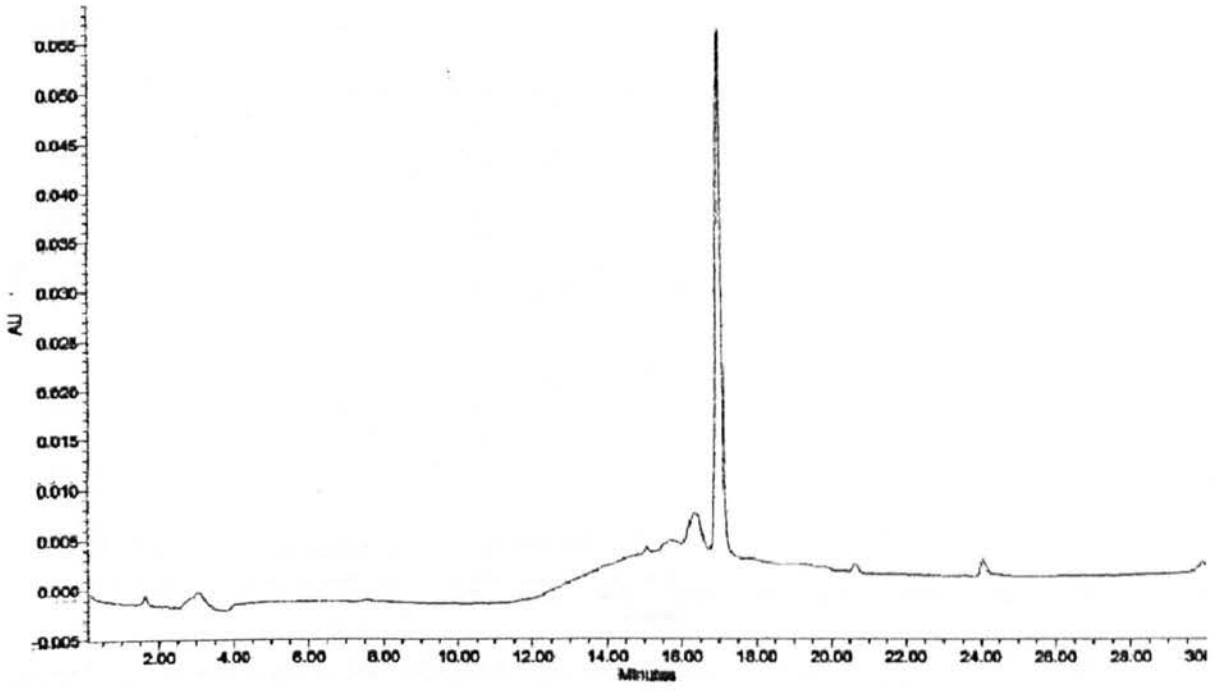


Figure 90. Chromatogram for UA on PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using M6 method.

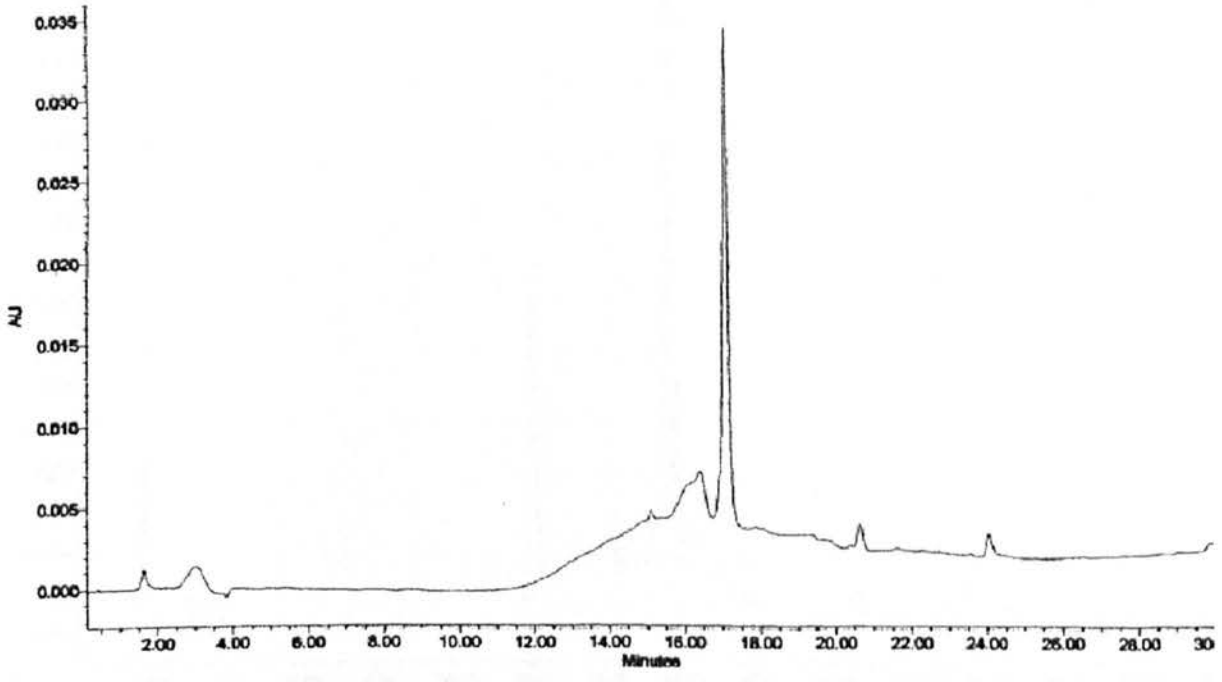


Figure 91. Chromatogram for OA on PFP(2) (250 x 4.60 mm, 5 µm) chromatographic column using M6 method.

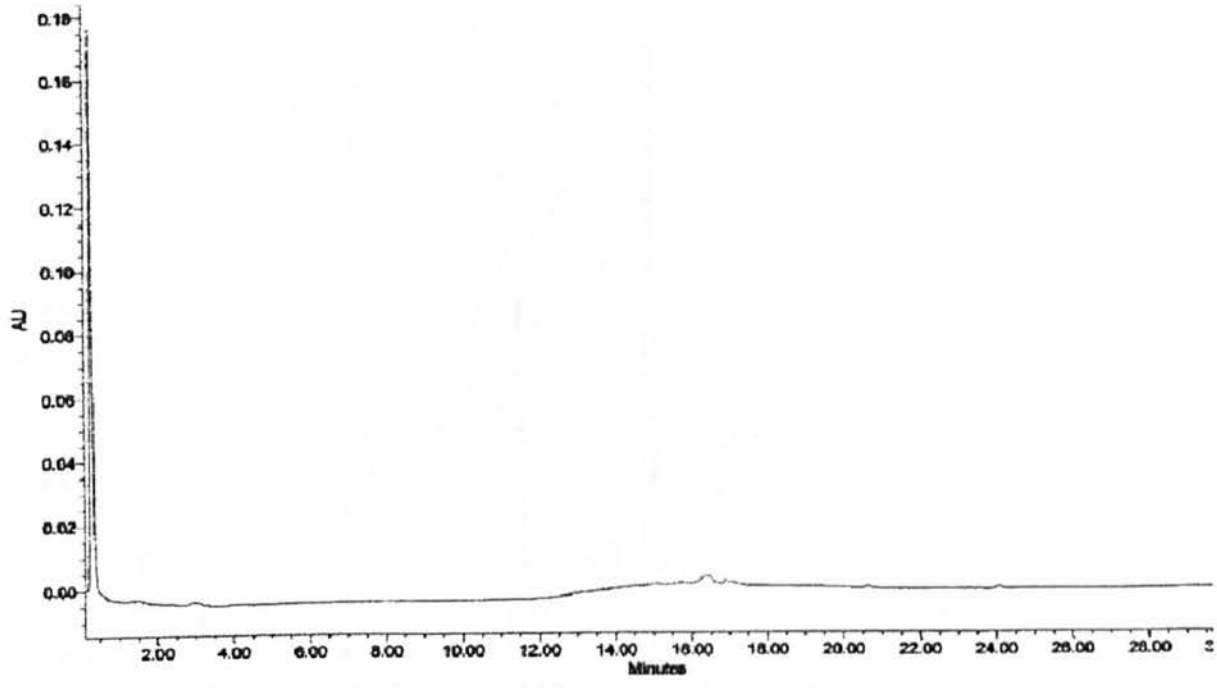


Figure 92. Chromatogram for solvent blank for OA and UA on PFP(2) (250 x 4.60 mm, 5 μm) chromatographic column using M6 method.

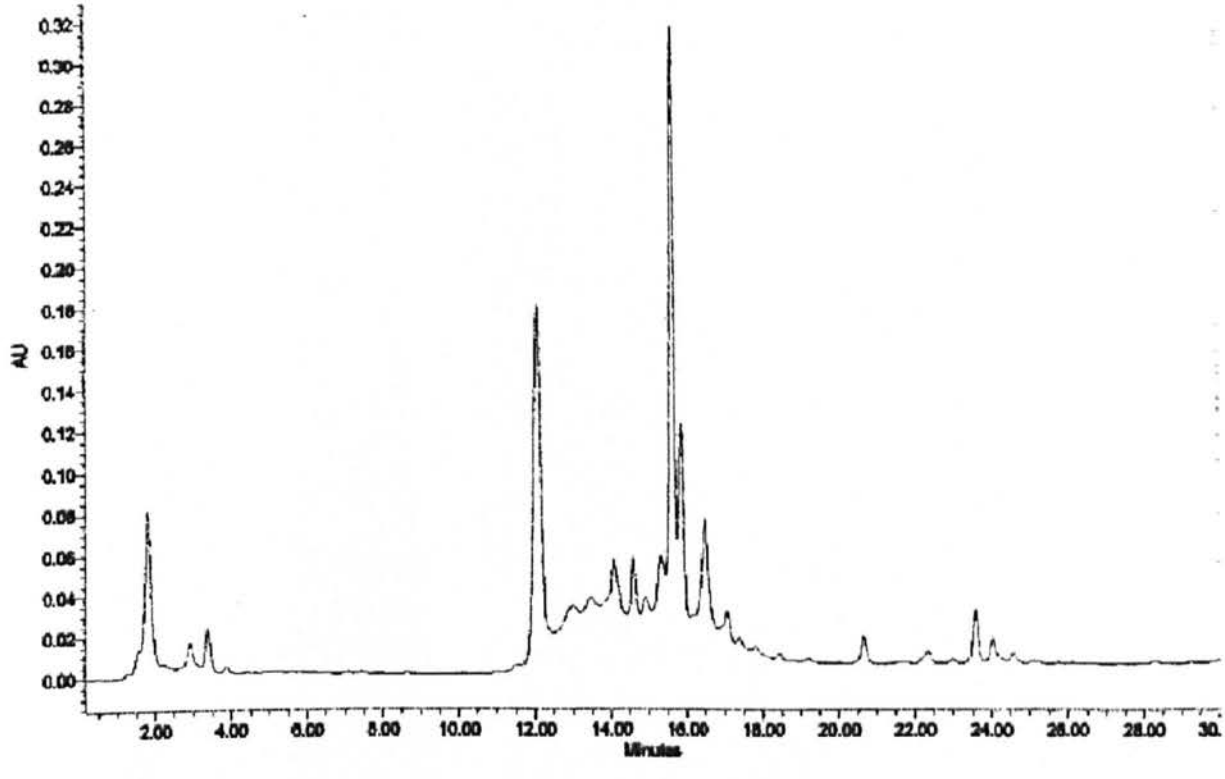


Figure 93. Chromatogram for fraction F9 on PFP(2) (250 x 4.60 mm, 5 μm) chromatographic column using M6 method. Rt of the peak is 17 min.

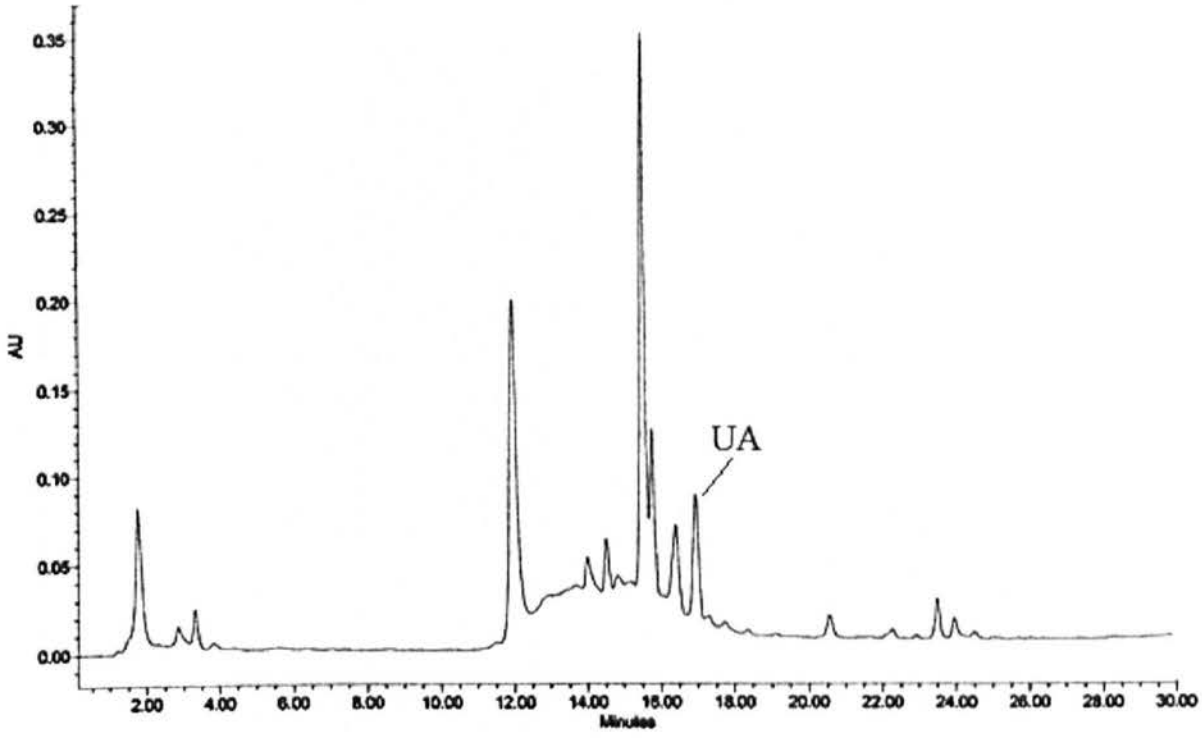


Figure 94. Chromatogram for fraction F9 with UA as an internal standard on PFP (2) (250 x 4.60 mm, 5 µm) chromatographic column using M6 method.

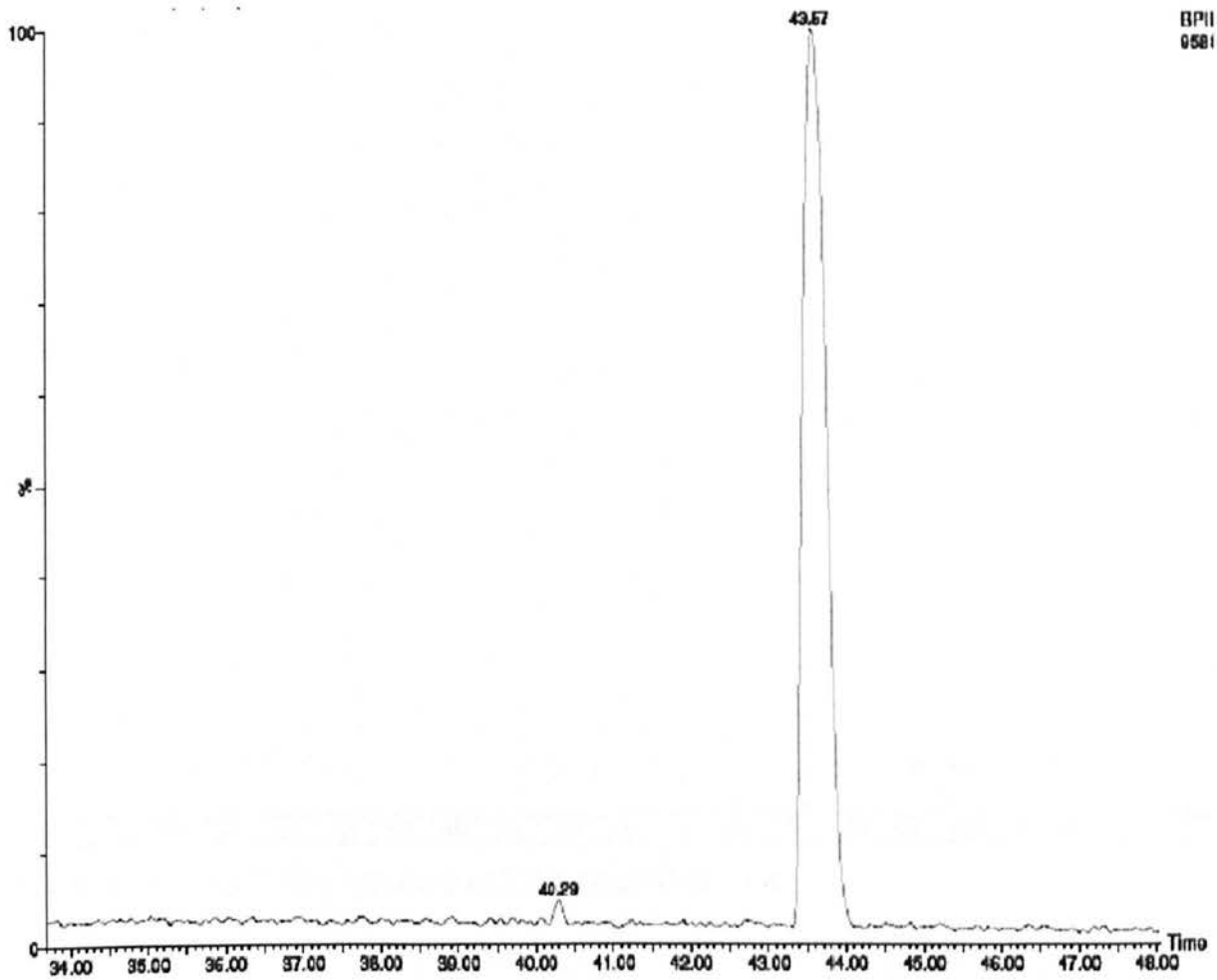


Figure 95. LC-MS (ESI-) chromatogram for UA (at 43.57 min) using M7 method.

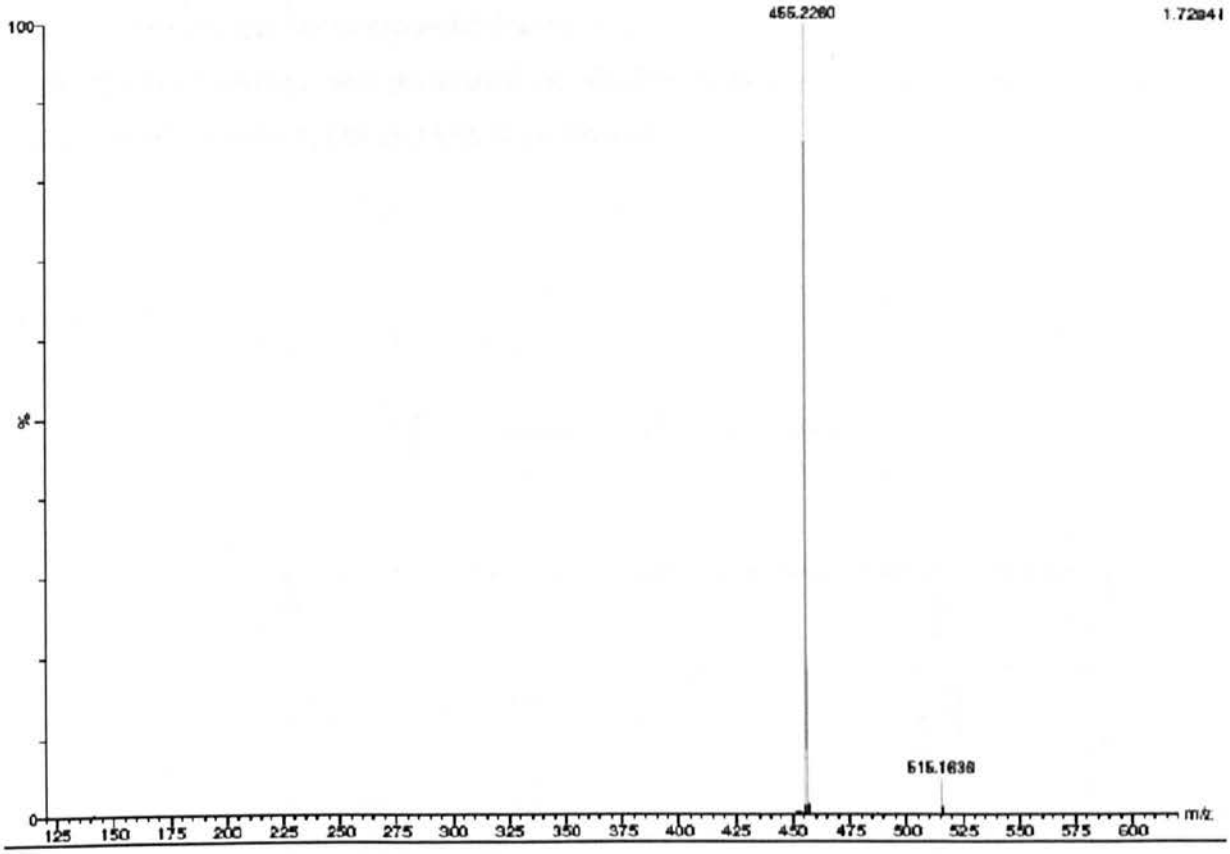


Figure 96. LC-MS (ESI-) spectrum of UA using M7 method.

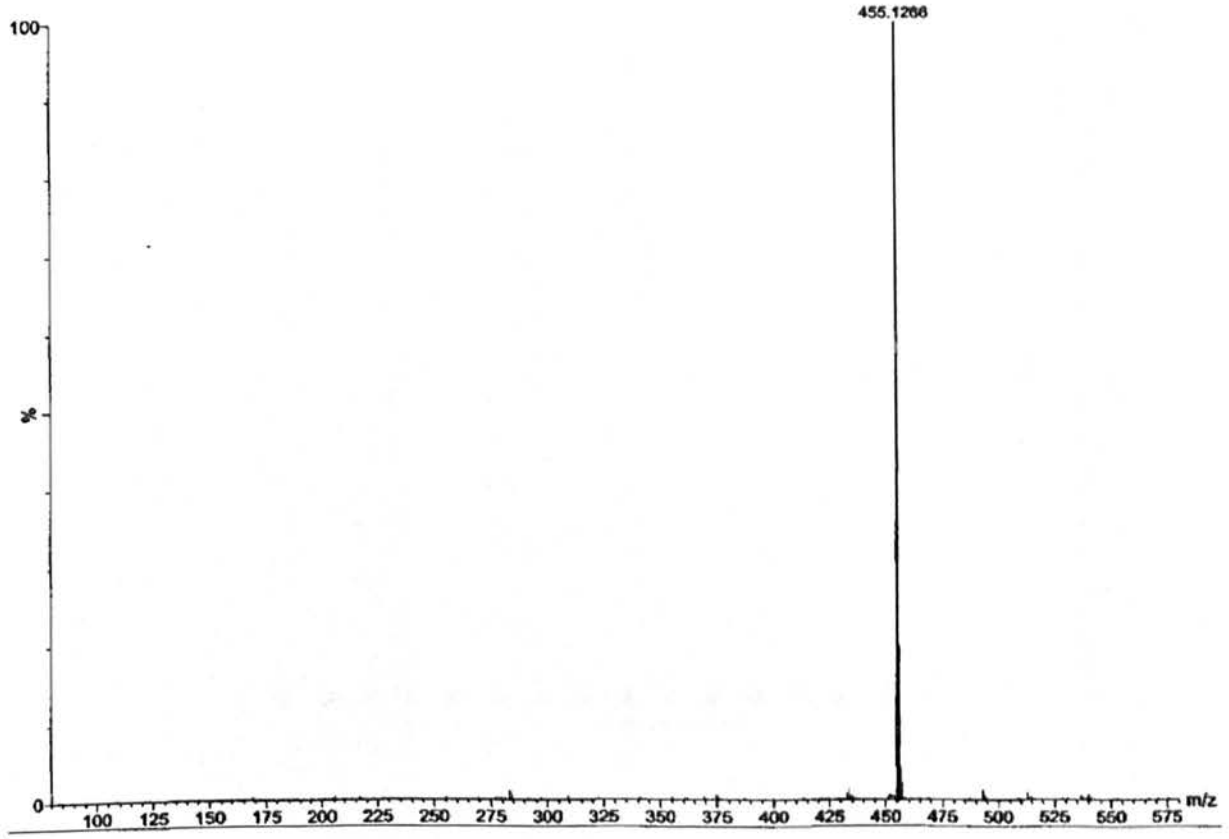


Figure 97. LC-MS (ESI-) spectrum for m/z 455 ion (compound-fraction-8) which eluted at 43.75 min from ethyl acetate extract using M7 method.

7.3.4. MS-MS results for compound-fraction-8

Fragment ion assessment was performed on MS-MS tandem spectroscopy and mass spectra for compound-fraction-8, OA and UA are followed.

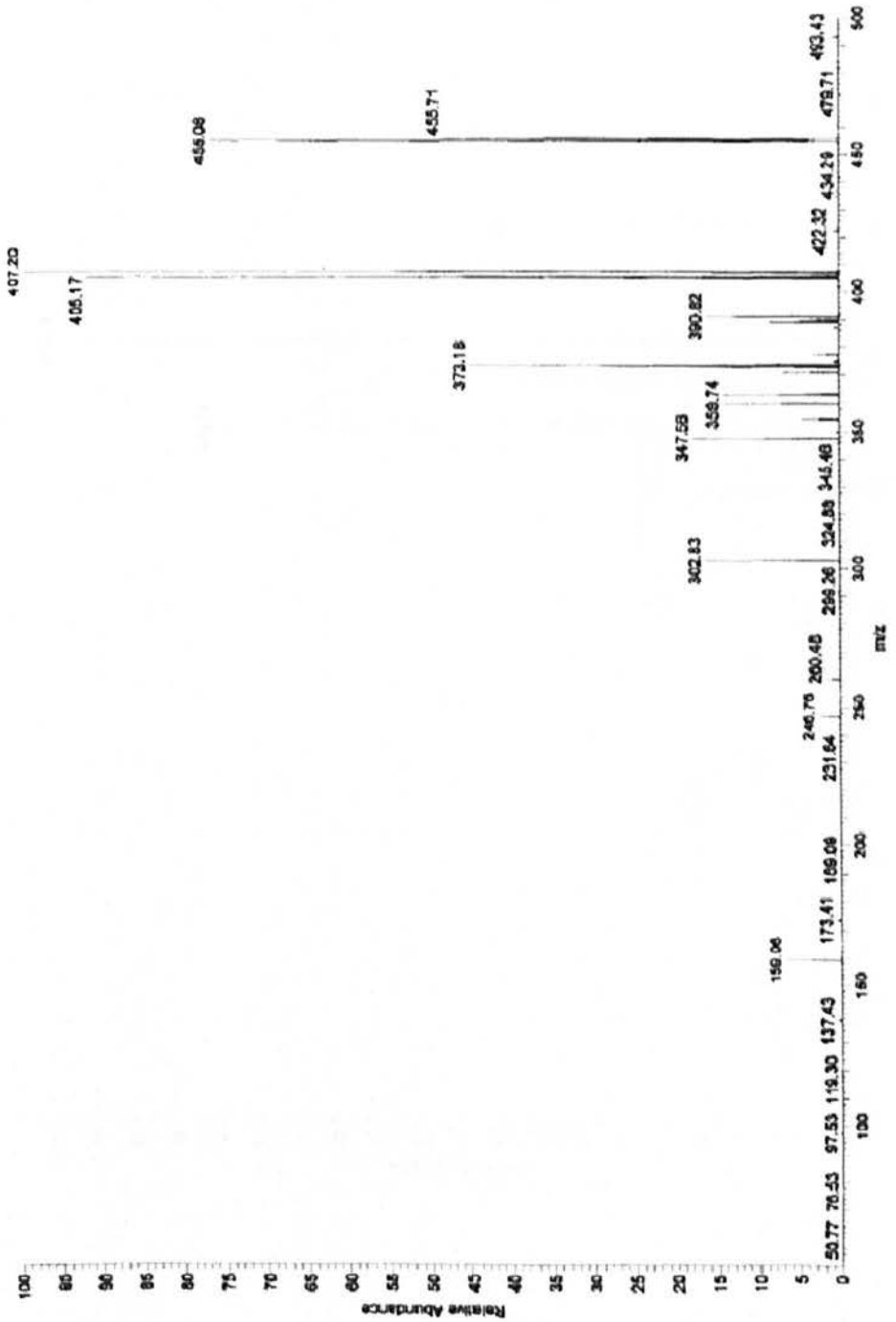


Figure 98. Mass spectrum for compound-fraction-8.

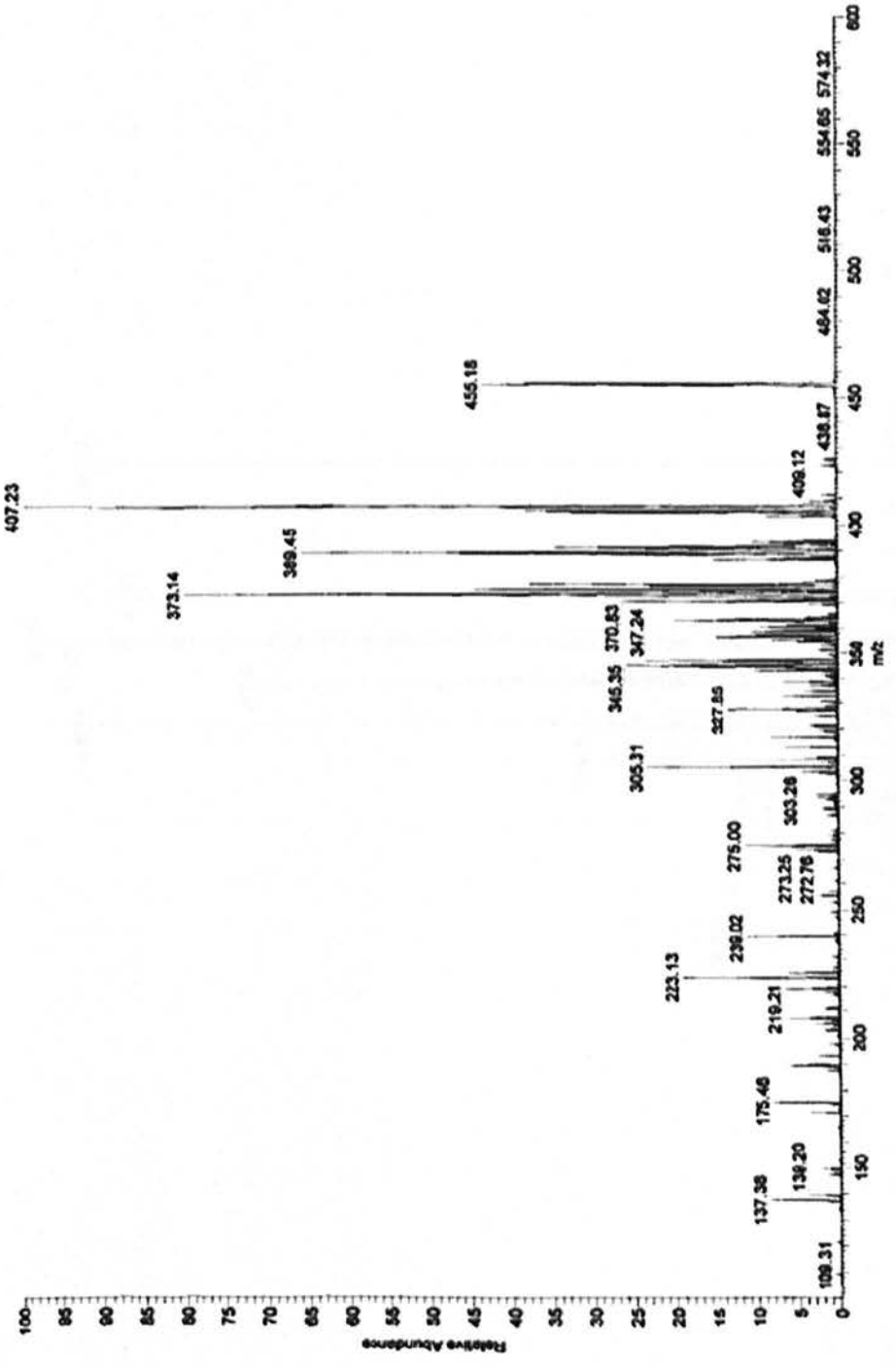


Figure 99. Mass spectrum for OA.

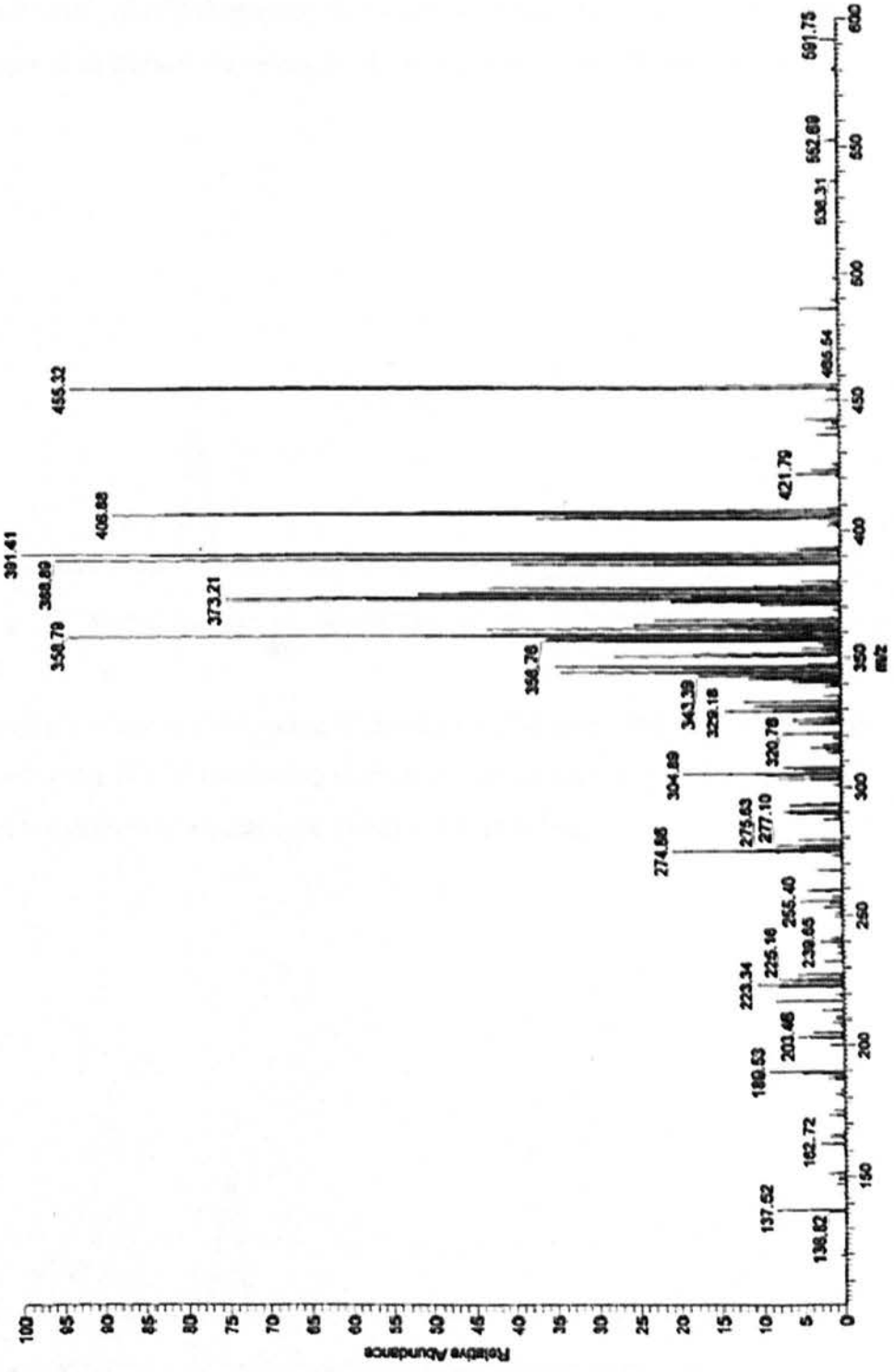


Figure 100. Mass spectrum for UA.

7.3.5. Comparison HPLC assessment of compound-fraction-8

Chromatograms of control OA and UA and their mixture as well as compound-fraction-8 (m/z 455 ion) from on C18 column (250 mm x 4.6 mm, 5 μm, Phenomenex, UK) with mobile phase methanol-water (83:17 containing 0.2% ammonium acetate, pH=6.7) at a flow-rate of 1.0 mL/min and at detection wavelength 210 nm [Liang *et al.*, 2009] are shown in Figures 101-104.

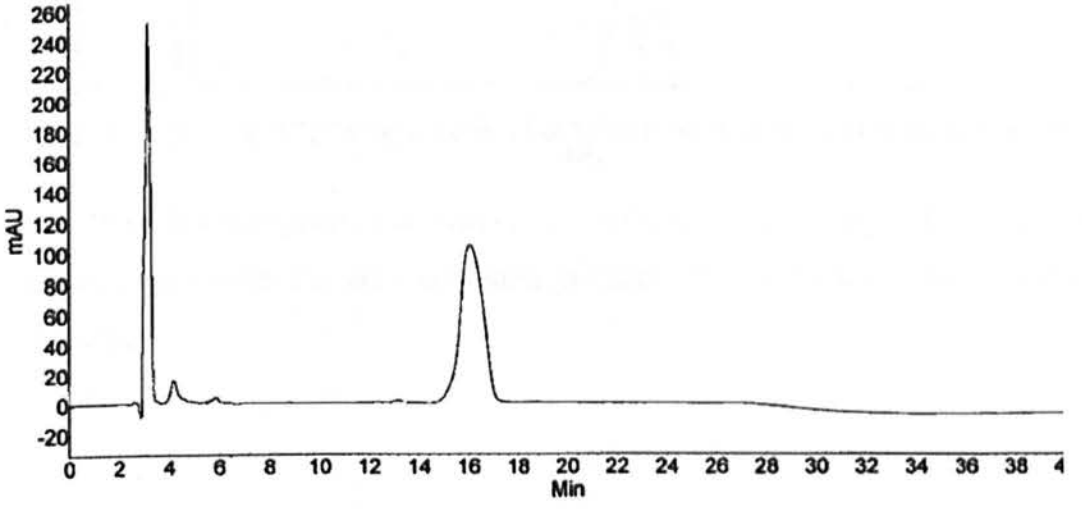


Figure 101. Chromatogram of control OA using C18 column (250 mm x 4.6 mm, 5 μm) with mobile phase methanol-water (83:17 containing 0.2% ammonium acetate, pH=6.7) at a flow-rate of 1.0 mL/min and at detection wavelength 210 nm. Rt=16.07 min.

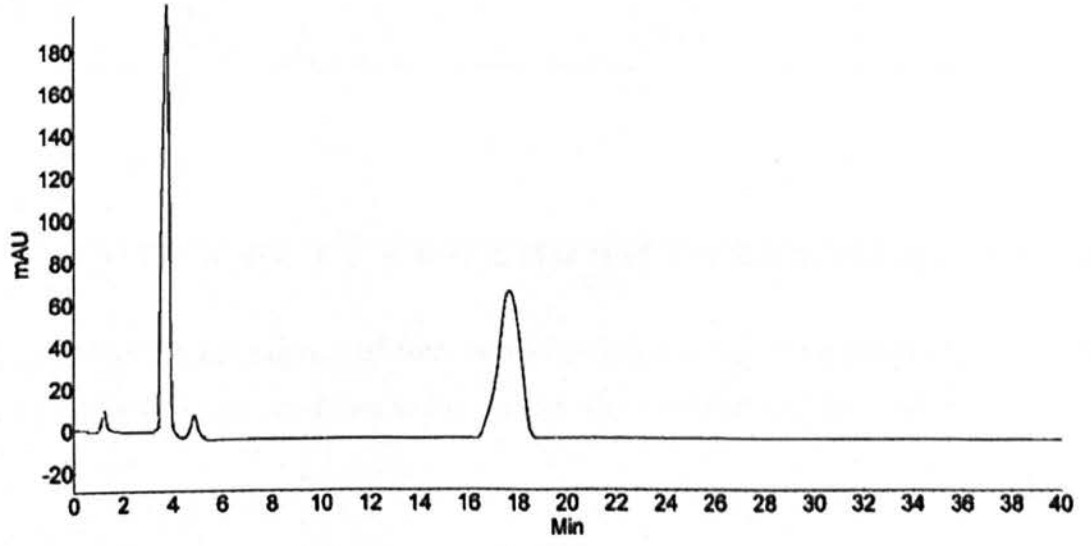


Figure 102. Chromatogram of control UA using C18 column (250 mm x 4.6 mm, 5 μm) under the same condition as Figure 101. Rt=17.64 min.

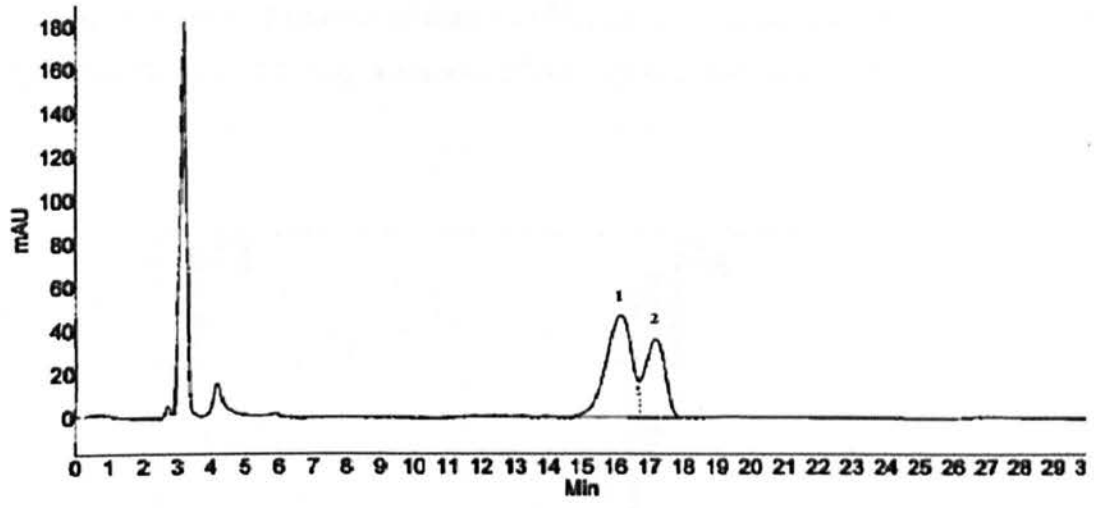


Figure 103. Chromatogram of control mixture of OA and UA using C18 column (250 mm x 4.6 mm, 5 μm) under the same condition as Figure 101. Rt (1-OA) =16.16 and Rt (2-UA) =17.20 min.

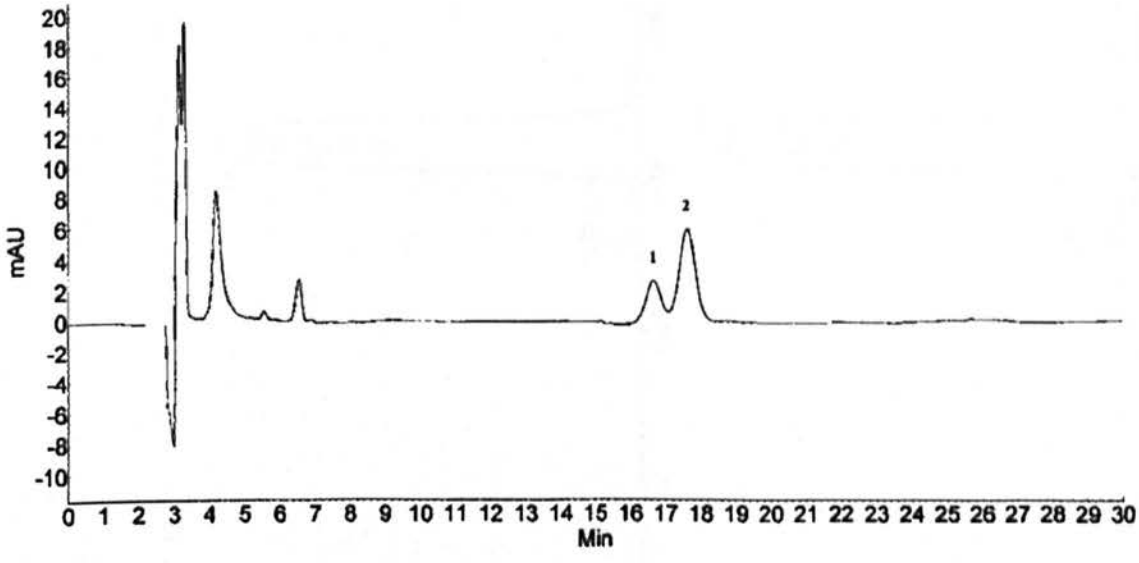


Figure 104. Chromatogram of compound-fraction-8 using C18 column (250 mm x 4.6 mm, 5 μm) under the same condition as Figure 101. Rt (1) =16.49 and Rt (2) =17.58 min.

7.3.6. Comparison LC-MS analysis of compound-fraction-8

Compound-fraction-8 and the mixture of OA and UA were investigated on LC-MS using the above (see Section. 7.2.2.6) mentioned HPLC method and results follow:

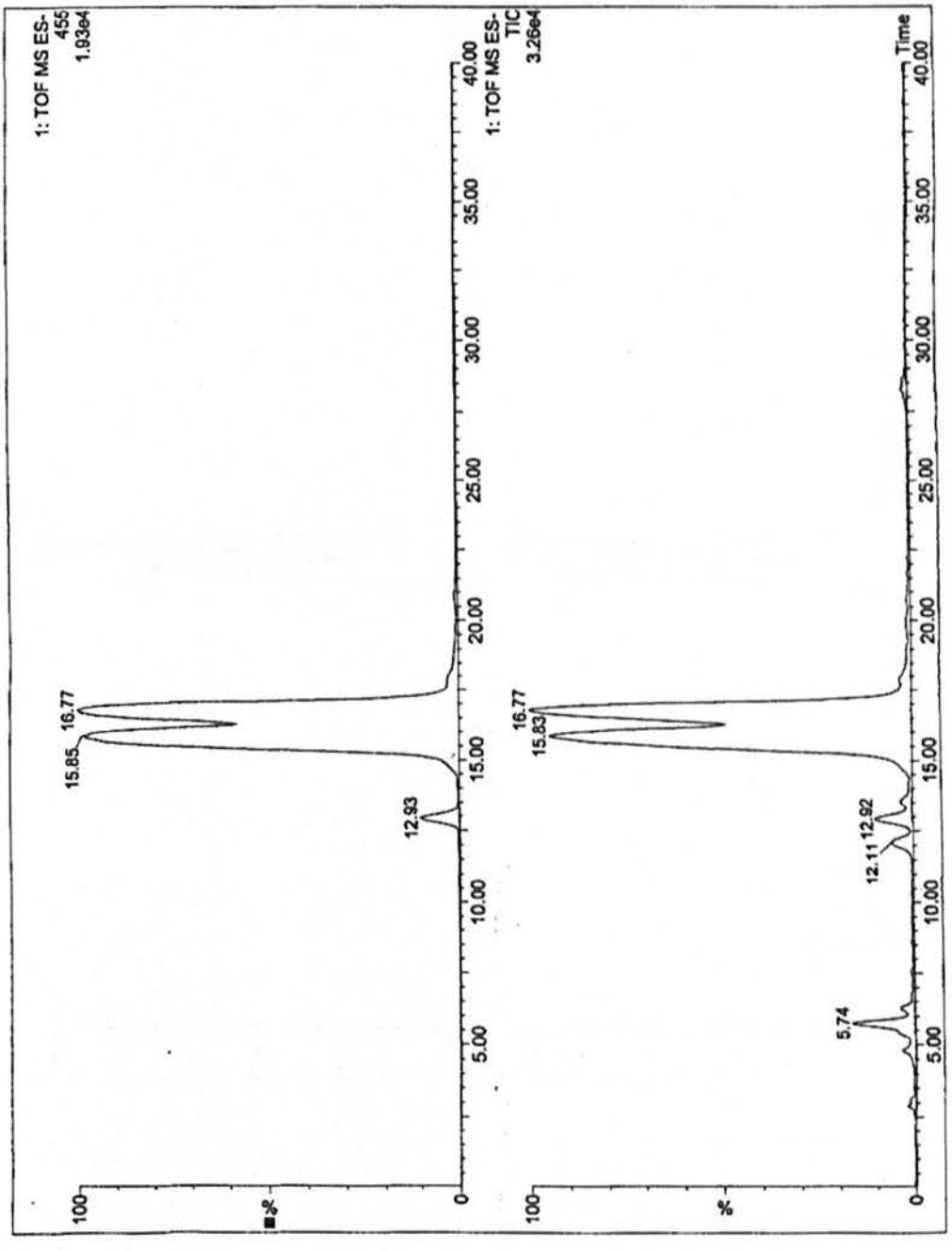


Figure 105. LC-MS (ESI-) spectra of extracted m/z 455 ion (top) and total ion chromatogram for mixture of standard OA and UA (bottom).

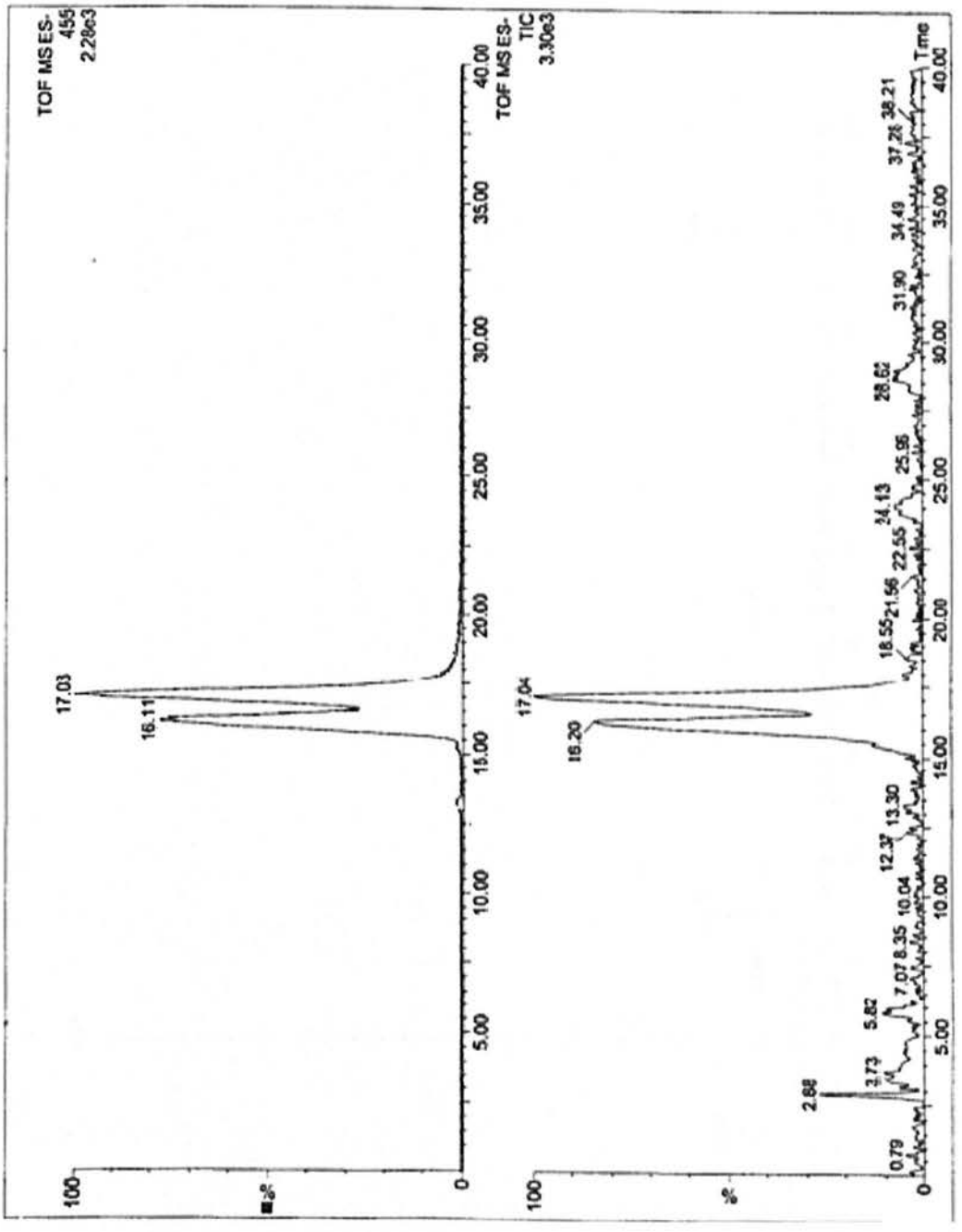


Figure 106. LC-MS (ESI-) spectra of extracted m/z 455 ion (top) and total ion chromatogram for compound fraction-8 (Bottom).

7.3.7. MS-MS assessment of fraction FEA-1 (m/z 549 ion)

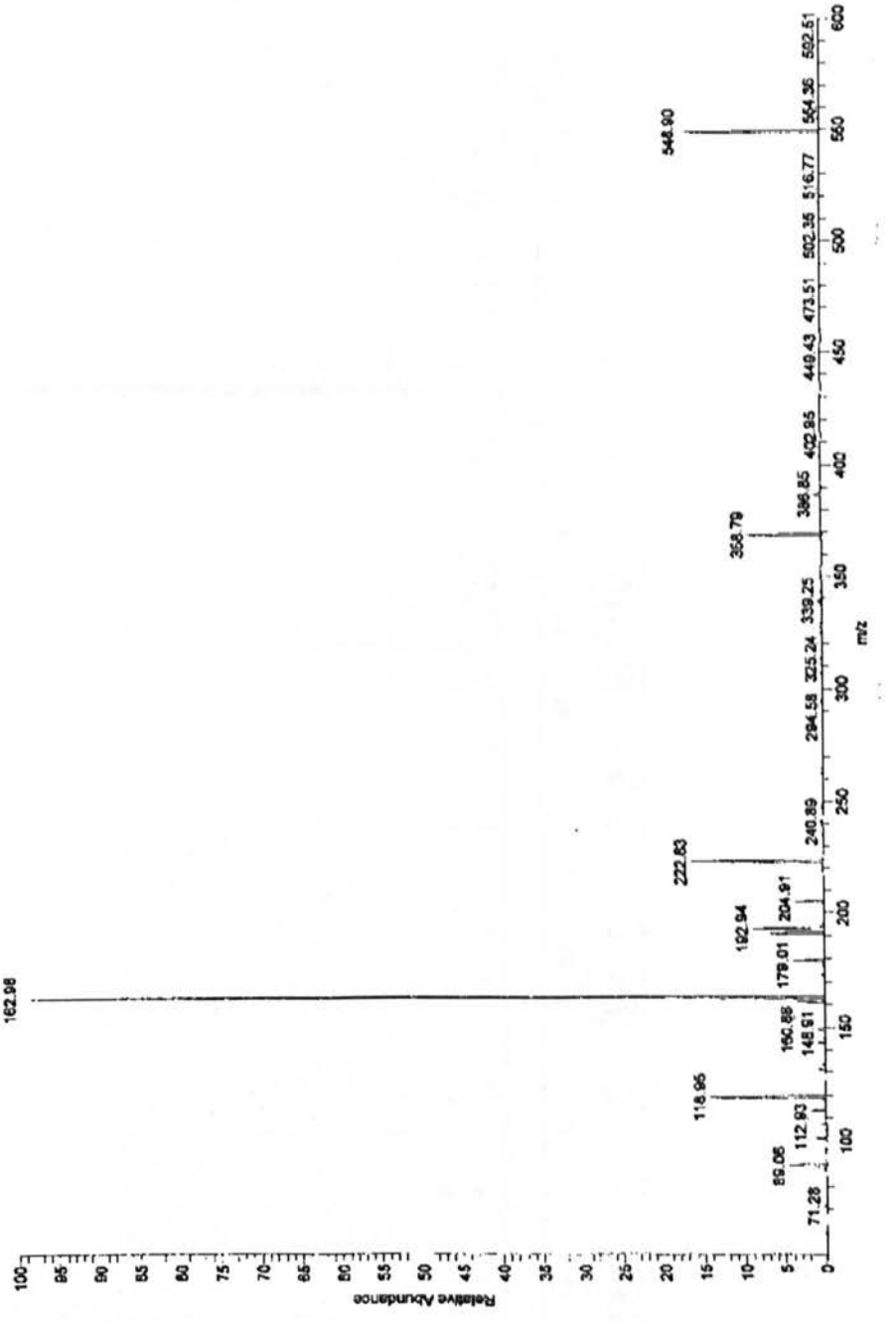


Figure 107 . Mass spectrum for fraction FEA-1 (m/z 549 ion).

7.3.8. Comparison NMR assessment of fraction FEA-1

Molecular weight of fraction FEA-1 (m/z 549) is 550.17. The recorded NMR spectra results for FEA-1 are followed.

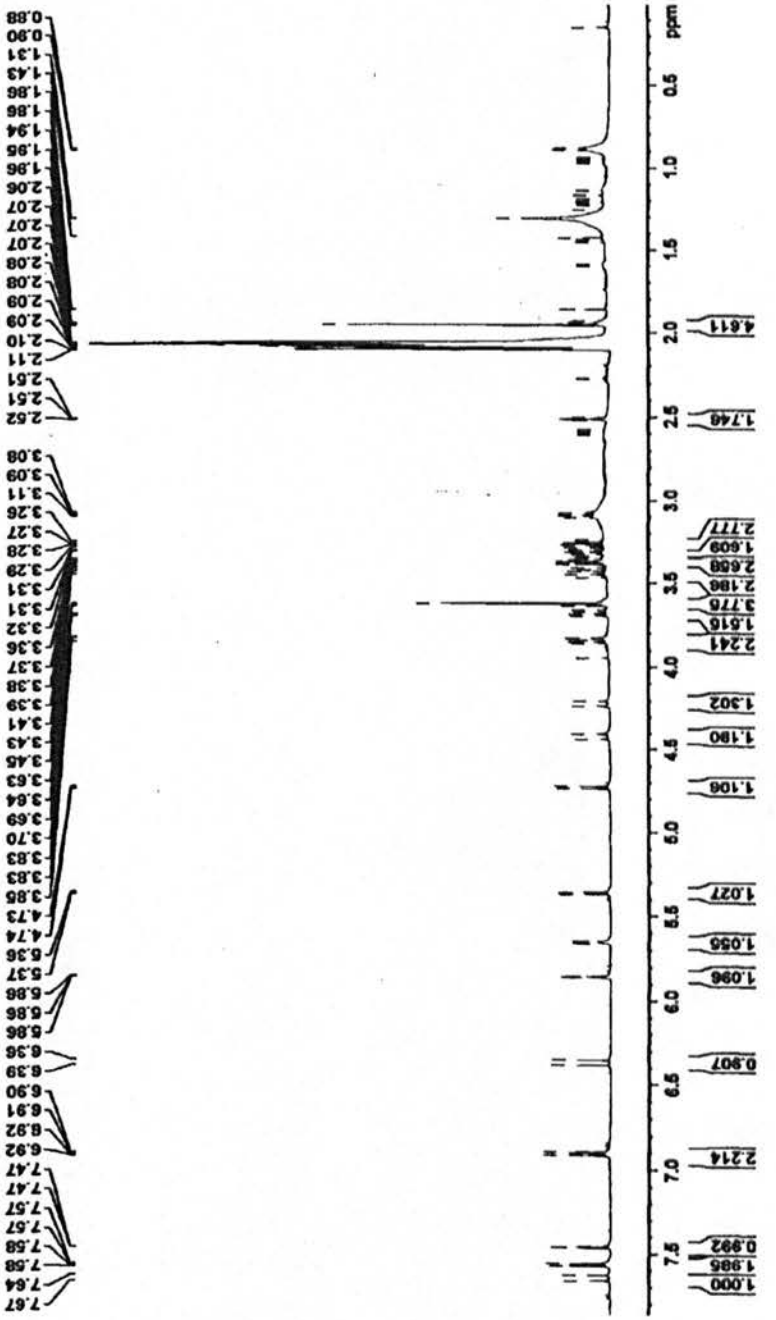


Figure 108. ^{1}H-NMR Data of fraction FEA-1.

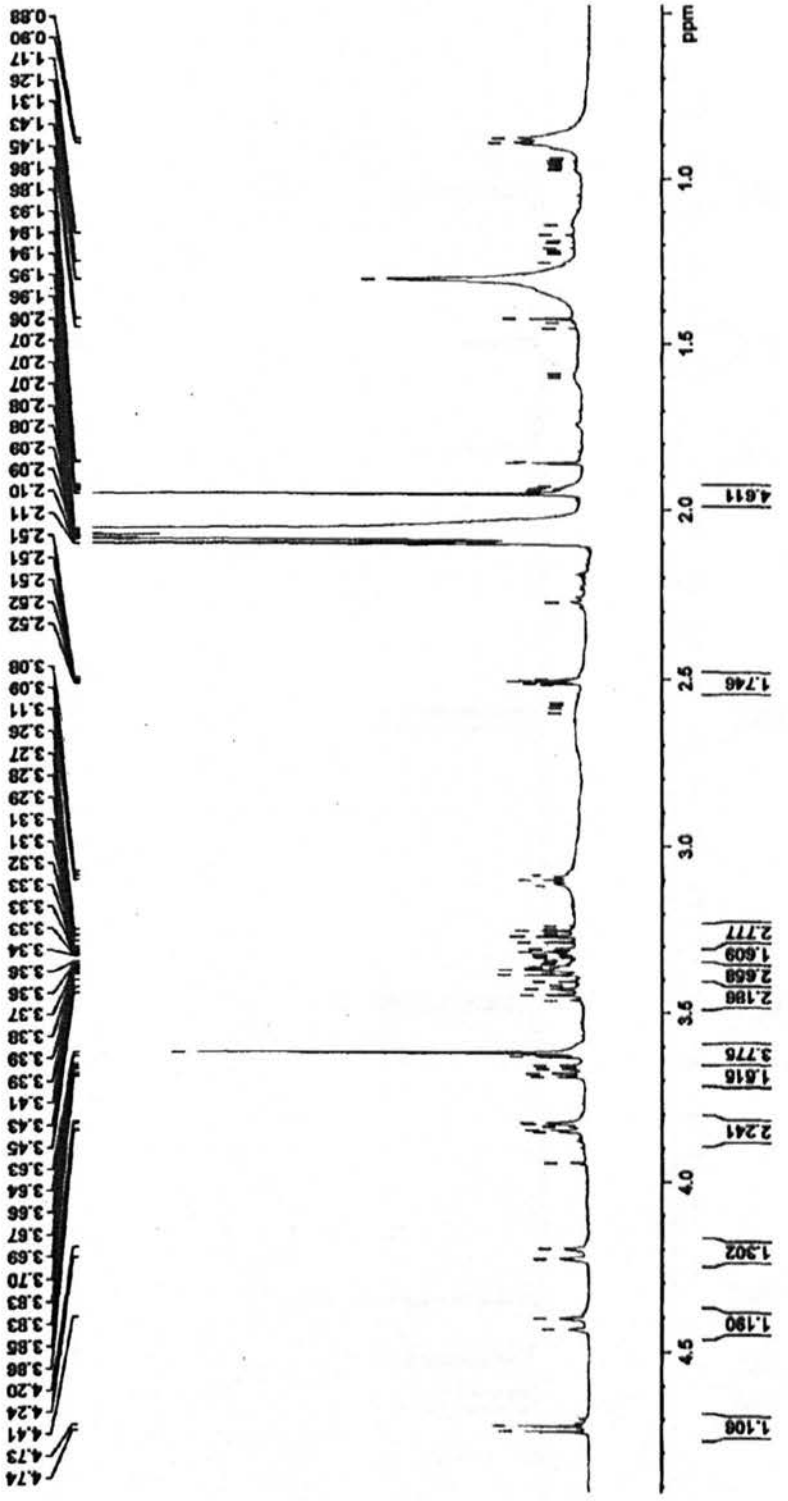


Figure 109. ^{1}H-NMR Data of fraction FEA-1, extension 0-5 ppm.

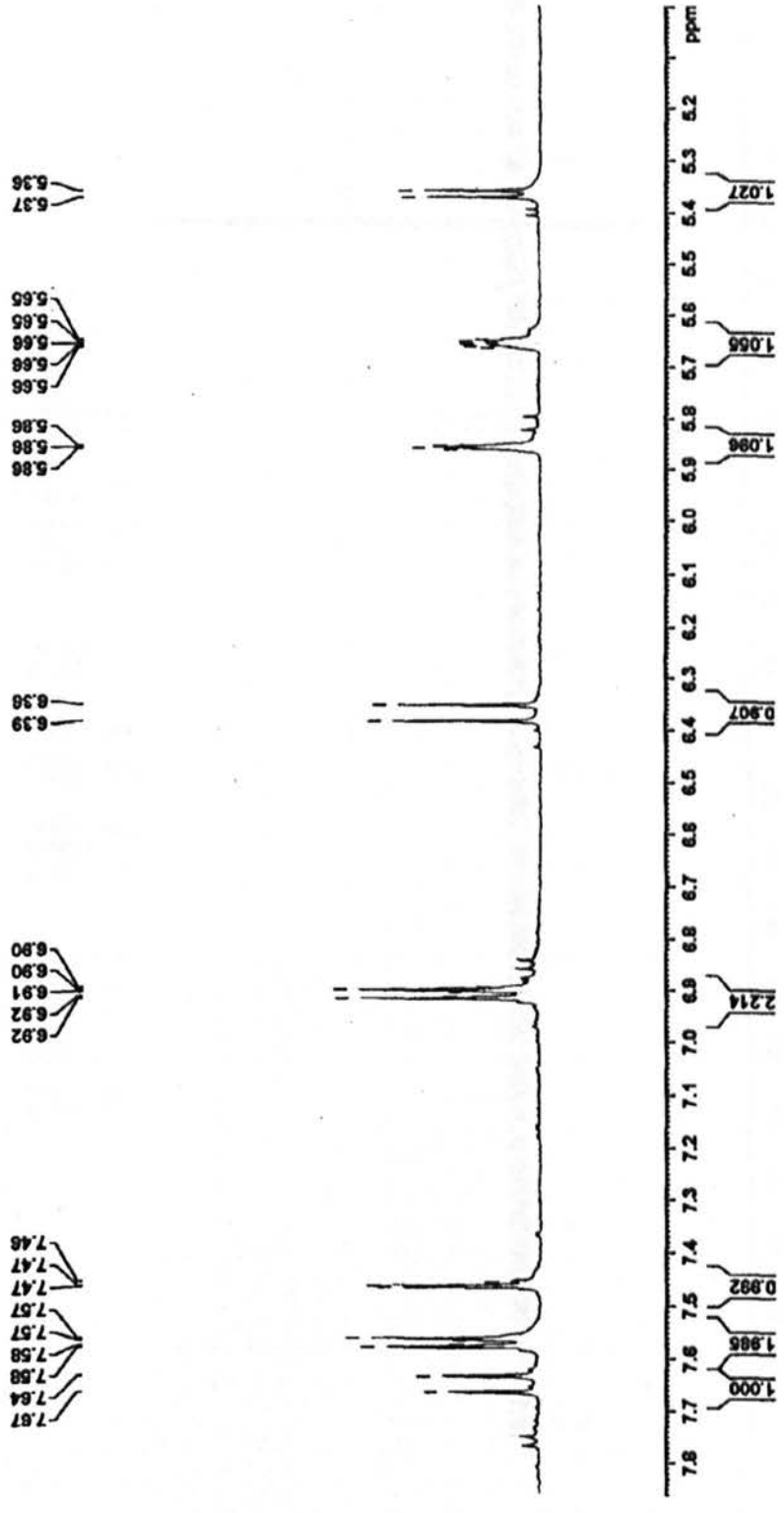


Figure 110. ^{1}H-NMR Data of fraction FEA-1, extension 5-8 ppm.

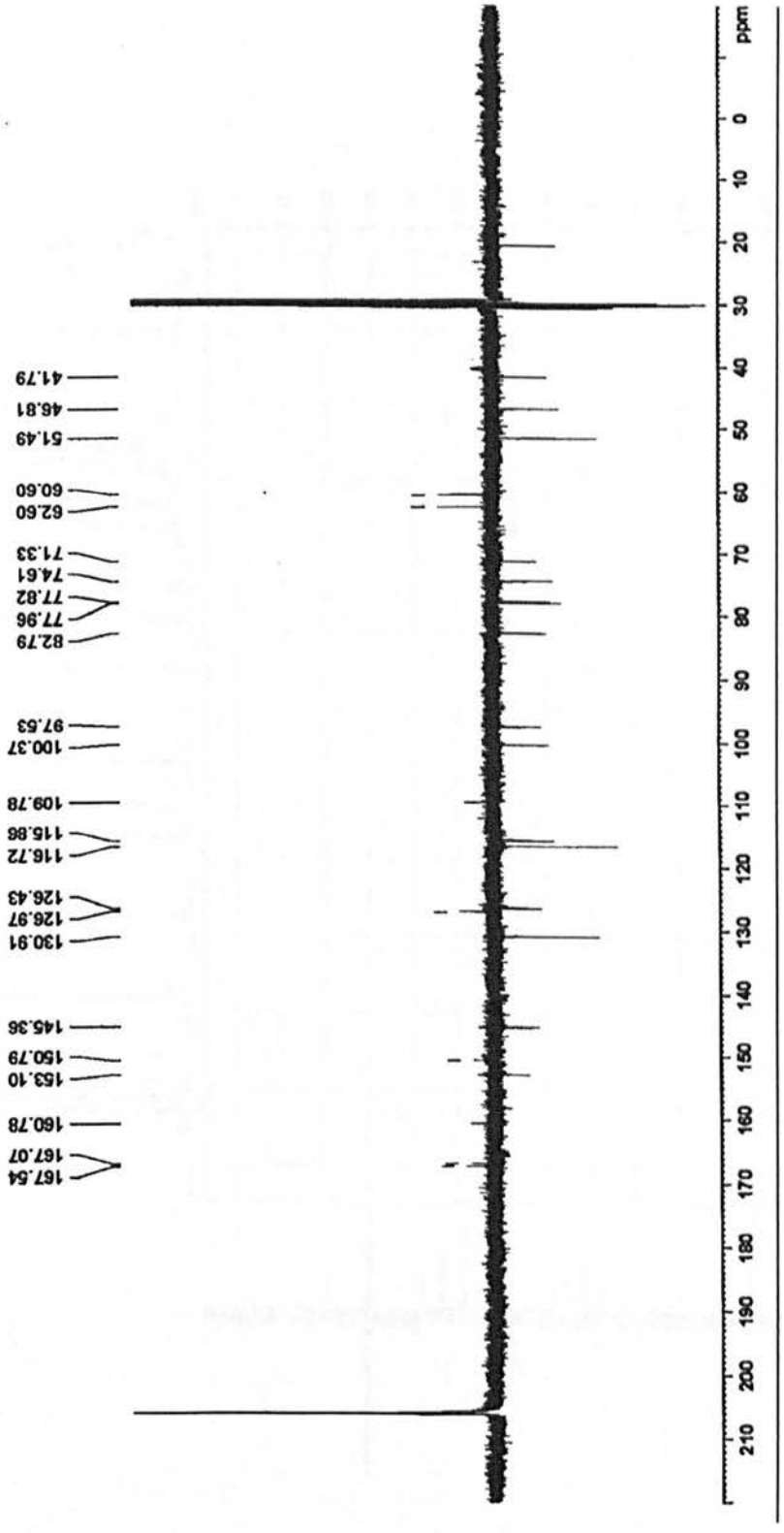


Figure 111. ^{13}C-NMR data for fraction FEA-1.

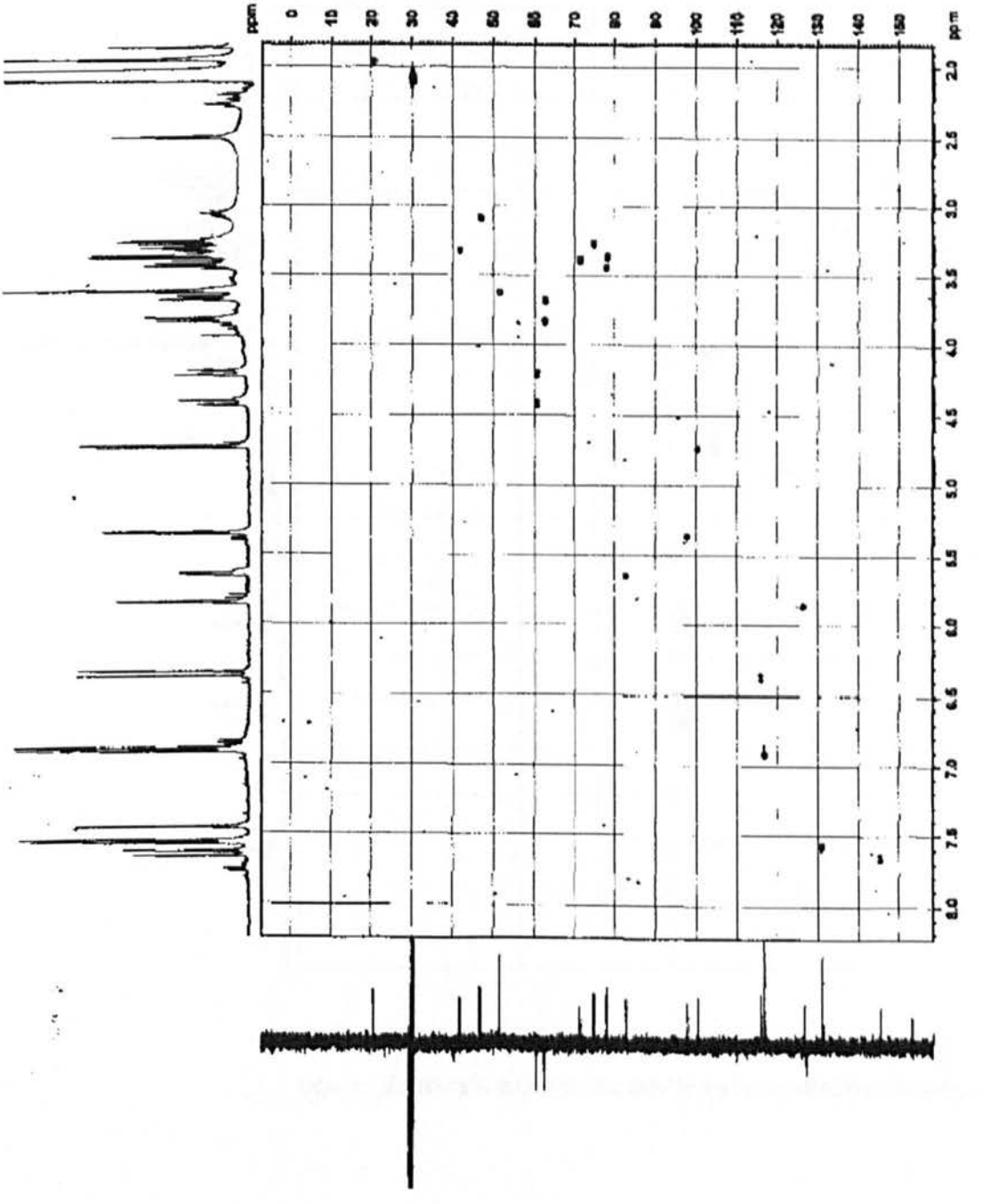


Figure 112. HSQC NMR data for fraction FEA-1.

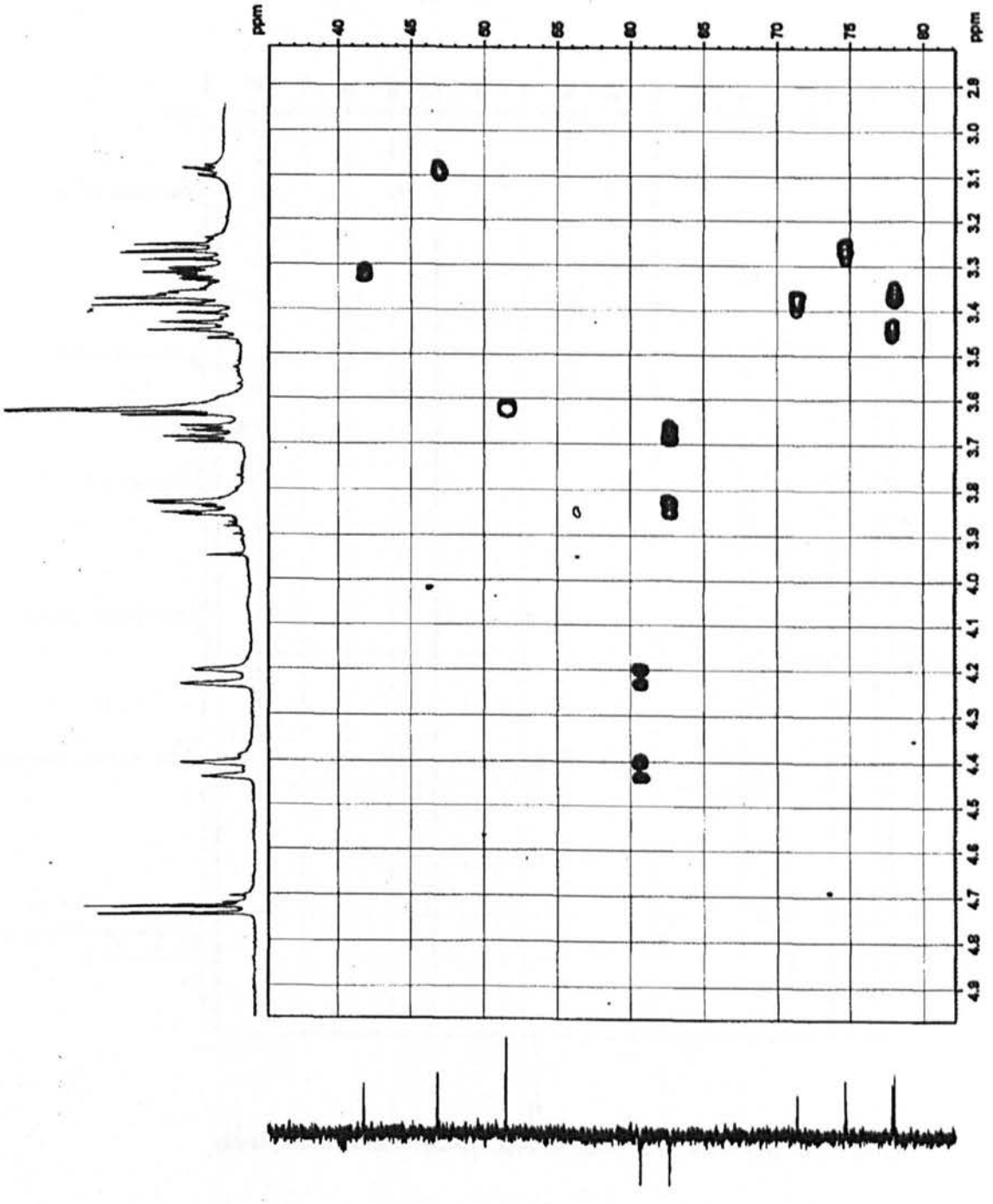


Figure 113. HSQC NMR data for fraction FEA-1, extension 0-90 ppm and 1-5 ppm.

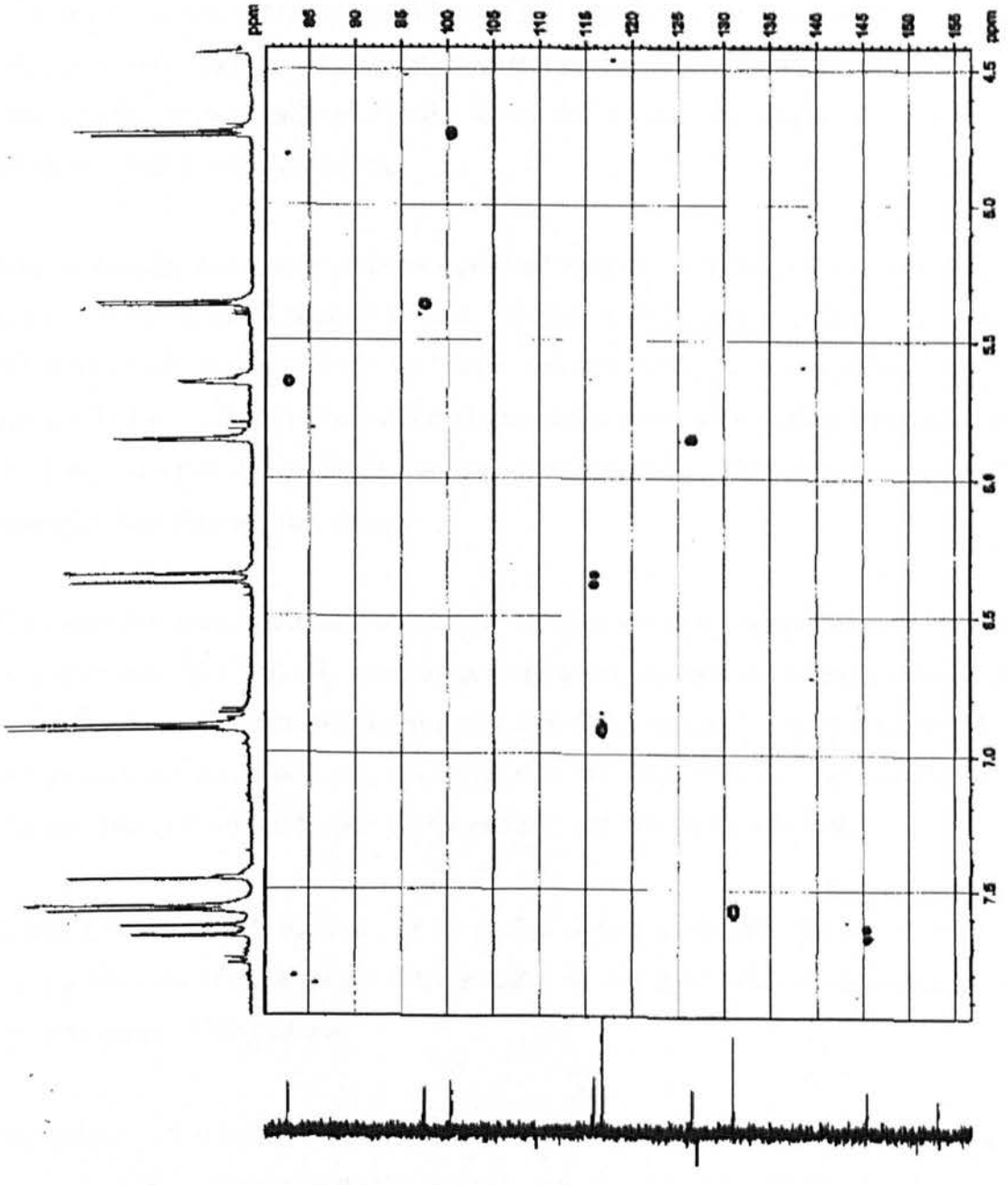


Figure 114. HSQC NMR data for fraction FEA-1, extension 90-170 ppm and 5-8 ppm.

7.4. Discussion

There were some difficulties in collecting pure compounds from the preparative column as there was poor resolution. Therefore, subfractions were manually collected from LC-MS. So, the column was connected to the MS (via an ESI interface). The tube was split into MS and a collecting point. Tube length was an important consideration and different lengths of tubes were attached to the machine in order to set the correct tube length. The optimised tube length was found to be 32 cm long.

After collection, each compound was examined back on the LC-MS to check the purity, and m/z 237 at 36.03, m/z 279 at 39.40 min, m/z 255 at 40.29 min, m/z 281 at 41.25 min, m/z 283 at 43.27 min and m/z 455 at 43.27 min, and m/z 549 at 10.92 min, m/z 327 at 7.98 min and m/z 329 at 12.28 min were collected successfully with pure quality, whereas m/z 267 at 34.78 min and m/z 241 at 38.52 min contained impurities. Therefore, for further analysis these nine subfractions were chosen.

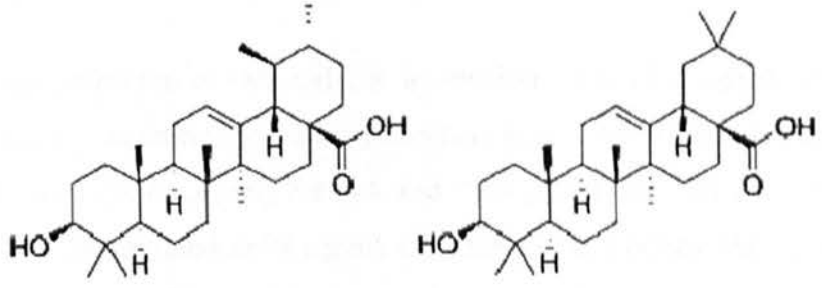
The molecular mass determination analysis was performed by high resolution-MS using 0.5 ppm quercetin (MW 302.24) solution as a reference. According to results, the determined molecular masses were 268.07 for m/z 267, 238.07 for m/z 237, 242.24 for m/z 241, 280.38 for m/z 279, 256.24 for m/z 255, 282.25 for m/z 281, 284.27 for m/z 283 and 456.36 for m/z 455, and 550.17 for m/z 549, 328.22 for m/z 327 and 330.24 for m/z 329.

Results from RP-HPLC showed that the peak that was expected as OA or UA from fraction F9, and the peak of the standard OA/UA had a similar Rt=17 min when analysing them by the same method (M6 method).

Furthermore, all samples were analysed on LC-MS using PFP(2) (250 x 4.60 mm, 5 μm) chromatographic column with M7 method, and the peak (M+455.12) eluted at 43.73 min from fraction F9 was matched to the UA peak (M+455.22) which was eluted at 43.57 min. Thus, the peak of the fraction F9 could possibly be the UA or OA.

On MS-MS with ESI ionisation at capillary temperature 400 °C, sheath gas pressure 10 mL/min and spray voltage 4500 V, the compound-fraction, OA and UA (MW=456) were ionised and detected. The compound-fraction-8 (MW=456) exhibited major peaks at m/z 455.08, 407.20, 390.82, 373.18, 359.74, 347.58, 302.83 and 159.06. The OA (MW=456)

exhibited major peaks at m/z 455.18, 407.23, 389.5, 373.14, 347.24, 305.31, 223.13 and 137.38. The UA (MW=456) exhibited major peaks at m/z 455.32, 406.88, 391.41, 373.21, 358.79, 304.89, 274.65, 223.34, 189.53 and 137.62. Therefore, this compound-fraction-8, may be UA or OA as the base peak at 407.20 and major matching fragment ions are 455.08, 390.82, 373.18, 359.74, 347.58 and 302.83, but more likely could be OA and UA together. OA and UA are isomers (see Figure 115) with similar chemical structures and therefore, they are difficult to separate, and were already isolated and identified in *Oldenlandia diffusa*. They are included in the main compounds of this herb, and have been suggested for use as marker compounds for the quality evaluation of *Oldenlandia diffusa* [Zhou *et al.*, 2002], [Luo *et al.*, 2004], [Zhang *et al.*, 2004].



Ursolic acid Oleanolic acid

Figure 115. Structure of UA and OA.

In addition other isolated compounds' (subfractions') fragment ions were studied on MS-MS and spectra are attached to Appendices (Figures A2-A9).

Furthermore, the comparison investigation of compound-fraction-8 to standards OA and UA were performed on C18 HPLC (see Figures 101-104). According to results, OA was eluted at 16.07 min, UA was eluted at 17.64 min, when they were injected separately. Then, when they were injected as a mixture, they were eluted at 16.16 min and at 17.20 min, respectively. When compound-fraction-8 was analysed, one peak was separated into two peaks (16.49 min (peak 1) and at 17.58 min (peak 2)). According to Rt, the first peak was OA and the second peak was UA.

To identify the ions of these two separated peaks in compound-fraction-8, the sample and the mixture of OA and UA were investigated on LC-MS (ESI-) (see Figures 105-106). In

compound-fraction-8 analysis, Rt of peaks were at 16.20 and 17.04, and extracted m/z 455 ion peak were at 16.11 min and 17.03 min, respectively. Whereas, on standards, Rt of peaks were at 15.83 min and 16.77 min, and extracted m/z 455 ion peak were at 15.85 min and 16.77 min, respectively. Therefore, it showed that both peaks that were separated from compound-fraction-8 are related to OA and UA.

The amounts of OA and UA in the decoction are 0.069 mg/g and 0.173 mg/g, respectively. Whereas, the amount of these acids that are determined in acetone (by sonication) extract were determined as 0.442 mg/g for OA and 1.897 mg/g for UA [Liang *et al.*, 2009]. Therefore, the amount of OA and UA appearing in the decoction is around 8 times lower than that in acetone extract.

Moreover, the concentration of OA and UA in fraction F9 is 17.2 μg/mL and 42.7 μg/mL, respectively, whereas, according to other researchers report, the IC50 value on HCT25 colon carcinoma cells were 54.72 μg/mL for OA and 27.4 μg/mL for UA [Jie *et al.*, 2002]. In addition, ED50 was determined as 72 μg/mL on HL60 for UA [Chao-Mei *et al.*, 2005]. Also, there were reported that on 10 μmol UA treatment, very effective suppressing of tumour cell growth occurred on the HM-SFME-1 cells [Hideaki *et al.*, 2008].

Some researchers examined the ability in inhibiting the tumour growth of those two triterpene acids, OA and UA. They showed remarkable inhibitory effect against implanted tumour growth with the doses of 50 and 100 mg/kg. A dose-dependent effect was also seen in the enhanced recovery of leukocytes [Hsue-Yin *et al.*, 1997]. Therefore, both OA and UA are giving the part of the anti-cancer activity of the most cytotoxic fraction of the *Oldenlandia diffusa* decoction.

In addition, according to the high resolution-MS analysis, the MW of fraction FEA-1 was determined as 550.17 and the formula is $C_{26}H_{30}O_{13}$. It was orange-yellow powder. Some researchers of Hong Kong Baptist University isolated and determined the structure of the compound with same molecular weight 550.168645 from *Oldenlandia diffusa* as an *E*-6-O-*p*-coumaroyl scandoside methyl ester [Liang *et al.*, 2006]. Therefore, the NMR assessment was performed for fraction FEA-1 and comparison results are shown in Tables 6-7.

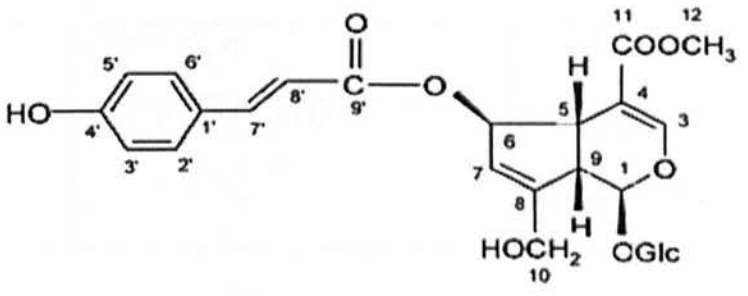


Figure 116. Structure of *E*-6-O-*p*-coumaroyl scandoside methyl ester, $C_{26}H_{30}O_{13}$ MW=550.168645 [Liang *et al.*, 2006]*.

Table 6. ^{1}H-NMR Data of *E*-6-O-*p*-coumaroyl scandoside methyl ester and fraction FEA- 1.

No	***E*-6-O-*p*-coumaroyl scandoside methyl ester***	**Fraction FEA- 1 (m/z 549 ion)**
H-1	5.30, d, *J*=6.0 Hz	5.36, d, *J*=6.0 Hz
H-3	7.50, s	7.46, s
H-5	3.20, d, *J*=9.0 Hz	3.31, m
H-6	5.67, m	5.66, m
H-7	5.85, m	5.86, m
H-9	3.11, m	3.09, m
H-10	4.39, d, *J*=15.6 Hz	4.40, d, *J*=15.6 Hz
H-10	4.22, d, *J*=15.6 Hz	4.22, d, *J*=15.6 Hz
H-12	3.65, s	3.63, s
H-2', 6'	7.46, d, *J*=8.4 Hz	7.55, d, J-8.6 Hz
H-3', 5'	6.80, d, *J*=8.4 Hz	6.90, d, *J*=8.6 Hz
H-7'	7.62, d, *J*=15.9 Hz	7.64, d, *J*=16.0 Hz
H-8'	6.33, d, *J*=15.9 Hz	6.36, d, *J*=16.0 Hz

Glc-1	4.69, d, J=7.8 Hz	4.73, d, J=7.8 Hz
Glc-2,3,4,5	3.20-3.42, m	3.26-3.45, m
Glc-6	3.88, d, J=11.1 Hz	3.82, dd, J=12, 2.3 Hz; 3.66 dd, J=12, 5.6 Hz

Table 7. ^{13}C-NMR Data of *E*-6-O-*p*-coumaroyl scandoside methyl ester and fraction FEA- 1 (MW=550).

No	***E*-6-O-*p*-coumaroyl scandoside methyl ester***	**Fraction FEA-1 (m/z 549 ion)**
C-1	97.8	97.53
C-3	153.9	153.1
C-4	109.8	109.8
C-5	42.3	41.8
C-6	83.5	82.8
C-7	127.1	126.4
C-8	150.1	150.7
C-9	46.9	46.8
C-10	60.9	60.6
C-11	168.9	167.1
C-12	52.0	51.8
C-1'	127.1	127.0
C-2', 6'	131.0	130.9
C-3', 5'	116.7	116.7
C-4'	161.0	160.8

C-7'	146.4	145.4
C-8'	115.3	115.9
C-9'	168.6	167.5
Glc-1	100.2	100.3
Glc-2	74.7	74.6
Glc-3	78.3	77.8
Glc-4	71.4	71.33
Glc-5	77.8	77.9
Glc-6	62.6	62.6

According to the ^{1}H-NMR spectrum, iridoid's main structure a proton signal of C-3 was revealed as a singlet at 7.46 (7.50 for Reference compound (Ref-comp)) (1H). Also, signals revealed at d 6.36 (6.33 for Ref-comp) and d 7.64 (7.62 for Ref-comp) with J =16 Hz (15.9 Hz for Ref-comp). Signals were at d 7.55 (7.46 for Ref-comp) and d 6.90 (6.80 for Ref-comp) J=8.6 Hz (8.4 Hz for Ref-comp). These data evident of the presence of *p*-coumaroyl group in fraction FEA-1 same as reference compound. In addition, carbomethoxy singlet was at 3.63 (3.65 for Ref-comp) (OCH3). Therefore, the structure of FEA-1 is identified (Table 6 and 7) as *E*-6-*O*-*p*-coumaroyl scandoside methyl ester.

In addition, this compound showed anticancer effect on HL60 and Caco-2 cancer cell lines (Chapter 6). Therefore, this compound is one of the anti-cancer effective compounds of decoction of *Oldenlandia diffusa*. As mentioned earlier, *E*-6-*O*-*p*-coumaroyl scandoside methyl ester which was isolated from 95% ethanol extract of *Oldenlandia diffusa* showed anti-proliferation effect on PC3 human androgen-independent prostate cancer cells a 50 μM [Li *et al.*, 2010].

7.5. Conclusion

From *Oldenlandia diffusa* decoction, 9 compounds in all, including compound-fraction-3 to 8 and fraction FEA-1 to 3 were successfully purely isolated. Their MW were determined as 267.07 (at 34.78 min), 238.07 (at 36.03), 242.24 (at 38.52 min), 280.38 (at 39.40 min), 256.24 (255 at 40.29 min), 282.25 (at 41.25 min), 284.27 (at 43.27 min), 456.36 (at 43.27 min), and 550.17 (10.92 min), 328.22 (at 7.98 min) and 330.24 (at 12.28 min).

The most cytotoxic fraction of *Oldenlandia diffusa* decoction F9 contains both of OA and UA in it with the amounts of 0.0677 mg/g and 0.1659 mg/g, respectively. These were close to levels reported as toxic in cancer cells.

E-6-*O*-*p*-coumaroyl scandoside methyl ester was isolated and identified from the decoction of *Oldenlandia diffusa* that has growth inhibition effect on HL60 and Caco-2 cancer cell lines.

Chapter 8. Study of the post-absorption sample of CHR *Oldenlandia diffusa* decoction

Chapter 8. Study of the post-absorption sample of CHR *Oldenlandia diffusa*

8.1. Introduction

Previous studies concerned the decoction of *Oldenlandia diffusa*, including the separation analysis, the investigation of its most cytotoxic contents and the cytotoxic potential of the decoction and its fractions on cancer cells *in vitro*.

In this part of the study, *Oldenlandia diffusa* decoction and its fraction F9's post-absorption samples were assessed on a created gut mimic wall using Caco-2 colon carcinoma cell line.

8.1.2. Drug absorption

The absorption, distribution, metabolism, excretion and toxicity (ADMET) are important characteristics of a pharmaceutical compound within an organism [Balani *et al.*, 2005]. From all of these steps, the most important one is absorption. It is the ability of a drug to cross the membrane barrier of the target cell from the administration point to the site of action. Drug absorption is determined by the drug's route of administration, physicochemical properties and formulation.

There are different types of dosage forms which depend on the route of administration, such as: inhaled dosage forms (aerosol, gas), oral dosage forms (solution, powder, tablet and capsule), parenteral dosage forms (injection), topical dosage forms (lotion, gel, ointment, liniment, cream, paste, powder and transdermal patch), ophthalmic dosage forms and optic dosage forms (drops), rectal dosage forms (suppository, enema) and vaginal dosage forms (tablet, pessary, intrauterine device). Drugs are formulated in different dosage forms and are consist of the drug and other ingredients to formulate and administer by various above-mentioned routes. However, drugs in the different dosage forms must be in solution to be absorbed.

The *Oldenlandia diffusa* decoction is in oral solution form. It is prepared in water and is homogenous, easier to swallow than drugs in solid form and ready for absorption.

For oral drug absorption, the main influencing factors are the physicochemical properties and physiology of the gastrointestinal tract. At first, oral drug absorption is affected by the drug properties such as drug dosage form, its dissolution, and the drug's interaction with

the aqueous environment and membrane, its permeability across membrane, as well as its irreversible removal by the intestine, liver and lung. In addition, oral drug absorption is influenced by the physiology of the gastrointestinal tract, and properties of patient [Pang, 2003]. To be absorbed, an orally administered drug has to be resistant to low gastric pH and gastrointestinal secretions such as degrading enzymes. Furthermore, absorption of oral drugs across the membranes of the epithelial cells in the gastrointestinal tract is affected by the surface area per luminal volume, blood perfusion, epithelial membranes and the presence of bile and mucus [Thanou *et al.*, 2001].

Functionally, the gastrointestinal tract is divided into four parts namely, preparative and primary storage region (mouth and stomach), secondary and absorptive region (the midgut, including, duodenum, jejunum, ileum), water reclamation system (ascending colon) and waste-product storage system (the descending and sigmoid colon regions and the rectum) [Dressman & Lennernas, 2000]. The stomach has a relatively large epithelial surface, but it has a thick mucus layer and a limited short transit time of absorption. The small intestine has the largest surface area for drug absorption in the gastrointestinal tract and its membranes are more permeable than the membranes of stomach [Naim, 1992]. Therefore, most absorption occurs in the small intestine. Before it reaches the systemic circulation, the drug must cross several cell membranes. These membranes are consists of a lipid bilayer and proteins, which determine membrane permeability characteristics. Drugs cross the cell membranes by passive and active transport processes.

8.1.3. Passive transport

Passive transport is the cellular process of moving molecules across the membranes. It does not involve any chemical energy (ATP). There are four main types of passive transport such as diffusion, facilitated diffusion, filtration and osmosis.

Diffusion

Diffusion is the movement of molecules from an area of higher concentration to an area of lower concentration. The concentration difference between the two areas is called the concentration gradient and the diffusion continues until the concentration gradient disappears. The rate of diffusion depends on the temperature, diameter of diffusing molecule, charge of diffusing molecule and the concentration of the gradient [Mottier *et al.*, 2006].

Facilitated Diffusion

Facilitated diffusion is the carrier-mediated transport which uses transport proteins embedded within the cell membrane [White *et al.*, 2001]. Large molecules move across the cell membrane on transport proteins from a higher concentration (outside the cell) to a lower concentration (inside the cell) without using any chemical energy [Mohamed *et al.*, 2002].

Filtration

Filtration is the movement of solute molecules and water across a membrane by normal cardiovascular pressure. The passing solute molecules should fit in the size of the membrane pores.

8.1.4. Active transport

Active transport is the movement of molecules across the membrane resulting directly from the expenditure of metabolic energy and transport against a concentration gradient [Swarbick & Boylan, 2002].

A schematic of passive and active transports are shown in Figure 117.

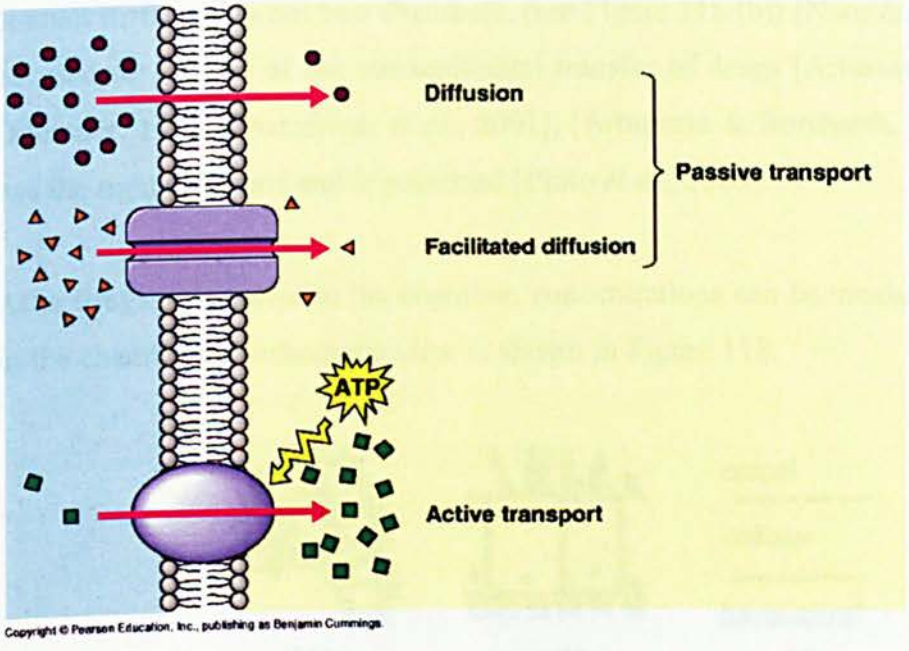


Figure 117. Schematic of passive and active transports mechanisms.

[Taken from http://www.college-cram.com/study/biology/presentations/38]

8.1.5. The use of Caco-2 cell line for mimicking the intestinal epithelia

Cell culture models can predict oral drug absorption. The absorption can occur through passive transcellular or paracellular diffusion, active carrier transport or active efflux mechanisms. The most commonly used method assesses potential intestinal permeability is the measurement of compound flux across a monolayer of Caco-2 cells. Dr. Jorgen Fogh and co-workers [Fogh & Trempe, 1975], [Fogh *et al.*, 1977] have established that the Caco-2 cell line exhibits morphological and functional characteristics of small intestinal cells. Also, it is proven by other researchers that Caco-2 cells are useful in this type of study, because these cells express various membrane transporters relevant to drug absorption [Smith & O'Donell, 2006]. The cells expresses typical small-intestinal microvillus hydrolases and nutrient transporters, as well as it shows well-differentiated brush border on the apical surface and tight junctions [Pinto *et al.*, 1983]. Therefore, the Caco-2 cell line is the most popular *in vitro* model and used for elucidation of the pathways of drug transport, determination of the optimal physicochemical characteristics for passive diffusion of drugs, assessment of the formulation to enhance membrane permeability and its toxic effects of drug on the biological barrier [Meunier *et al.*, 1995].

Also, when Caco-2 cells are cultivated as monolayers on permeable filters, they form a contact surface between two chambers, (see Figure 118 (b)) [Niko & Raul, 2009] and it can be used for studies of the transepithelial transfer of drugs [Artursson & Karlsson, 1991], [Meunier, 1995], [Artursonn *et al.*, 2001], [Artursson & Borchardt, 1997]. The monolayer has the tight junctions and is polarized [Pinto *et al.*, 1983].

After drugs are applied to the chamber, concentrations can be measured by the time period in the chambers. A schematic view is shown in Figure 118.

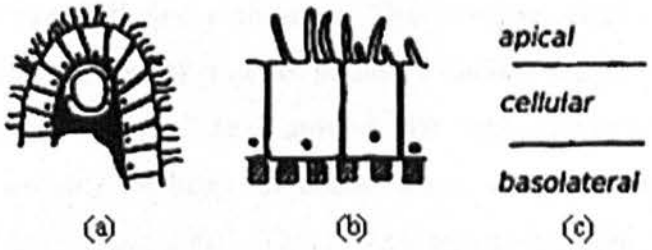


Figure 118. Schematic view of intestinal wall with microvilli (a), an epithelial cell monolayer grown on a permeable support in a cell culture insert (b), and schematic model of monolayer growing chambers (c) [Adopted from Komin & Toral, 2009].

8.1.6. Permeability pathways

As mentioned earlier, cultured Caco-2 cells on filters achieve a monolayer density and exhibit morphological characteristics similar to enterocytes such as tight intercellular junctions and highly developed microvilli (Figure 119). The majority of drugs cross the biological barriers by a passive diffusion mechanism. Some compounds, e.g. di- and tripeptides, use active transport systems [Camenisch *et al.*, 1997].

There are two possible pathways for permeation:

1- The paracellular route (Across tight junctions between epithelial cells).

2- The transcellular route (Across the plasma membrane of the epithelial cells)

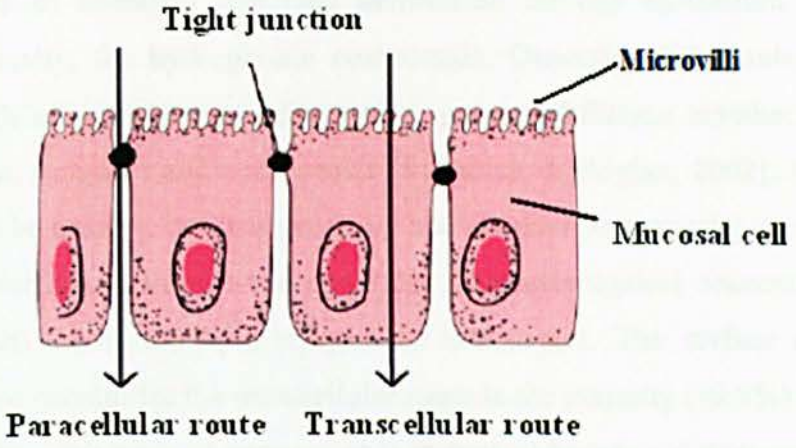


Figure 119. Schematic of paracellular and transcellular route of absorption.
[Adopted from http://www.life-enthusiast.com/condition/liver/images/intestinal_permeability.gif]

1. The paracellular route

Paracellular transport is a passive diffusional transport pathway. It occurs between the cells according to concentration gradients, electrical potential and hydrostatic pressure between the two sides of the epithelial membrane. The junctions between cells are leaky and it allows paracellular transport of water or solutes without passage through the epithelial cells themselves. It can access small (e.g. around MW 200) hydrophilic molecules. Therefore, this route is not suitable for large molecular drugs, which are too large to cross between cell junctions [Hillary *et al.*, 2001]. This route refers to the tight junction between the cells [Tanaka *et al.*, 1995]. So, the barrier restricting the passive movement of solutes through the paracellular pathway is the tight junction.

Passive permeation through a biological membrane depends on physicochemical properties, such as lipophilicity, polarity and molecular size [Gian *et al.*, 1997]. For example, negative charges at the membrane near the paracellular route supply an ion-selective permeation [Conradi *et al.*, 1996]. The surface area of the tight junctions represents only 0.01% of the total surface area of the intestinal epithelial layers. Generally, if the permeability of compound is limited to the paracellular pathway, the amount of absorption of compounds is low, and if the compound is readily permeable through the paracellular pathway, the amount of absorption of compounds is high.

2. The transcellular route

The main route of intestinal epithelial permeation through epithelium is transcellular transport, especially, for hydrophobic compounds. Generally, this route involves three processes which are simple passive transport, passive diffusion together with an efflux pump and active transport and endocytosis [Swarbick & Boylan, 2002]. So, transcellular transport is can be passive, but it is generally active which is requiring energy and cellular facilities. Transcellular transport can move the molecules against concentration gradients (active transport) across the lipid bilayers of enterocytes. The surface area of the cell membrane which constitutes the transcellular route is the majority (99.9%) of the epithelial surface area. Thus, this route of permeation is important because of its magnitude.

Therefore, the human intestinal epithelial cell line Caco-2 has been recommended for such biological *in vitro* studies, since these cells express various biological membrane properties, including enzymatic and transporter systems [Arturrson & Karlsson, 1991], [Hillgren *et al.*, 1995].

8.1.7. A Transepithelial electrical resistance measurement

A method for the permeability *in vitro* includes the measurement of electrical resistance to determine the transepithelial electrical resistance (TEER) [Trine *et al.*, 2005].

Ion transport and ionic equilibrium across cell membranes are physiological processes and essential in all living organisms. Mammalian epithelial cells possess polarized plasma membrane surfaces, and cell-to-cell tight junctions prevent movement of solutes and ions across the epithelium.

A TEER is a measure of the movement of ions across the paracellular pathway. Measurement of a TEER of cells grown on permeable membranes can give an indirect assessment of tight junction establishment and its stability [Shaw, 2002], [Pedemonte, 1995]. Because the epithelium separates cellular compartments with very different fluid composition and the maintenance of its stability and electrical resistance is important for essential physiological processes. Therefore, changes in a TEER can represent an early damage of cell [Carlos, 1997]. The confluence of the cellular monolayer is quickly determined by a sharp increase in a TEER. In this study, the manual EVOM-Epithelial voltohmmeter (Figure 120) which was introduced from Millipore was used in order to measure the health and quantitative confluence of Caco-2 cell monolayer by measuring electric resistance.

Figure 120. EVOM-Epithelial voltohmmeter. [Taken from http://www.pharmaceutical-int.com/categories/teer-measurement/trans-epithelial-electric-resistance-teer-measurements.asp]

Epithelial voltohmmeter is used for a TEER measurement of epithelial cells in 24 and 96 well plates. Before taking the resistance measurement using the epithelial voltohmmeter, the electrode has to be immersed in electrolyte solution (e.g. 0.1-0.15 M KCl) and then let to equilibrate. Then, the electrodes have to be sterilized by immersing it in 70% methanol for 20 minutes. To take the measurements, electrodes have to be placed in the sample well.

The blank resistance must be measured and then subtracted from the resistance reading across tissue in order to obtain the true tissue resistance [WPI, 2000]. A TEER measurement is the most convenient, reliable and non-destructive method to evaluate and monitor the growth of epithelial cell cultures *in vitro*.

8.2. Method and materials

8.2.1. Materials

Epithelial voltohmmeter was purchased from World Precision Instruments, Inc, Florida, USA. Hank's balanced salt solution, 2-(4-morpholino)-ethane sulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), CyQUANT NF assay kit, trypan blue and UV quartz cuvette were purchased from Sigma-Aldrich Inc, Poole, UK. 0.20 μm and 0.45 μm sterile filters were (Nalgene, Hereford, UK), UV spectrometer, Varian, UK, C18 SPE Fisher Scientific, UK. Chemicals such as methanol, acetonitrile (HPLC grade), hexane ethyl acetate, dichloromethane, n-butanol (analytical grade), and extractor were supplied by Fisher Scientific Inc, UK, and ammonium acetate (HPLC grade) was purchased from Sigma-Aldrich Inc, Poole, UK. The others were as mentioned in Chapter 4 and 7.

8.2.2. Methods

8.2.2.1. Sample preparation

Preparation of Oldenlandia diffusa decoction

A decoction of *Oldenlandia diffusa* was prepared as mentioned in Chapter 2.

Preparation of transport buffers

Transport buffer Hank's balanced salt solution (HBSS) was prepared dissolving 9.8 g of salt in 1 L deionised water. From this buffer different pHs of buffers were prepared by adding: 10 mM MES for HBSS-MES (pH=6) and 25 mM HEPES for HBSS-HEPES (pH=7.4). All prepared buffers were sterilised by filtering through 0.20 μm sterile filter.

8.2.2.2. Cell culture

Caco-2 cells were grown in complete growth medium DMEM containing 20% FBS [Berherns & Kissel, 2003], 5 mM L-glutamine and 2 mM non-essential amino acids at 37 °C in an atmosphere containing 5% CO_2. Cells were subcultured every 5-6 days. In each monolayer building experiment, the same passage numbers of growing cells were used.

8.2.2.3. Growth rate study of Caco-2 cells

Growth inhibition investigation of Caco-2 cells was continued for 7 days. Cells were seeded onto 6 well plates with 2.0 x 10^5 cells/mL density and every 24 hours 1 well of each plate was investigated by trypan blue exclusion method.

8.2.2.4. Creation of Caco-2 cell monolayers

Caco-2 cells ($2x10^5$ cells) were seeded onto three wells of each 6 well plates with collagen-coated insert (24 mm in diameter, 4.7 cm^2 with growth area and 0.4 μm pore size), and monolayers were grown up to 21 and 28 days. To check the integrity of the monolayer, TEER measurements were taken every week. Experiments were done five times.

8.2.2.5. TEER measurement

TEER measurements were taken on 2^{nd}, 7^{th}, 14^{th}, 21^{st} and 28^{th} days from each well using the epithelial voltohmmeter. Before take the resistance measurement, the voltammeter's performance availability was checked. Its value was 1000 Ω. Then zero was adjusted. Before use the electrodes, they were equilibrated by immersing in electrolyte solution (0.15 M KCl). After that, the electrodes were immersed in 70% methanol for 20 minutes in order to be sterilized, and then equilibrated in medium for 15 minutes.

For resistance measurement, electrodes were placed in sample well, and kept in stable position until the measurement was obtained. From each well there were taken three measurements from three different positions. The electrodes were sterilized in 70% methanol for 20 minutes and equilibrated with medium for 15 minutes for subsequent measurements.

8.2.2.6. Cytotoxic effect of *Oldenlandia diffusa* decoction on the created Caco-2 monolayer

Different concentrations (0.75 mg/mL-3.5 mg/mL) of the decoction were investigated at 30, 60, 120 and 180 min time periods on Caco-2 monolayers, in order to study their cytotoxic effect on monolayers. Cell viability was determined using MTT assay. Experiments were done three times.

8.2.2.7. The post-absorption sample collection from the decoction and fraction F9 via the created Caco-2 monolayers.

When creation process for Caco-2 monolayer finished, all old medium were removed and inserts were washed twice with warm PBS. After that, freshly prepared buffer HBSS with MES was added to apical side and HBSS with HEPES was added to basal side of each well and equilibrated for 30 min. After equilibration, different concentrations of decoctions were added to the apical side of wells which have created monolayers, and buffer was

added to the control wells. Final volumes kept in each well were 1500 µL and 2500 µL in apical and basal chambers, respectively [Markowska *et al.*, 2001].

Post-absorption samples from the decoction and the fraction F9 were collected after 30, 60, 120 and 180 min time intervals from the basal chambers. Samples were kept at -20 °C for next analysis.

8.2.2.8. Determination of concentrations of the collected post-absorption samples

Concentrations of the collected post-absorption samples were determined using a UV spectrometer. Each sample collected from each chamber was placed in UV cell and measured against water.

8.2.2.9. The post-absorption sample collection of *Oldenlandia diffusa* decoction via cultured Caco-2 monolayers for its cytotoxicity study

The post-absorption samples (PAS) were collected from 2.5 mg/mL of decoction at 30, 60, 120 and 180 min time intervals using 21 day built monolayers. Cytotoxcity was examined on HL60 (human promyelotic leukaemia) cell line using the CyQUANT NF assay.

HL60 cells were grown in RPMI 1640 medium and were seeded to 96 well plates with $1x10^4$ cells/well density. Collected post-absorption samples of *Oldenlandia diffusa* decoction (0.5 mg/mL) and controls were added to HL60 cells and incubated for 24 hours at 37 °C in an atmosphere containing 5% CO_2.

After the incubation, cells were transferred to eppendorf tubes and were sedimented by centrifugation at 300 x g for 7 minutes. Then, the supernatant was removed and the cells resuspended in 100 µL of CyQUANT NF assay kit. The 96 well microplate was covered and incubated at 37 °C for 45 minutes as this incubation period is required for equilibration of dye-DNA binding. After that, the fluorescence intensity of each sample was measured using a fluorescence microplate reader with excitation at 485 nm and emission detection at 530 nm. Fluorescence measurements were performed at ambient temperature.

8.2.2.10. Content(s) of the most cytotoxic compounds in the post-absorption sample

In order to check the possible content of the most cytotoxic compounds in post-absorption samples, it was investigated on LC-MS using PFP(2) (250x4.60 mm, 5 µm)

chromatographic column, methanol (A)/10 mM ammonium acetate buffer (B) as a mobile phase with 50% B for 0-10 min, 100% A at 50 min, 50% A 55-60 min (M7 method), at 210 nm wavelength.

8.3. Results

8.3.1. Growth rate study of Caco-2 cells

For growth rate study, Caco-2 cells were incubated onto 6 well plates with $2.0x10^5$ cells/mL density for six days, and each day one well of each plate was investigated using trypan blue exclusion method. Results are followed:

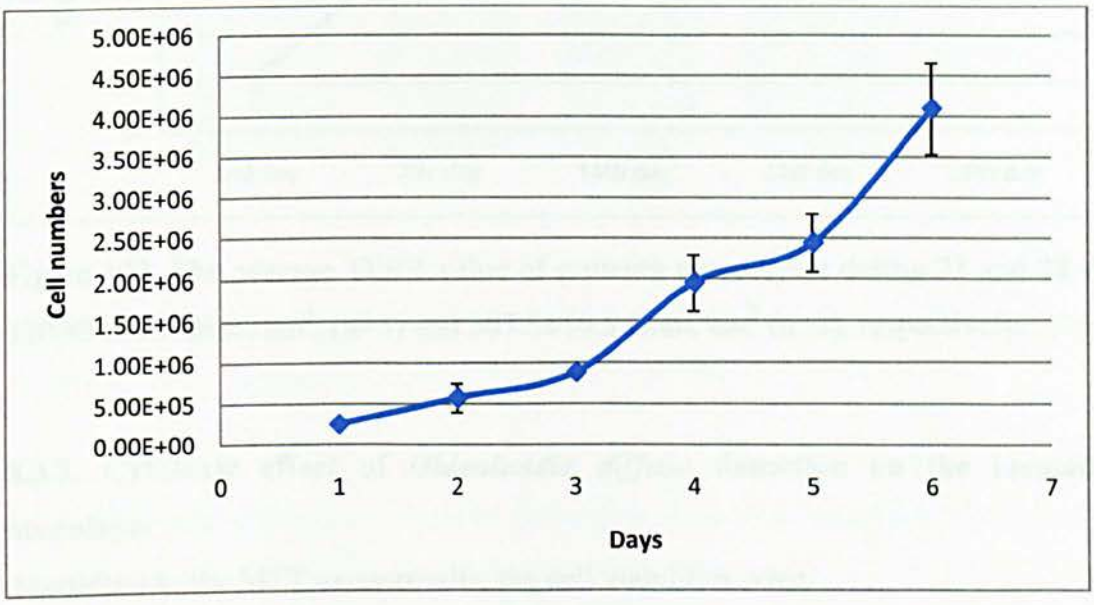


Figure 121. Growth of Caco-2 cell for 6 days, by cell numbers.

Growth rates were 1 for Day-1, 3.88 for Day-2, 1.54 for Day-3, 2.21 for Day-4, 1.25 for Day-5 and 1.68 for Day-6.

8.3.2. Creation of gut mimicking wall (monolayer) using Caco-2 cells

Gut mimic wall was created using Caco-2 cells ($2x10^5$ cells/well) by seeding onto 6 well plates with collagen-coated insert. Cells were growing for 21 and 28 days. During this period to check the integrity of the monolayer, TEER measurements were taken after 2, 7, 14, 21 and 28 days. Results for TEER measurement for gut mimic wall integrity are below:

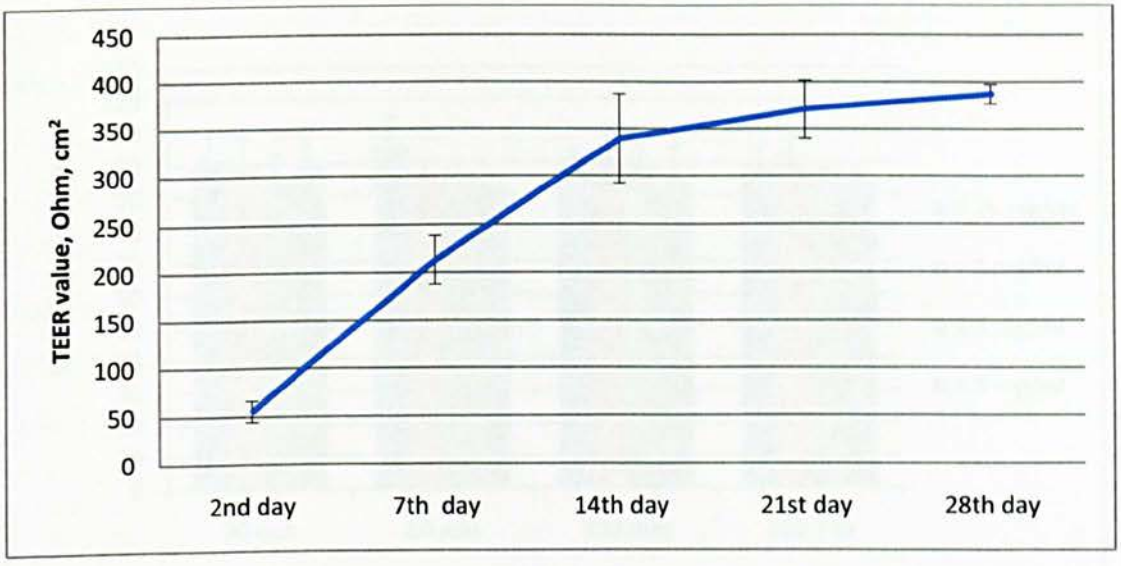


Figure 122. The average TEER value of growing monolayers during 21 and 28 days were 370.85±30.7 Ohm. cm^2, (n=5) and 387.5±10.3 Ohm. cm^2 (n=3), respectively.

8.3.3. Cytotoxic effect of *Oldenlandia diffusa* decoction on the created Caco-2 monolayer

According to the MTT assay results, the cell viabilities were:

- For 0.75 mg/mL decoction: V=101.33±8.26% (30 min), V=105.20±9.43% (60 min), V=100.88±3.38% (120 min), V=99.68±4.94% (180 min);
- For 1.5 mg/mL decoction: V=98.53±9.94 (30 min), V=97.69±3.83% (60 min), V=99.81±9.55% (120 min), V=102.39±6.45% (180 min);
- For 2.5 mg/mL decoction: V=97.65±6.18% (30 min), V=97.34±3.63% (60 min), V=98.87±9.55% (120 min), V=97.86±4.40% (180 min);
- For 3.5 mg/mL decoction: V=98.26±14.55% (30 min), V=93.05±7.36% (60 min), V=94.78±10.21% (120 min) and V=94.56±5.15% (180 min).

Generally, cell viability of Caco-2 ranged from V=94.56±3.92% to 105.7±6.45%, n=3 for 0.75 mg/mL-3.5 mg/mL concentrations of the decoctions at 30, 60, 120 and 180 min time intervals on Caco-2 cells (see Figure 123).

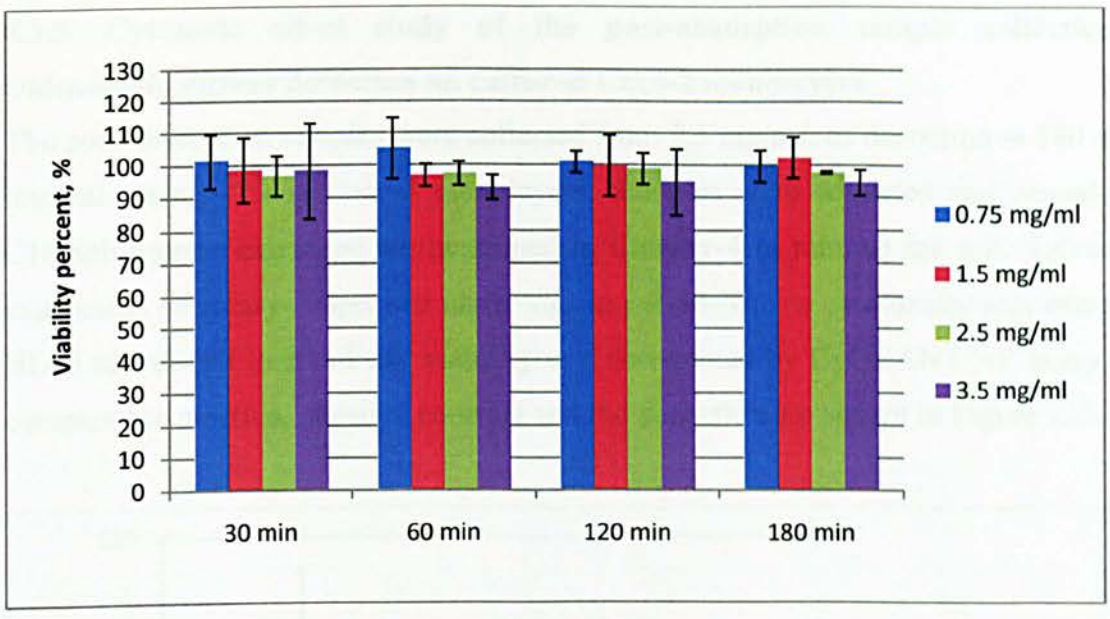


Figure 123. Cytotoxicity of different concentrations of decoction at different time intervals on created monolayers. V=94.56±3.92%-105.7±6.45%, n=3.

8.3.4. The post-absorption sample collection from the decoction via created Caco-2 monolayers and their concentration determination

Post absorption samples were collected from 2.5 mg/mL concentration of the decoction at 30, 60, 120 and 180 min time periods using 21 day created monolayers. After collecting, concentrations of those samples were determined by UV spectrometer and sum of the results is shown in Figure 124.

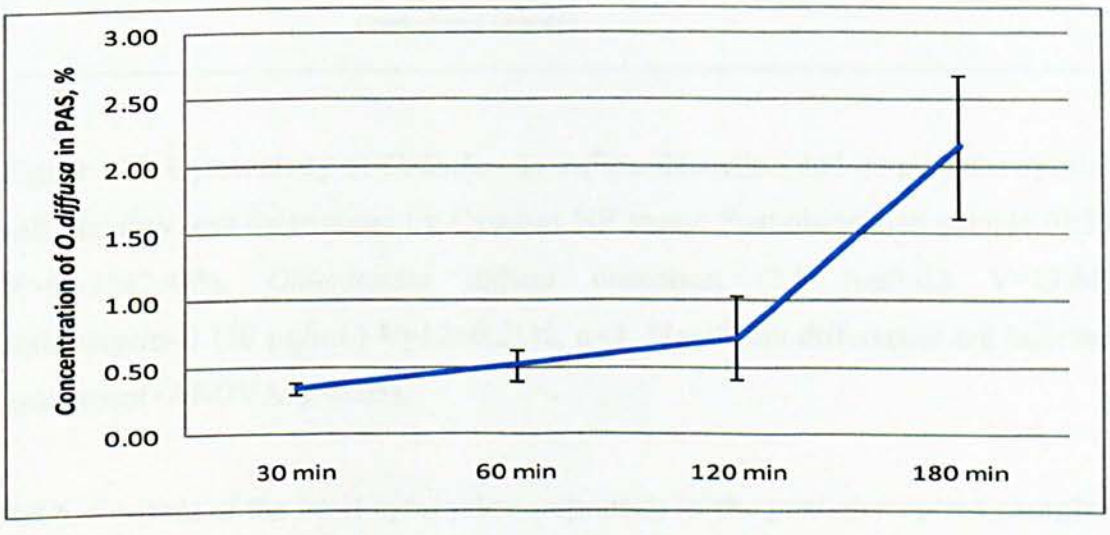


Figure 124. The average concentration of the post-absorption samples of 2.5 mg/mL concentration of *Oldenlandia diffusa* decoction at 30, 60, 120 and 180 min time intervals, n=4.

8.3.5. Cytotoxic effect study of the post-absorption sample collection from *Oldenlandia diffusa* decoction on cultured Caco-2 monolayers

The post-absorption samples were collected from 2.5 mg/mL of decoction at 180 min time intervals using 21 day Caco-2 monolayers. Samples were collected and passed through C18 solid phase extraction as mentioned in Chapter-4 to remove the salt. Solvents were evaporated on rotary-evaporator under vacuum at 40 °C. Its cytotoxcity was examined on HL60 cancer cell line and cell viability was determined by CyQUANT NF assay. Results compared to positive, negative controls and the decoction are shown in Figure 125.

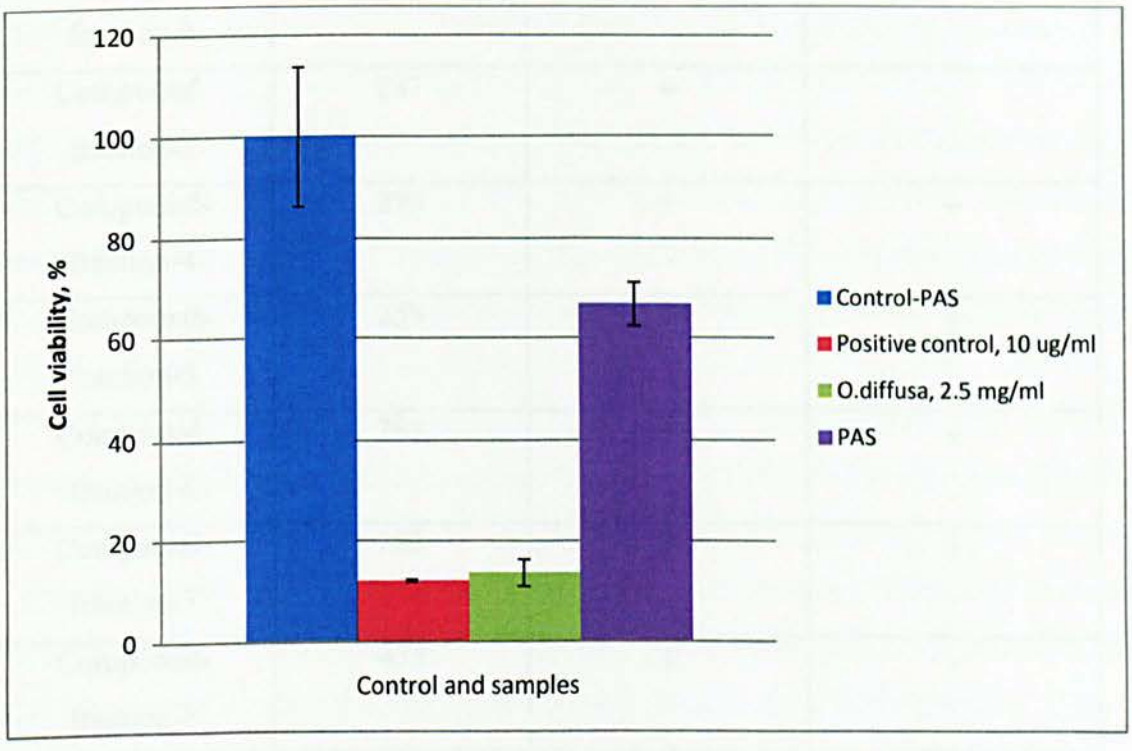


Figure 125. Cytotoxicity of *Oldenlandia diffusa* decoction and its post-absorption sample; cell viability was determined by Cyquant NF assay: Post-absorption sample (0.5 mg/mL) V=66.97±7.46%, *Oldenlandia diffusa* decoction (2.5 mg/mL) V=13.65±2.71%, actinomycin-D (10 µg/mL) V=12±0.21%, n=3. Significant differences are indicated in this assessment (ANOVA, $p<0.05$).

8.3.6. Content of the most cytotoxic compounds in the post-absorption sample

To check the possible content of the most cytotoxic compounds in the post-absorption sample of the decoction and fraction F9, sample was investigated on LC-MS using M7 method. Results are shown in Table 8 and Figures A10-A21 (Appendix).

Table 8. m/z of compounds in the post-absorption sample of the *Oldenlandia diffusa* decoction and fraction F9.

Subfractions	**Isolated ions, m/z**	**In PAS of decoction**	**In PAS of fraction F9**
Compound-fraction-1	241	+	+
Compound-fraction-2	267	-	-
Compound-fraction-3	237	+	-
Compound-fraction-4	279	+	+
Compound-fraction-5	255	+	+
Compound-fraction-6	281	+	+
Compound-fraction-7	283	+	+
Compound-fraction-8	455	+	-
FEA-1	549	+	-
FEA-2	327	+	-
FEA-3	329	+	-

8.4. Discussion

In order to use the Caco-2 cells for creation of the gut mimic wall (monolayer), its growth inhibition was investigated by incubating $2.0x10^5$ cells/mL densities of cells on 6 well plates for seven days. Cell numbers were determined using trypan blue exclusion method [Lodish *et al.*, 2001], [Doyle & Griffiths, 2001], [Shaw, 1996], [Sian *et al.*, 1990].

According to results, Caco-2 cells were growing exponentially in 20% FBS and 5 mM L-glutamine containing complete medium as the cell numbers were increased from $1.52x10^5 \pm 7.07x10^3$ (Day-1) to $3.69x10^6 \pm 5.59x10^5$ (Day-7). The growth rates were 1 for Day-1, 3.88 for Day-2, 1.54 for Day-3, 2.21 for Day-4, 1.25 for Day-5 and 1.68 for Day-6.

As growth rate of Caco-2 cells were exponential, this cell line was used for creation of the gut mimic wall. For this, Caco-2 cells ($2x10^5$ cells/well) were grown as a monolayer on 6 well plates with collagen-coated insert up to 21 and 28 days, however, in most other publications mentioned that the monolayer is created for 21 days [Doyle & Griffiths, 2001], [Shaw, 1996].

In order to check the health and integrity of cells, its TEER measurements were taken on 2^{nd}, 7^{th}, 21^{st} and 28^{th} days (Figure 122). TEER measurements until 14^{th} day were sharply increased to 339.68±47.69 Ohm.cm^2, then steady increased to 370.85±30.7 Ohm.cm^2, n=5 and 387.5±10.3 Ohm.cm^2, n=3, on 21^{st} and 28^{th} days, respectively. So, this shows that it is not necessary to let the monolayer grow up to 28 day, the 21 day growing is the optimal period for monolayer creation.

In addition, if the TEER results are above 300 x cm^2, this means the microparticles are non-toxic to the cells [Beck *et al.*, 2007]. In these experiments, TEER measurements were 370.85±30.7 Ohm.cm^2 after 21 day growing, thus it suggests that these monolayers can be used for post absorption sample collecting experiments.

Before using these created Caco-2 monolayers for post-absorption sample collection, it was necessary to check the cytotoxic effect of the *Oldenlandia diffusa* decoction on it. For that, 0.75 mg/mL, 1.5 mg/mL, 2.5 mg/mL and 3.5 mg/mL concentrations of the decoction were investigated at 30, 60, 120 and 180 min time intervals on Caco-2 cells. These times

were the periods which the post-absorption samples will be collected via the Caco-2 monolayers.

The results of MTT assay showed that the cell viabilities of Caco-2 cells which were exposed to 0.75 mg/mL-3.5 mg/mL concentrations of the decoctions at 30, 60, 120 and 180 min time intervals ranged from V=94.56±3.92% to 105.7±6.45%, n=3 (see Figure 123). The cell viability of 3.5 mg/mL concentrations were generally lower than the other concentrations of decoction, however, no considerably lower cell viability occurred in all those concentrations at all the time intervals. Therefore, 2.5 mg/mL concentration of decoction was chosen for further analysis.

Once the gut mimic wall (monolayer) was created and the loading concentration of decoction was chosen, the collecting time period needed to be determined in order to obtain highest possible amount of the post-absorption samples.

Post-absorption samples were collected from the 2.5 mg/mL decoction after 30, 60, 120 and 180 min time intervals from the Caco-2 monolayer. The concentration of each post absorption sample collected at each time point was determined by UV spectrometer. The highest concentration occurred was after 180 min with 2.15±0.54% (see Figure 124). So, the post-absorption sample can be collected from 2.5 mg/mL decoction at 180 min time intervals.

Apparent permeability coefficient (centimeters/second) (P_{app}) was calculated by the following equation

$$P_{app}= V_R x(dC/dt)x(1/AC_0) \quad (4) \quad \text{[Beck } et\ al.\text{, 2007]}$$

V_R- volume of receiver chamber

dC/dt- cumulative concentration of the compound in the receiver chamber over time

A- surface area of membrane

C_0- initial concentration of compound in donor chamber

So,

P_{app}=2.6 ml x (0.17 mg/mL/10800 sec) x (1/4.7 cm^2x2.5 mg/mL) = =3.48x10^{-6} cm/s

There are comparable values for apparent permeability coefficient. If $P_{app} > 1x10^{-6}$ cm/s, it means good permeability, if $P_{app} < 1x10^{-6}$ cm/s, this means low permeability [Beck *et al.*, 2007]. Therefore, if we compare these values to our result, $3.48x10^{-6}$ is more than $1x10^{-6}$ cm/s which means it had a good permeability at intestinal pH.

Furthermore, collected post absorption sample's cytotoxic effect was investigated on HL60 cancer cell line by comparing to positive, negative controls and *Oldenlandia diffusa* decoction. Cell viabilities were determined by CyQUANT NF assay and viability percents were for the post-absorption sample (0.5 mg/mL) V=66.97±7.46%, for *Oldenlandia diffusa* decoction (2.5 mg/mL) V=13.65±2.71%, and for actinomycin-D (10 μg/mL) V=12±0.21%, n=3 (see Figure 125). Thus, the post-absorption sample has a cytotoxic effect on HL60 cell line.

In previous studies, the most cytotoxic compounds in the decoction and fraction F9 were studied. In this part of the study, we investigated that are these most cytotoxic contents remained in it in the same form or as a metabolite. For that reason, collected post-absorption samples from the decoction and fraction F9 were analysed on LC-MS using the same method which had been established method for separation of fraction F9. The most cytotoxic contents which were determined in fraction F9, including m/z 267, 237, 241, 279, 255, 281, 283 and m/z 455 were searched in the post-absorption samples collected from the decoction and the fraction F9. Results showed that in the post-absorption sample from the decoction, there were ten ions such as m/z 283, 242, 279, 255, 281, 455, 237, 327, 329 and 549 detected, while from the post-absorption sample collected from the fraction F9 five ions were detected, including m/z 279, 255, 283, 281 and 241. Some ions were not detected in both post-absorption samples from the decoction and the fraction F9, and they were m/z 267, 327 and 267, 237 ions, respectively.

Also, the amount of OA and UA that transferred through the gut mimicking wall (Caco-2 monolayers) were calculated and were 0.0689 μg/mL for OA and 0.1729 μg/mL for UA, respectively. Therefore, this suggests cytotoxic amounts of bioavailable OA and UA are less than that in the decoction.

8.5. Conclusion

Caco-2 cells were growing exponentially as the cell numbers were increased from $1.52x10^5 \pm 7.07x10^3$ (Day-1) to $3.69x10^6 \pm 5.59x10^5$ (Day-7).

For the post-absorption sample collection of the decoction, monolayers were successfully created using Caco-2 cells on collagen-coated insert for 21 days (TEER=370.85±30.7 Ohm. cm^2, n=5).

Also, during the post-absorption sample collection, up to 3.5 mg/mL concentration of *Oldenlandia diffusa* decoction will not destroy the created monolayers (V=94.56±3.92%-105.7±6.45%, n=3). The highest post-absorption sample concentration (2.15±0.54%) of 2.5 mg/mL decoction was obtained at 180 min time interval. It has a good permeability (P_{app}=3.575xE-06 cm/s). So, the post-absorption samples can be collected from 2.5 mg/mL decoction after 180 min using the monolayers which grew Caco-2 cells on collagen-coated insert for 21 days.

In addition, the collected post-absorption sample of *Oldenlandia diffusa* decoction has cytotoxic effect on HL60 cells (V=66.97±7.46%, n=3).

The amount of OA and UA that transferred through the gut mimicking wall were calculated and this corresponds to a concentration of OA 0.0689 μg/mL and UA 0.1729 μg/mL, respectively.

The post-absorption samples of the decoction and the fraction F9 contain most of the ions which were detected in *Oldenlandia diffusa* decoction and its ethyl acetate extract (fraction F9) by LC-MS. The detected ions were m/z 283, 241, 279, 255, 281, 455, 237, 327, 329 and 549 in the post-absorption sample of the decoction, and m/z 279, 255, 283, 281 and 241 in the post-absorption sample of the fraction F9.

Chapter 9. General Discussion

Chapter 9. General discussion

9.1. General discussion

Oldenlandia diffusa is one of the most popular CHR used in complementary anti-cancer treatment and it is officially listed in the Chinese Pharmacopoeia [Pharmacopoeia of China, 2005], [Li *et al.*, 1986], [Islam *et al.*, 2009]. This herb has been studied by number of researchers. Some researchers have reported that *Oldenlandia diffusa* has antitumor, immunomodulatory [Shan *et al.*, 2001], [Yoshida *et al.*, 1997], [Chung *et al.*, 2002], anti-inflammatory, hepatoprotective [Lin *et al.*, 2002], anti-oxidative [Lu *et al.*, 2000], [Kim *et al.*, 2005] and neuroprotective [Kim *et al.*, 2001] activities. Its antitumor activity has been identified as the main pharmacological effect [Kim *et al.*, 1998], [Wong *et al.*, 1996], [Shan *et al.*, 2001], [Gupta *et al.*, 2004], [Li *et al.*, 2002].

Thus, the aim of this investigation was to separate and determine the most cytotoxic constituents of the decoction and study the bioavailability. This chapter focused upon relating the results obtained in this investigation to the objectives of the project.

An initial objective of this study was to set up the separation method for *Oldenlandia diffusa* decoction using HPLC. For assessments of the cytotoxic potential of the decoction and its responsible contents, the separation method was set up by investigating parameters (columns, mobile phase, isocratic and gradient elution, flow rate, pH of buffer and detection wavelength, etc) using HPLC. The method that was set up for the separation of *Oldenlandia diffusa* decoction was:- PFP(2) (250 x 4.60 mm, 5 µm) column, methanol (A)/10 mM ammonium acetate buffer (pH=6.5) (B) as a mobile phase with B 100% for 0-10 min, A 100% for 40-50 min, B 100% for 52-60 min gradient elution at 1 mL/min flow rate and 226 nm (Ms method). Using this method, eleven fractions (F1-F11) were manually collected from a preparative HPLC at various time intervals.

The next objective was to assess the direct cytotoxic potential of the decoction and collected fractions against cancer cells using an *in vitro* system and then to select the most cytotoxic fraction. In order not to give an additional effect to cell cytotoxicity, collected fractions were passed through the C18 SPE and freeze-dried to remove the salt. Then, dried decoction and fractions were dissolved in a medium for cancer cell cytotoxicity investigations on HL60 (leukaemic) and Caco-2 (colonic carcinoma) cancer cell lines by

comparison to positive and negative controls using trypan blue exclusion method, CyQUANT NF cell proliferation assay and neutral red uptake assay. Results showed that the *Oldenlandia diffusa* decoction had a cytotoxic effect on both cancer cell lines. From the collected fractions, F3-F9 fractions showed low growth inhibition effects on HL60 and Caco-2 cancer cell lines, but the fraction F9 had the most cytotoxic activity. However, some other researchers studied the anti-cancer effect of the aqueous extract of *Oldenlandia diffusa* on the HL60 leukaemic cell line, and then compared this effect to primary human blood lymphocytes (PBLs). It was shown that the aqueous extract of *Oldenlandia diffusa* was found to be toxic to the HL60 cell line, inducing apoptosis via some form of activity specific to these cells [Willimott *et al.*, 2007].
Anti-cancer activity of the decoction on Caco-2 cells is for the first time revealed by our study.

Cytotoxicity assays cannot distinguish between necrosis and apoptosis. Therefore, the next objective was to characterize the chemotherapeutic potential of *Oldenlandia diffusa* decoction and the most cytotoxic fraction against the cancer cells. To characterize the cancer mode of cells death, the decoction and chosen fraction's ability to induce the apoptosis in cancer cells was examined using DAPI staining on confocal fluorescence microscopy (morphological) and Western blotting (detection of cleaved-PARP). There are several pathways of caspases to proceed such as from death receptors through caspase-8 to caspase-3 and then to caspase-6 [Takahashi *et al.*, 1997], [Stennicke *et al.*, 1998], from the cytochrome *c* through caspase-9 to caspases-3 and caspases-7 and then to caspase-6 [Srinivasula *et al.*, 1998], [Kuida *et al.*, 1998], [Srinivasula *et al.*, 1996] and caspase-6 can activate caspase-3 [Srinivasula *et al.*, 1996], [Liu *et al.*, 1996]. Some caspases e.g. caspase-3 and caspase-7 can both cleave PARP [Deveraux *et al.*, 1998]. Therefore, untreated and decoction, chosen fraction and positive control treated HL60 and Caco-2 cells were assessed after 24 and 48 hours incubations. Results provided the evidence that HL60 and Caco-2 cancer cells exposed to *Oldenlandia diffusa* decoction and its fraction F9 die by apoptosis which is mediated by a caspase cascade.

Once it was revealed that the decoction and the most cytotoxic fraction F9 have cytotoxic effects which induce apoptosis, the investigation was directed to fraction F9. To determine the constituents of the fraction F9, the fifth objective was to set up the separation method for it using HPLC. Again, different parameters were examined namely, different types of

chromatographic columns, flow rates, different wavelengths, isocratic and gradient elutions of different mixtures of mobile phases and some methods were developed. The separation method which was set up for fraction F9 contents was:- PFP(2) (250 x 4.60 mm, 5 μm) chromatographic column, methanol (A)/10 mM ammonium acetate buffer (B) as a mobile phase with 50% B for 0-10 min, 100% A at 50 min, 50% A 55-60 min with flow rate 1 mL/min, at 210 nm (M7 method).

During this assessment period, a greater amount of fraction F9 was needed. Therefore, fraction collecting HPLC method was changed to a liquid-liquid extraction method. There were 4 different polarities of organic solvents used. After the examination, the ethyl acetate fraction was chosen as it contained whole constituents of the fraction F9. Using the method which was set up for fraction F9, eight compound-fractions (compound-fraction 1 to 8) were manually collected. In ethyl acetate fraction, some interesting compounds were observed, thus, there were three fractions (fraction-ethyl acetate-1 (FEA-1) to FEA-3) collected, additionally. So, all together eleven subfractions (compound-fractions and FEAs) were collected from ethyl acetate fraction. FEA-1 to FEA-3 and ethyl acetate fractions were examined on HL60 and Caco-2 cancer cell lines and they have a cytotoxic effect that induced apoptosis.

The sixth objective of this investigation was to isolate these separated compounds and identify them. To collect the pure compounds and to identify them, LC-MS, tandem MS and NMR experiments were performed. Eleven subfractions were isolated and their molecular weights were determined using high resolution-MS, but 9 of them, including compound-fraction-3 to 8 and FEA-1 to 3 were collected purely.

As the MW of compound-fraction-8 was 456, it was predicted that it could be oleanolc acid (OA) or ursolic acid (UA). Using HPLC, LC-MS, MS-MS investigations it was proved that the compound-fraction-8 contains both of OA and UA. The approximate amounts in the decoction are 0.069 mg/g and 0.173 mg/g, respectively from empirial measurement of areas. While, the amount of these acids that were determined in acetone extract of *Oldenlandia diffusa* were 0.442 mg/g for OA and 1.897 mg/g for UA [Liang *et al.*, 2009]. Therefore, the amount of OA and UA appearing in the decoction is around 8 times lower than that in the acetone extract that these researchers found.

In addition, the concentration of OA and UA in fraction F9 is approximately 17.2 μg/mL and 42.7 μg/mL, respectively. According to other researchers report, the IC50 value on HCT25 colon carcinoma cells were 54.72 μg/mL for OA and 27.4 μg/mL for UA [Jie *et al.*, 2002]. Also, ED50 was determined as a 72 μg/mL on HL60 for UA [Chao-Mei *et al.*, 2005]. The IC_{50} values of OA on Caco-2 cells were 37.5 μg/ml, which indicated that it has antiproliferative effect [Liang *et al.*, 2008]. By comparing these data, the concentration of these acids, in fraction F9 are reasonable amounts to give cytotoxic activity on cancer cell lines.

Also, some researchers examined these acids' ability to inhibit tumour growth and they showed a remarkable inhibitory effect against implanted tumour growth with doses of 50 and 100 mg/kg. A dose-dependent effect was also seen in the enhanced recovery of leukocytes [Hsue-Yin *et al.*, 1997]. Also, UA which was isolated from a methanol extract of *Oldenlandia diffusa* demonstrated a significant inhibition on the proliferation of human lung, ovary, skin, brain, colon, stomach, murine leukaemia and murine melanoma tumour cells. Results suggested that UA had an apoptotic effect on HL60 cells [Shan *et al.*, 2001], [Lacikova, 2006]. Some research revealed that OA and UA that were identified in methanol extract are the main antiproliferative constituents [Liang *et al.*, 2008].

Our investigation showed that both OA and UA, which were identified and included in the most cytotoxic fraction F9 of the decoction gave a certain amount of anti-cancer activity.

Furthermore, *E*-6-O-*p*-coumaroyl scandoside methyl ester was identified in the decoction. Its MW is 550.17 and the formula is $C_{26}H_{30}O_{13}$. Other researchers of Hong Kong Baptist University isolated and determined the structure of this compound [Liang *et al.*, 2006]. Therefore, the NMR assessments were performed on FEA-1 and compared to *E*-6-O-*p*-coumaroyl scandoside methyl ester results (see Tables 6, 7). These results revealed that FEA-1 is *E*-6-O-*p*-coumaroyl scandoside methyl ester. In addition, this compound showed a cytotoxic effect on HL60 and Caco-2 cancer cell lines (see Chapter 6). Therefore, this compound is one of the anti-cancer effective compounds of the decoction. Also, other researchers proved that *E*-6-*O*-*p*-coumaroyl scandoside methyl ester which was isolated from 95% ethanol extract of *Oldenlandia diffusa* has anti-proliferation effect on PC3 human androgen-independent prostate cancer cells [Li *et al.*, 2009].

Phytochemical studies reported that the main five compounds of *Oldenlandia diffusa* are OA and UA [Chung *et al.*, 1998], 6-O-p-coumaroyl scandoside methyl ester, asperuloside [Nishihama *et al.*, 1981], [Takagi *et al.*, 1982] and *E*-6-O-p-coumaroyl scandoside methyl ester-10-O-methyl ether [Gu & Weng, 2001].

So, according to our anti-cancer activity based separation study, OA and UA and *E*-6-O-p-counaroyl scandoside methyl ester were isolated and identified in the traditionally prepared *Oldenlandia diffusa* decoction as part of those responsible compounds for anti-cancer effects.

Previous work concerned the separation, fractionation, anti-cancer potential of *Oldenlandia diffusa* decoction and its fractions, and separation, purification and content identification of the cytotoxic fraction. Therefore, the next objective was to investigate the bioavailability after ingestion of the decoction, using Caco-2 monolayers as a mimic of intestinal absorption and to some degree metabolism.

Cell culture models are predictors of oral drug absorption. It is proven by other researchers that Caco-2 cells are useful for oral drug absorption studies, because these cells express various membrane transporters relevant to drug absorption [Smith & O'Donell, 2006], [Fogh & Trempe, 1975], [Pinto *et al.*, 1983]. After the cultivation of Caco-2 cells on permeable filters, they form a contact surface between two chambers [Niko & Raul, 2009], and this can be used for studies on the transepithelial transfer of drugs [Artursson &. Karlsson, 1991], [Meunier, 1995], [Artursonn *et al.*, 2001], [Artursson & Borchardt, 1997]. The monolayer has tight junctions and is polarized [Pinto *et al.*, 1983], therefore, it has been recommended for such biological *in vitro* studies [Arturrson & Karlsson, 1991], [Hillgren *et al.*, 1995]. Therefore, Caco-2 cells were used to create the gut mimicking wall (monolayer). Before the creation of the monolayer using Caco-2 cell line, its growth inhibition was assessed. Cells were growing exponentially, thus, Caco-2 cells were cultivated on collagen-coated inserts for 21 to 28 days under controlled conditions. However, most other publications mentioned that a monolayer is created for 21 days [Doyle & Griffiths, 2001], [Shaw, 1996]. In order to check the health and integrity of cells, its TEER measurements were taken every week (see Figure 120). Also, before using the created Caco-2 monolayers for the post-absorption sample collection, the cytotoxic effect of the decoction was investigated and it was shown that up to 3.5 mg/mL concentrations of the decoctions at up to 180 min time intervals is not

harmful for monolayers. Thus, the method for the post-absorption sample collection was set up by investigating the optimum collecting time intervals and concentrations of the decoction. The method which was set up was:- from a 2.5 mg/mL decoction after 180 min using monolayers of Caco-2 cells on collagen-coated inserts for 21 days. In addition, the decoction has good permeability at intestinal pH. In addition, post-absorption samples of the decoction were tested for effects on cell growth and it has a cytotoxic effect on HL60 cell line.

We also calculated the amount of OA and UA that transferred through the gut mimicking wall (Caco-2 monolayers) and this corresponds to a concentration of OA 0.0689 μg/mL and UA 0.1729 μg/mL, respectively. Therefore, this suggests cytotoxic amounts of bioavailable OA and UA are maybe less than that has been previously reported.

The final objective of this investigation was to assess the participation of these determined cytotoxic compounds in the post-absorption sample and whether they are in original or in a metabolised form. In previous studies, the contents of the most cytotoxic fraction of the decoction were studied. The post-absorption samples of the decoction and fraction F9 were collected and comparison analyses were performed using LC-MS. The contents of the subfractions (compound-fractions (1-8) and FEAs (1-3)) were studied in the post-absorption samples collected from the decoction and fraction F9. The post-absorption sample of the decoction contained 10 out of 11 compounds, while the post-absorption sample of fraction F9 contained 5 out of 8 compounds. So, in post-absorption samples most compounds were found in their original form (non-metabolized).

9.2. Conclusion

This study has revealed that traditionally prepared decoction of the aerial parts of the *Oldenlandia diffusa* has a cytotoxic potential *in vitro* on HL60 and Caco-2 cancer cell lines, inducing apoptosis. This investigation was based on an anti-cancer activity study. After the separation, which was directed by the cancer cell inhibition effect from the most cytotoxic (apoptosis induced) fraction F9, two of the main compounds OA and UA [Chung *et al.*, 1998] were identified. The approximately determined amounts of OA and UA in the decoction are 0.069 mg/g and 0.173 mg/g, respectively. Compared to others researchers' reports, these concentrations were in reasonable amounts to show cell inhibition effects on cancer cell lines.

In addition, another compound *E*-6-O-p-coumaroyl scandoside methyl ester was isolated and identified in the ethyl acetate fraction of the *Oldenlandia diffusa* decoction. It had cytotoxic effect on HL60 and Caco-2 cancer cell lines.

Bioavailability results revealed that the decoction has a good permeability through the intestinal pH (Caco-2 monolayers) and the cytotoxic effect still remained after the absorption. Isolated compounds (10 out of 11), including above 3 compounds were found in the post-absorption sample in non-metabolized forms.

9.3. Further work

Further work could be to isolate the other compounds from *Oldenlandia diffusa* decoction and characterize them and investigated each compound's anti-cancer activity on cells using some cytotoxicty assays, and investigate their cell death mode and apoptotic mechanisms by Western blotting, Flow cytometry (FACS) or other modern methods and facilities.

References

References

Agilent Application Note, Optimizing Fraction Collection with LC/MSD Systems Using an Active Splitter and Delay Sensor, (2002) publication number 5988-7610EN.

Ahuda, S., Editor, Handbook of Bioseparations, Academic Press, New York (2000), pp. 19.

Ahuja, S., Dong, M.W., Handbook of pharmaceutical analysis by HPLC, (2005), ISBN 0-12-088-547-6, pp. 55.

Arellano, M. and Moreno, S., Regulation of CDK/cyclin Complexes During the Cell Cycle, (1997), Int. J. Biochem. Cdl Bid. ,4, pp. 559-573.

Artursson, P. and Borchardt, R.T., Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond, Pharmaceutical Research, 12, (1997), pp. 1655–1658.

Artursson, P. and Karlsson, J., Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal (Caco-2) cells, Biochem. Biophys. Res. Commun. 175, (1991), pp. 880–885.

Artursson, P., Palm, K. and Luthman, K., Caco-2 monolayers in esperimental and theoretical predictions of drug transport, Advanced Drug Delivery Reviews 46, (2001), pp. 27-43.

Arpino, P., Mass Spectrometry Reviews, Combined liquid chromatography mass spectrometry, Part I. Coupling by means of a moving belt interface, Vol 8, (1989), pp. 35-55.

Arpino, P., Mass Spectrometry Reviews, Combined Liquid chromatography mass spectrometry. Part III. Application of thermospray, (2005), Vol 11, pp. 3-40.

Balani, SK., Miwa, K., Gerald, TG., Liang-Shang, W., Jing-Tao, L., Frank, W., Strategy of Utilizing In Vitro and In Vivo ADME Tools for Lead Optimization and Drug Candidate

Selection, Source: Current Topics in Medicinal Chemistry, Number 11, (September 2005), pp. 1033-1038(6).

Barker, J., Mass spectrometry, 2nd Edition, (1998), John Willey & Sons Ltd., ISBN 0471 9671 629, pp. 331.

Beck, R.C.R., Pohlmann, A.R., Hoffmeister, C, Gallas, M.R, Collnot, E., Schaefer, U.F., Guterres, S.S., Lehr, C.M., Dexamethasone-loaded nanoparticle-coated microparticles: Correlation between in vitro drug release and drug transport across Caco-2 cell monolayers, European Journal of Pharmaceutics and Biopharmaceutics 67, (2007), pp. 18–30.

Bellavite, P., Semizzi, M., Lussignoli, S., Andrioli, G., Bartocci, U., A computer model of the 'five elements' theory of traditional, Chinese medicine, Complementary Therapies in Medicine (1998), 6, pp. 133-140.

Berherns, I., Kissel, T., (2003), Do cell culture conditions influence the carrier-mediated transport of peptides in Caco-2 cell monolayers, 19, pp. 433-442.

Berne, L.J. and Alan, SM., The Amino-Acid Sequence of a Purothionin from Triticum monococcum, a Diploid Wheat, Eur. 5. Biochem. 133, (1983), pp. 621-625.

Biji, TK. and Hal Sco-Weld, R., Western blotting, Methods, 38, (2006), pp. 283–293.

Borenfreund, E. and Puerner, J., Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett., (1985), 24, pp. 119-124.

Bown, D., *Encyclopaedia of Herbs and their Uses.* Dorling Kindersley, London. (1995), ISBN 0-7513-020-31.

Bruce, A., Alexander, J., Lewis, J., Raff, M., Roberts, K., Walter, P., Molecular biology of the cell, 5th Edition, New York : Garland Science, (2008) ISBN – 0815341067, pp. 1115-1130.

Bruce, A., Alexander, J., Lewis, J., Raff, M., Roberts, K., Walter, P., Molecular Biology of The Cell, (2008), Garland Science, Taylor & Francis, ISBNN 978 0 8153 4105 5, pp. 1062-164.

Bruce, A., Cell Movemenets and Shaping of the Vertebrate Body, Molecular Biology of the Cell, 4th Edition, (2002), Chapter 21.

Bruins, A.P., Mechanistic Aspects of Electrospray Ionization, J. Chromatogr. A (1998), 794, pp. 345–357.

Bump, N.J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenberg, A.H., Miller, L.K., and Wong, W. W., (1995) Science 269, pp. 1885–1888.

Burgard, R.D., Kuznicki, T.J., Chemometrics: Chemical and Sensory Data, (2000), ISBN 0-8493-4864-1, pp. 14.

Bupa's health information team, Herbal remedies, (2009), accessed on October, 2009 from http://hcd2.bupa.co.uk/fact_sheets/html/herbal_medicine.html

Campbell, N. A., Williamson, B., Robin, J. H., Biology: Exploring Life. Boston, Massachusetts: Pearson Prentice Hall, ISBN 0-13-250882-6, 2006.

Camenisch, G., Folkers, G., Waterbeemd, H van de., Comparison of passive drug transport through Caco-2 cells and artificial membranes, International Journal of Pharmaceutics 147, (1997), 1, pp. 61-70.

Cancer Cells in Culture, (2007), http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/CancerCellsInCulture.html, accessed August, 2007.

Cancer research UK, (2009), http://info.cancerresearchuk.org/cancerstats/mortality/?a=5441 accessed by October, 2009.

Carlos, H.P., Tracy, C.Y., Penny, K.R., Space Radiation Environment and Its Biological Effects, UH, (1997), from http://www.isso.uh.edu/publications/A9697/9697-9.html, accessed on October, 2009.

Cech, N., Enke, C., Practical implicationsof some recent studies in electrospray ionization fundamentals. Mass Spectrometry Reviews, (2001), 20, pp. 362-387.

Chandler, J.M., Alnemri, E.S., Cohen, G.M. and MacFarlane, M., Activation of CPP32 and Mch3a in wild-type p53-induced apoptosis, (1997), Biochem. J., 322, pp. 19±23.

Chao-Mei, M., Shao-Qing, C., Jing-Rong, C., Rui-Qing, W., Peng-Fei, T., Masao, H., Mohsen, D., The cytotoxic activity of ursolic acid derivatives, European Journal of Medicinal Chemistry 40 (2005), pp. 582–589.

Chen, G.Q., Zhu, J., Shi, X.G., Ni, J.H., Zhong, H.J., Si, G.Y., Jin, X.L., Tang, W., Li, X.S., Xong, S.M., et al. In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia: As_2O_3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. *Blood.* (1996), 88(3), pp. 1052–1061.

Chen, H. and Horvath, Cs., High Speed High-Performance Liquid Chromatography of Peptides and Proteins, J. Chromatogr. A, 705, (1995), pp. 3-20.

Cheng, K.F., Leung, K.S., Leung, P.C., Interactions between modern and Chinese medicinal drugs: a general review. *Am J Chin Med.* (2003), 2, pp. 163–169.

Cheng, Y.L., Chang, W.L., Lee, S.C., Liu, Y.G., Lin, H.C., Chen, C.J., Yen, C.Y., Yu, D.S., Lin, S.Z., Harn, H.J., Acetone extract of Bupleurum scorzonerifolium inhibits proliferation of A549 human lung cancer cells via inducing apoptosis and suppressing telomerase activity, Life Science, (2003 Sep), 73(18), pp. 2383-2394.

Cheng, J.T., Review: drug therapy in Chinese traditional medicine, Journal of Clinical pharmacology 40 (2000), pp. 445–450.

Cheng, K.F., Leung, K.S,, Leung, P.C., Interactions between modern and Chinese medicinal drugs: a general review. *Am J Chin Med.* (2003), 2, pp.163–169.

Chinnaiyan, A.M., Hanna, W.L., Orth, K., Duan, H., Poirier, G.G., Froelich, C.J. and Dixit, V. M., Cytotoxic T-cell-derived granzyme B activates the apoptotic protease ICE-LAP3, (1996), Curr. Biol., 6, pp. 897±899.

Choi, D.H., Shin, S., Park, I.K., (1999). Characterization of antimicrobial agents extracted from *Asterina pectinifera*. Int. J. Antimicrob. Agents 11, pp. 65–68.

Chung, K.S., Takeatsu, K., Paul, P.H., But, International Collation of Traditional and Folk Medicine, Northern Asia, (1998), 3, pp. 119.

Chung, H.S., Jeong, H.J., Hong, S.H., Kim, M.S., Kim, S.J., Song, B.K., Jeong, I.S., Lee, E.J., Ahn, J.W., Baek, S.H., Kim, H.M., 2002. Induction of nitric oxide synthase by Oldenlandia diffusa in mouse peritoneal macrophages. Biol. Pharm. Bull. 25, pp. 1142–1146.

Cohen, G.M, Caspases: the executioners of apoptosis, (1997), Biochem. J. 326, pp. 1–16.

Collins, S.J., et al. Continuous growth and differentiation of human myeloid leukemic cells in suspension culture, (1977), Nature 270, pp. 347-349.

Collins, S.J., Molecular Medicine Program, Fred Hutchinson Cancer Research Center, Seattle, The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression, Journal of The American Society of Hematology, (Nov 1987), 5, pp. 1233-1244.

Collins, S.J., Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc. Natl. Acad. Sci. USA, (1978), 75, pp. 2458-2462.

Davila, J.C., Rodriguez, R.J., Melchert, R.B., Acosta, D., (1998) Predictive value of in vitro model systems in toxicology Annu. Rev. Pharmacol. Toxicol, 38, pp. 63–96.

Decker, T., Lohmann-Matthes, M.L., A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity, (November 1988), J. Immunol. Methods, 1, pp. 61–9.

Deepak, A. and Anshu, S., Indigenous Herbal Medicines: Tribal Formulations and Traditional Herbal Practices, Aavishkar Publishers Distributor, Jaipur-India, (2008), ISBN 9788179102527, pp. 440.

Demsey, A., Kawka, D. and Stackpole, W.C., Application of Freeze-Drying Intact Cells to Studies of Murine Oncornavirus Morphogenesis, Journal Of Virology, (Jan. 1977), 1, pp. 358-365.

Dennis, R.A., Mans, A., Rocha, B., Schwartsmann, G., Anti-Cancer Drug Discovery and Development in Brazil: Targeted Plant Collection as a Rational Strategy to Acquire Candidate Anti-Cancer Compounds, The Oncologist, (June 2000), 3, pp. 185-198.

Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S., and Reed, J.C.. IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. EMBO J., (1998), 17, pp. 2215–2223.

Dharmananda, S., Herbal Remedies, (2002), http://www.senhealth.co.uk/cms/supplements/tabid/1301/Default.aspx, accessed September, 2007.

Donald, L.P., Gary, M.L., George, S.K., Vyvyan, J.R., Introduction to Spectroscopy, 4th Edition, (2009), ISBN- 10 0 495 11478 2, pp. 430.

Dong-Hyun, K., Hyo-Jung, L., Young-Jun, O., Min-Jung, K., Sung-Hoon, K., Tae-Sook, J., and Nam-In, B., Graduate School of Biotechnology, Kyung Hee University, Korea, Iridoid Glycosides Isolated from Oldenlandia diffusa Inhibit LDL-Oxidation, Arch Pharm Res, (2005), 10, pp. 1156-1160.

Dong W. Michael, Modern HPLC for practicing scientists, a John Willey & Sons Inc., (2006), ISBN -13: 978-0-471-72789-7, pp. 83.

Doyle, A. and Griffiths, J.B., Cell and Tussue Culture for Medical Research, (2001), ISBN 0471852139, John Wirley&Sons Ltd, Chapter II.

Dressman, B.J., Lennernas, H., Oral Drug Absorption: Prediction and Assessment, Published by Informa Health Care, (2000), ISBN 0824702727, 9780824702724, pp. 15-18.

Duan, H,. Chinnaiyan, A.M., Hudson, P.L., Wing, J.P., He, W.W., Dixit, V.M., ICE-LAP3, a novel mammalian homologue of the Caenorhabditis elegans cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis. *J Biol Chem.* (1996 Jan 19), 3, pp. 1621–1625.

Duckworth, H.E., Barber, R.C., Venkataubramanian, 2nd Edition, (1990), Cambridge University Press, ISBN 0 521 38689 6, pp. 110.

Eaton, D.A., Clesceri, S.L., Rice, W.E., Greenberg, E.A., Standard method for Examination of Water and Wastewater, American Water Works Association, (2005), ISBN 0-87553-047-8, pp. 5-42.

ECCAC, Caco-2 cell line, http://www.biotech.ist.unige.it/cldb/cl1689.html, accessed on May, 2008.

ECCAC, HL60 cell line http://www.biotech.ist.unige.it/cldb/totcl619.html, accessed on November, 2008.

Ergil, K.V., Kramer, E.J., Ng, A.T., Chinese herbal medicines. *West J Med.* (2002) 176, pp. 275-279.

Elena, K., Eksteen, R., Schoenmakers, P., Miller, N., Handbook of HPLC, Volume 78, (1998), ISBN-0-8247-94-44-3, pp. 26.

Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F., Whitehouse, C.M,. Electrospray Ionization-Principles and Practice. Mass Spectrom. Rev. (1990), 9, pp. 37–70.

Fernandes-Alnemri, T., Armstrong, R.C., Krebs, J., Srinivasula, S.M., Wang, L., Bullrich, F., Fritz, L.C., Trapani, J.A., Tomaselli, K.J., Litwack, G., Alnemri, E.S., In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains, (1996 Jul), 15, pp. 7464–7469.

Fifield, F.W. and Kaelay, D., Princeples of practice of Analytical Chemistry, 5th Edition, (2000), ISBN 0-632-05384-4, pp. 95.

Fogh, J. and Trempe, G., New human tumor cell lines. In Human Tumor Cells *in vitro*, New York and London, Plenum Press, (1975), pp. 115-159.

Fogh, J., Wright, W.C., Loveless, J.D., Absence of HeLa cell contamination in 169 cell lines derived from human tumors, J Natl Cancer Inst. (1977), 2, pp. 209–214.

Freshney, R., Culture of Animal Cells: A Manual of Basic Technique, Alan R. Liss, Inc, New York (1987), pp. 117.

Fritza, J.S. & Macka, M., Solid-phase trapping of solutes for further chromatographic or electrophoretic analysis, Journal of Chromatography A, 902 (2000) 137–166.

Gallagher, R., et al., Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. Blood (1979), 54, pp. 713-733.

Fuh, R.A., Du. H, Li. J, Corkan. A, Lindsey. J. S., "PhotochemCAD: A computer-aided design and research tool in photochemistry, 1998, Photochemistry and photobiology, 68, pp.141-142.

Giddings, C., Grishka, E., Cazes, J., Brown, R.P., Advances in Chromatography, Volume 22, (1983), ISBN 0-8247-7049-8, pp. 45.

Gordon, M.H., Weng, X.C., Antioxidant properties of extracts from tanshen (Salvia miltiorrhiza Bunge), Food Chemistry 44 (1992), pp. 119–122.

Gooding, M.K., Regnier, E.F., HPLC of Biological macromolecules, 2nd Edition, ISBN 0-8247-0665-X, (2002), pp. 501.

Green, F.J., The Sigma-Aldrich Handbook of Stains, Dyes & Indicators, Aldrich Chemical Co. Milwaukee, WI: (1990), pp. 721-722.

Greenberg, A.H., Granzyme B-induced apoptosis, (1996), Adv. Exp. Med. Biol. 406, pp. 219-228.

George Rendina, Experimental Methods in Modern Biochemistry W. B. Saunders Company: Philadelphia, PA. (1976). pp. 46-55.

Gu, L.W., Weng, X.C., Antioxidant activity and components of Salvia plebeia R.Br., Chinese herb, Food Chemistry 73 (2001), pp. 299–305.

Gupta, S., Zhang, D., Yi, J., Shao, J., (2004) Anticancer activities of *Oldenlandia diffusa*. Journal of Herbal Pharmacotherapy 1, pp. 21-33.

Halasz, I., Endele, R., Asshauer, J., HPLC system, J. Chromatogr. 112 (1975), pp. 37.

Hammarton, T.C., et al., Stage-specific differences in cell cycle control in *Trypanosoma brucei* revealed by RNA interference of a mitotic cyclin. J. Biol. Chem., (2003), 25, pp. 22877-86.

Hewlett Packard, API-electrospray LC/MS system, User's Guide, (1994).

Hideaki, Y., Toshira, N., Yumi, K., Isolation of Ursolic acid from Apple Peels and Its Specific Efficacy as a Potent Antitumour Agent, Journal of Health Science, (2008), 6, pp. 654-660.

Hillary, M.A., Lloyd, W.A., Swarbrick, J., Drug Delivery and Targetting for Pharmasits and Pharmaceutical scientists, (2001), Taylor and Francis, (2001), ISNB-0 145 27198 3, pp. 14.

Hillgren, K.M., Kato, A., Borchardt, R.T., In vitro systems for studying intestinal drug absorption. Medicinal Research Reviews, (1995), 15, pp. 82-109.

Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., et al., (1998), J.Exp. Med. 187, pp. 587–600.

Ho, Y.S., Wu, C.H., Chou, H.M., Wang, Y.J., Tseng, H., Chen, C. H., Chen, L. C., Lee, C. H., Lin, S.Y., (2005) Molecular mechanisms of econazole-induced toxicity on human colon cancer cells: G0/G1 cell cycle arrest and caspase 8-independent apoptotic signalling pathways. Food and Chemical Toxicology 43, pp. 1483-1495.

Hobart, H.W., Lynne, L.M., John, A.D., Frank, A.S., Instrumental Methods of Analysis, 7th Edition, (1998), Wadsworth Inc., ISBN 0 534 8142 8, pp. 593.

Hogendoorn, E., Van Zoonen, P., Recent and Future Developments of Liquid Chromatography in Pesticide Trace Analysis. Journal of Chromatography, (2000), 892, pp. 435–453.

Hoffmann, E. De., Vincent Stroobant (2003). Mass Spectrometry: Principles and Applications (2nd Edition). Toronto: John Wiley & Sons, Ltd, ISBN 0-471-485667, Chapter III.

Hong-Fen, L., Waisman, T., Maimon, Y., Shakhar, K., Rosenne, E., Ben-Eliyahu, S., The effects of a Chinese herb formula, anti-cancer number one (ACNO), on NK cell activity and tumor metastasis in rats, Int Immunopharmacol., (2001 Oct), 11, pp. 1947-56.

Hsieh, T.C., Lu, X., Guo, J., Xiong, W., Kunicki, J., Darzynkiewicz, Z., Wu, J. M., Effects of herbal preparation Equiguard on hormone-responsive and hormone-refractory prostate carcinoma cells: mechanistic studies, Int J Oncol. (2002 Apr), 4, pp. 681-9.

Hsue-Yin, H., Jenq-Jer, Y., Chun-Ching, L., Effects of oleanolic acid and ursolic acid on inhibiting tumor growth and enhancing the recovery of hematopoietic system postirradiation in mice, Cancer Letters III (1997) pp. 7- I3.

Huh, J.E., Kang, K.S., Ahn, K.S., Kim, D.H., Saiki, I., Kim, S.H., Mylabris phalerlata induces apoptosis by caspase activation following cytochrome c release and Bid cleavage, Life Science, (2003 Sep 12), 17, pp. 2249-62.

Ikezoe, T., Chen, S., Saito, T., Asou, H., Kyo, T., Tanosaki, S., Heber, D., Taguchi, H., Koeffler, H.P., PC-SPES decreases proliferation and induces differentiation and apoptosis of human acute myeloid leukemia cells, Int J Oncology, (2003 Oct), 4, pp. 1203-11.

Ikezoe, T., Chen, S.S., Yang, Y., Heber, D., Taguchi, H., Koeffler, H.P., PC-SPES: Molecular mechanism to induce apoptosis and down-regulate expression of PSA in LNCaP human prostate cancer cells, Int J Oncol. (2003 Nov), 5, pp. 1461-70.

Islam, M.S., Akhtar, M.M., Rahman, M.M., Rahman, M.A., Sarker, K.K. and Alam, M. F., Antitumor and Phytotoxic Activities of Leaf Methanol Extract of Oldenlandia diffusa (Willd.) Roxb, Global Journal of Pharmacology, (2009), 2, pp. 99-106.

IUPAC, Compendium of Analytical Nomenclature. Second Edition, Blackwell Scientific Publications, Oxford, (1987), pp. 93.

Jandera, P, Journal of Chromatography A, 845 (1999), Simultaneous optimisation of gradient time, gradient shape and initial composition of the mobile phase in the high-performance liquid chromatography of homologous and oligomeric series, pp. 133–144.

Jahnke, R., "The Most Profound Medicine, Part II: Physiological Mechanisms Operating in the Human System During the Practice of Qigong and Yoga/Pranayama," Townsend Letter for Doctors, (1991), pp. 126.

Janson, J.C., Pettersson, T., In: Ganestos, G. and Barker, P.E., Editors, Preparative and Production Scale Chromatography, Marcel Dekker, New York (1992), pp. 559.

Jiangsu, New Medical College, (1977), *Chinese Materia Medica Dictionary*, Shanghai People's Publishing House, Shanghai, pp. 2016.

Jie, L., Wei-Jian Guo, Qing-Yao, Y., Effects of ursolic acid and oleanolic acid on human colon carcinoma cell line HCT15, World J Gastroenterol (2002), 3, pp. 493-495.

Joan, S., PhD, Alan, H., Roenneburg, L., Hegeman, T., (Gilson, Inc.), Craig Esser, PhD, Regina Black, Derek Von Langen, PhD (Merck & Co., Inc.), Optimizing Purified Sample Recovery on Reverse-Phase HPLC Systems, (2003).

Joe, H.K.C., bai hua she she cao, Complementary and Alternative Healing University, (2006), from http://alternativehealing.org/bai_hua_she_she_cao.htm, accessed on November, 2007.

Joel, K.S., HPLC- Practical and IndustrialApplications, 2d Edition, ISBN0-8493-0003-7, (2000), pp. 151.

John, E.M., Kamlesh, D. and Jorgenson, J.W., Anal. Chem. (1999), Ultrahigh-Pressure Reversed-Phase Liquid Chromatography: Isocratic and Gradient Elution Using Columns Packed with 1.0-um Particles, 71, pp. 700-708.

Jones, P.A., Bracher, M., Marenus, K. and Kolma, H., Performance of the Neutral Red Uptake Assay in the COLIPA International Validation Study on Alternatives to the Rabbit Eye Irritation Test, Toxicology *in Vitro* 13, (1999), pp. 325-333.

Jones, T.D., Ganeshaguru, K., Virchis, E.A., Folarin, I.N., Lowdell, M., Mehta, B.A., Prentice, H.G., Hoffbrand, A.V. and Wickremasinghe, R.G., Caspase 8 activation independent of Fas (CD95/APO-1) signaling may mediate killing of B-chronic lymphocytic leukemia cells by cytotoxic drugs or γ radiation, Abstract, Blood, (1 November 2001), 9, pp. 2800-2807.

Jumarie, C., Malo, C., Caco-2 cells cultured in serum-free medium as a model for the study of enterocytic differentiation in vitro, Department of Physiology, Faculty of Medicine, University of Montrial, Quebec, Canada, PubMed, (1991 Oct), 149(1), pp. 24-33.

Karp, G., Cell and molecular biology: concepts and experiments, 5th Edition, Hoboken, N.J, John Wiley, (2008), ISBN 978-0-470-04217-5, John Willey & Sons, pp. 660.

Karp, G., Cell and molecular biology, concepts and experiments, Fifth Edition, (2008) ISBN 978-0-470-04217-5, John Willey & Sons, Inc., pp. 485.

Kerr, J.F.R, Wyllie, A.H., Currie, A.R., (1972), Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer, 26, pp. 239–257.

Kerr, J.F.R. & Harmon, B.V., Definition and incidence of apoptosis: an historical perspective. In: Tomei L.D. and Cope F.O Editors, The Molecular Basis of Cell Death vol. 82, Cold Spring Harbor Laboratory Press, New York (1991), pp. 5–31.

Kim, Y., Park, E.J., Kim, J., Kim, S.R. and Kim, Y.Y., Neuroprotective constituents from *Hedyotis diffusa*, *J. Nat. Prod.* 64 (2001), pp. 75–78.

Kinzler, K.W., Vogelstein, B., The Genetic Basis Of Human Cancer, 2nd Edition, New York: McGraw-Hill, Medical Pub. Division, (2002), ISBN 978-0-07-137050-9, pp. 5.

Ko, R.J., Adulterants in Asian patent medicines. *N Engl J Med.* (1998), pp. 339-847.

Komin, N. and Toral, R., Drug absorption through a cell monolayer: A theoretical work on a non-linear three-compartment model, European Journal of Pharmaceutical Sciences 37 (2009), pp. 106–114.

Krstulovic, A.M., Lee, C.R., Firmin, S., Jacquet, G., Van Dau, C. N., Tessier D. Applications of LC-MS Methodology in the Development of Pharmaceuticals. Lc Gc Europe (2002), 15, pp. 31.

Kuida, K., Haydar, T.F., Kuan, C.Y., Gu, Y., Karasuyama, H. T., Su, M.S., Rakic, P. and Flavell, R.A., Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9 (1998). Cell 94, pp. 325–37.

Kuo-Yen, L., Haohua, T. and Asit, K.R., (2005), Charge Limits on Droplets during Evaporation, Langmuir, (2005), 9, pp. 3786–3794.

Kuwana, T., Smith, J.J., Muzio, M., Dixit, V.M., Newmeyer, D.D., Kornbluth, S., Apoptosis Induction by Caspase-8 Is Amplified through the Mitochondrial Release of Cytochrome *c*, (1998), J. Biol. Chem. 273, pp. 16589–94.

Lacikova, L., Abdel-Hamid, M. E., Novotny, L., Masterova, I. and Grancai, D., A Rapid Tandem Mass Spectrometric Assay for Determination of Ursolic Acid – Application to Analysis of Ursolic Acid in Four Species of *Staphylea* L. and Leaves of *Staphylea Pinnata* L. Gathered During Ontogenesis, Chromatographia, 3-4 (2006), pp. 117-122.

Lai, J., et al., Loss of HSulf-1 up-regulates heparin-binding growth factor signaling in cancer. J. Biol. Chem., (2003), 278(25), pp. 23107-17.

Lai, J., Hwang, J., Chen, H., Wang, J., Finished Herbal Product as an Alternative Treatment for Menopausal Symptoms in Climacteric Women, J Altern Complement Med. (2005 Dec), 6, pp. 1075-84.

Lansing, T.D., Jeffrey, R.H., Kenneth, A.G., High content screening: a powerful approach to systems cell biology and drug discovery, Edition: illustrated, Published by Humana Press, (2006), ISBN 1588297314, 9781588297310, pp. 444.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.C., (1994) Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371, pp. 346–347.

Lee, S.M., LC-MS applications in drug development, (2002), John Wiley and Sons, ISBN 10158-0012, pp. 5-10.

Legge, J., The Chinese Classics: The Ch'un Ts'ew, with Tso Chuen. Taipei: SMC Publishing Inc., 1994.

Leo, M.L.N., Handbook of Food Analysis: Residues and other food component analysis, 2nd Edition, (2004), ISBN 0-8247-5039-X, pp. 1223.

Li, R., Zhao, H.R., Lin, Y.N., (2002). Anti-tumor effect and protective effect on chemotherapeutic damage of water soluble extracts from Hedyotis diffusa. J. Chin. Pharm. Sci. 11, pp. 54–58.

Li, M., Jiang, R.W., Po-Ming, H., Ling, C., Ling-Lin, L., Jin-Rong, Z., Pang-Chui, S., Paul, P.B., Authentication of the anti-tumor herb Baihuasheshecao with bioactive marker compounds and molecular sequences, Food Chemistry 119 (2010), pp. 1239-1245.

Liang, Z., Jiang, Z., Fong, W. D. and Zhao, Z., Determination of Oleanolic Acid and Ursolic Acid in *Oldenlandia diffusa* and Its Substitute Using High Performance Liquid Chromatography, Journal of Food and Drug Analysis, 2, (2009), pp. 69-77.

Liang, Z., Mingfang, H., Fong, W., Jiang, Z., Zhao, Z., A comparable, chemical and pharmacological analysis of the traditional Chinese medicinal herbs *Oldenlandia diffusa* and *O.corymbosa* and a new valuation of their biological potential, Phytomedicine 15 (2008), pp. 259–267.

Liang, Z., Mingfang, H., Fong, W., Jiang, Z., Zhao, Z., Determination of Iridoid Glucosides for Quality Assessment of Herba Oldenlandiae by High-Performance Liquid Chromatography, Chem. Pharm. Bull., (2006), 8, pp. 1131—1137.

Lillie R.D., Williams and Wilkins Conn's Biological Stains, 9th ed., Lillie, R. D., Williams and Wilkins, Baltimore, MD: (1977), pp. 158.

Lin, C.C., Ng, L.T., Yang, J.J. and Hsu, Y. F., Anti-inflammatory and hepatoprotective activity of peh-hue-juwa-chi-cao in male rats, Am. J. Chin. Med. 30 (2002a), pp. 225–234.

Lin, S.Y., Liu, J.D., Chang, H.C., Yeh, S.D., Lin, C.H., Lee, W.S., Magnolol suppresses proliferation of cultured human colon and liver cancer cells by inhibiting DNA synthesis and activating apoptosis, J cell Biochem. (2002b), 3, pp. 532-44.

Lisk, D.J., Secor, C.L., Rutzke, M., Kuntz, T.H., (1989), Element composition of municipal refuse ashes and their aqueous extracts from 18 incinerators. Bull Environ Contam Toxicol 42, pp. 534–539.

Liss, R., Alan, Inc., Detection of Caspase Activation Combined with Other Probes of Apoptosis, Eurekah Bioscience Collection, New York, 1987.

Liu, X., Kim, C.N., Pohl, J. and Wang, X., Purification and characterization of an interleukin-1β-converting enzyme family protease that activates cysteine protease P32 (CPP32), (1996), J. Biol. Chem. 271, pp. 13371-13376.

Loconto, R.P., Trace environmental quantitative analysis: principles, techniques, and applications, 2nd Edition, (2006), ISBN 10-0-8247-5853-6, pp. 154.

Lodish, H., Berk, A., Zipursky, S. L., Matsudaria, P., Baltimore, D., Molecular Cell Biology, 6th Edition, (2008), ISBN 0 7167 7601 4, W. H. Freeman and Company, pp. 882.

Lodish, H., Baltimore, D., Barnell, J., Molecular Cell Biology, 3rd Edition, (2001), ISBN 0716723808, Scientific Merforbass, Inc, pp. 1247-1287.

Lorenzo, K.H. and Susin, A.S., Therapeutic potential of AIF-mediated caspase-independent programmed cell death, Drug Resistance Updates 10 (2007), pp. 235–255.

Lowdell, A., Mehta, B., Prentice, H.G., Hoffbrand, A.V., and Wickremasinghe, R. G., Caspase 8 activation independent of Fas (CD95/APO-1) signaling may mediate killing of B-chronic lymphocytic leukemia cells by cytotoxic drugs or g radiation, Blood, (1 November 2001), 9, pp. 2800-2807.

Lu, C.M., Yang, J.J., Wang, P.Y. and Lin, C. C., A new acylated flavonol glycoside and antioxidant effects of Hedyotis diffusa, Planta Med. 66 (2000), pp. 374–377.

Luo, X.G., Jiang, L.L. and Zhao, S.J., (2004). Simultaneous determination of oleanolic acid and ursolic acid in *Hedyotis diffusa* with HPLC. China Pharmacy 15, pp. 175-176.

Majno, G. and Joris, I., Apoptosis, oncosis, and necrosis: an overview of cell death. Am. J. Pathol. 146 (1995), pp. 3–15.

Markowska, M., Oberlea, R., Juzwina, S., Hsua, C., Gryszkiewiczb, M., Streeter, J. A., Optimizing Caco-2 cell monolayers to increase throughput in drug intestinal absorption analysis, Journal of Pharmacological and Toxicological Methods 46, (2001), pp. 51– 55.

Marquet, P., Progress of Liquid Chromatography-Mass Spectrometry in Clinical and Forensic Toxicology. Therapeut. Drug Monit. (2002), 24, pp. 255–276.

Marvin, C.M., LC/MS A Practical User's Guide, (2005), John Wiley & Sons, ISBN 13-978-0-471-65531-2, pp. 50-52.

Mann G., Some considerations on the use of preparative liquid chromatography in the pharmaceutical industry, Analysis 26 (1998), pp. 76–82.

Meunier, V., Bourrie, M., Berger, Y., Fabre, G., The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications, Cell Biology and Toxicology, 3-4, August, (1995), pp. 187-194.

Minitret, J.F., Schaletzky, J., Clemons, W. M., "Ribosome binding of a single copy of the SecY complex: implications for protein translocation", (December 2007). Mol. Cell, 6, pp. 1083–92.

Mohamed, H.Al-Marzouqi, Kees, J.A., Hogendoorn, G.F., Versteeg, Analytical solution for facilitated transport across a membrane, Chemical Engineering Science 57 (2002), pp. 4817 - 4829.

Molecular Probes, Inc., CyQUANT NF Cell Proliferation Assay (2006), from http://probes.invitrogen.com/media/pis/mp35006.pdf accessed on 04.03.2008.

Morgan, O.D., The cell cycle: Principles of Control, New Science Press, (2007), ISBN-13 978 0 9539181 2 6, pp. 5, 34.

Mottier, L., Alvarez, L., Ceballos, L., Lanusse, C., Drug transport mechanisms in helminth parasites: Passive diVusion of benzimidazole anthelmintics, Experimental Parasitology 113 (2006), pp. 49–57.

Murphree, A.L., Benedict, W.F., (March 1984), Retinoblastoma: clues to human oncogenesis, *Science* 223 (4640), pp. 1028–33.

Murray, K.K., Mass Spectrometry Reviews, Coupling of matrix assisted laser desorption/ionization to liquid separations, (1998), 16, pp. 283-299.

Naim, Y.H., Angiotensin-converting enzyme of the human small intestine Subunit and quaternary structure, biosynthesis and membrane association, Biochem. J. (1992) 286, pp. 451-457.

National Institutes of Health, (August 2001), Guidance document on using in vitro data to estimate in vivo starting doses for acute toxicity, NIH Publication No. 01-4500.

Nicholson, D.W., Thornberry, N. A., Caspases: killer proteases. Trends Biochem Sci., Abstract, (1997 August), 8, pp. 299–306.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., (1995 Jul 6). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376, pp. 37–43.

Niessen, W.M.A., Liquid Chromatography Mass Spectrometry, 2nd Edition, by Marcell Dekker, (1999), ISBN 0-8147-1936, pp. 85.

Nijel, J., Simpson, K., Solid-Phase Extraction Principles, Techniques, and Applications, (2000), ISBN 0-8247-0021-X, pp. 282.

Niko, K., Raul, T., Drug absorption through a cell monolayer: A theoretical work on a non-linear three-compartment model, European Journal of Pharmaceutical Sciences 37, (2009), pp. 106–114.

Ninghon, L., Chinese Medicinal Herbs of Hong Kong, Vol. 5 vol., (1986), Chinese Medical Research Institute, Hong Kong.

Nishihama, Y., Masuda, K., Yamaki, M., Takagi, S. and Sakina, K., Three new iridoid glucosides from Hedyotis diffusa, Planta Med. 43 (1981), pp. 28–33.

Oldenlandia (bai hua she she cao), All Rights Reserved, Nutritional Wellness, (2007), http://www.nutritionalwellness.com/nutrition/herbs/o/oldenlandia.php, accessed on 18.11.07

Oldenlandia diffusa, image, http://www.dkimages.com/discover/previews/825/25012996.JPG, accessed on 04.02.10.

Ouyanga, J., Delanghea, J., Baeyens, W.R.G., Langlois, M., Application of Western-blotting technique with chemiluminescence imaging to the study of haptoglobin type and haptoglobin complexes, Analytica Chimica Acta 362 (1998) , pp. 113±120.

Pang, K.S., Modelling Of Intestinal Drug Absorption: Roles of Transport and Metabolic Enzymes, The American Society for Pharmacology and Experimental Therapeutics, (2003), 12, pp. 1507-1519.

Pedemonte, C.H., Inhibition of Na-Pump Expression by Impairment of Protein Glycosylation Is Independent of the Reduced Sodium Entry Into the Cell, J. Membrane Biol. 147, (1995), pp. 223-31.

Pavia, L.D., Lampman, M.G., Kriz, S. G., Vyvyan, R. J., Introduction to Spectroscopy, (2009), Brooks/Cole, ISBN -13-978-0-495-1148-9, Chapter 8.

Pham, C.T. and Ley, T.J., (1997). The role of granzyme B cluster proteases in cell-mediated Cytotoxicity, S*eminars in* immunology, 9, pp. 127–133.

Pharmacopoeia of China, (2005). Chemical Industry Publishing House, Beijing, p. 22.

Phenomenex, (2008), http://www.phenomenex.com/productcfg/pc/viewCategories.asp?idCategory=25, accessed on 10.01. 2008.

Pinto, M., Robine-Leon, S., Appay, M. D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J., Zweibaum, A., Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture, (1983), Biol. Cell 47, pp. 323–330.

Plants for a Future, (2007), http://www.ibiblio.org/pfaf/cgi-bin/arr_html?Oldenlandia+diffusa, accessed on 19.11.07.

Polinsky R. Katherine, Tumour suppressor genes, Nova Science Publisher, (2007), ISBN-13 978 1 60021 693 0, pp. 35-38.

Pollard, D.T., Earnshaw, C.W., Cell Biology, (2004), ISBN 0 72-16-3360-9, Elseveir Science (USA), Inc., pp. 777.

Promega Corporation, (2007), Protocols and Application Guide. Apoptosis, from http://www.promega.com/paguide/chap4.htm, accessed on 05 November, 2009.

Pui, K.W., William, C.S.T., Liang, L., Zhao, Z.Z., Wen, L.W.H., Oleanolic acid isolated from Oldenlandia diffusa exhibits a unique growth inhibitory effect against ras-transformed fibroblasts, Life Sciences 85 (2009) pp. 113–121.

Quan, L.T., Tewari, M., O'Rourke, K., Dixit, V., Snipas, S. J., Poirier ,G. G., Ray, C., Pickup, D.J. and Salvesen, G.S., Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B, (1996), Proc. Natl. Acad. Sci. USA 93, pp. 1972-1976.

Rainer, S., Nehls-Sahabandu, M., Grabowsky, B., Dehlinger-Kremer, M., Schulz, I. and Georg, W.M., Max-Planck., Synthesis and application of photoaffimity analogues of inositol 1,4,5-trisphosphate selectively substituted at the 1-phosphate group, Biochem. J., (1990), 272, pp. 817-825.

Resnicoff, M., Valentinis, B,. Herbert, D., Abraham, D., Friesen, P.D., Alnemri, E. S., Baserga, R., (1998). The Baculovirus Anti-apoptotic p35 Protein Promotes Transformation of Mouse Embryo Fibroblasts. J. Biol. Chem. 273, pp. 10376-10380.

Riss, T.L., Moravec, R.A., Use of multiple assay endpoints to investigate the effects of incubation ime, dose of toxin, and plating density in cell-based cytotoxicity assays, (February 2004), Assay Drug Dev Technol 1, pp. 51–62.

Roos, W.P. and Kaina, B., DNA damage-induced cell death by apoptosis, Trends Mol Med. (2006 Sep), 9, pp. 440-50.

Rouessac, F. and Rouessac, A., Chemical Analysis Modern Instrumentation Methods and Techniques, 2nd Edition, (2007), John Wiley & Sons, ISBN 978 0 470 85 903 2, pp. 402-404.

Sadava, D., Ahn, J., Zhan, M., Pang, M-L., Ding, J., Kane, S., E. (2002) Effects of four Chinese herbal extracts on drug sensitive and multidrug-resistant small-cell lung carcinoma cells. Cancer Chemoether Pharmacol 49, pp. 261-266.

Satyajit, D.S., Zahid, L., Alexander, I.G., Natural products isolation, 2nd Edition, illustrated, Published by Humana Press, (2006), ISBN 1588294471, 9781588294470, pp. 515.

Satyajit, D.S., Zahid, L., Alexander, I.G., Natural products isolation, 2nd Edition, (2006), Library of Congress cataloging, ISBN -1 59259 955 9, pp. 214.

Scaffidi, C., Fulda, S., Srivinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H. and Peter, M. E., (1998). Two CD95 (APO-1)/Fas) signaling pathways, EMBO J. 17, pp. 1675–1687.

Scott, H.K. and William, C.E., Induction of Apoptosis by Cancer Chemotherapy, Experimental Cell Research (2000), 256, pp. 42–49.

Shan, B.E., Yoshida, Y., Sugiura, T., Yamashita, U., Stimulating activity of Chinese medicinal herbs on human lymphocytes in vitro, International Journal of Immunopharmacology 21 (1999), pp. 149–159.

Shan, B.E., Zhang, J.Y., Du, X.N., Immunomodulatory activity and anti-tumor activity of *O.diffusa* in vitro., Zhongguo Zhong Xi Yi Jie He Za Zhi., (May 2001), 5, pp. 370-374.

Shaw, A.J., Epithelial Cell Culture, A Practical Approach, Oxford University, (2002), ISBN 0 19 962572 2, pp. 114.

Shimadzu Scientific, http://www.ssi.shimadzu.com/products/literature/HPLC/Prominence_SIL-20a.pdf, (2007), accessed July 2007.

Sian, W., Williamson I., Hassan, I. and Mackay, M., Isolation and characterisation of clones from the Caco-2 cell line displaying increased taurocholic acid transport, (September 1990) from http://jcs.biologists.org/cgi/reprint/98/3/323.pdf, accessed on 15.12.2009.

Simal-Gandara, J., Damant, A.P., Castle, L., The Use of LC-MS in Studies of Migration from Food Contact Materials: A Review of Present Applications and Future Possibilities. Crit. Rev. Anal. Chem. (2002), 32, pp. 47–78.

Skoog, A. D., West, M. D., Holler, F. J., Fundamentals of analytical chemistry, 8th Edition, (2004), Belmont, Calif, London : Thomson/Brooks/Cole, ISBN 0534417973, pp. 983.

Skoog, A. D., West, M. D., Holler, F. J., Princeples of Instrumental Analysis, 6th Edition, (2007), ISBN 10:0-495-01201-7, pp. 763.

Somenath, M., Sample preparation techniques in Analytical Chemistry, (2003), ISBN 0-471-32845-2, pp. 109.

Srinivasula, S.M., Ahmad, M., Fernandes, A.T., Alnemri, E.S., Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization, (1998), Mol. Cell, 1, pp. 949–57.

Srinivasula, S.M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R. C., Wang, L., Trapani, J.A., Tomselli, K.J., Litwack, G., Alnemri, E.S., The Ced-3/interleukin 1β converting enzyme homolog Mch6 and the lamin-cleaving enzyme Mch2a are substrates for the apoptotic mediator CPP32. J. Biol. Chem.(1996), 271, pp. 27099–27106.

Smith, G.C., O'Donell, T. J., The process of new drug discovery and development, 2nd Edition, (2006), by Informa Healthcare USA, Inc, ISBN-13 978 0 8493 2779 7, pp. 173.

Snyder, R.L. and Dolan, W.J., High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model, Wiley Interscience. (2006), ISBN 0-471706469.

Stavros, K., HPLC Made to Measure Practicle handbook for Optimization, (2006), Wiley-VCH, ISBN 3-527-31377, pp. 68.

Stennicke, H.R., Jurgensmeier, J.M., Shin, H., Deveraux, Q., Wolf, B.B. and Yang, X., et al., Pro-caspase-3 is a major physiologic target of caspase-8, J Biol Chem 273 (1998), pp. 27084–27090.

Subhuti Dharmananda, Director, Institute for Traditional Medicine, Portland, Oregon, *Oldenlandia* and *Scutellaria* Antitoxin and Anticancer Herbs, (2004), http://www.itmonline.org/arts/oldenlandia.htm, accessed on 20.12.2007.

Sung, H.K., Byung-Zun, A. and ShiYong, R., Antitumour Effects of Ursolic Acid Isolated from *Oldenlandia diffusa,* Phytother. (1998). Res. 12, pp. 553–556.

Swarbick, J., Boylan, C.J., Encyclopedia of Pharmaceutical Technology, 2nd Edition, Volume 3, Marchel Dekker, ISBN 0 8247 2825 4, (2002), pp. 202.

Takagi, S., Yamaki, M., Nishihama, Y. and Ishiguro, K., On the iridoid glucosides of the Chinese drug "bai hua she she cao", Shoyakugaku Zasshi, (1982), 36, pp. 366–369.

Takahashi, A., Hirata, H., Yonehara, S., Imai, Y., Lee, K.K., Moyer, R.W., Turner, P.C., Mesner, P.W., Okazaki, T., Sawai, H., (1997) Affinity labeling displays the stepwise activation of ICE-related proteases by Fas, staurosporine, and CrmA-sensitive caspase-8. Oncogene 14, pp. 2741–2752.

Takayuki, S., Liquid Chromatographic Analysis, (1994), ISBN 0-8247-8941-5, pp. 6.

Takayuki, S. and Shibamoto, S., Chromatographic Analysis of Environmental and Food Toxicants, Vol. 77, Publisher: CRC Press, (1998), ISBN-13: 9780824701451, pp. 97.

Teng, L., Shaw, D., Barnes, J., Traditional Chinese herbal medicine, (2006), The Pharmaceutical Journal, 276, pp. 361-363.

Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G.S., Dixit, V.M., (1995) Yama/CPP32, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81, pp. 801–809.

Thanou, M., Verhoef, J. C. and Junginger, H. E., Oral drug absorption enhancement by chitosan and its derivatives, (November 2001), 2, pp. 117-126.

Thomas, R., Recent developments in LC-MS-MS for the Identification and Measurement of Nanoscale Amounts of Proteins and Peptides. Spectroscopy (2001), 16, pp. 28.

Todd, M.D., Lin, X, Stankowski, L.F., Desai, M. and Wolfgang, G.H I., J Biomolec, (1999), Screen 4, pp. 259-268.

Triglia, D., Wegener, P.T., Harbell, J., Wallace, K., Matheson, D. and Shopsis, C., Interlaboratory validation study of the keratinocyte neutral red bioassay from Clonetics Corporation. In Alternative Methods in Toxicology, 7, (1989), A.M. Goldberg, pp. 357-365.

Trine, D.K., Pedersen, M.H., Avrelija, C. and Birgitte, B.B., Application of Measurements of Transepithelial Electrical Resistance of Intestinal Epithelial Cell Monolayers To Evaluate Probiotic Activity, Appl Environ Microbiol. (2005 November), 11, 7528–7530.

Tuschl, H. and Schwab, C.E., Flow cytometric methods used as screening tests for basal toxicity of chemicals., (2004), Toxicology *in vitro*, 18, pp. 483-491.

Ulasiman-kalat, *Oldenlandia Diffusa (Willd.) Roxb.*, from http://www.bpi.da.gov.ph/Publications/mp/pdf/u/ulasiman-kalat.pdf, accessed on 18.06.07.

Velarde, G., Ait-Aissa, S., Gillet, C., Rogerieux, F., Lambre, C., Vindimian, E., Prcher, J. M., Use of Transepithelial Electrical Resistance in the Study of Pentachlorophenol Toxicity, Toxicology in Vitro 13, (1999), pp. 723-727.

Verweij, J. and de Jonge, M.J.A., (2000) Review: Achievements and future of chemotherapy. European Journal of Cancer 36, pp. 1479-1487.

Voet, D., Voet, G. J., Pratt, W., Charlotte, Fundamentals of Biochemistry Life at the molecular level, 2nd Edition, (2006), ISBN 0 471-21495-7, John Willey & sons, Inc., pp. 1054.

Wang, L., Tang, Y., Rubin, D.. C. and Levin, M. S., Chronically administered retinoic acid has trophic effects in the rat small intestine and promotes adaptation in a resection model of short bowel syndrome, American journal of physiology. Gastrointestinal and liver physiology, (2007), 6, pp. G1559-69.

Wang, R.R., Yinyang (Yin-yang), The internet Encyclopedia of Philosophy, Loyola Marymount University, (2006), http://www.iep.utm.edu/yinyang/, accessed on 20.10.09.

Wartenberg, M., Budde, P., De Marees, M., Grunheck, F., Tsang, S.Y., Huang, Y., Chen, Z. Y., Hescheler, J., Sauer, H., Inhibition of tumor-induced angiogenesis and matrix-metalloproteinase expression in confrontation cultures of embryoid bodies and tumor spheroids by plant ingredients used in traditional chinese medicine, Lab Invest., (2003 Jan), 1, pp. 87-98.

Wayne, M.B., Reece, G. B., Poenie, M.F., The world of the cell, Third Edition, ISBN 0-8053-0880-6, The Benjamin/Cummings Publishing Company, Inc., pp. 2.

Weng, N., Bioanalytical liquid chromatography tandem mass spectrometry methods on underivatized silica columns with aqueous/organic mobile phases, Journal of Chromatography B, 796 (2003), pp. 209–224.

White, M.K., Smith, D.B., Dugganb, J. P., Sheahan, L. S., Tyndall, M. E., Mechanism of facilitated saccharide transport through plasticized cellulose triacetate membranes, Journal of Membrane Science 194 (2001), pp. 165–175.

Willet, G., Christopher, S.H., Abrams, A. R., Regine, F. W., Rich, A. T., Clinical Research In Pancreatic Cancer: The Radiation Therapy Oncology Group trials, Int. J. Radiation Oncology Biol. Phys., (2003), 4, Supplement, pp. 31–37.

Willoughby, R., Sheehan, E., Mitrovich, S., A global View of LC/MS. Pittsburgh: Global View Publishing, (1998); W.M.A. Niessen, Liquid Chromatography-Mass Spectrometry, 2nd Ed. New York: Dekker, pp. 1999.

Williams, A., Taylor, K., Dambuleff, K., Persson, O. and Kennedy, R.M., Journal of Chromatography A, 1-2, (25 January 2002), Maintenance of column performance at scale, pp. 69-75.

Willimott, S., Barker, J., Jones, A. L. and Opara, I. E., Apoptotic effect of *O.diffusa* on the leukemic cell line HL60 and human lymphocytes., Journal of Ethnopharmacology, (2007), 3, pp. 290-299.

Wong, B.Y., Lau, B.H., Jia, T.Y., Wan, C.P., (1996). Oldenlandia diffusa and Scutellaria barbata augment macrophage oxidative burst and inhibit tumor growth. Cancer Biother. Radiopharm. 11, pp. 51–56.

World Health Organization, General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine, (2000).

World Health Organization, General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine, (2003).

World Health Organization, Traditional Medicine: Definitions, (2010), Health System Governance and Service Delivery, Geneva.

WPI, TEER Measurement, (18 Feb, 2000), from http://www.wpiinc.cn/pdf/evom-im-021308.pdf, accessed on October, 2007.

Xuesong, L., Caryn, N. K., Jan, P., Xiaodong, W., Purification and Characterization of an Interleukin-1β-converting Enzyme Family Protease That Activates Cysteine Protease P32 (CPP32), (2009), from http://www.jbc.org/content/271/23/13371.abstract?ijkey=af28372dfd88230ece43798fe75a83b83b8cfaaa&keytype2=tf_ipsecsha, accessed on 01.11.09.

Yao, X., An Introduction to Confucianism, Cambridge University Press, (2010) ISBN-13: 9780521643122, pp.7.

Yoshida, Y., Wang, M.Q., Liu, J.N., ,Shan B.E. and Yamashita, U., Immunomodulating activity of Chinese medicinal herbs and *O.diffusa* in particular., International Journal of Immunopharmacology, (April 1998), 7, pp. 359-370.

Yuri, K., Rosario, L., Contributor, Y. K., Rosario, L., HPLC for pharmaceutical scientists, Edition: illustrated, Published by John Wiley and Sons, (2007), ISBN 0471681628, 9780471681625, pp. 1104.

Yuri, K., Rosario, L., HPLC for Pharmaceutical Scientists, John & Willey, (2007), ISBN-10 0 471 68162 8, pp. 415.

Zhang, J.W., Chen, L.B., Gu, J.K., Ge, W.H., Yang, M., Novel Theory and Methods for Chemomic Multi-component Release/Dissolution Kinetics of Traditional Chinese Medicine, Chinese Journal of Natural Medicines 6 (2008). pp. 0048-0052.

Zhang, D.Y., Wu, J., Ye, F., Xue, L., Jiang, S., Yi, J., Zhang, W., Wei, H., Sung, M., Wang, W., Li, X., Inhibition of cancer cell proliferation and prostaglandin E2 synthesis by Scutellaria baicalensis, Cancer Res, (2003 Jul 15), 14, pp. 4037-43.

Zhang, C.H., Guo, X.J., Li, F., Xue, X. F. and Li, F. M., (2004). HPLC determination of the contents of oleanolic acid and ursolic acid in *Hedyotis diffusa* Willd. J. Shenyang Pharm. Uni. 21, pp. 358-370.

Zhou, C., Wang, L. and Feng, X. Y., (2002). Comparison of the contents of oleanolic acid and ursolic acid in *Hedyotis diffusa* Willd. and *H. corymbosa* (L.) Lam. Zhongyaocai 25, pp. 313-314.

Appendices

Appendices

Pathway of nuclear apoptosis

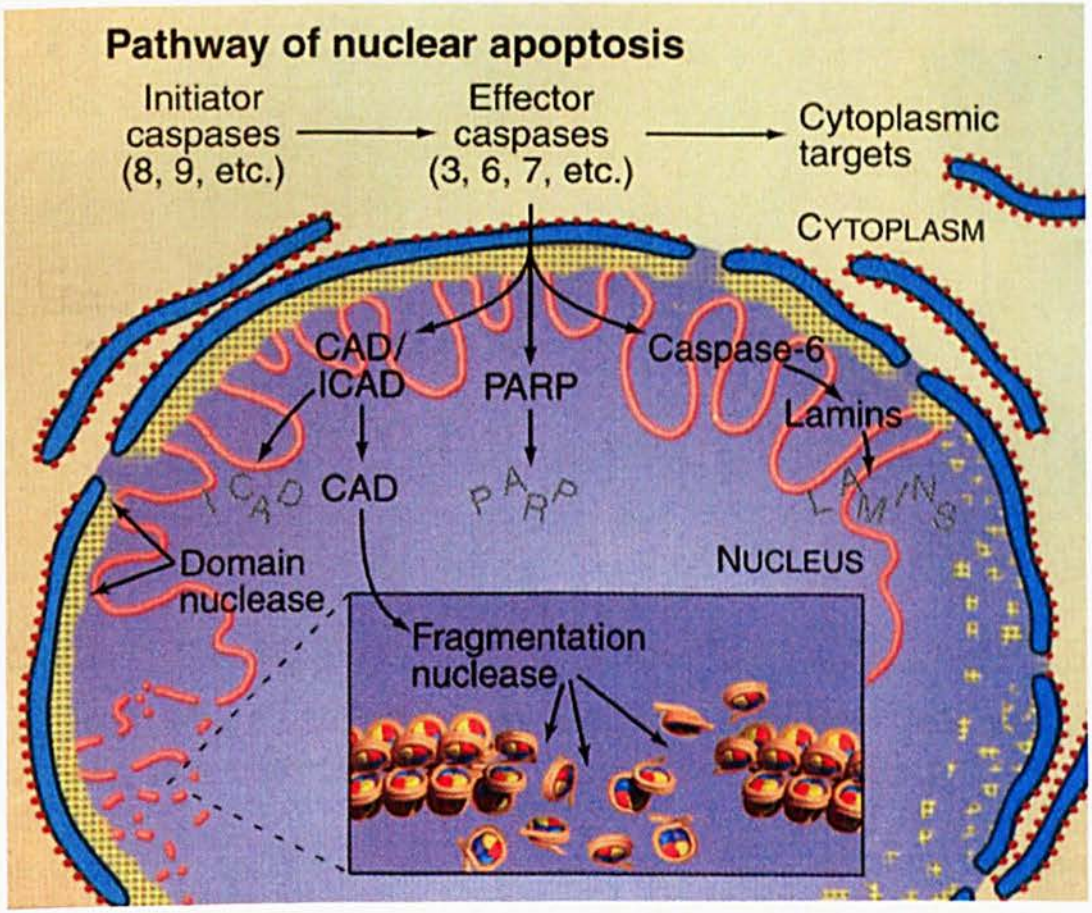


Figure A1. Some of the roles of caspases in disassembly of the nucleus [Pollard *et al.*, 2004].

MS-MS results for compound-fractions and fraction FEA 2, 3 (refer to Section 7.2.2.5) (Figures A2-A9).

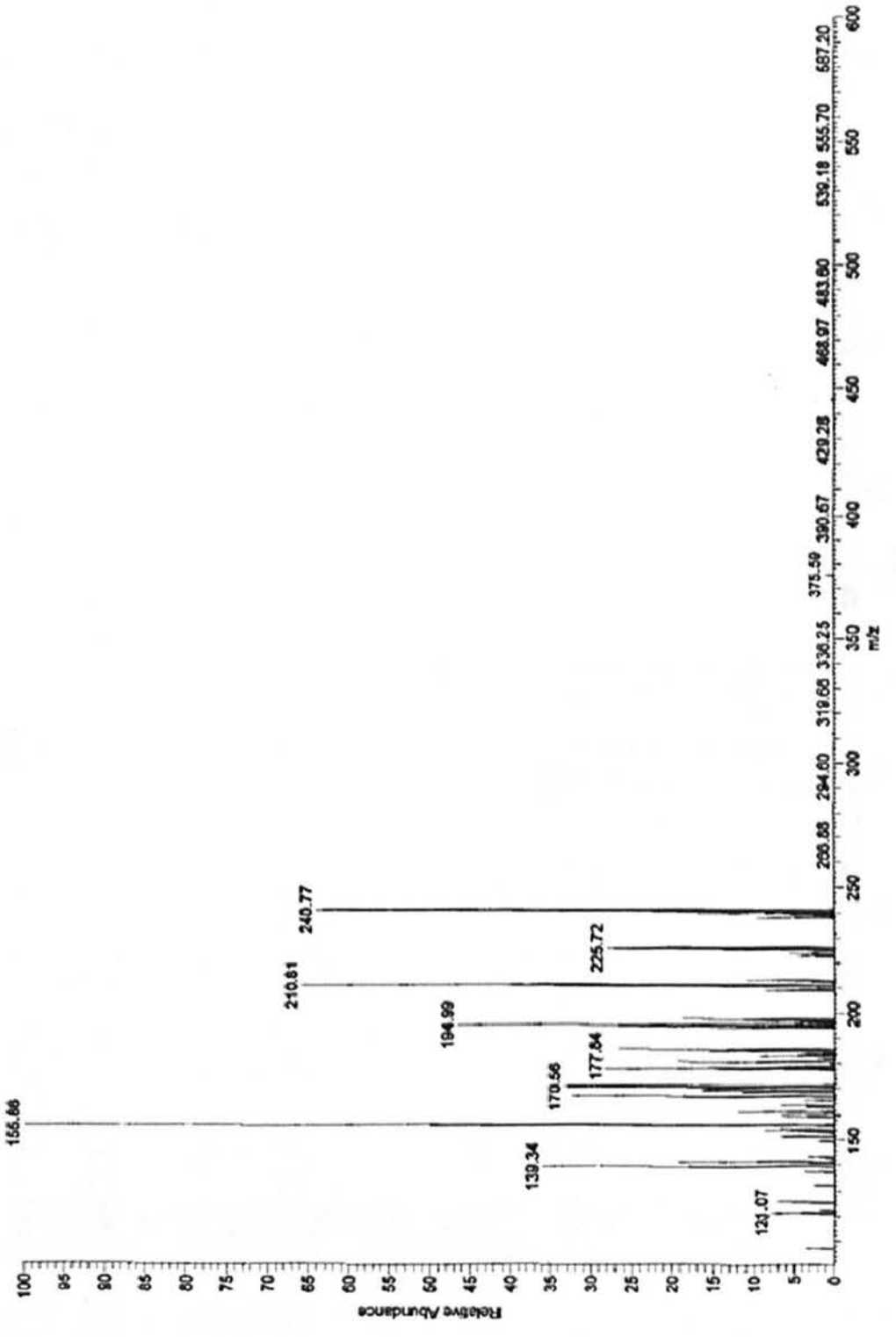


Figure A2. Mass spectrum (ESI-) for m/z 241.

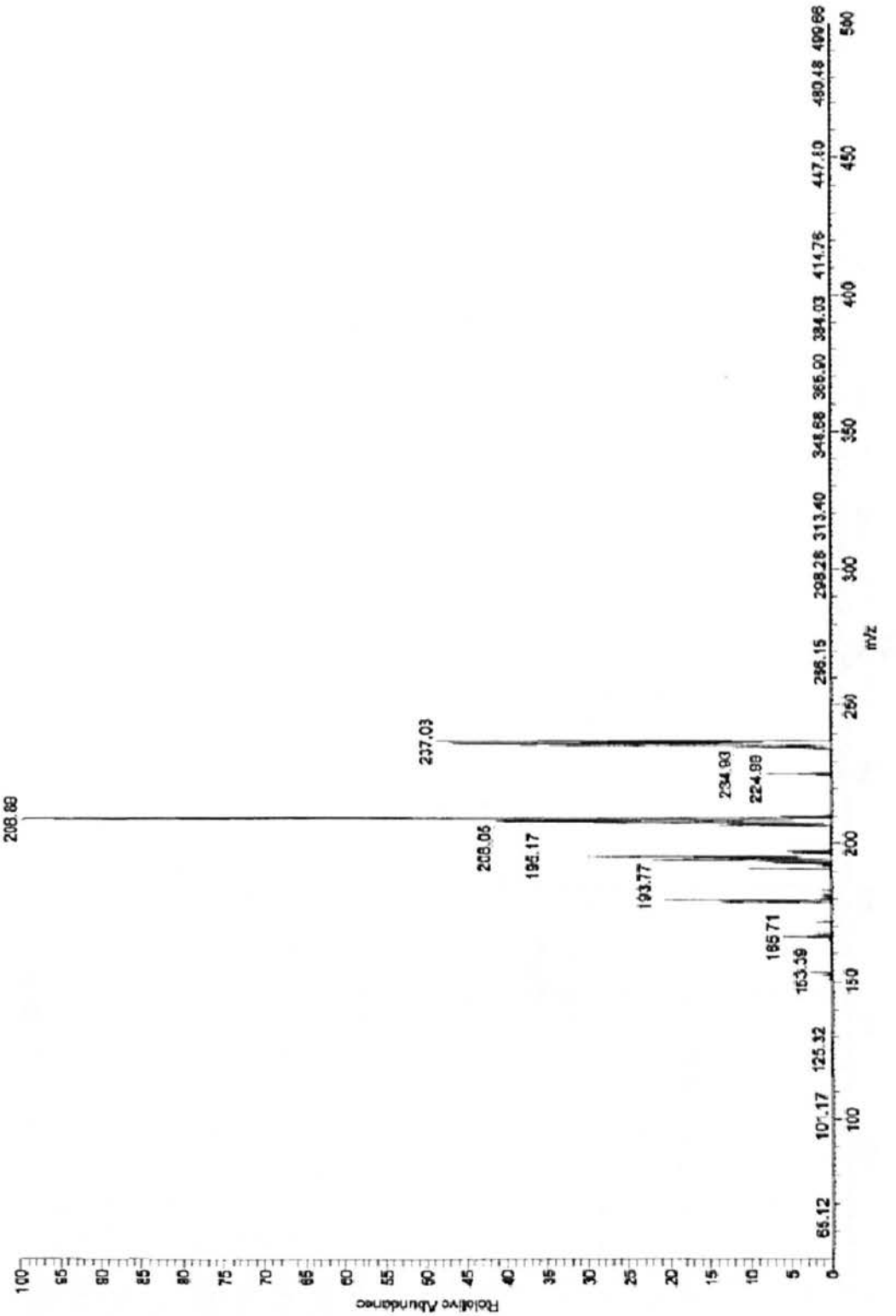


Figure A3. Mass spectrum (ESI-) for m/z 237.

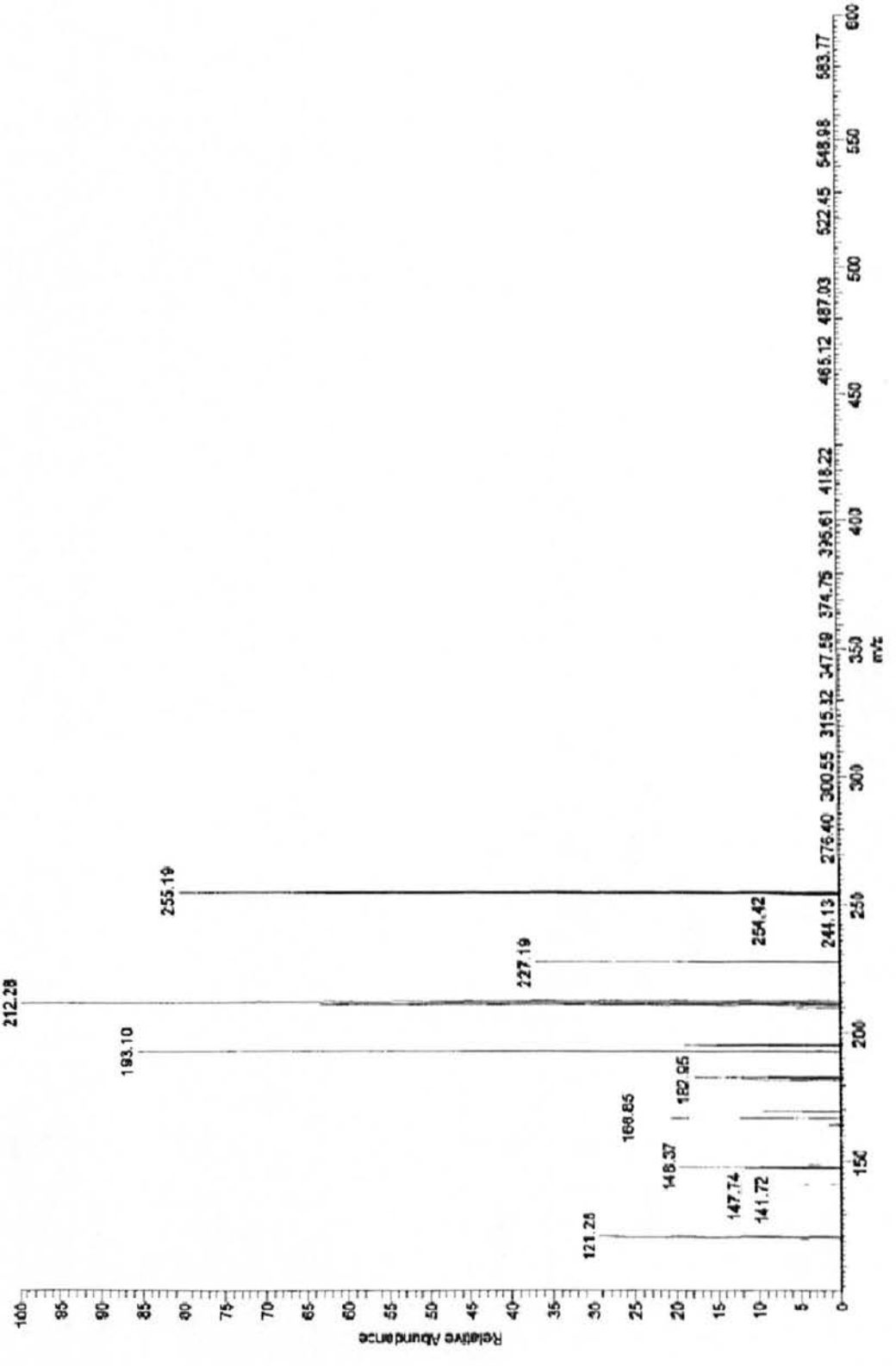


Figure A4. Mass spectrum (ESI-) for m/z 255.

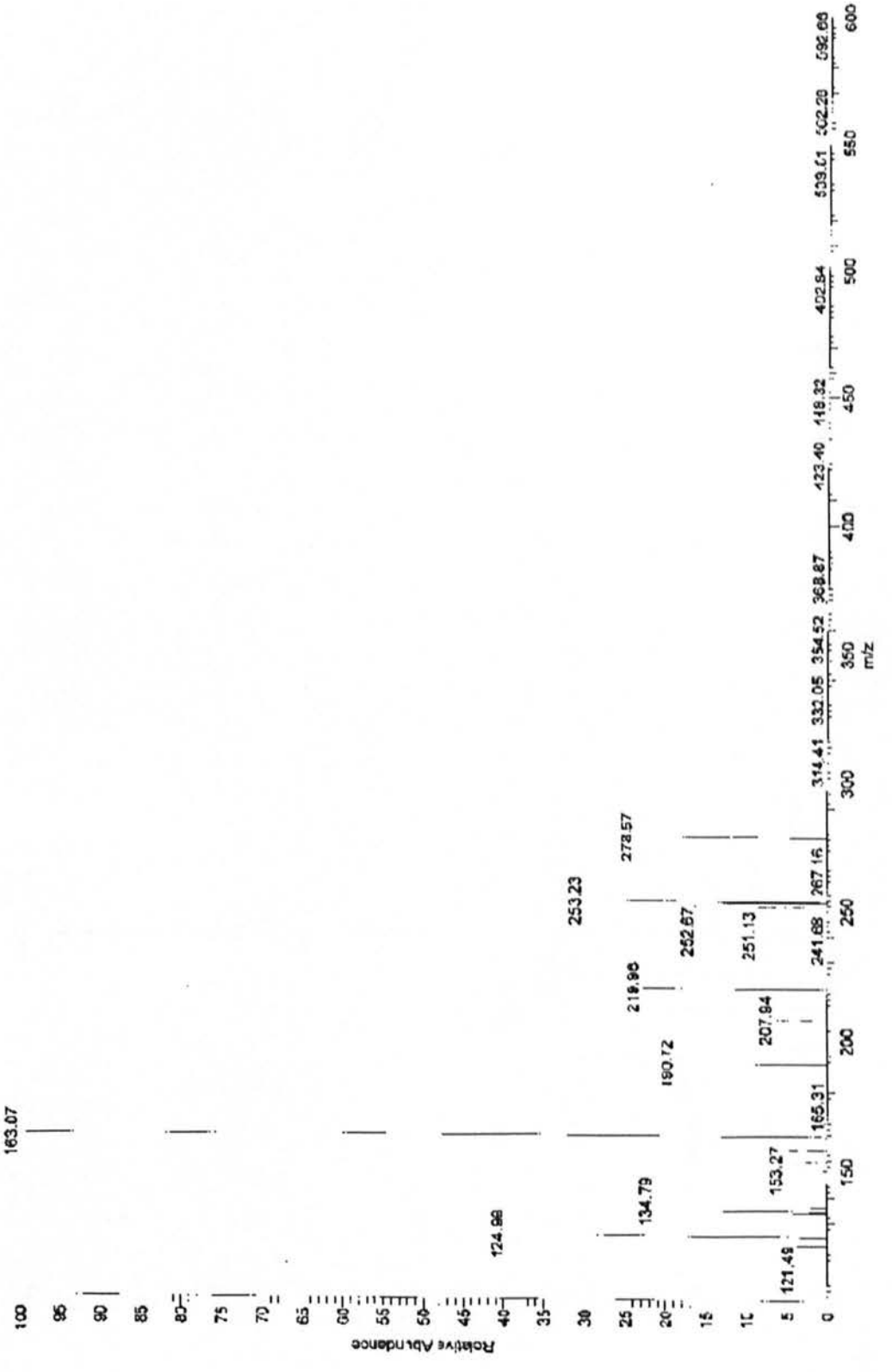


Figure A5. Mass spectrum (ESI-) for m/z 279.

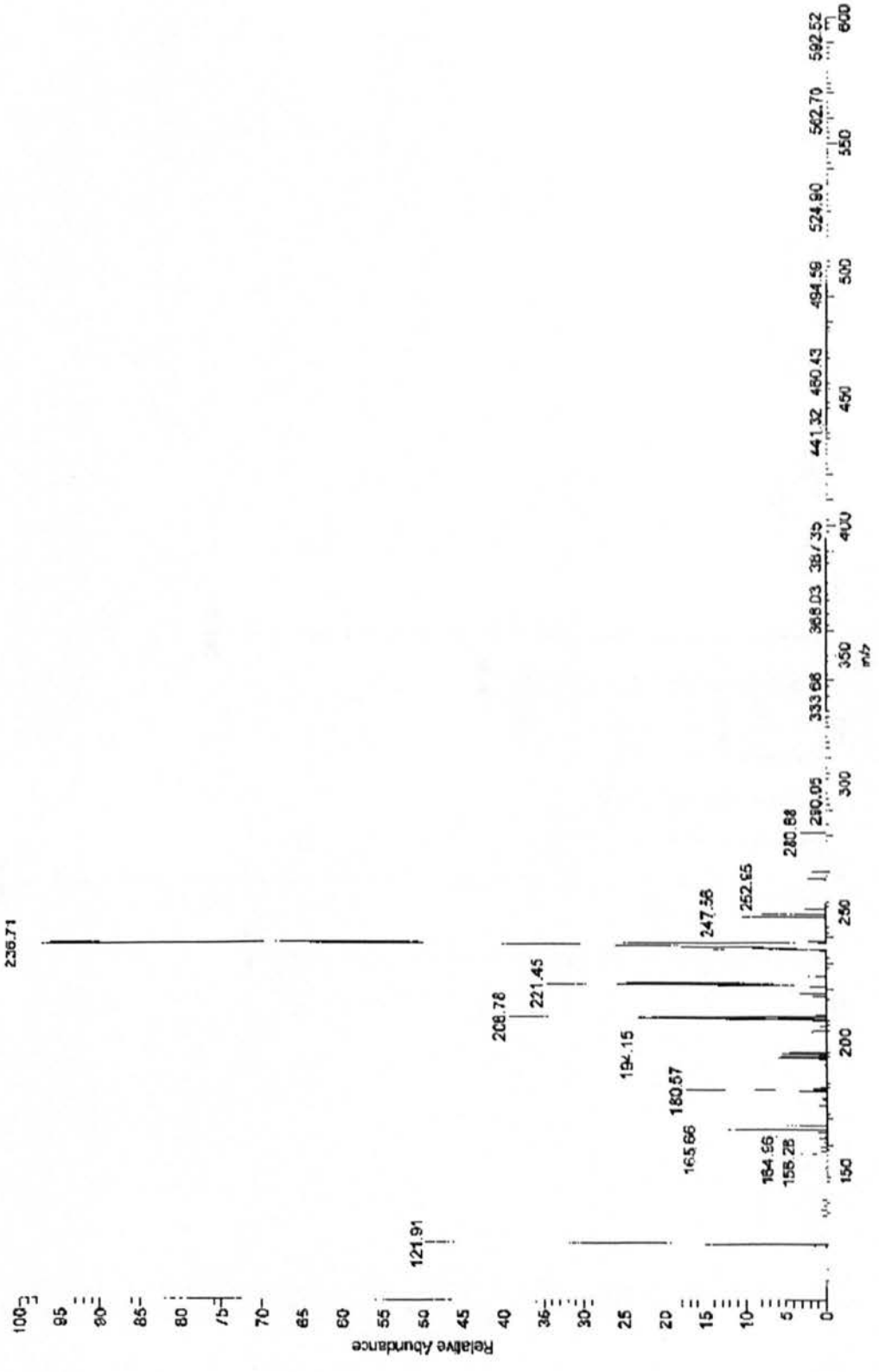


Figure A6. Mass spectrum (ESI-) for m/z 281.

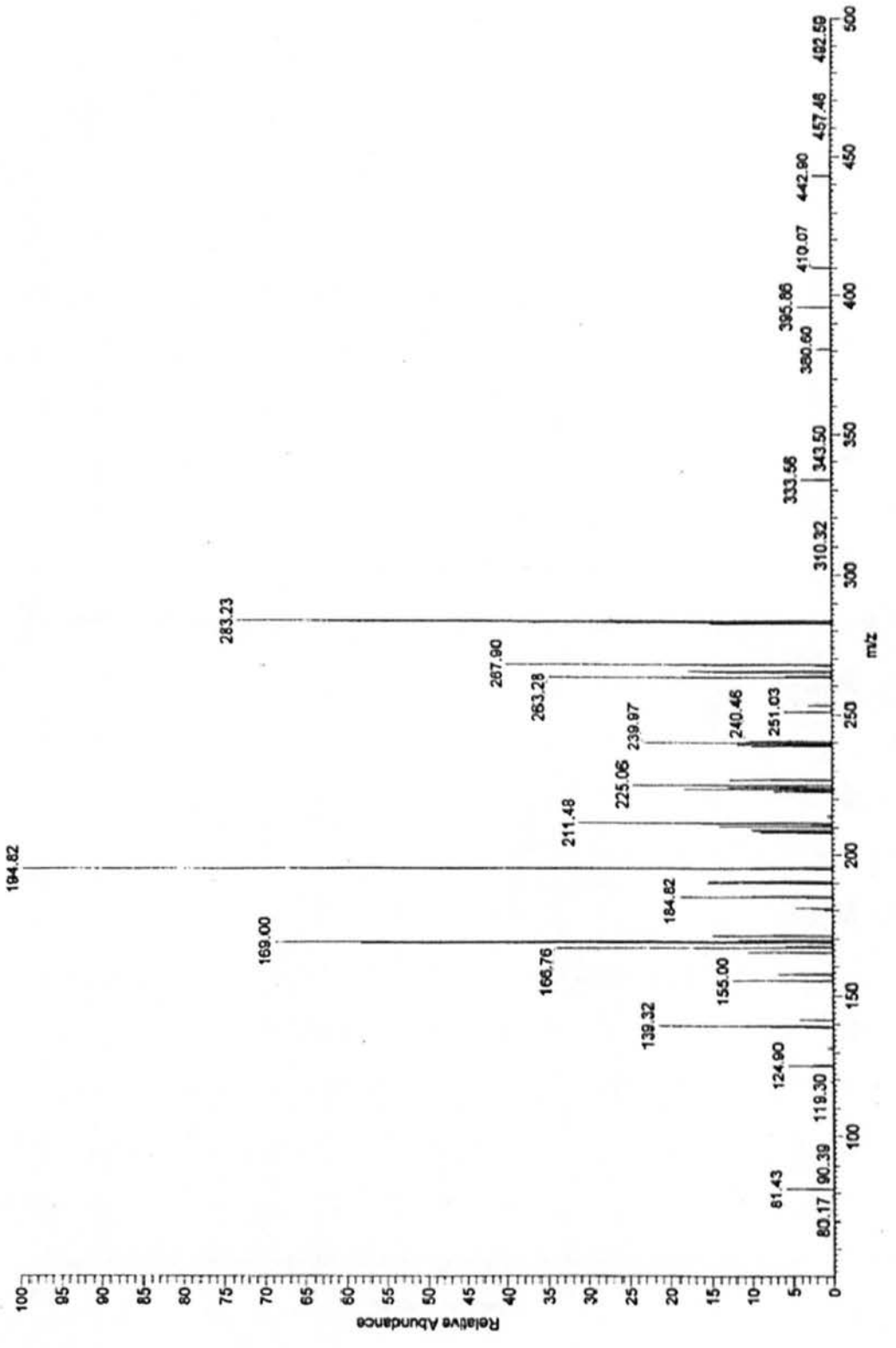


Figure A7. Mass spectrum (ESI-) for m/z 283.

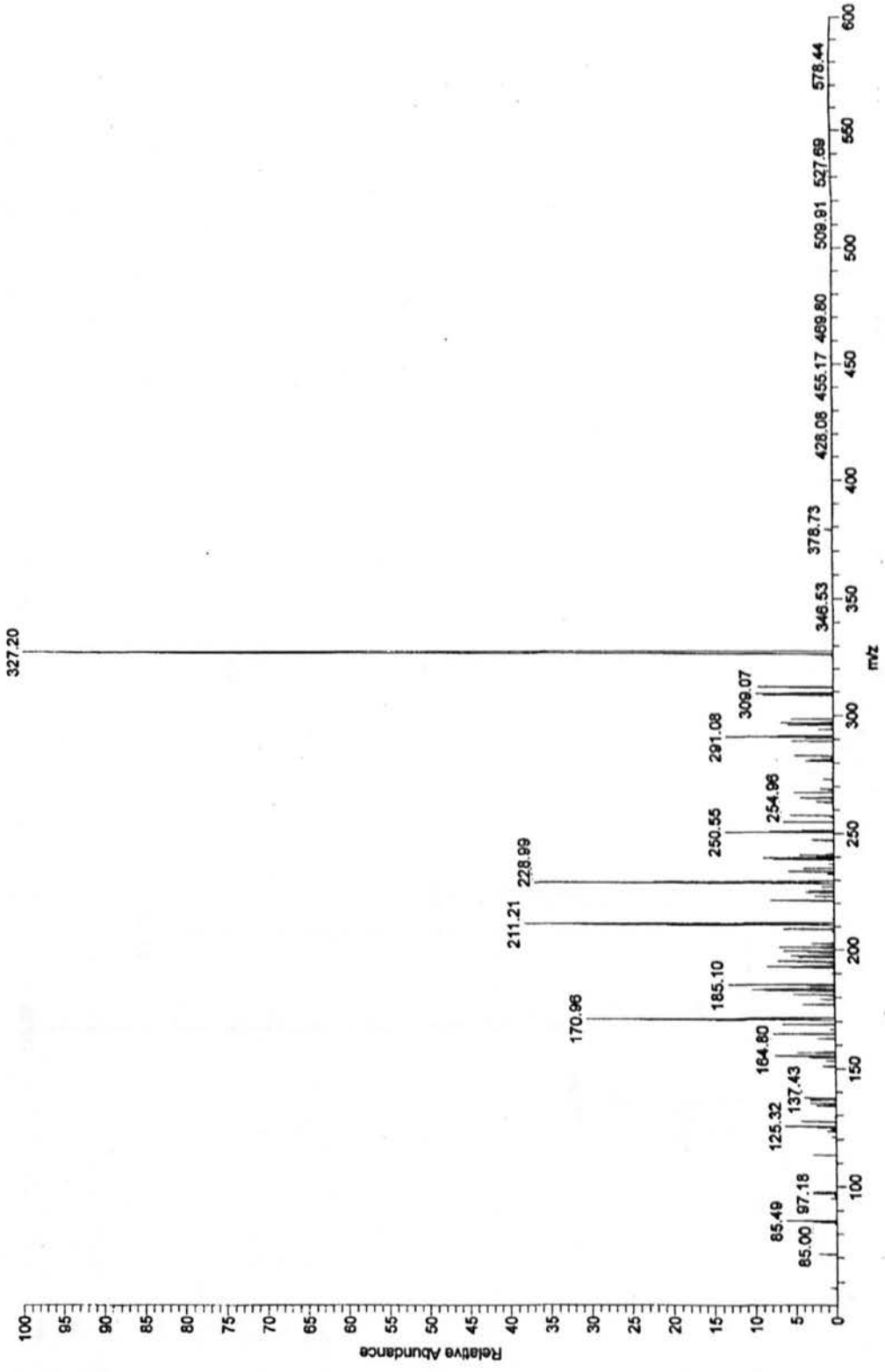


Figure A8. Mass spectrum (ESI-) for m/z 327.

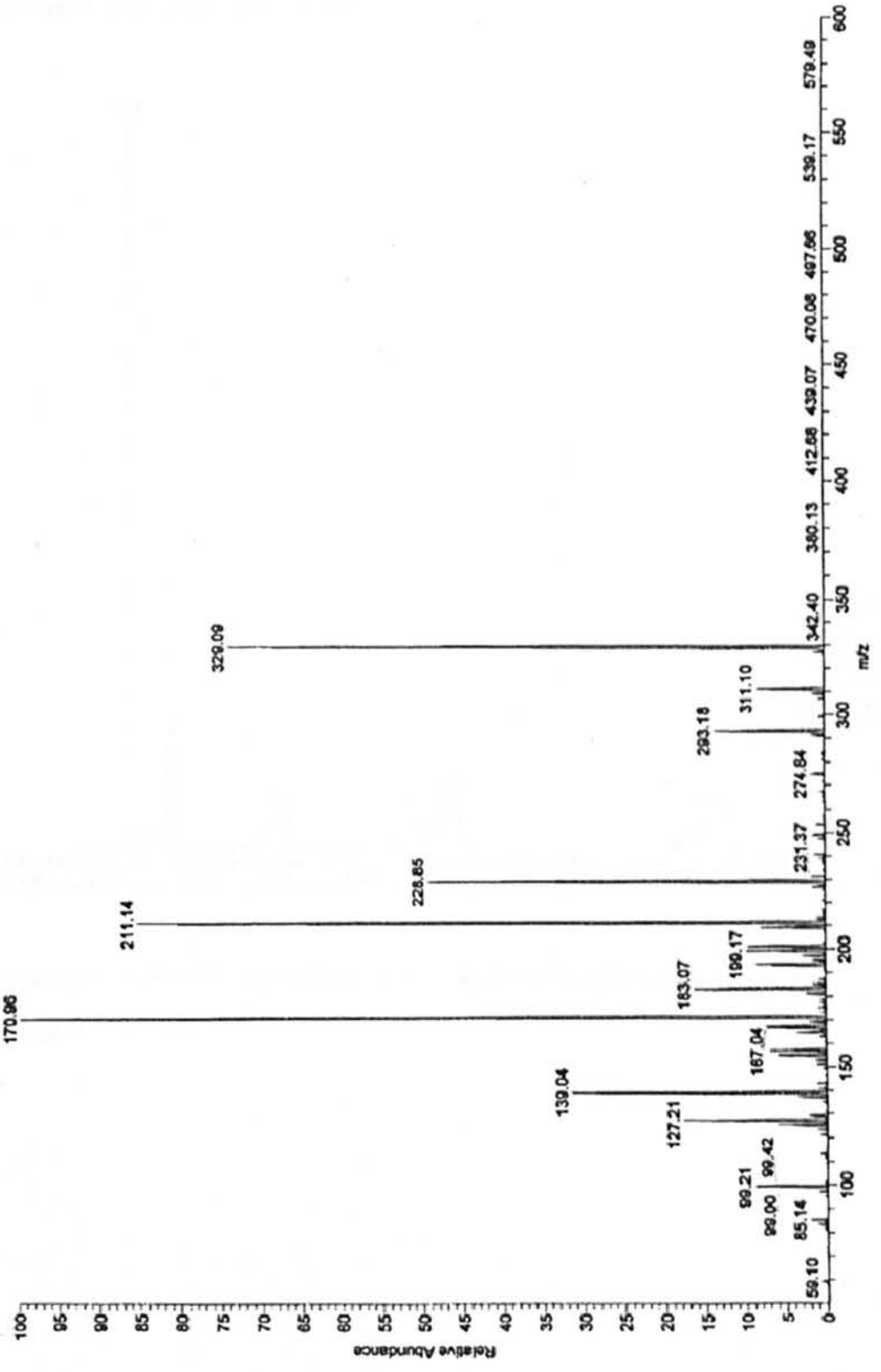


Figure A9. Mass spectrum (ESI-) for m/z 329.

m/z of compounds in the post-absorption sample of *Oldenlandia diffusa* decoction and fraction F9 (refer to Section 8.3.6) (Figures A10-A21).

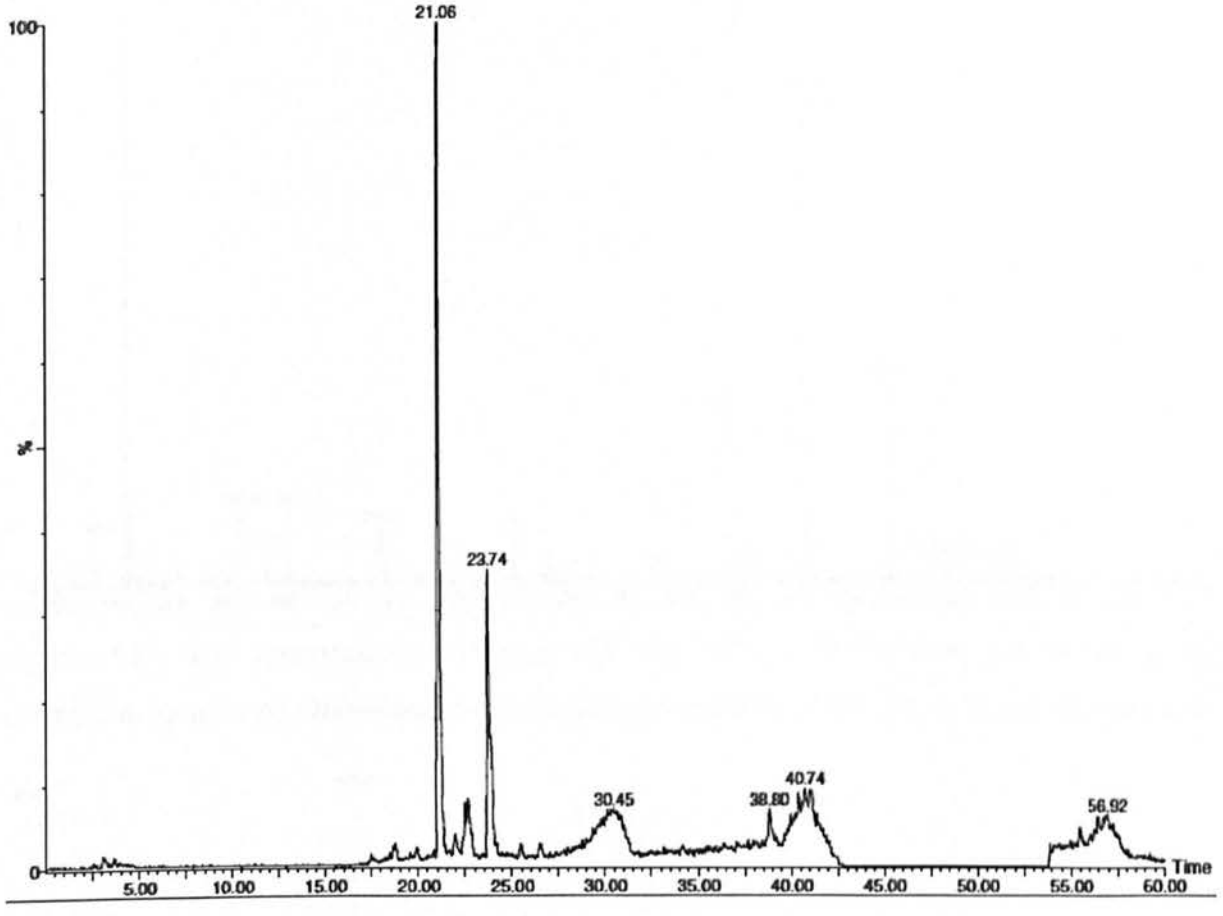


Figure A10. The chromatogram for the post-absorption sample of *Oldenlandia diffusa* decoction on LC-MS using M7 method.

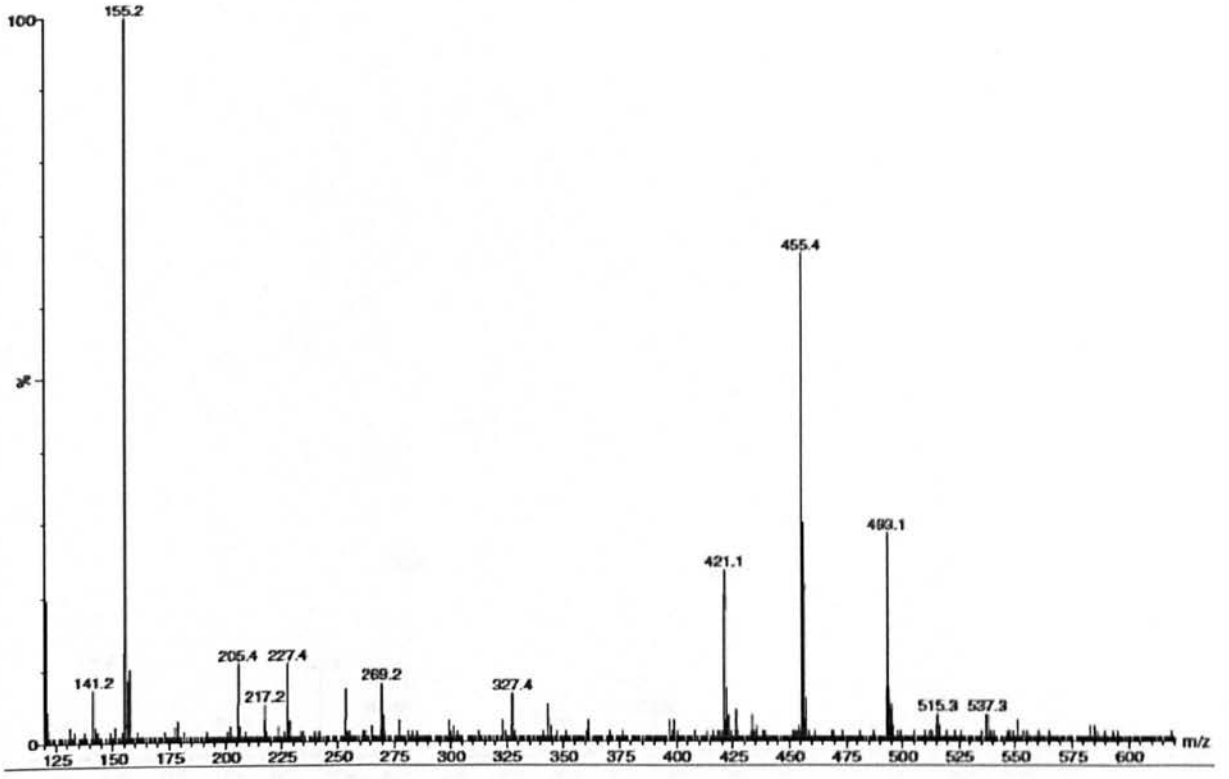


Figure A11. The spectrum for the m/z 455 and 327 (at 39.39 min) ion of the post-absorption sample of *Oldenlandia diffusa* decoction on LC-MS (ESI-) using M7 method.

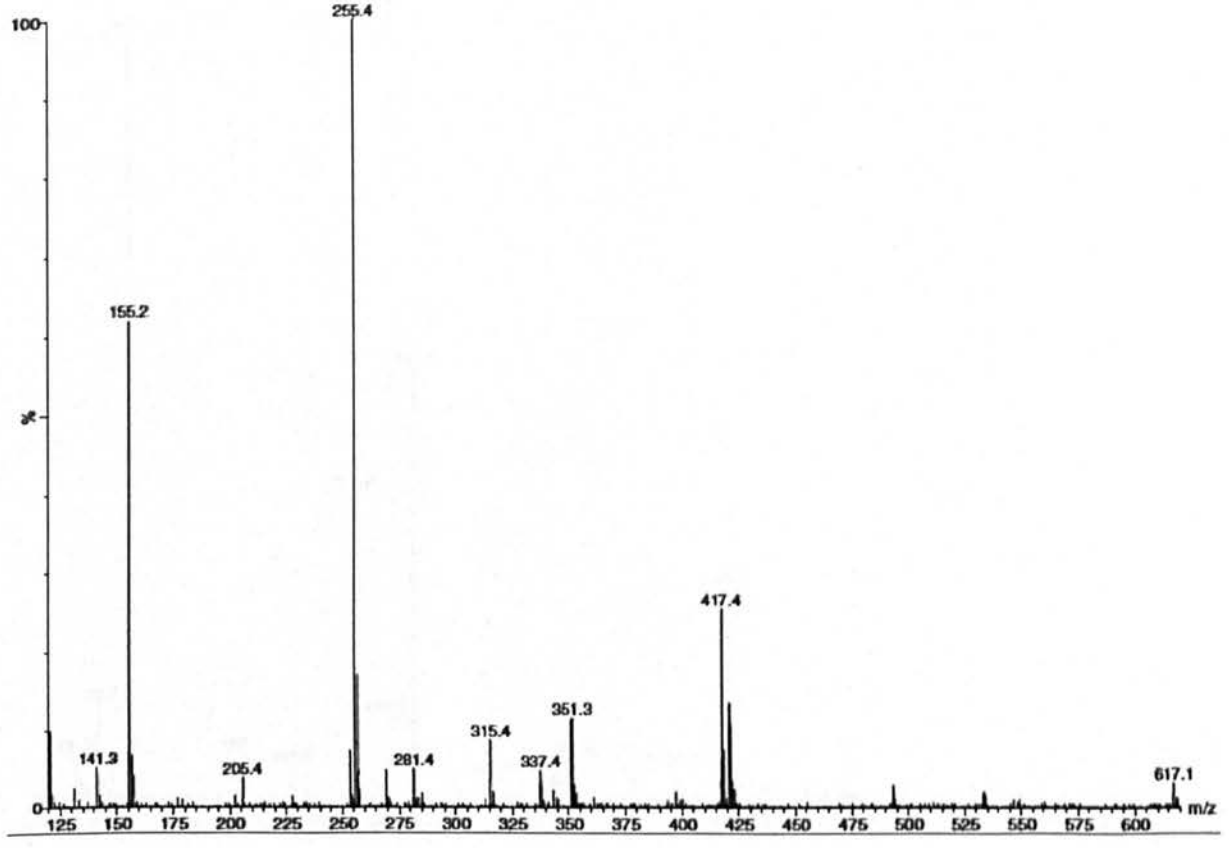


Figure A12. The spectrum for the m/z 255 ion (40.03 min) of the post-absorption sample of *Oldenlandia diffusa* decoction on LC-MS (ESI-) using M7 method.

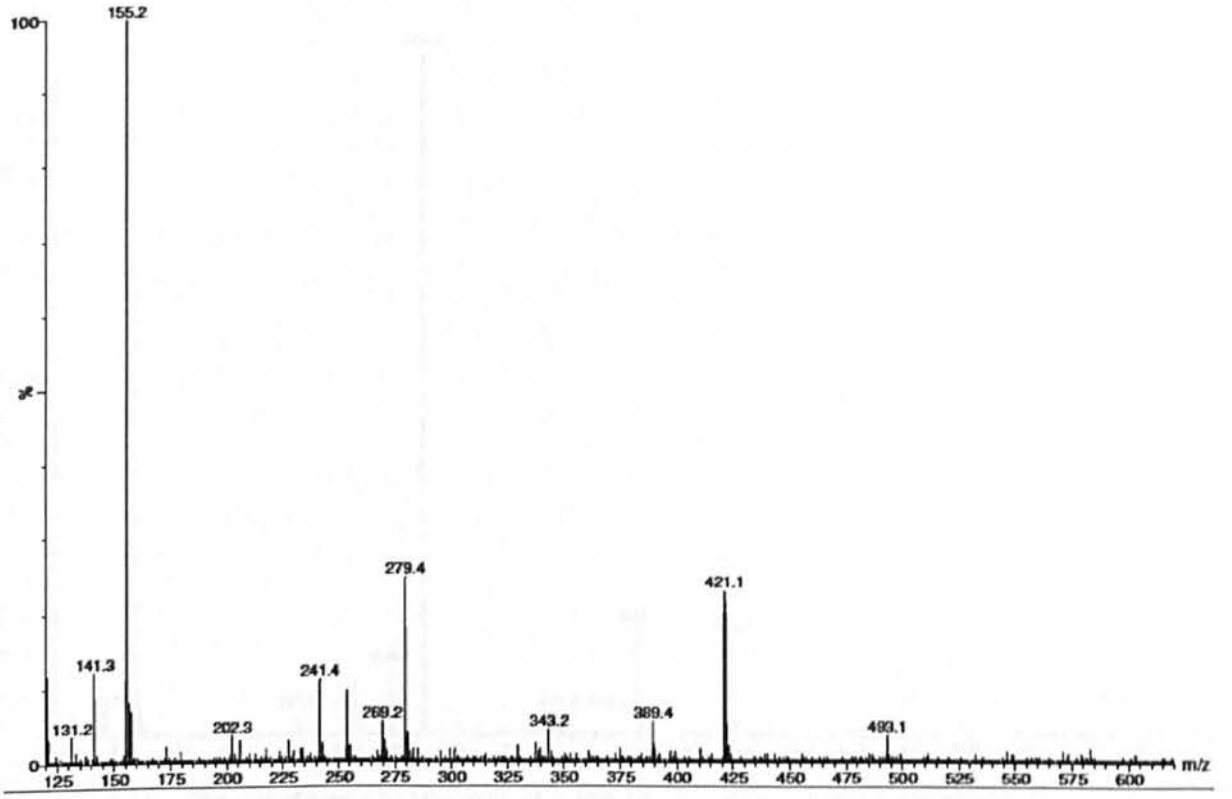


Figure A13. The spectrum for the m/z 241 and 279 ions (at 37.6 min) of the post-absorption sample of *Oldenlandia diffusa* decoction on LC-MS (ESI-) using M7 method.

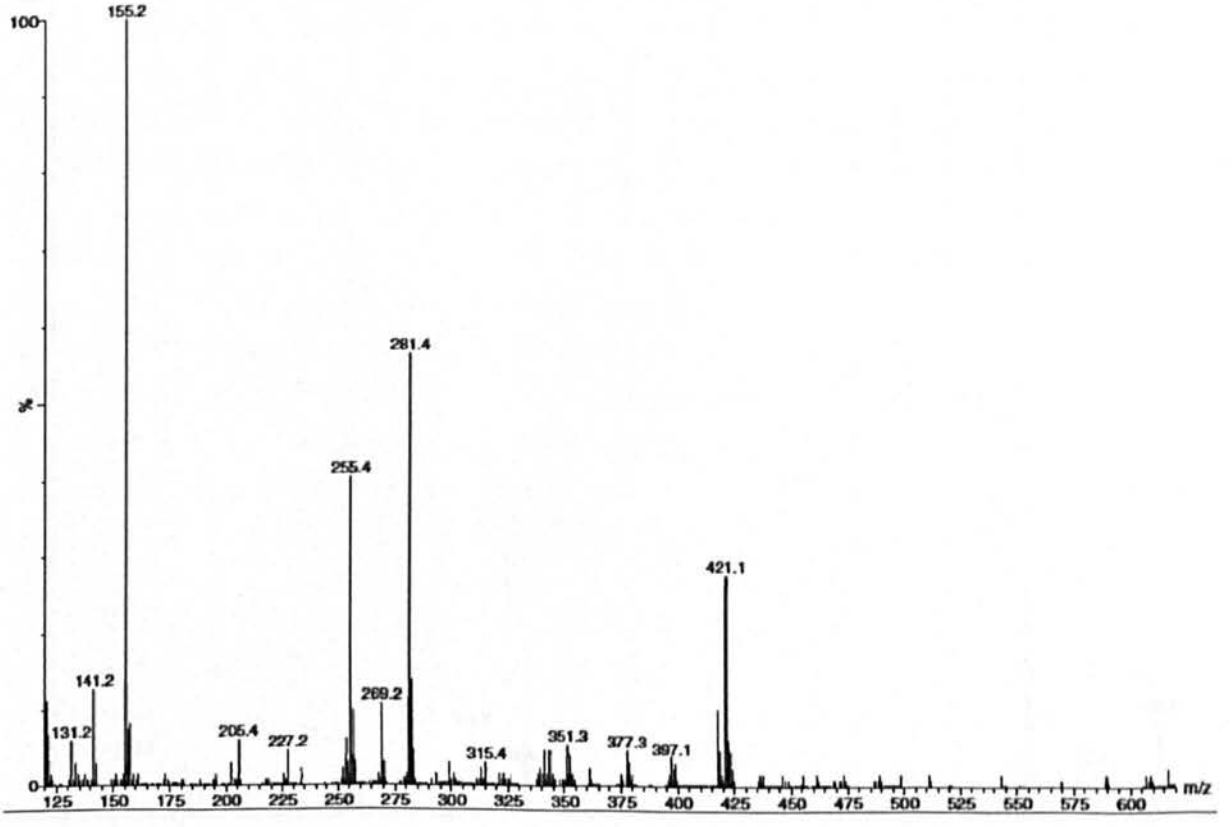


Figure A14. The spectrum for the m/z 281 and 255 ions (40.25 min) of the post-absorption sample of *Oldenlandia diffusa* decoction on LC-MS (ESI-) using M7 method.

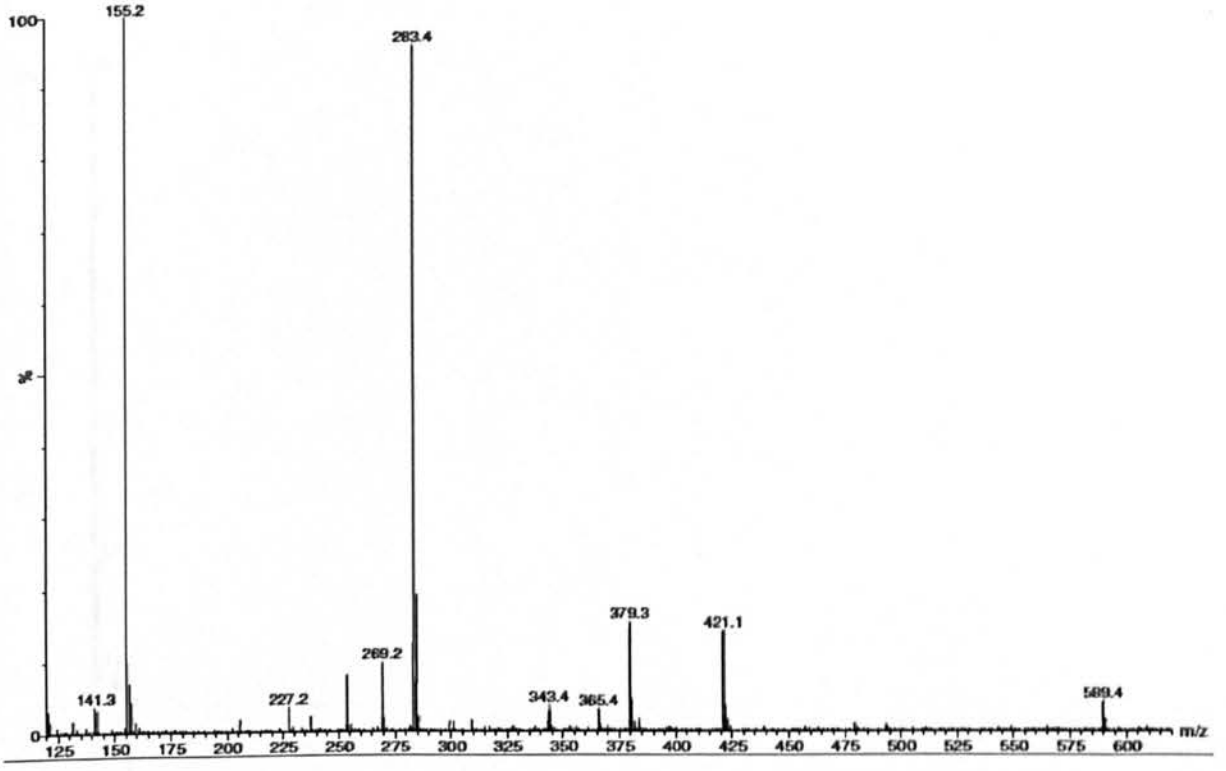


Figure A15. The spectrum for the m/z 283 ion (41.01 min) of the post-absorption sample of *Oldenlandia diffusa* decoction on LC-MS (ESI-) using M7 method.

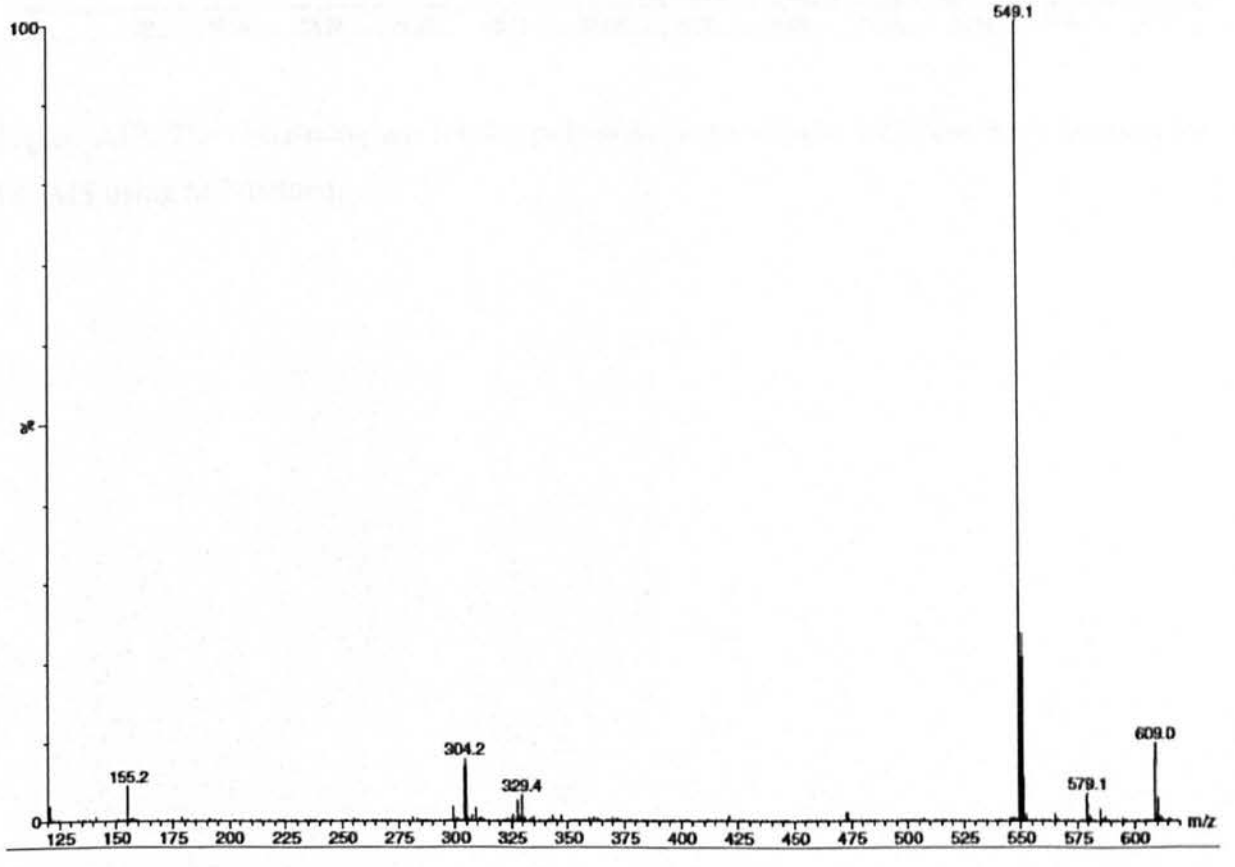


Figure A16. The spectrum for the m/z 329 and 549 ions (23.76 min) of the post-absorption sample of *Oldenlandia diffusa* decoction on LC-MS (ESI-) using M7 method.

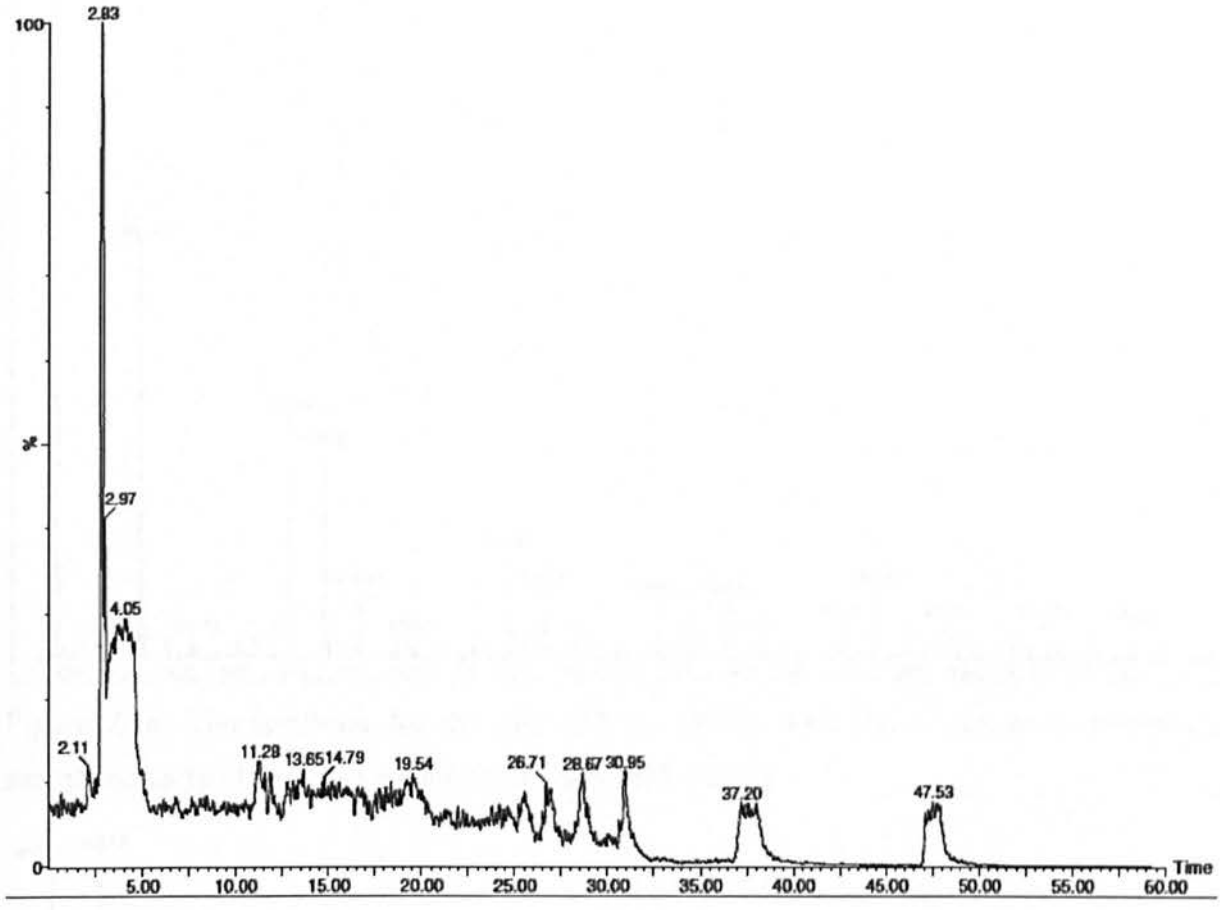


Figure A17. The chromatogram for the post-absorption sample collected from fraction F9 LC-MS using M7 method.

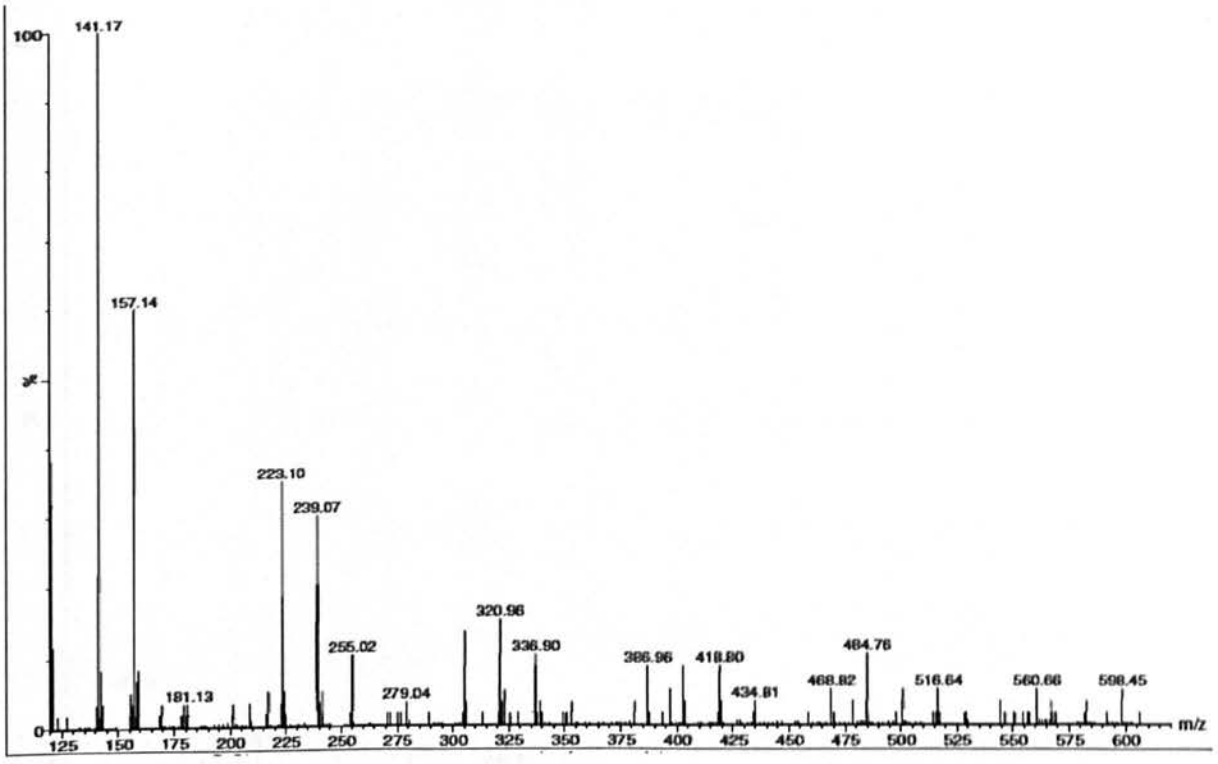


Figure A18. The spectrum for the m/z 255 (at 38.025 min) ion of the post-absorption sample collected from fraction F9 on LC-MS (ESI-) using.

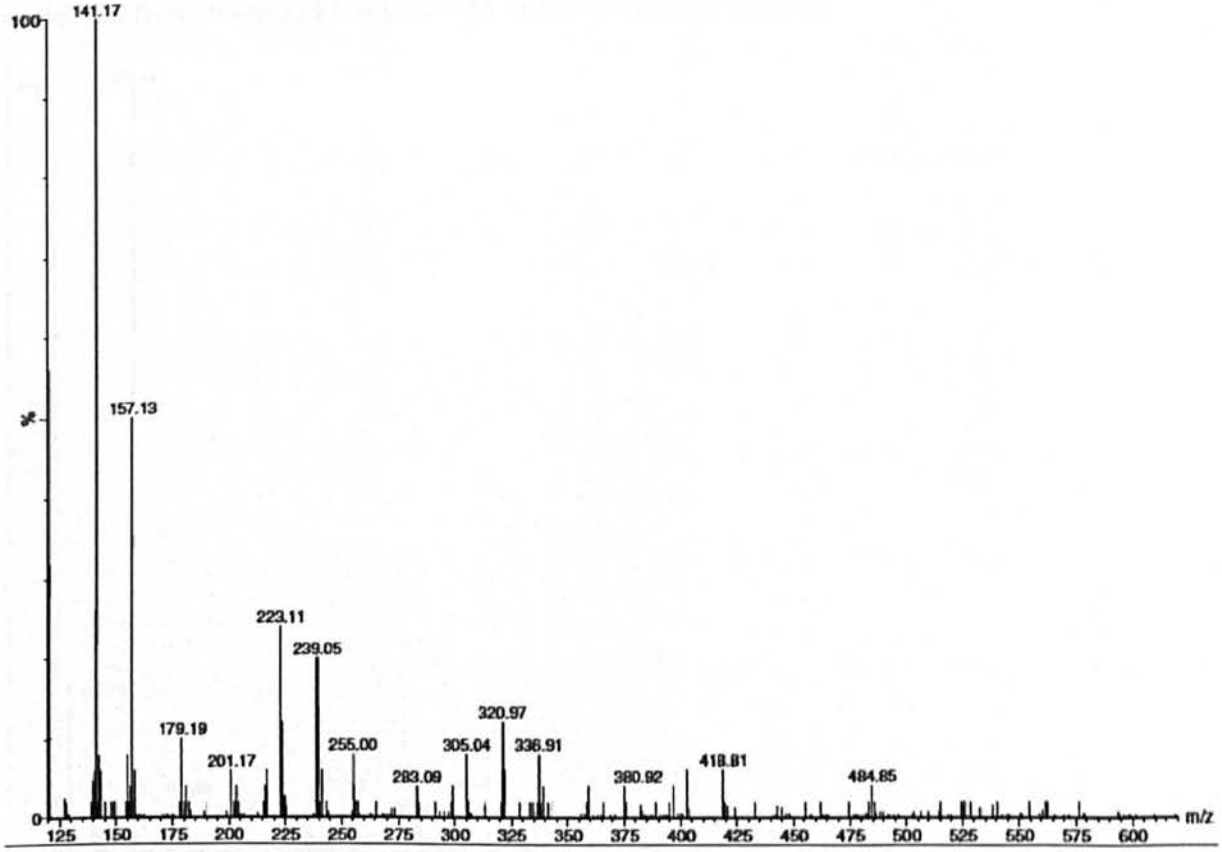


Figure A19. The spectrum for the m/z 283 and 255 (at 4.953 min) ions of the post-absorption sample collected from fraction F9 on LC-MS (ESI-) using M7 method.

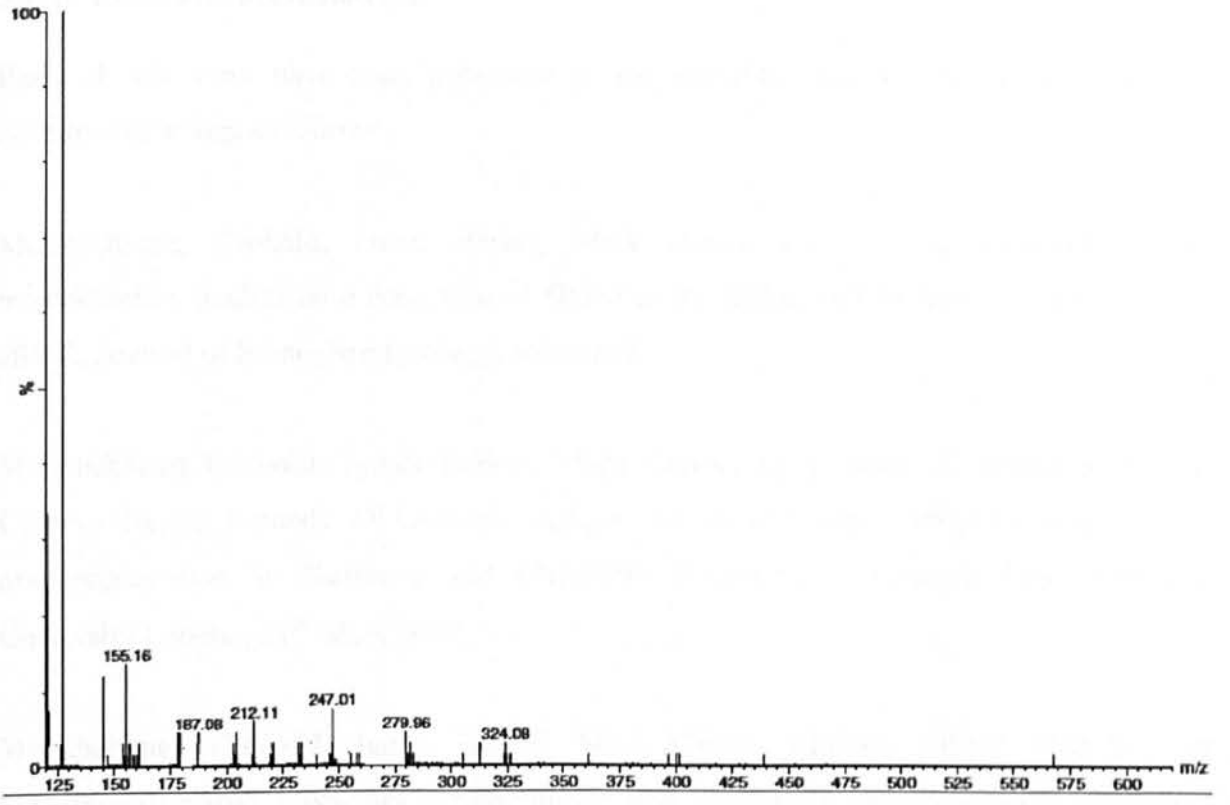


Figure A20. The spectrum for the m/z 279 (at 26.71 min) ion of the post-absorption sample collected from fraction F9 on LC-MS (ESI-) using M7 method.

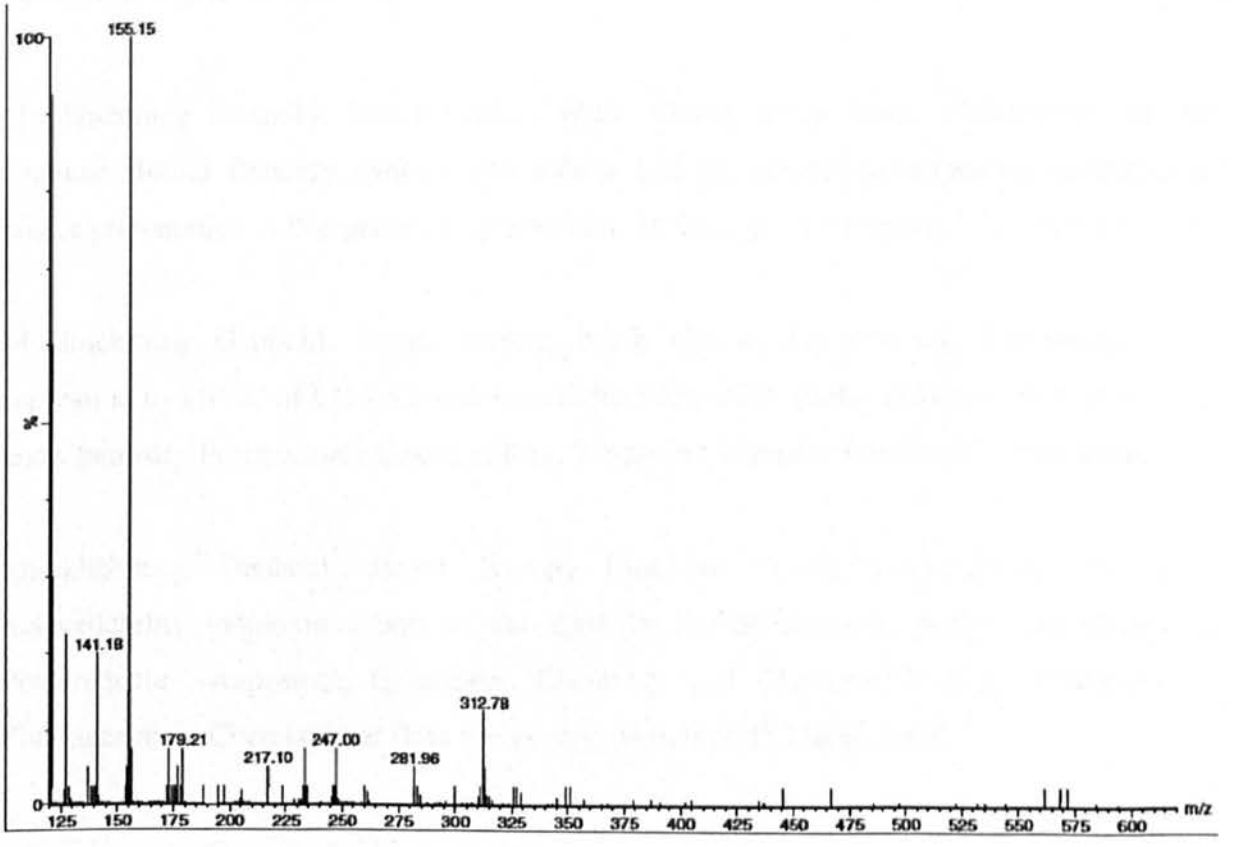


Figure A21. The spectrum for the m/z 281 (19.5 min) ion for the post-absorption sample collected from fraction F9 on LC-MS (ESI-) using M7 method.

Publications and presentations

Parts of this work have been published in the scientific literature and/or presented at scientific meetings as follows.

Munkhchimeg Ganbold, James Barker, Mark Carew, Lucy Jones, Cytotoxicity and bioavailability studies on a decoction of *Oldenlandia diffusa* and its fractions separated by HPLC, Journal of Ethnopharmacology, submitted.

Munkhchimeg Ganbold, James Barker, Mark Carew, Lucy Jones, Cytotoxicity of the Chinese Herbal Remedy *Oldenlandia diffusa* and its anti-cancer effective constituents, oral presentation at Pharmacy and Chemistry Postgraduate Research Day, Kingston University London, 21nd May, 2009.

Munkhchimeg Ganbold, James Barker, Mark Carew, Chinese Herbal Medicine, at "Traditional herbal medicines: opportunities and challenges in a changing regulatory environment" Conference, meeting at the Royal Pharmaceutical Society of Great Britain, London, 6th April 2009.

Munkhchimeg Ganbold, James Barker, Mark Carew, Lucy Jones, Cytotoxicity of the Chinese Herbal Remedy *Oldenlandia diffusa* and its anti-cell proliferation constituents, poster presentation at Postgraduate Symposium, St. George's University, 3rd December 2008.

Munkhchimeg Ganbold, James Barker, Mark Carew, Lucy Jones, Cytotoxicity and separation by HPLC of CHR *Oldenlandia diffusa* decoction, poster presentation at Pharmacy and Chemistry Postgraduate Research Day, Kingston University London, 8th May, 2008.

Munkhchimeg Ganbold, James Barker, Elizabeth Opara, Lucy Jones, The post bioavailability cytotoxic action of the Chinese Herbal Remedy, poster presentation at Postgraduate Symposium, Bioorganic Chemistry and Chemical Biology, Centenary of Pharmaceutical Chemistry, at Bath University, Monday 31st March 2008.