THE POTENTIAL OF *CANNABIS SATIVA* L. AERIAL PLANT PARTS EXTRACTS TO REVERSE DRUG RESISTANCE IN SELECTED RESISTANT LUNG- AND COLON CANCER CELL LINES

INNOCENSIA M. MANGOATO

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Faculty of Health Sciences

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Supervisor: Prof. M.G. Matsabisa

ABSTRACT

A major problem related to the successful application of chemotherapy in human cancer is anti-cancer drug resistance. Verapamil is one of the first drugs known to circumvent multidrug resistance (MDR), but its clinical application is limited by lack of efficacy in clinical trials, enhanced toxicity to normal cells and inhibition of cytochrome P450 enzymes resulting in pharmacokinetic interactions with increased host toxicity, thereby leading to severe adverse effects.

Thus, this study was designed to evaluate the potential reversal of doxorubicin resistance by *Cannabis sativa* L. extracts using selected MDR expressing lung- and colon cancer cells in an *in vitro* test model. Firstly, the pulverized plant material was sequentially extracted with four organic solvents, in order of increasing polarity, starting with hexane, dichloromethane (DCM), DCM: methanol (1:1; v/v) and methanol, respectively. A water extract was prepared to simulate traditional preparation of the plant. Crude extracts were further fractionated by means of solid phase extraction (SPE) using the following eluting concentrations: 100% H_2O, 25% acetonitrile (ACN), 50% ACN, 75% ACN and 100% ACN. The SPE yielded five fractions from each of the extracts. Qualitative phytochemical analysis performed on the pulverized crude plant material indicated the presence of glycosides, saponins, terpenoids, tannins, phytosterols and no flavonoids.

Chemical fingerprinting of the *C. sativa* L. crude extracts, SPE fractions and cannabis standards was determined by liquid chromatography tandem mass spectrometry (LC-MS). The DCM- and methanol extracts were subjected to ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS) analysis while the DCM: methanol crude extract, SPE fractions, and cannabis standards (CBD and THC) were analysed using high performance liquid chromatography tandem mass spectrometry (HPLC-MS). Compound separation was achieved with a gradient mobile phase of distilled H_2O with 0.1% formic acid (A): ACN (B) at a flow rate of 0.4ml/min. The mass spectrometer with electrospray ionization was operated in both negative and positive

mode for the DCM- and methanol extracts to avoid the destruction of chemically sensitive compounds, negative mode for the DCM: methanol extract and positive mode for the SPE fractions.

UPLC-MS analysis showed that the negative mode detected more peaks compared to the positive mode. The major peaks in the DCM extract with retention times of 10.38- (327.1967m/z), 11.31- (359.2227m/z), and 12.76 minutes (353.1766m/z) were also observed in the methanol extract, with only slight variation in the retention times at 10.37- (327.2158m/z), 13.68 (359.2227m/z), and 14.67 minutes (353.1758m/z). In the positive mode, only one peak in the DCM extract, with retention time of 12.96 minutes (282.2805m/z), was similarly observed in the methanol extract at a retention time of 14.06 minutes (282.2798m/z). Analysis of the DCM: methanol extract, SPE fractions, THC, and CBD revealed the presence of different compounds with different molecular weights. Some of the major peaks observed in both the DCM- and methanol extracts were also seen in the DCM: methanol extract. Chemical characterization of these peaks was not attempted but left for another project.

Anticancer and cytotoxicity assays were conducted against a panel of human lung- and colon cancer cells, namely; HT-29, Caco-2, NCI-H146 [H146], HCT-15 MDR, LS513 MDR and H69AR MDR cells; and human normal colon (CCD-18Co) cells. According to the American National Cancer Institute (NCI) guidelines, plant extracts with IC_{50} values of less than 20µg/ml, between 20-100µg/ml and more than 100µg/ml are considered active, moderately active and inactive, respectively. Cytotoxicity results showed that DCM: methanol extract potently inhibited the growth of Caco-2, whilst moderately inhibiting the HCT-15, LS513 and NCI-H146 [H146] cells growth. The methanol extract showed moderate growth inhibition of LS513 and NCI-H146 [H146] cells, and potently inhibited the Caco-2 cells. The hexane extract showed good growth inhibition of Caco-2 cells; and moderately inhibited LS513, NCI-H146 [H146] and H69AR cells. Similarly, the DCM and H_2O extracts showed good growth inhibition of Caco-2 and HT-29 cells, whilst moderately inhibiting the growth of HCT-15, LS513, NCI-H146 [H146], and H69AR cells growth. All the extracts appeared to be more cytotoxic towards all the lung- and colon cancerous cell lines than the normal colon cells as indicated by their selectivity indices.

The resistant reversal effect of doxorubicin by *C. sativa* L. extracts was determined on Caco-2, HCT-15, LS513 and H69AR cells through combination of the extracts with doxorubicin. *C. sativa* L. extracts showed MDR reversal activities in HCT-15, LS513 and H69AR cells characterized by decreased IC_{50} values of the extracts. In Caco-2 cells, the hexane-, DCM-, DCM: methanol- methanol- and H_2O extracts showed an increase in their IC_{50} values from 0.64-, 0.65-, 0.67-, 0.02- and 0.55µg/ml to 2.0-, 1.92-, 5.67-, 8.72- and 1.56µg/ml, respectively, and were 0.32-, 0.34-, 0.12-, 0.002- and 0.35-fold more sensitive to doxorubicin compared to verapamil with a 4.80-fold reversal factor. In contrast, the same extracts showed a reduction in their IC_{50} values from 180.5-, 140.4-, 47.08-, 140- and 25.6µg/ml to 39.33-, 40.13-, 1.45-, 1.89-and 12.3µg/ml and increased doxorubicin sensitivity in HCT-15 cells by 4.59-, 3.50-, 32.97-, 74.07- and 2.08-fold, respectively, compared to verapamil, which showed a 1.41-fold reversal factor. These extracts showed 2.2-, 300.7-, 9.1-, 4.3- and 11-fold more sensitivity to doxorubicin than verapamil with a 0.05-fold reversal factor in LS513 cells. These extracts were 0.32-, 0.34-, 0.12-, 0.002- and 0.35-fold sensitive to doxorubicin compared to verapamil with a 4.80-fold reversal factor. The same extracts also increased doxorubicin sensitivity in H69AR cells by 8.60-, 7.09-, 11.34-, 20.51- and 11.42-fold compared to verapamil that showed 0.87-fold reversal factor.

The combination index (CI) analysis demonstrated that both the control and extracts yielded a normal to very strong synergistic interaction (CI<1) in Caco-2 cells, normal to strong synergistic interaction (CI <1) in HCT-15 cells, moderate to strong synergistic interaction (CI <1) in LS513 cells and nearly additive (CI=1) to antagonistic interaction (CI >1) in H69AR cells. Based on this evidence, the extracts were successful in increasing the sensitivity of HCT-15, LS513 and H69AR cells to doxorubicin *in vitro*. Future research is warranted to purify the most active extract and study the biological mechanisms involved in reversing doxorubicin resistance both *in vitro* and *in vivo*.

DECLARATION OF INDEPENDENT WORK

I, Innocensia Mangoato, hereby declare that this dissertation hereby submitted by me for the M.Med.Sc degree in Pharmacology at the University of the Free State is my own independent work and has not previously been submitted by me at another university or faculty for admission to a degree or diploma. I furthermore cede copyright of the dissertation to the University of the Free State.

…… ……

Signature Date

SUPERVISOR'S DECLARACTION

I, Professor Gilbert Motlalepula Matsabisa, the supervisor of the master's research dissertation entitled: **The potential of *Cannabis sativa* L. aerial plant parts extracts to reverse drug resistance in selected resistant lung and colon cancer cell lines**, hereby certify that the work in this project was done by Innocensia Mangoato at the Department of Pharmacology, University of the Free State.

I hereby approve submission of this thesis and also affirm that this has not been submitted previously, either in part or in its entirety, to the assessors, neither to this or any other institution for admission to a degree or any other qualification.

…… ……

Signature Date

DEDICATION

This work is dedicated to my family, my parents (Abram and Asteria), and my brothers (Guillermo and Jaffet) for having faith in me and for their support and encouragement throughout the project.

Bwana Mungu ametubariki kwa namna ya ajabu na atazidi kutubariki.

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Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ACN	Acetonitrile
BCRP	Breast cancer resistance protein
CANSA	Cancer Association of South Africa
CBC	Cannabichromene
CBCA	Cannabichromenic acid
CBCV	Cannabichromevarin
CBD	Cannabidiol
CBDA	Cannabidiol acid
CBDV	Cannabidivarin
CBE	Cannabielsoin
CBG	Cannabigeraol
CBGAM	Cannabigerolic acid monomethylether
CBL	Cannabicyclol
CBV	Cannabivarin
CI	Combination index
CSA	Cyclosporin A
CYP	Cytochrome P450
DAD	Diode array detector

DCM	Dichloromethane
D:M	Dichloromethane: methanol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Docetaxel
DOX	Doxorubicin
DPBS	Dulbecco’s phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EMEM	Eagle’s minimum essential medium
ESI	Electrospray ionization
FBS	Fetal bovine serum
FLD	Fluorescence detector
g	Grams
H_2O	Water
HEX	Hexane
HI-FBS	Heat inactivated fetal bovine serum
HPLC	High performance liquid chromatography
IC_{50}	Concentration that results in inhibition of 50% of biological activity
LC-MS	Liquid chromatography mass spectrometry
LCRP	Lung cancer resistance protein

MDR	Multidrug resistance
MDR 1	Multidrug resistance drug 1
MEOH	Methanol
MRP	Multidrug resistance associated protein
MS	Mass spectrum
MSDs	Membrane-spanning domains
m	Mass
m/z	Mass per ratio
mg/ml	Milligram per milliliter
µg/ml	Microgram per milliliter
µl	Microliter
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MXR	Multixenobiotic
NBDs	Nucleotide-binding domains
NCR	National Cancer Registry
nm	Nanometer
NMR	Nuclear magnetic resonance
NSCLC	Non-small cell lung cancer
PDA	Photodiode array
***P*-gp**	P-glycoprotein
PARP	Poly (ADP-ribose) polymerase

QTOF	Quadrupole time of flight
RNA	Ribonucleic acid
r.p.m	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SCLC	Small cell lung cancer
SD	Standard deviation
SI	Selectivity index
SPE	Solid phase extraction
THC	∆9-Tetrathydrocannabinoidal
THCA-C4	Tetrahydrocannabinolic acid
THCV	Tetrahydrocannabivarin
TLC	Thin layer chromatography
TMDs	Transmembrane-binding domains
UPLC-MS	Ultra performance liquid chromatography mass spectrometry
UV	Ultra violet
v/v	Volume per volume
v/v/v	Volume per volume per volume
WHO	World Health Organization
w/v	Weight per volume ratio

CHAPTER ONE: INTRODUCTION

Cancer is the leading cause of death worldwide in both developing- and non-developing countries (1). The International Agency for Research in Cancer revealed that Africa, Asia and South America not only account for 60% of the world's cancer cases, but that 70% of the deaths caused by cancer globally are also from these regions (2). Lung- and colon cancer are the most diagnosed and leading cause of cancer deaths in both men and women worldwide (3,4). Moodley *et al*. (2016) reported that both lung- and colon cancer are amongst the most commonly diagnosed cancers in South Africa (5).

A major common cause of failure of standard chemotherapeutic agents, is drug resistance (6). The ability of cancer cells to simultaneously develop resistance against structurally- and functionally unrelated anticancer drugs is known as multidrug resistance. Multidrug resistance (MDR) results in lower intracellular drug levels by limiting the uptake of drugs, which requires transporters to enter the cells and enhancing the efflux of drugs. These changes in return inhibit the apoptotic effect activated by most anticancer drugs (7).

Overexpression of ATP-binding cassette (ABC) transporters is one of the prominent mechanisms of MDR. Several members of these transporters, namely, p-glycoproteins (P-gp), multidrug resistance associated proteins (MRP1, MRP2, MRP3, and MRP7) and breast cancer resistance protein (BCRP), are involved in the efflux of toxic endogenous substances and xenobiotics out of cells (8). One of the approaches developed to overcome resistance to anticancer treatment, is by either blocking or inactivating ABC transporters, using three generations of multiple agents. Examples of agents belonging to these categories include verapamil, cyclosporin A (CSA), quinine, valspodar, biricodar, zosuquidar and tariquidar (8,9).

However, the use of these compounds are limited by intolerable toxicity at clinical doses; lack of significant efficacy in clinical trials when used in conjunction with other anticancer drugs, and inhibiting the cytochrome P450 enzymes, thereby resulting in decreased metabolism and clearance of substrates, leading to systemic toxicity (8,9).

Apart from the clinical adverse effects, other factors associated with the need to develop new compounds to circumvent MDR in cancer, as stipulated by the World Health Organization (WHO), included: the evolution of the disease, treatment failure, the inability of patients from rural areas to access treatment as well as the cost of treatment. This results in patients resorting to traditional herbal medicine as means of primary health care. Research on traditional medicines has revealed a variety of natural compounds such as curcumin, epigallocatechin, flavonoids and terpenoids in the quest to inhibit the overexpression of certain ABC- transporters (6,10–12).

Globally, Cannabis is the most widely used illicit drug for a variety of ailments including cancer treatment. Cancer patients use it therapeutically for its anti-emetic-, analgesic- and appetite stimulant properties (13,14). Interestingly, numerous studies have shown that ∆9-tetrathydrocannabinoidal (THC), the primary psychoactive component of cannabis plants, inhibits tumour growth and decreases *P*-gp expressions to a similar extend as flavonoids and curcumin (14). However, there is insufficient scientific knowledge of its ability to inhibit other efflux drug transporters that play a role in multidrug resistance in cancer.

The present study was therefore designed to demonstrate the potential of *Cannabis sativa* L. extracts to reverse resistance, using selected lung- and colon cancer cells, and also to scientifically characterize the traditional formulation of these *Cannabis sativa* L. extracts to ensure efficacy and safety.

CHAPTER TWO: LITERATURE REVIEW

SECTION ONE: OVERVIEW OF MULTIDRUG RESISTANCE IN CANCER

2.1. INTRODUCTION

Diseases related to cancer are estimated to being the second largest cause of death globally, killing more than 8.2 million individuals every year, especially in developing countries (15,16). Cancer is a condition characterized by invasive abnormal proliferation of cells with a rapid growth rate that is usually accompanied by the spread to other, distant points in the body. When malignant tumours are left untreated, they inevitably lead to death of the host. The genes that regulate the process that forms these tumour cells are called proto-oncogenes. They code for the proteins that indirectly regulate apoptosis, mitosis and deoxyribonucleic acid (DNA) repair. When these proto-oncogenes are defective, they are referred to as oncogenes because their abnormal proteins cannot regulate normal tissue formation. If this balance is disrupted, tumour development follows (2).

2.2. CANCER

Over 200 different types of cancers are known to affect humans. A recent review on cancer research in South Africa reported that 1 in 9 women and 1 in 8 men are at risk of developing cancer in their lifetime (5). Based on data of the Cancer Association of South Africa (CANSA) data, there are many factors known to increase the risk of cancer in humans, such as tobacco use, alcohol consumption, dietary factors, certain viral- and bacterial infections, exposure to radiation, lack of physical activity, obesity, and environmental pollutants. These factors can directly damage genes or combine with existing genetic mutations within cells to cause cancer (17).

2.3. ANATOMY AND PHYSIOLOGY OF THE LUNGS

The lungs (see Figure 2.1) form part of the respiratory system, which also includes the nose and mouth, the trachea and bronchi. These structures are located in the chest and surrounded by the chest wall. As air is breathed in, it passes from the nose or mouth, through the trachea, bronchi and bronchioles into the alveoli. The main functions of the

lungs are to transfer oxygen from air to the blood, and then to release carbon dioxide from the blood to the air. The lungs also play a role in the body's defences against harmful substances in the air, such as smoke, pollution, bacteria or viruses (18).

2.3.1. Lung cancer

Lung cancer is the leading cause of cancer related deaths globally, accounting for 1.6 million deaths annually with an estimated 1.8 million new cases reported annually worldwide (19,20). According to data from the South African National Cancer Registry data, lung cancer is the second most common cancer in men (5). The American Cancer Society also reported that more people die of lung cancer in the United States than of colon-, breast- and prostate cancers combined (3).

2.3.2. Classification of lung cancer

Lung cancer forms in the tissue of the lung, usually in the cells lining the air passages. It is classified into two categories: non-small cell lung cancer (NSCLC), which accounts for 80% of all lung cancer reports; and small cell lung cancer (SCLC), which is normally more aggressive as compared to NSCLC (3,20–22). The most common types of NSCLC are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma, but there are several other types that occur less frequently (22). SCLC consists of pure small cell-, mixed small cell-, and large cell carcinoma (21,22). The classification of lung tumours has an important purpose for patient care, since treatment varies greatly depending on the type- or stage of lung cancer diagnosed. Furthermore, lung cancer classification enables physicians and surgeons to choose the best treatment for each patient (21).

2.3.3. Risk factors and symptoms of lung cancer

Smoking is a major risk factor for lung cancer, as tobacco contains cancer-causing agents. Other known risk factors include: exposure to radon; diesel exhaust fumes; radioactive ores, such as uranium; previous radiation therapy to the lungs; and a personal- or family history of lung cancer (3).

The most common symptoms of lung cancer are: coughing which is present in 65 – 75% of patients; chest discomfort or pain; hemoptysis (coughing up of blood); dyspnea,

that develops early in about 60% of patients; loss of appetite accompanied by weight loss; fatigue; infections, such as bronchitis pneumonia; Cushing's syndrome; hypercalcemia; severe headaches; wheezing; and seizures (21,23).

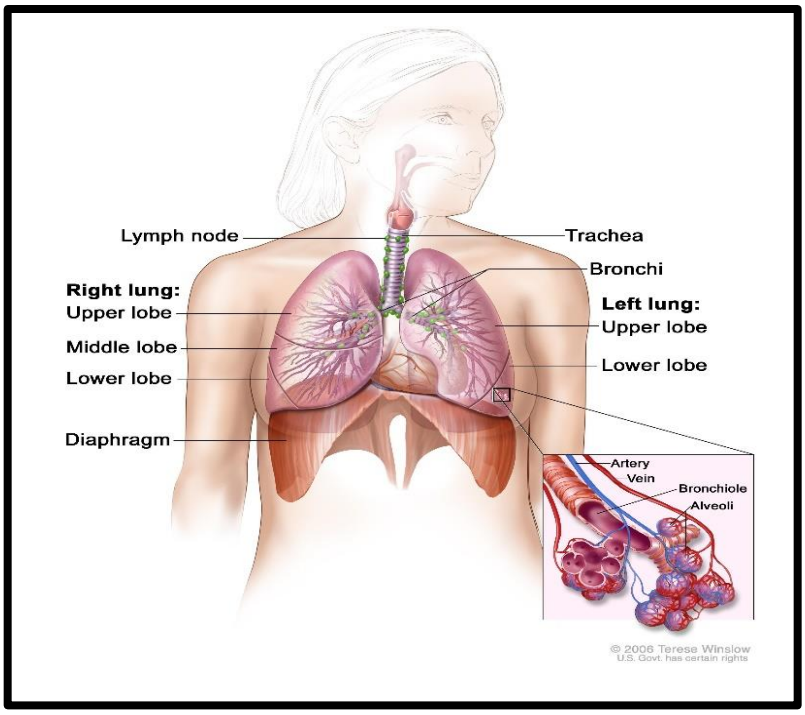

Figure 2.1: Anatomy of the respiratory system [Available from: (22)]

2.4. ANATOMY AND PHYSIOLOGY OF THE COLON

The colon (see Figure 2.2) (24) forms the largest part of the gastrointestinal tract. It is composed of the ascending colon, transverse colon, descending colon, and an S-shape towards the end of the descending colon forming the sigmoid colon, the rectum and the anal canal. A primary function of the large intestine is to store feces before defecation. The colon also acts as a reservoir for indigestible food residues, unabsorbed biliary components and remaining fluid contents delivered from the small intestines; and absorbs water and electrolytes to assist with the elimination of fecal matter (18,25).

2.4.1. Colon cancer

Colorectal cancer is the third most commonly diagnosed cancer, and the fourth leading cause of oncological deaths in men, while third in women, worldwide (26,27). In South Africa, colon cancer is the third most diagnosed cancer, after breast- and cervical

cancer (5). Colorectal cancer develops either in the colon or rectum, slowly over a period of 10 - 20 years. The incidence and mortality rates of colorectal cancer increase with age and are about 30 - 40% higher in men than in women (4,26).

2.4.2. Risk factors and symptoms of colon cancer

Risk factors associated with colorectal cancer include: a personal- or family history of chronic inflammatory bowel disease (23); and other behavioral factors such as physical inactivity (28), heavy alcohol consumption, unhealthy diet involving high consumption of red and/or processed meat, smoking prevalence and being overweight and obese (4,26).

The symptoms associated with colorectal cancer often appear as the tumour increases in size, resulting in bleeding and obstruction of the intestines. These include: bleeding from the rectum; blood in the stool or in the toilet after having a bowel movement; dark or black stools; a change in the shape of the stool (e.g. more narrow than usual); cramping or discomfort in the lower abdomen; an urge to have a bowel movement when the bowel is empty; constipation and diarrhea that last for more than a few days; decreased appetite; anemia; and unintentional weight loss (23).

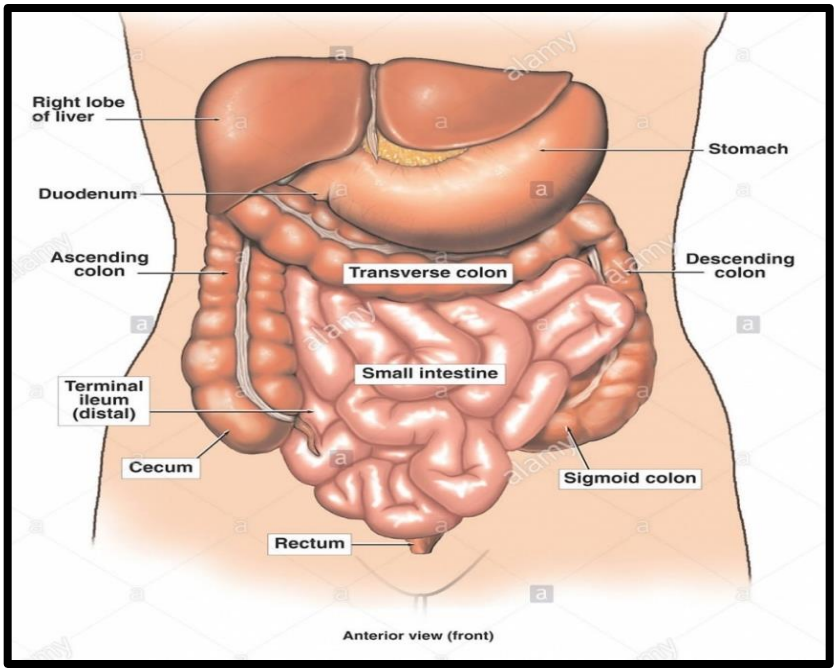

Figure 2.2: Anatomy of the large and small intestines [Available from: (24)]

2.5. CHEMOTHERAPY

Chemotherapy is one of the principle modes of treatment of cancer. It involves the use of toxic substances to destroy the DNA in tumour cells, by directly damaging it or inducing faulty division. However, this mode of treatment also destroys normal proliferating cells thus resulting in serious adverse events (1,2). During chemotherapy, the cytotoxic drugs used, induce a tolerable effect on the cells, which initially causes a decrease in the growth of the tumour. After a while, the anti-tumour effect fades and the growth of the tumour continues at its initial high rate. This development of tolerance to the drugs is caused by a group of tumour cells which are resistant to the drug (2). Resistance of these tumour cells to anti-cancer drugs is one of the major reasons why chemotherapy fails to treat cancer (1,29).

2.6. DRUG RESISTANCE IN CANCER

A major obstacle associated with chemotherapy, is drug resistance, albeit cellular drug resistance or multidrug resistance. The ability of these tumour cells to become resistant to certain anticancer drugs may be of acquired- or intrinsic nature. Acquired resistance presents after exposure to anti-cancer drugs with a targeted mutation, whereas intrinsic resistance has been present prior to the treatment with an anti-cancer drug. Furthermore, a number of factors such as altered pharmacokinetics, limited penetration of the drug into the tumour, and administration of inadequate doses, justify the causes of drug resistance in cancer (1,12). Figure 2.3 shows the entry of an anti-cancer drug into a cancer cell, through the membrane and under normal circumstances, whereas Figure 2.4 shows a cancer cell with increased P-gp expression across the membrane, which actively extrudes the anti-cancer drug resulting in low intracellular concentrations below the therapeutic level.

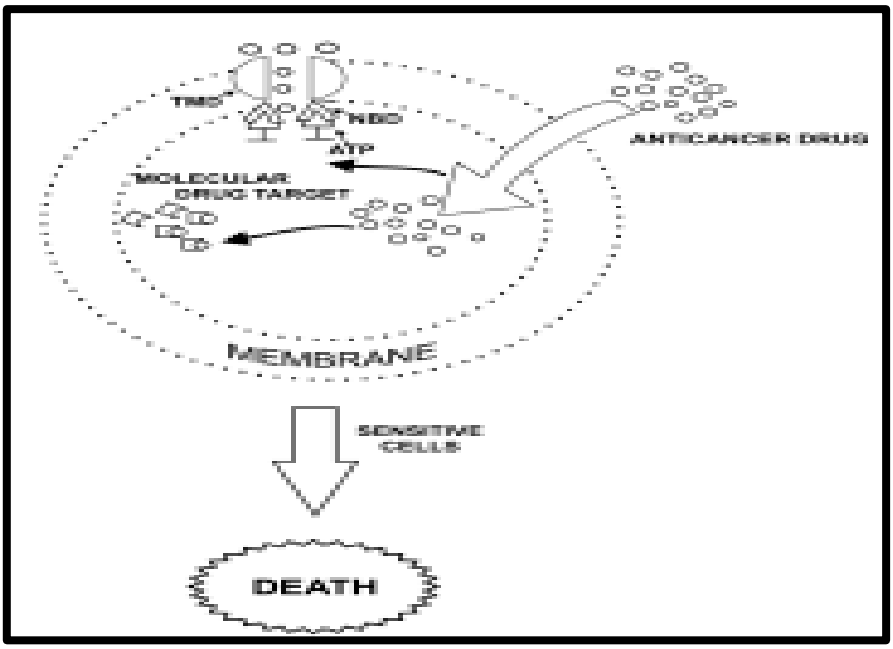

Figure 2.3: Schematic presentation of the entry of anticancer drugs into a cancer cell via the membrane [Available from: (6)]

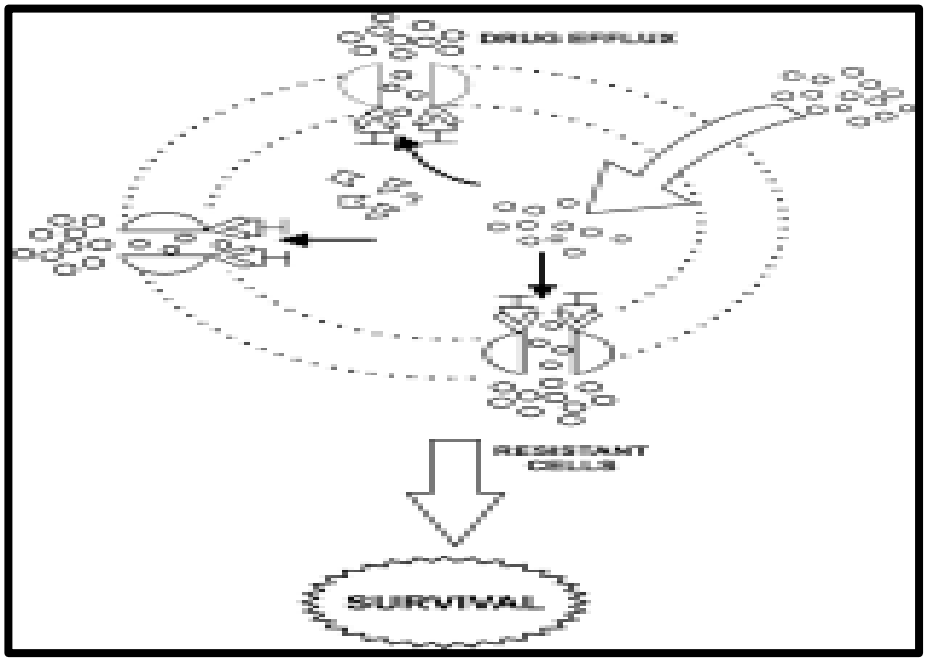

Figure 2.4: Schematic presentation of the phenomenon of MDR in cancer cells [Available from: (6)]

2.7. MULTIDRUG RESISTANCE IN CANCER

Multidrug resistance is a phenomenon, mirrored by cross-resistance, to a variety of anti-cancer drugs with different chemical structures and intracellular mechanisms of action (11). The underlying mechanisms by which cancers elude treatment, also described in Figure 2.5, includes the overexpression of ATP-binding cassette (ABC) efflux transporters, reduced uptake of drugs, diminished apoptotic signaling, alteration of drug targets, detoxifying enzymes, and genetic responses which may occur before or during therapy (7,11,30). Of all, the main mechanism that constitutes the development of MDR is the presence and overexpression of ABC transporters (29).

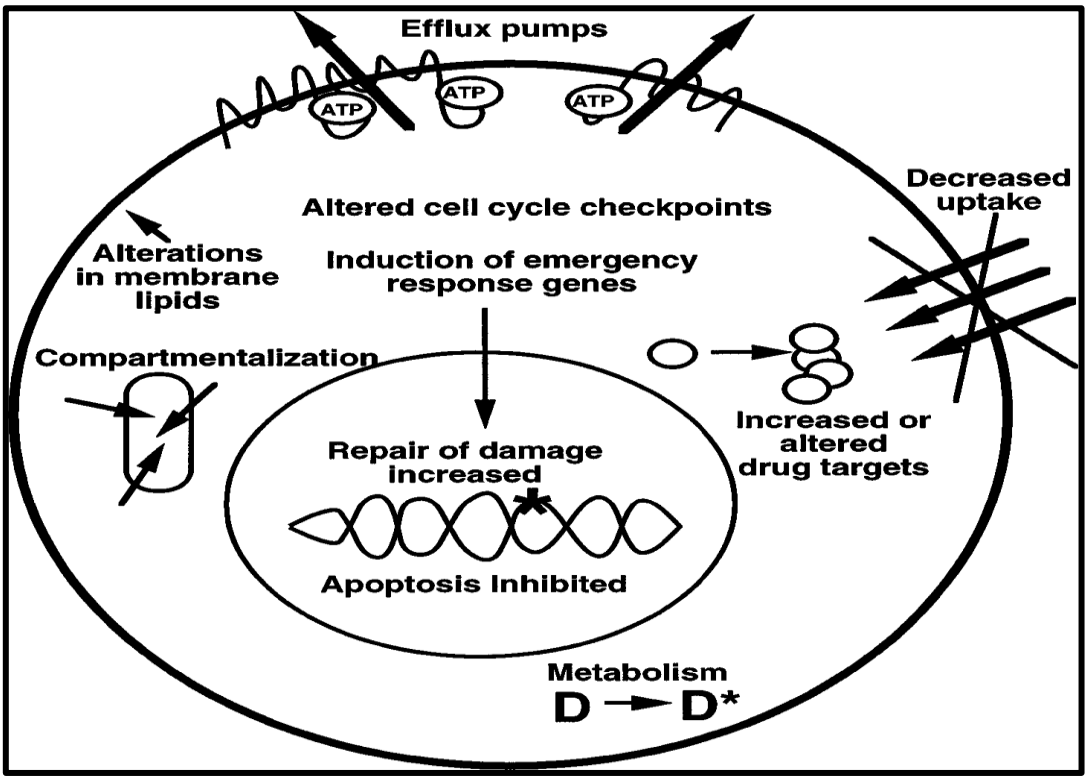

Figure 2.5: Schematic summary of ways in which cultured cancer cells have been shown to become resistant to cytotoxic anti-cancer drugs. The efflux pump shown in the plasma membrane includes MDR1, MRP family members, and BCRP [Available from: (7)]

2.8. MECHANISMS OF MULTIDRUG RESISTANCE

There are a number of mechanisms that mediate the development of MDR, and they can be classified as either non-cellular or cellular, depending on the factors that result in MDR (6).

2.8.1. Non-cellular multidrug resistance

Multidrug resistance in cancer by this mechanism is mainly due to extracellular factors, such as limited cell growth environment or vascular accessibility. Extracellular factors are often observed in solid tumours, where MDR is characterized by increased interstitial fluid pressure in comparison to normal tissues. This phenomenon leads to reduced drug access to areas within the solid tumour and protects the tumours cells from cytotoxicity. This mechanism normally holds for certain types of cancer, which portray intrinsic- or acquired resistance to the anticancer drugs at their initial exposure to the tumour (6,31).

2.8.2. Cellular multidrug resistance

This mechanism involves factors which can either be transport-based or non-transport-based. The non-transport-based mechanism of multidrug resistance involves enzymes which do not alter the drug's effective intracellular concentration, but rather limits its desired activity. On the other hand, transport-based cellular multidrug resistance ensures the expulsion of chemotherapeutic drugs from tumour cells by various energy-dependent membrane transport proteins, resulting in intracellular drug concentrations below the killing threshold (6).

2.9. THE MECHANISM OF TRANSPORT-BASED MULTIDRUG RESISTANCE

Transporter proteins are mainly located on the lipid bilayer of biological membranes. They are crucial determinants of the pharmacokinetics and pharmacodynamics of many drugs (32). The mechanisms of classical transport-based MDR are related to the ABC family of membrane transporters, which are responsible for the ATP-dependent movement of a wide variety of xenobiotics (including cytotoxic drugs), lipids and metabolic products across the plasma membrane (32,33). Cytotoxic drugs that are mostly associated with classical MDR are hydrophobic, amphipathic natural products, such as: taxanes, paclitaxel and docetaxel; vinca alkaloids, vincristine and vinblastine;

anthracyclines, doxorubicin, daunorubicin and epirubicin; epipodophyllotoxins, etoposide and teniposide; and antimetabolites, methotrexate, fluorouracil and 6-mercaptopurine (32).

2.10. ATP-BINDING CASSETTE TRANSPORTERS

ABC drug transporters are a group of active transporters located in the plasma membrane of cells involved mainly in MDR *in vitro*. They utilize energy, obtained from the hydrolysis of ATP, to transport their substrates across the membrane against a concentration gradient (8,29,34). ABC transporters constitute a large family of 49 members, which are divided into seven subfamilies, ABCA through ABCG. Structurally, they have two nucleotide-binding domains (NBDs) and two transmembrane binding domains (TMDs) (8). Amongst which, the overexpression of P-gp, MRP1, MRP7, BCRP or multixenobiotic resistance (MXR), and lung cancer resistance protein (LCRP) seriously affect chemotherapy (11). Four MDR-ABC transporters are discussed below.

2.10.1. ABCB1/MDR1/P-gp transporters

P-gp is one of the first MDR transporters to be discovered, and has been extensively studied and characterized (6,8). The transporter is encoded by the MDR1 gene located on chromosome 7q21, and consists of 28 exons that encode a 1280-amino acid glycoprotein. Its structure is composed of a drug-binding cavity with two ATP-binding sites formed by two bundles of six transmembrane helices which bind electrically neutral and positively charged hydrophobic drugs (7,10).

Therefore, exposure of tumour cells to chemotherapy would result in induction of MDR1 ribonucleic acid (RNA) (2). P-gp is expressed in the transport epithelium of the liver and kidney, in adult stem cells, assorted cells of the immune system and in the blood-brain barrier (8,10,11). It plays a major role in mediating resistance to a number of pharmacologically unrelated anti-cancer drugs, such as vinblastine, vincristine, daunorubicin, epirubicin and paclitaxel (Figure 2.6) (8,10).

In one study, it was shown that paclitaxel resistance occurs by hepatic metabolism involving the cytochrome P450 (CYP) enzymes CYP3A4 and CYP2C8, where several

single nucleotide polymorphisms in the ABCB1 gene are positively correlated with progression-free survival after paclitaxel treatment (7).

2.10.2. ABCC1/MRP1 transporters

MRP1 is a second member of the ABC transporter family encoded by the ABCC1 gene (35). It was first found in the anthracycline-resistant cell lines, H69AR and HL60/Adr (8). MRP1 is composed of 17 transmembrane domains, and two binding sites with1531 amino acids expressed in almost in various organs and cell types (11,35). In contrast to MDR1, MRP1 transports negatively charged natural-product drugs that have been modified by glutathione, conjugation, glucosylation, sulfation, and gluconoylation (7,11).

The increased expression of ABCC1 transporters confers resistance to a wide range of anti-cancer drugs, such as the anthracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins, methotrexate and saquinavir (Figure 2.4) (8,11). However, MRP1 does not confer resistance to the taxanes, which is an important component of the P-gp resistance profile, though many of the anti-cancer drugs that are *P*-gp substrates are also substrates of MRP1 (8,29). Ultimately, discovery of MRP1 led to the search for other members within this family and resulted in the discovery of 9 - 10 MRP genes, of which at least 6 are transporters of anti-cancer- and antiviral compounds (7).

2.10.3. ABCG2/MXR/BCRP transporters

The third ABC transporter is ABCG2 (BCRP; MXR) protein. It is the first known half-transporter with one TMD and one NBD encoded by 655 amino acids to mediate MDR (8,11). ABCG2 is active upon homodimerization or oligomerization with itself or other transporters (8).

BCRP is highly expressed in a variety of stem-cells and human tissues, including the placenta synctiotrophoblasts, liver canalicula, kidney, blood-brain barrier and apical surface of intestinal epithelium, protecting them from endogenous- and exogenous toxins (8,11,29). Hypoxic conditions induce BCRP expression in tissues, where it plays a role in protecting cells and tissues from protoporphyrin accumulation by interacting with heme and porphyrins (29). Similar to *P*-gp and MRP, BCRP is overexpressed in

cells selected for resistance to epipodophyllotoxins, anthracyclines and tyrosine kinase inhibitors (Figure 2.4) (7,11).

2.10.4. ABCC10/MRP7 transporters

The ABCC10 transporter is an ABCC10 encoded gene product localized to chromosome 6p21.1, consisting of two NBDs and three membrane-spanning domains (MSD1, MSD2 and MSD3). MRP7 is localized in the basolateral cell surface and highly expressed in the pancreas, followed by the liver, placenta, lungs, kidneys, brain, ovaries, lymph nodes, heart and colon (8).

Similar to MRP1, MRP7 substrates are also restricted to modification by glutathione and gluconoylation. Furthermore, the MRP7 transcript also confers resistance to various anti-cancer drugs including paclitaxel, docetaxel, vincristine, vinblastine, vinorelbine, cytarabine and gemcitabine (Figure 2.4) (8).

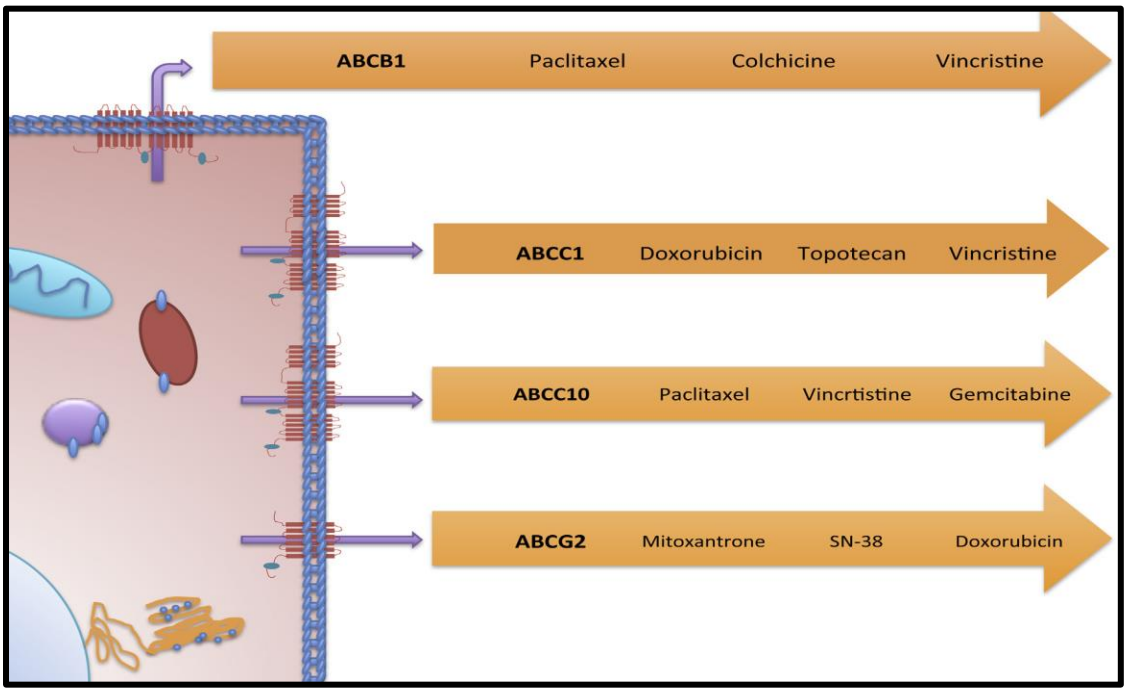

Figure 2.6: Anticancer drugs as substrates of MDR-ABC transporters located on the cell surface that extrudes anti-cancer drug substrates from the cells [Available from: (6)]

2.11. ABC TRANSPORTERS IN NORMAL CELLS

In generally**,** the main function of ABC transporters main function is to aid in the protection of cells from a variety of endogenous- and exogenous toxins, as described below in Figure 2.7. Not only do they protect normal cells, but effectively do so with cancerous cells as well. This protective trait needs to be taken in consideration when determining the bioavailability of oral drugs and should also be circumvented in order to effectively destroy malignant cells (32).

More specifically, the P-gp transporter assists by preventing cytokines from crossing the endothelium and attacking the brain. It also protects the testes by transporting toxins to the capillary lumen, and the liver by transporting toxins to the bile. As for MDR1, the transporter situated in the placenta plays a role in protecting the fetus against cationic xenobiotics. Additionally, its location in the apical membrane assists in determining oral drug bioavailability (32). Finally, MRP proteins localized in the basolateral membrane ensure that substrates are transported directly to the blood, rather than across the apical surface into the intestinal lumen. Those found in the basolateral membrane of the choroid plexus, pumps metabolic waste products of the cerebrospinal fluid into the blood. In addition, MRP1 and ABCG2 transporters located in the placenta have protective functions for fetal blood (32).

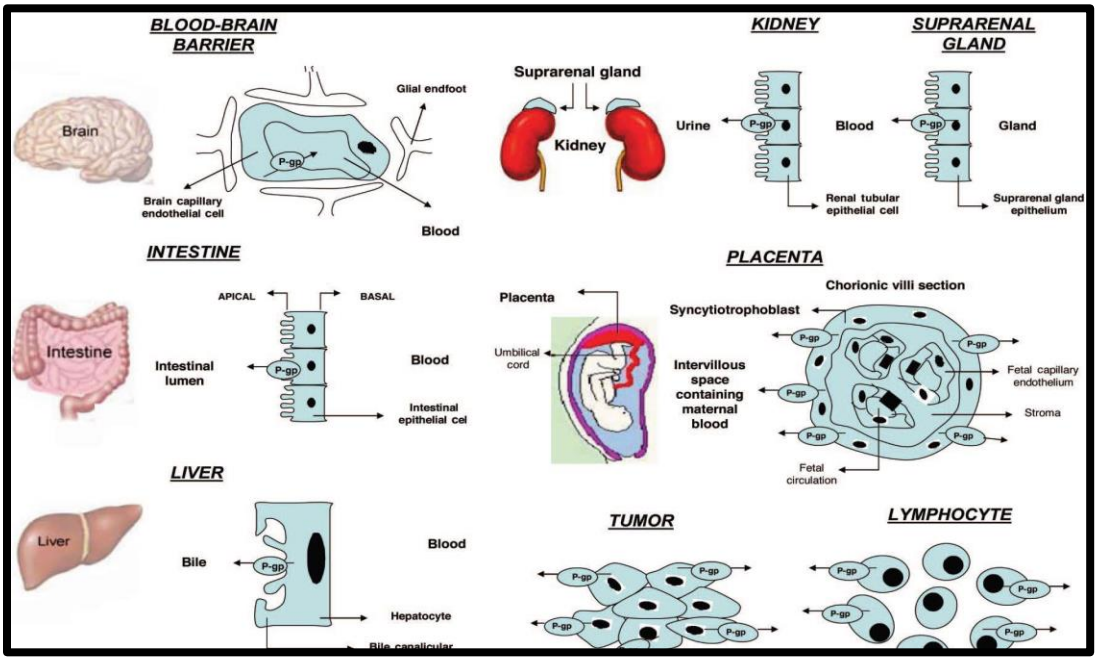

Figure 2.7: Schematic representation of the main sites of localization of ABC transporters in the body [Available from: (32)]

2.12. MODULATORS OF ABC TRANSPORTERS

Over the years, many efforts have been made to develop approaches that could either block or inactivate these transporters in order to achieve enhanced drug penetration, -distribution, -accumulation, and restoration of drug sensitivity(8,29). Currently, three generations of agents are used as chemosensitizers to reverse drug resistance in cancer (Figure 2.8), which in return all cause toxicity (29).

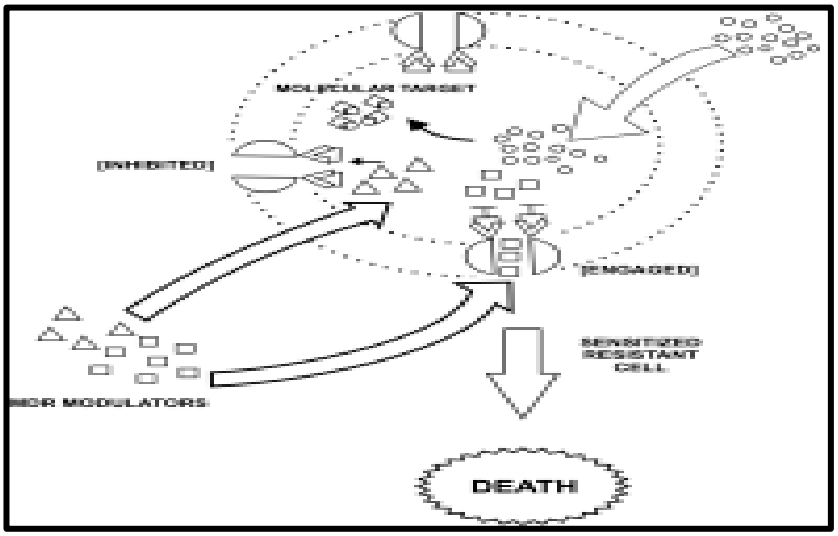

Figure 2.8: Schematic presentation of MDR reversal strategies using MDR modulators [Available from: (6)]

2.12.1. First generation agents

First generation agents were the first modulators to be discovered in the 1980's, and include the following: verapamil, cyclosporin A (CSA), tamoxifen, calmodulin antagonists, antimalarial quinine and anti-arrhythmic quinidine (8,31,36). Unfortunately, these compounds failed as cancer treatment due to causing non-specific toxicity in patients at doses required to inhibit P-gp (8,33,36).

Calmodulin antagonists, such as chlorpromazine and trifluorperazine, have the ability to reverses MDR at concentrations ranging from 1 - 10μM, while quinine and quinidine were reported to reverse MDR in conjunction with doxorubicin. In addition, CSA is still regarded as the most effective MDR agent of this group. It is an immunosuppressant

used after organ transplants and in the treatment of psoriasis. Previous clinical trials have shown that when CSA was used in conjunction with vincristine, doxorubicin and dexamethasone to reverse MDR, there was lack of significant efficacy, which was surprising since most of these agents present with excellent reversal activities *in vitro* (8,31).

In 1981, Tsuruo *et al*. reported that the calcium channel blocker, verapamil, reverses MDR by inhibiting active drug efflux, and restoring drug sensitivity in MDR cells. Doxorubicin is one of the anti-cancer drugs whose intracellular levels is increased by verapamil (31,37). Subsequent studies demonstrated that verapamil and other calcium blockers, such as felodipine, nifedipine, bepridil and nicardipine modulate MDR at high doses, but result in verapamil-induced cardiotoxicity and enhanced toxicity to normal cells (8,31). Furthermore, another study reported that verapamil also showed anti-proliferative effects against colon adenocarcinoma HCT cells, achieved through apoptotic- and cell cycle blockage mechanisms (38).

2.12.2. Second generation agents

The second generation agents, also known as chemosensitizers, are structural analogues of verapamil and CSA, respectively, such as d`exverapamil, valspodar, cyclosporin D and biricodar. These agents were reported to be less toxic, more efficacious with improved bioavailability, compared to their predecessors. Verapamil analogues reversed MDR *in vitro* displaying marginal toxicity *in vivo* while analogues of CSA demonstrated effective MDR reversal in numerous cancer cell lines *in vitro.* However, all lacked efficacy in clinical trials and showed to inhibit cytochrome P450 enzymes, resulting in pharmacokinetic interactions and subsequent host toxicity (8,29,31).

2.12.3. Third generation agents

Compounds in this category were designed using quantitative structure-activity relationship approaches to obtain molecules with specific physico-chemical traits that function at nanomolar range (29,36). As such, elacridar, laniquidar, zosquidar and tariquidar have significantly inhibited ABCB1 with less signs of toxicity (6). Furthermore,

others agents have dual ABCB1- and MRP blocking effects, such as biricodar and timcodar (36).

SECTION TWO: NATURAL PRODUCTS IN DRUG DISCOVERY

2.13. INTRODUCTION

Over a number of years, natural products from plants, animals, microorganisms etc. have been playing a critical role in the treatment and prevention of diseases (32). Since 1994, almost half of FDA approved drugs have been based on natural products, and cover a range of therapeutic indications such as: anti-cancer, antidiabetic, antibacterial, anti-infective, immunostimulatory and antimalarial, amongst others. Currently, there are more than 100 new products in clinical development, particularly as anti-cancer agents and anti-infectives (39).

Recently, researchers have been investigating the active ingredients from natural medicinal products (called secondary metabolites) to develop MDR modulators for putative low toxicity drug resistance reversal agents when used in combination with anticancer drugs. Figure 2.9 portrays the effect of natural products in different MDR mechanisms (29).

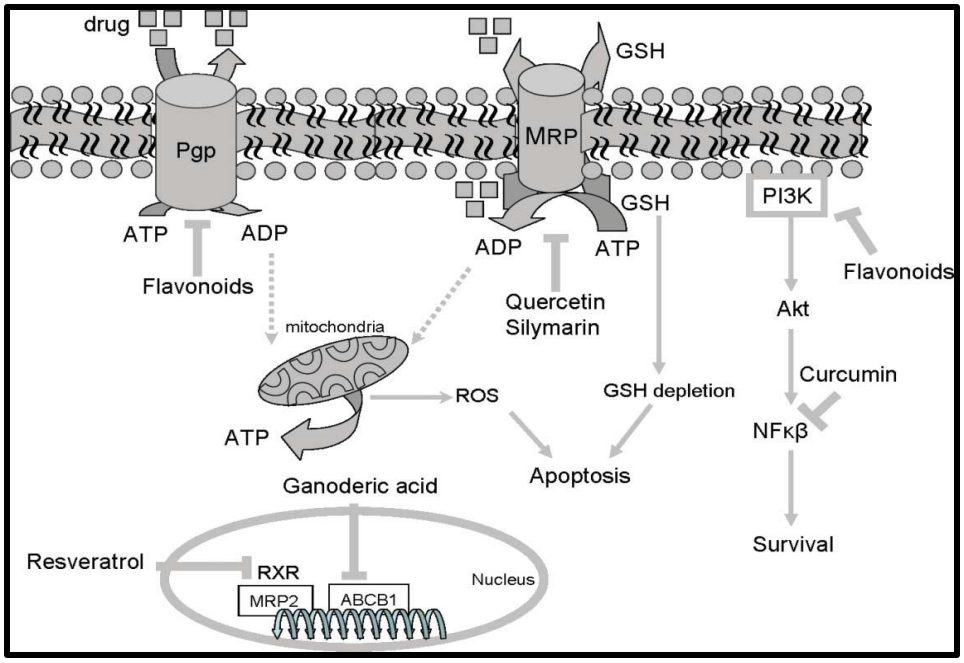

Figure 2.9: Schematic diagram of natural products affecting MDR mechanisms [Available from: (29)]

2.14. NATURAL PRODUCTS AND CANCER

The increasing number of deaths related to cancer, diseases and its high cost of treatment, spur a continued search for new anti-cancer drugs. In the recent decades, natural compounds have attracted considerable attention as anti-cancer agents. To date, the most effective anti-cancer drugs are derived from natural products (Table 2.1). The first ever, natural product to be used as an anti-cancer agent is known as podophyllotoxin, which was isolated from *Podophyllum peltatum* L. (29). Later, paclitaxel and its analogue docetaxel, were discovered from *Taxus brevifolia* L. and the analogues, vincristine and vinblastine, isolated from *Catharanthus roseus* L. (20).

Table 2.1: Plant derived drugs in research and clinical trials (40)

Anticancer agent	Plant isolated from:	Research and clinical development
Sulphoraphane	Isotiocynanate in cruciferous vegetables *Brassica*	Clinical trials with oral administration of cruciferous vegetable preparation with sulphoraphane
Paclitaxel (Taxol)	Taxane; *Taxus brevifolia* L.	In clinical use; Phase I-III clinical trials; early treatment settings; non-small lung cancer, breast cancer, ovarian cancer, Kaposi sarcoma. Research and development in alternative drug administration using nanoparticles, naocochealtes and nanoliposomes.
Epipodophyllotoxin	*Podophyllum peltatum* L.; Podophyllotoxin isomer	Lymphomas and testicular cancer trials
Vincristine, Vinblastine, Vinorelbine, Vindesine	*Catharanthus roseus* G. Don; Vinca alkaloids	Lymphomas, sarcomas and leukaemias; in clinical use; combination trials
Pomiferin	Isoflavonoid isolatated from *Maclura pomifera; Dereeis Malaccensis*	Growth inhibition in six human cancer cell lines: ACHN (kidney), NCI-H23 (lung), PC-3 (prostate), MDA-MB-231 (breast), LOX-IMVI (Melanoma), HCT-15 (colon)
Epigallacotechin-3-gallate	Catechin; green tea	Clinical trials in prostate cancer treatment ; Phase I clinical study for oral dose administration
Combretastatin A-4 phosphate	Water-soluble analogue of combretastatin; *Combretum caffrum*	Early trials; mimics developed; clinical and preclinical trials

2.15. SECONDARY METABOLITES OF PLANTS

Phytochemicals are secondary active constituents found in plants, and cover a broad range of structurally diverse compounds, including phenols, terpenes, alkaloids and organosulfur (41). Therefore, through phytochemical screening, active compounds responsible for certain medicinal activities can be isolated from medicinal plants (32). The major classes of secondary metabolites with their anti-cancer properties are discussed in the next session.

2.15.1. Flavonoids

Flavonoids are the largest group of plant phenols present in most food and herbal products (Figure 2.10). It has been stated that they possess multiple pharmacological properties, including cytotoxic anti-tumour activity of different natures (42,43). Their antimutagenic effects are mediated by the ability of polyphenols to absorb ultraviolet radiation (41). Numerous studies have demonstrated that ABC transporters can be influenced by this new class of chemosensitizers through an effective on their ATPase activity or inhibition of ABC transporters (6,11).

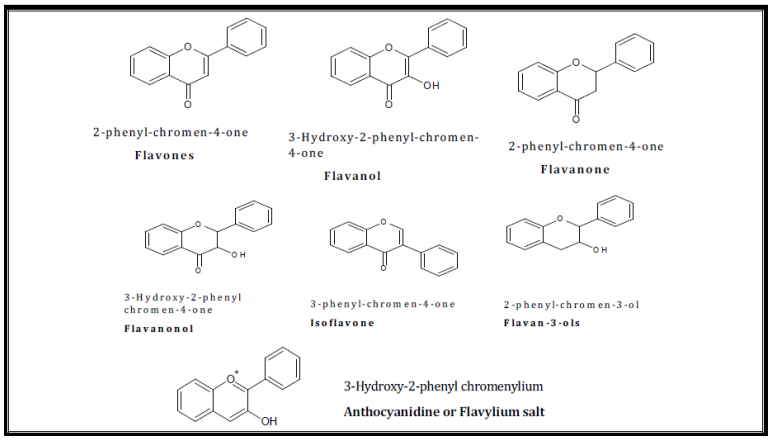

Figure 2.10: Chemical structures of some representative flavonoids [Available from: (50)]

2.15.2. Tannins

Tannins are described as a group of polyphenols with a high molecular weight which have the ability to form carbohydrates and protein complexes (44). They are mainly

found in fruits such as grapes, strawberries, blueberries and persimmon, tea, chocolate, legume forages, sorghum, corn, etc. (42). For example; the green tea polyphenol, epigallocatechin gallate (EGCG), is a major constituent of *Camellia sinensis*. It increases the efficacy of doxorubicin and enhances the doxorubicin concentrations in drug-resistant KB-A1 cells (6). Moreover, it also increases the accumulation of rhodamine 123 and daunorubicin in KB-C2 cells at concentrations ranging from 10 – 100µM, suggesting that it reverses ABCB1-mediated MDR (8,44).

In one specific report, the effects of tannins on cardiotoxicity resulting from the administration of doxorubicin for breast cancer treatment, were evaluated. Here, the results showed that tannins were successful in preventing doxorubicin-induced cardiotoxicity, and was due to over-activation of poly (ADP-ribose) polymerase (PARP), in mammary tumours in a MDA-MB-321 breast cancer cell line. Furthermore, the results also proved that tannins are potentiators of doxorubicin during treatment of mammary tumours, as tannic acid is an inhibitor of PARP and also prevents PARP-1 mediated cell death which is known to cause anti-cancer effects (45).

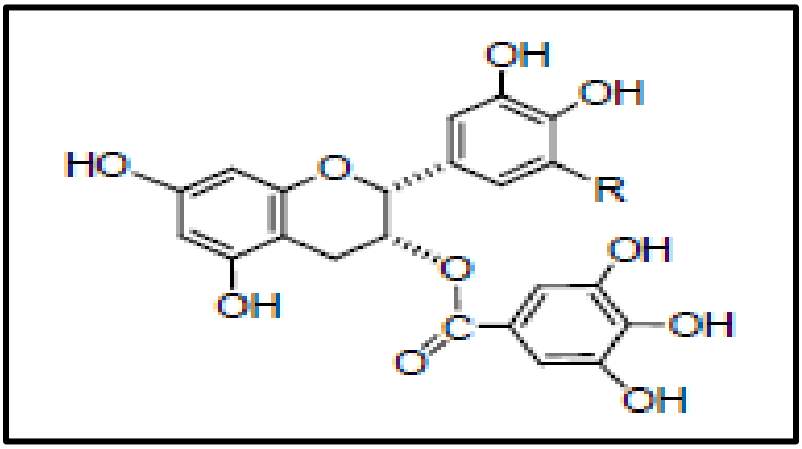

Figure 2.11: Chemical structure of epigallocatechin gallate, a green tea tannin [Available from: (73)]

2.15.3. Saponins

Saponins are a large group of glycosides which give persistent foam when dissolved in aqueous solutions, after being shaken (46). There are 150 different kinds of saponins

within the plant kingdom that possess remarkable anti-cancer properties, and enhance the therapeutic effect of anti-tumour drugs when used in conjunction with conventional tumour treatment (47). Some of these saponins (Figure 2.12) such as spirostones, oleananes, dammaranes, and cycloartones portray very strong anti-tumour reactions through different pathways (42,47). They are generally classified into two categories, namely, steroidal saponins which occur in monocotyledonous angiosperms, and triterpenoid saponins which are mainly found in dicotyledonous angiosperms, according to their aglycone skeleton, which refers to the remaining compound after the hydrogen atom replaces the glycosyl group on a glycoside (46). These two groups of saponins are classified into 11 more categories such as dammaranes, tirucallanes, lupanes, hopanes, oleananes, cycloartanes, ursanes, cucurnitanes and taraxasterones (47).

2.15.3.1. Cycloartones

Cycloartones are a group of saponins which mainly have antitumour effects on human colon cancer cells. They can be used with other orthodox chemotherapeutic drugs to lessen the adverse effects (47).

2.15.3.2. Dammaranes

Dammaranes are a group of saponins with anticancer effects which are carried out using the OWI-1 compound that directly damages the mitochondrial membrane and the cristae, in both leukaemia and pancreatic cancer (47).

2.15.3.3. Oleananes

Oleananes also shown in Figure 2.12 (C), have various pathways through which they work, pathways such as the anticancer route by signalling transduction, antimetastasis, stimulating the immune system and chemoprevention mostly in breast cancer (47).

2.13.3.4. Spirostones

Spirostones are a group of saponins with very potent anticancer and immunostimulation effects. They are strong inducers of cell death by disrupting the proper functioning of the mitochondria and enforcing stress on the endoplasmic reticulum (47).

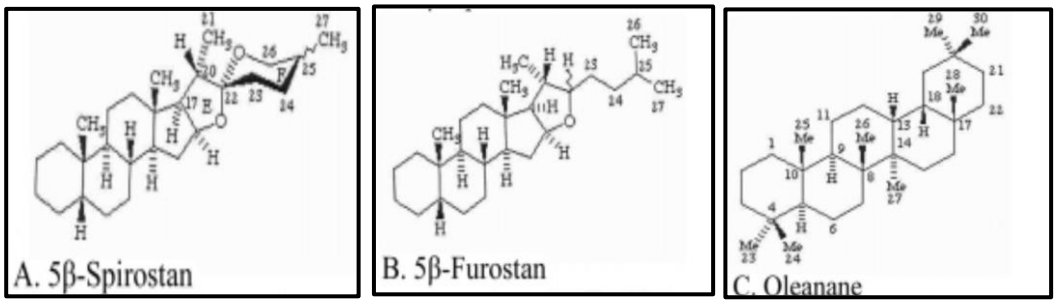

Figure 2.12: Basic structure of steroidal (A&B) and triterpenoid (C) saponins [Available from: (50)]

2.15.4. Glycosides

Glycosides are secondary metabolites, found in virtually every medicinal plant, and are composed of a glycone (sugar) and aglycone (non-sugar) components, which determines the solubility of glycosides in organic and aqueous solvents (39,48). The most encountered glycosides of medicinal importance are: polyphenolic (anthraquinone) glycosides, coumarin glycosides and steroidal (cardiac) glycosides (48).

2.15.4.1. Anthraquinone glycosides

Anthraquinone glycosides are present in a fairly limited distribution in plants (39). Upon ingestion, the anthraquinone glycosides are hydrolized in the large intestines (colon) to free aglycones, which stimulate peristalsis and increase water retention in the colon (48). However, the persistent use of anthraquinone glycosides, for other than its potent antibacterial, antidiabetic and antioxidant effects could result in fluid- and water loss, renal failure and acute hepatic failure (39).

2.15.4.2. Coumarin glycosides

Coumarin glycosides are substances which are antibacterial and often antioxidant, and are synthesized by plants in response to a bacterial- or fungal infection, physical damage, chemical injury or a pathogenic process (Figure 2.13). Pharmacologically, coumarin glycosides have shown to exert haemorrhagic, antitumor and antifungicidal activities (48).

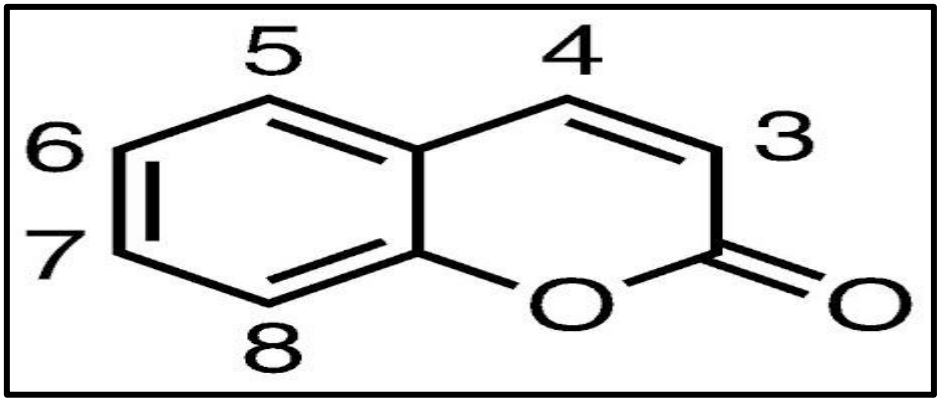

Figure 2.13: Chemical structure of a coumarin glycoside [Available from: (49)]

2.15.4.3. Steroidal (cardiac) glycosides

Cardiac glycosides are naturally occurring drugs with both beneficial- and toxic effects (at high doses) on the heart (48). Foxglove is the source of two strong glycosides used as heart stimulants, namely, digoxin and digitoxin, which are both used in the modern treatment of congestive heart failure, atrial fibrillation and -flutter (39,48).

2.15.5. Terpenoids

Terpenoids are the largest class of secondary plant metabolites, and have molecular structures consisting of carbon backbones made of isoprene units. The classification of terpenoids is characterized by the number of isoprene units present in each compound (49). Terpenoids exhibit numerous pharmacological properties such as anti-inflammatory, anticancer, antimalarial and antibacterial activities (50). Structures of the most common terpenoids found in medicinal plants are shown in Figure 2.14.

Terpenoids isolated from methanolic extracts of *Euphorbia* species, were evaluated against different MDR variants of pancreatic- (EPP85-181), colon- (HT-29) and gastric (EPG85-257) cell lines with over-expression of MDR1. Results revealed that terpenoids exhibited high antineoplastic activities against the drug-resistant subline EPG85-257RDB derived from gastric and HT29RNOV colon carcinoma (51).

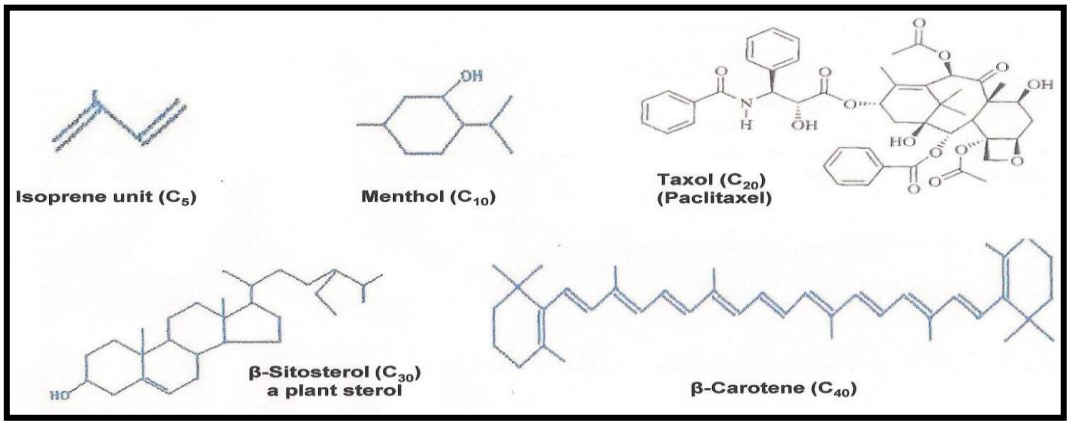

Figure 2.14: Structures of the most common terpenoids found in plants [Available from: (49)]

2.15.6. Phytosterols

Phytosterol is a term used to describe a subclass of natural compounds possessing a steroidal nucleus (32). Plant sterols consist of sterols, steroid saponins, steroid alkaloids etc., and play analogue roles in plants. The major dietary phytosterols are β-sitosterols, campesterol and stigmasterol, and their contents are higher in edible oils, seeds and nuts (Figure 2.15) (32,35). Experimental studies suggest that dietary plant sterols have anti-cancer properties in prostate-, colon- and breast cancer (32).

One study investigated the effect of dietary sterols on human MDR1 and MRP1 using P-gp human carcinoma KB-C2 cells and MRP1 gene-transfected KB/MRP cells, respectively. The results showed that dietary plant sterols inhibited the efflux of daunorubicin from KB-C2 cells and KB/MRP cells (35).

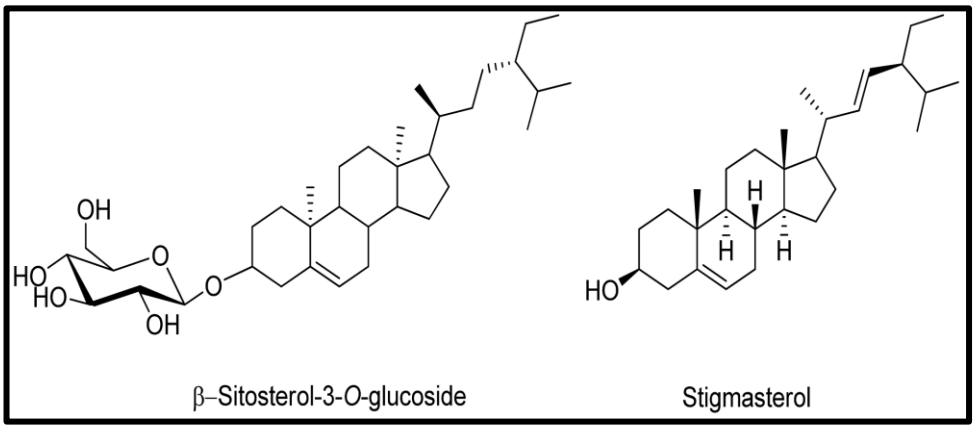

Figure 2.15: Chemical structures of major phytosterols [Available from: (33)]

2.16. *CANNABIS SATIVA* L. (Linnaeus)

2.16.1. Description

C. sativa L.**,** commonly known as “marijuana”, “weed” or “hemp” is an annual, herb belonging to the *Cannabaceae* family. It is native to western and central Asia and has been long cultivated worldwide for over 4500 years as a recreational drug or as medicine, albeit unauthorized (52,53). In medieval times, it was brought to North Africa where it is currently cultivated exclusively for cannabis resin (harshish) (53).

Cannabis is an erect bush, which grows to 5 m in height and has variable stems that are sometimes hollow, especially above the first pair of true leaves (Figure 2.16). Its flowers are monoecious or dioecious with the staminate (male) being taller but less robust than the pistilate (female). Fruit is generally brown and tightly embraces the seed with its fleshy endosperms. The drug-producing part of the plant is depended on environmental- and hereditary factors, as well as the method of cultivation (51,52).

Figure 2.16: Picture of *C. sativa* L. plant [Available from: (54)]

2.16.2. Chemical constituents of *C. sativa* L.

Cannabis is very complex in its chemistry due to the vast number of constituents and their possible interactions with one another (52). It consists of three major classes of bioactive molecules namely, flavonoids, terpenoids and more than 60 types of cannabinoids (54). Cannabinoids are the active chemical components of the *C. sativa* L. plant and are classified into three categories based on their source of production; known as, endogenous cannabinoids (endocannabinoids), phytocannabinoids and synthetic cannabinoids (54,55). So far, about 419 chemical constituents have been identified in cannabis such as flavonoids, terpenes, amino acids, proteins, sugars, steroids, glycosides, to name a few (52).

2.16.2.1. Endocannabinoids

Our bodies produce endocannabinoids through the endocannabinoid system, where they act as neuromodulators or retrograde messengers that affect the release of various neurotransmitters in the peripheral- and neural tissues. They regulate a wide variety of physiological process such as, energy metabolism, mood, appetite, pain sensation, inflammation response, memory and reproduction, through two of their major

endocannabinoids known as N-arachidonylethanolamine (AEA-anandamine) and 2-arachidonylglycerol 2-AG (54,56).

2.16.2.2. Phytocannabinoids

Phytocannabinoids are cannabinoids that occur naturally in the cannabis plant, mainly in the resin of the plant (54). More than 60 phytocannabinoids have already been identified. Δ^9-Tetrahydrcannabinol (Δ^9-THC), cannabidiol (CBD) and cannabinol (CBN) are the major and most prevalent cannabinoids (53,57). Other known cannabinoids include cannabigerol (CBG), cannabichromene (CBC), cannabicyclol (CBL), cannabivarin (CBV), tetrahydrocannabivarin (THCV), cannabidivarin (CBDV), cannabichromevarin (CBCV), and cannabielsoin (CBE) (52,55).

2.16.2.3. Synthetic cannabinoids

Synthetic cannabinoids are “designer drugs”, referred to as herbal highs that were synthesized to release similar functional effects as Δ^9-THC, in order to evaluate their potential clinical use (54,58,59). These drugs are sold and advertised on the internet as legal alternatives to cannabis (59). Synthetic cannabinoids do not contain tobacco or cannabis in their mixtures, and their consumption mimics that of Δ^9-THC (58). An important group of synthetic cannabinoids would consist of aminoalkylindoles, e.g. WIN 55,212-2 and JWH-018; eicosanoids anandamide, e.g. methanandamide; classical e.g. HU-210/308, JWH-139/133 and non-classical cannabinoids e.g. CP55940, CP55244 and CP47497 (54,59).

2.16.3. Traditional uses of *C. sativa* L.

The different parts of *C. sativa* L. have been traditionally used to treat an assortment of human ailments. An extract of the entire plant in hot water, is taken orally for asthma, as a narcotic, and to relieve pain of dysmenorrhea. A decoction of the leaves is administered orally as an anthelmintic, and externally to relieve muscular pain, whereas the seeds are used for cancer, indigestion, migraines, and are indicated as well for induction of abortion, insomnia, labour and menstruation, as well. Arial parts of the plant are taken orally as treatment for malaria and smoked for recreational and religious purposes (53).

2.16.4. Pharmacological scientific study of *C. sativa* L. in cancer

Over the years, studies were concentrated on the use of cannabis in cancer symptom palliation (55). These investigations have led to the approval of a few cannabinoid drugs such as dronabinol and nabilone, which were found to be more efficacious in the management of pain, nausea and vomiting resulting from chemotherapy. Nabiximols, which is only available in the United States through clinical trials, is also used in Canada and the United Kingdom for treatment of spasticity in multiple sclerosis and pain (55,60)

Recent investigations have also demonstrated that cannabis can reduce tumour growth in various *in vitro* and *in vivo* models (13,54–56) (see Table 2.2.). An *in vitro* study was conducted using a cannabis extract with high contents of CBD to potentially inhibit colon carcinogenesis using HCT-116 and DLD-1 human colon adenocarcinoma cells. The study proved that a high content of CBD exerts an anti-proliferative effect on colorectal cells, and attenuated colon carcinogenesis through CB_1 and CB_2 receptor activation (61). A similar effect was observed when CBD inhibited cell growth and induced cell death in ME-180 and SiHa cervical cells (62). Cannabinoids have also been reported to inhibit tumour growth in breast-, lung-, brain- and reproductive system cancer (13,54).

Moreover, a recent study showed that combined administration of gemcitabine and various cannabinoid agonists, synergistically reduced cell viability in pancreatic cancer cells. It was also observed that combined administration of THC and CBD enhances the efficacy of THC, and reduces the dose of THC needed to induce its tumour growth-inhibiting activity (56).

Table 2.2: Role of cannabinoids in different cancers

Cannabinoids	Anticancer effects
Anandamine	Breast cancer, colon cancer and prostate cancer (13,54)
THC	Breast cancer, prostate cancer, lung cancer, glioma, and lymphoma (54)
HU 120	Prostate cancer (54)
WIN 55,212-2	Breast cancer, prostate cancer, skin cancer, glioma, thyroid cancer, bone cancer and lymphoma (13,54,56)
JWH 133	Breast cancer, lung cancer, thyroid cancer and skin cancer (54,56)
JWH 015	Breast cancer, bone cancer and prostate cancer (54)
Δ^9-THC	Breast cancer, prostate cancer, lung cancer, oral cancer and glioma (13,54)
CBD	Breast cancer, prostate cancer, colon cancer, glioma, lung cancer and cervical cancer (54,61,62)
Cannabidiol acid (CBDA)	Breast cancer (54)

SECTION THREE: PHARMACOLOGICAL INVESTIGATION OF MEDICINAL PLANTS

2.17. EXTRACTION OF MEDICINAL PLANTS

Extraction is the most important step when analyzing pharmacological properties of medicinal plants (63). During extraction, potential active constituents are separated from the medicinal plant using specific solvent systems. This takes place after various parts of the plant are collected and basic plant preparation steps such as the pre-washing, drying of the plant material, grinding to obtain a homogenous sample, are followed (62,64).

2.17.1. Extraction solvents

Aqueous- and organic solvents are commonly used to extract the compounds of interest. Methanol is mainly used in the extraction of hydrophilic compounds, whereas the extraction of lipophilic compounds is achieved using dichloromethane or a mixture of dichloromethane/methanol in a ratio of 1:1. Hexane is the preferred solvent for the removal of chlorophyll from the plant material and extraction of low polar compounds such as fats and lipids (64). Water is generally used in many traditional herbal extraction protocols to mimic, as closely as possible, the traditional preparations by the traditional health practitioners (39).

2.17.2. Maceration

The simplest and most inexpensive mode of extraction in medicinal plants is by maceration. This conventional extraction technique involves soaking the finely ground plant material in a cold solvent that is added in a closed vessel. The procedure may be performed at room temperature for a specific period, sometimes through occasional shaking, until the soluble portions are dissolved in the solvent. Lastly, the extract is filtered, whereas the residue is pressed to recover a large amount of occluded solutions (63,64).

2.17.3. Solid phase extraction

Once sufficient crude extracts have been obtained, the biological analysis of the extracts can be demonstrated through evaluating either the crude extracts, or fractions

isolated from the crude extracts (65). Since plant crude extracts contain large amounts of different chemical compounds, different chromatographic techniques are used to fractionate and isolate these chemical compounds. Solid phase extraction (SPE), as one of the chromatographic techniques, is required to concentrate and obtain fractions rich with certain chemical compounds for further analysis (66). SPE can also be used for extraction or cleanup of samples that is, to remove unwanted molecules from the samples (67).

2.18. CHARACTERIZATION AND CHEMICAL FINGERPRINTING OF MEDICINAL PLANTS

Methods for quality control of herbal medicines involve the use of chromatographic techniques such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and column chromatography (39,68).

2.18.1. Thin layer chromatography

TLC was the common method of choice for herbal analysis before the introduction of HPLC (68). This method uses glass or aluminum plates pre-coated with the sorbent (e.g. silica gel) to varying thickness, depending on the amount to be loaded. The compound mixture is loaded on the TLC plates and eluted to separate the compounds. The plates are visualized under UV light or through the use of spray reagents. Several reagents are available for visualization of the separated materials (65).

TLC has the advantage of being simple, convenient and highly cost-effective in as much as a large number of samples that can be analyzed or separated simultaneously. The few drawbacks include poor detection and control, or elution compared to HPLC (62,69).

2.18.2. High Performance Liquid Chromatography

HPLC is a very popular and widely used method for the analysis and isolation of bioactive natural products, because it may be easy to use and is not limited by the volatility or stability of the sample compound (65,68). One of the advantages of HPLC is that many detectors can be connected to it such as UV, diode array detector (DAD), fluorescence detector (FLD), mass spectrum (MS) and nuclear magnetic resonance

(NMR) etc., and even connect with two or more of them to enable the detection of a variety of chemical compounds (70).

However, the analytical sensitivity is enhanced depending on the detector that is being used. UV detection such as a photodiode array (PDA) enables the acquisition of UV spectra of eluting peaks between 190 - 800 nm. PDA UV detection has the advantage of detecting even compounds with poor UV characteristics which is particularly useful in the analysis of natural products such as terpenoids or polyketides, which may not necessarily have chromophores that will rise to a characteristic UV signature (65).

CHAPTER THREE: STUDY OVERVIEW

3.1. SUMMARY

- Diseases related to cancer are estimated to be the second largest cause of death globally.
- Resistance to anticancer drugs is one of the major reasons why chemotherapy fails in the treatment of cancer.
- Multidrug resistance is a phenomenon mirrored by cross-resistance where patients who develop resistance to the first chemotherapy are much more likely to develop resistance to the second chemotherapy.
- The most common mechanism to mediate multidrug resistance is the increased efflux of anticancer drugs from the cell by ABC transporters.
- Current treatment comprises of the use of three generations of multiple agents that modulates ABC transporters, but is associated with toxicity and interactions with other transporters.
- Therefore, there is a need to investigate alternative forms of treatment, such as Traditional Medicine.
- Extracts of the aerial part of *C. sativa* L., plant of interest in this study, have been used globally for the treatment of cancer.
- However, the lack of scientific evidence on the ability of *C. sativa* L. extracts to reverse drug resistance resulted in the need to gain knowledge on its potential anticancer drug resistance reversal effects *in vitro.*

3.2. AIM OF THE STUDY

To investigate the potential anticancer drug resistance reversal effect of *C. sativa* L. extracts *in vitro*.

3.3. OBJECTIVES

- Qualitative phytochemical analysis of the powdered aerial plant parts.
- Fractionation of the crude extracts using SPE.
- Characterization of *C. sativa* L.

- Fingerprinting the crude extracts and fractions using liquid chromatography-mass spectrometry (LC-MS).

❖ Evaluation of the cytotoxic effect of the extracts against selected lung and colon cancer cells

- Evaluation of the cytotoxic effect of the extracts against normal human colon CCD-18CO (ATCC® CRL-1459) cells *in vitro*.
- Evaluation of the anticancer effect of the extracts against human colorectal adenocarcinoma cells HT-29 (ATCC® HTB-38™), epithelial colorectal adenocarcinoma cells Caco-2 (ATCC® HTB-37™), human colorectal adenocarcinoma MDR cells HCT-15 (ATCC® CCL-225™) and colorectal carcinoma LS513 (ATCC® CRL-2134™) MDR cells *in vitro*.
- Evaluation of the anticancer effect of the extracts against human small cell lung cancer cells NCI-H146 [H146] (ATCC® HTB-173™) and small cell lung cancer H69AR (ATCC® CRL-11351™) MDR cells *in vitro.*

❖ To evaluate the reversal of MDR by determining the IC_{50} values of the extracts, standard drugs alone and standard drugs in combination with extracts against H69AR, Caco-2, HCT-15 and LS513 MDR cell lines *in vitro.*

- To evaluate the co-treatment of doxorubicin and crude extracts in MDR cell lines.
- To evaluate the co-treatment of doxorubicin and verapamil in MDR cell lines.

CHAPTER FOUR: PLANT COLLECTION AND EXTRACTION

4.1. SUMMARY

Successful extraction of the aerial plant parts of *C. sativa* L. was achieved. The pulverized plant material (600g) was sequentially extracted in 3L each of four organic solvents, in order of increasing polarity, starting with hexane, dichloromethane (DCM), DCM: methanol (1:1; v/v) and methanol, respectively every 24 hours for 2 days; whereas 50g of the powdered plant material was extracted in 250ml of distilled water every 24 hours for 3 days to simulate the traditional preparation of the plant. Hexane delivered a higher yield of crude extracts, followed by DCM, than the other solvents. Methanol and distilled water delivered equal yields of crude extracts.

4.2. MATERIALS AND REAGENTS

4.2.1. Apparatus

A balance (Scaltec Instruments, UK) was used to weigh the plant samples and other chemicals. A rotary evaporator (Buchi 189013, Glasappatefabrik Flawil, Switzerland) was used to concentrate the organic extracts, while a freeze dryer (Labconco, USA) was used to concentrate the water extract. A hammer mill (Staalmeester, SA) was used to grind the dried plant material into fine powder. A horizontal shaker (FMH Instruments, SA) was used to mix and blend the extracts by shaking them. A centrifuge (Rousselet Robatel, France) was used to filter the water extract. Filter papers (Whatmann International Ltd., England) were used to filter the organic extracts. Scintillation vials (Kimble, USA) were used to store crude extracts. A sonicator bath (Fisher Scientific, UK) was used to speed up the dissolution process.

4.2.2. Chemicals and reagents

All organic solvents used were analytical grade and purchased from Merck (SA). Distilled water was prepared by a Millipore water system (Milli –Q™).

4.3. METHODS

4.3.1. Plant collection

Fresh aerial parts (leaves and stems) of *C. sativa* L. were collected from Mohale's Hoek area in Lesotho. The plant was identified and authenticated by the Geo Potts herbarium (BLFU), University of the Free State. A permit (permit numbers: POS 064/2016/2017 and POS 172/2017/2018) was issued by the Department of Health to acquire, possess and use schedule 6 and 7 substances (recently amended to schedule 4) for academic purposes.

4.3.2. Plant preparation

Following the harvest, the collected plant material was washed and removed of any debris, cut into smaller pieces, air-dried at room temperature and ground to a uniform powder using an electric hammer mill. The pulverized plant material was stored in a well-closed container at room temperature, away from direct sunlight until further use.

Figure 4.1: A photograph showing the finely grounded plant material (from: Author)

4.3.3. Extraction procedures

Both extraction procedures were carried out according to the method described by Harbone (1984) (69).

4.3.3.1. Sequential extraction of dried plant material

Approximately 600g of the pulverized plant material was extracted sequentially with 3L each of four solvents in order of increasing polarity starting with hexane, DCM, DCM: methanol (1:1, v/v) and methanol, respectively. Each extraction was performed in duplicate at a solid-to-solvent ratio of 1:5 (wt./v). For each extract, the mixture was

covered and left overnight at room temperature, to allow the solvent to extract the soluble molecules from the plant material through constant stirring on a horizontal shaker. Every 24 hours, each extract was filtered through qualitative filter paper and the residual plant material was subsequently immersed in 3L of fresh solvent. After 48 hours, for each of the solvents, the two batches of extracts were pooled together and concentrated as described in Section 4.3.4.

4.3.3.2. Dried plant material extraction with water

The pulverized plant material (50g) was extracted with 250ml of distilled water at a solid-to-solvent ratio of 1:5 (w/v). The mixture was extracted at room temperature, in a covered beaker, overnight through vigorous stirring on a horizontal shaker. The process was done every 24 hours in triplicates. Every 24 hours, the extract was filtered using a centrifuge and the retentate was subsequently immersed in 250ml of distilled water. After 72 hours, the three batches were pooled together, and concentrated as described in Section 4.3.4.

4.3.4. Concentration of the extracts

The concentration of extracts was aimed to reduce the amount of solvent from the extract. Here, the organic extracts were concentrated to dryness under reduced pressure, at 40°C in order to minimize the degradation of thermolabile components (71). Thereafter, the dried crude extracts were stored in scintillation vials, covered in foil and away from direct sunlight until required for the experiments. The water extract was concentrated by a freeze drier under vacuum at -82°C to remove all the residual aqueous content from the extract, and yield a completely dry pulverized crude extract. The dried crude extract was then stored in an airtight container until further use. Ultimately, all the dried crude extracts obtained were weighed using an analytical balance and the yield percentage was determined as described in Section 4.3.5.

4.3.5. Determination of percentage yield

For each extract, the extraction yield was calculated using the following equation:

$$\text{Percentage yield} = \frac{mass\ of\ crude\ extract}{mass\ of\ initial\ powdered\ sample} \times 100$$

4.4. RESULTS

4.4.1. Sequential extraction using organic solvents and extraction using water

Table 6.1 shows the mass of crude extract obtained with the respective solvents, as well as the percentage yield of each extract, which ranged from 1.48% to 7.08%. Hexane delivered the highest amount of crude extract with a percentage yield of 7.08%, followed by DCM with 4.92%. In contrast, DCM: methanol (1:1; v/v) recorded the lowest extraction yield at 1.48%. Methanol and distilled water yielded equal amounts.

Table 4.1: Percentage yield of the respective *C. sativa* L. extracts

Extraction solvents	Mass extracted (g)	Percentage yield (%)
Hexane	42.5	7.08
DCM	29.5	4.92
DCM: Methanol	8.9	1.48
Methanol	17.26	2.88
dH_20	1.39	2.78

DCM: Dichloromethane; dH_2O: Distilled water

4.5. COMMENT

Sufficient quantities of the respective crude extracts were obtained through sequential extraction using organic solvents in order of increasing polarity, as well as distilled water to mimic the traditional preparation of the plant. Hexane delivered the highest amount of crude extract with a percentage yield of 7.08%, followed by DCM with 4.92%. In contrast, DCM: methanol (1:1; v/v) had the lowest yield at 1.48% while methanol and distilled water, respectively, provided equal yields.

CHAPTER FIVE: BIOLOGICAL ANALYSIS OF CANNABIS SATIVA L. EXTRACTS

5.1. SUMMARY

HT-29, Caco-2 MDR, NCI-H146 [H146], HCT-15 MDR, LS513 MDR and H69AR MDR cells, as well as normal human colon (CCD-18Co) cells, were exposed to the crude extracts of *C. sativa* L. at concentrations of 1µg/ml, 10µg/ml and 100µg/ml, respectively. Cytotoxic activity of the plant extracts was considered potently active, moderately active and/or inactive using the American National Cancer Institute (NCI) guidelines. The cytotoxicity assay showed that the DCM: methanol extract potently inhibited the growth of Caco-2 cells, whilst moderately inhibiting the growth of HCT-15, LS513 and NCI-H146 [H146] cells. The methanol extract showed moderate growth inhibition of LS513 and NCI-H146 [H146] cells with IC_{50} values between 30-100µg/ml. This extract potently inhibited the Caco-2 cells and showed inactivity in HCT-15, HT-29 and H69AR cells. The hexane extract showed good growth inhibition of Caco-2 cells; and moderately inhibited LS513, NCI-H146 [H146] and H69AR cells. Similarly, the DCM and H_2O extracts showed good growth inhibition of Caco-2 and HT-29 cells, whilst moderately inhibiting the growth of HCT-15, LS513, NCI-H146 [H146], and H69AR cells. All the extracts were more cytotoxic towards all the lung- and colon cancerous cell lines than the normal colon cells as indicated by their selectivity indexes. Furthermore, the *C. sativa* L. extracts were evaluated for reversal of doxorubicin resistance in Caco-2, HCT-15, LS513 and H69AR cells. The hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts showed an increase in their IC_{50} values from 0.64±0.36-, 0.65±0.15-, 0.67±0.13-, 0.02±0.15- and 0.55±0.18µg/ml, respectively, to 2.0±0.03-, 1.92±0.34-, 5.67±0.05-, 8.72±0.06- and 1.56±0.05µg/ml, respectively, in Caco-2 cells. These extracts were 0.32-, 0.34-, 0.12-, 0.002- and 0.35-fold more sensitive to doxorubicin compared to verapamil with a 4.80-fold reversal factor. In the HCT-15 cells, the hexane-, DCM-, DCM: methanol- methanol- and H_2O extracts showed a reduction in their IC_{50} values from 180.5±0.09-, 140.4±0.32-, 47.08±0.51-, 140±2.20- and 25.6±0.03µg/ml,

respectively, to 39.33±0.04-, 40.13±0.07-, 1.45±0.06-, 1.89±0.04- and 12.3±0.05µg/ml, respectively, and increased the doxorubicin sensitivity by 4.59-, 3.50-, 32.97-, 74.07- and 2.08-fold in comparison to verapamil (control), which showed a 1.41-fold resistance reversal factor. Hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts displayed decreased IC_{50} values from 23.2±0.13-, 30.07±0.10-, 40.1±0.45-, 30.8±0.07- and 40.7±0.05µg/ml, respectively, to 10.52±0.08-, 0.1±0.02-, 4.4±0.02-, 7.2±0.06- and 3.7±0.07µg/ml in all extracts, and also showed increased sensitivity to doxorubicin of 3.6-, 300.7-, 4.6-, 4.4- and 6.3-fold, versus verapamil with a 0.03-fold reversal factor in LS513 cells. The same extracts also increased doxorubicin sensitivity in H69AR cells by 8.60-, 7.09-, 11.34-, 20.51- and 11.42-fold compared to verapamil that showed a reversal factor of 0.87-fold. These extracts showed a decrease in the IC_{50} values from 80±0.06-, 68.8±0.10-, 110±0.07-, 160±0.12- and 70.8±0.08µg/ml, respectively, to 9.3±0.01-, 9.7±0.06-, 9.8±0.05-, 7.8±0.01- and 6.2±0.03µg/ml, respectively. Combination index (CI) analysis demonstrated that both the control and extracts yielded a normal to moderate synergistic (CI <1) interaction in HCT-15 cells, moderate to strong synergistic interaction in LS513 cells and nearly additive (CI=1) to antagonistic (CI >1) interaction in H69AR cells. In conclusion, the extracts showed growth inhibitory effects against the screened cancer cell lines and were successful in increasing the sensitivity of HCT-15, LS513 and H69AR cells to doxorubicin *in vitro.*

5.2. MATERIALS AND REAGENTS

5.2.1. Apparatus

T-75 cm^3 cell culture flasks (Highveld Biologicals, SA) were used for growth of the cells. A 96-well microplate was used to seed the cells during the assays. A 5% CO_2 incubator (Thermo Scientific, SA) was used for culturing of the cells at 37°C. Acrodisc PF Syringe filters (Pall Life Sciences, SA) were used to sterilize the test samples' stock solutions. A 5810 R centrifuge (Eppendorf, Germany) was used for centrifuging and quick spinning of samples. A platefuge (Benchmark Scientific, USA) was used to centrifuge the 96-well plate. A Cell Countess™ automated cell counter (Invitrogen, USA) was used to count cells and determine the cell viability. A vortex mixer (Scaltec Industries Inc., USA) was

used for mixing. A MultiscanGo ascent plate reader (Thermo Scientific, SA) was used to obtain photometric measurement performance of the 96-well plate.

5.2.2. Chemicals and reagents

Eagle's Minimum Essential Medium (EMEM), McCoy's 5A Medium, and Roswell Park Memorial Institute-1640 (RPMI) cell culture media, heat inactivated fetal bovine serum (FBS), doxorubicin hydrochloride; docetaxel hydrochloride and verapamil hydrochloride were purchased from Sigma Aldrich (USA). Trypsin-ethylenediaminetetraacetic acid (EDTA), trypsin neutralizer, trypan blue solution and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Thermo Scientific (SA). Dimethyl sulfoxide (DMSO) was purchased from Merck (SA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) was purchased from Melford Biolaboratories Ltd. (UK).

5.2.3. Cell material

- Normal human colon CCD-18Co (ATCC® CRL-1459) cells (American Type Culture Collection, USA) was used for the cytotoxicity assay (see Table 5.1).
- Human epithelial small cell lung cancer NCI-H146 [H146] (ATCC® HTB-173™), colorectal adenocarcinoma HT-29 (ATCC® HTB-38™) and human epithelial colorectal adenocarcinoma Caco-2 (ATCC® HTB-37™) cell lines (American Type Culture Collection, USA) were used for the anticancer assay (see Table 5.1).
- Human small cell lung cancer multidrug resistant H69AR (ATCC® CRL-11351™) and human colorectal carcinoma LS513 (ATCC® CRL-2134™) and HCT-15 (ATCC® CCL-225™) multidrug resistant cell lines (American Type Culture Collection, USA) were used for the resistant reversal assay (see Table 5.1).

Table 5.1: General information on selected colon and lung cell lines

Cell type	Organ	Gender	Ethnicity	Age	Expression of ABC transporters
Normal cell line					
CCD-18Co	Colon	Female	Black	2.5 months	-
Non-resistant cell lines					
HT-29	Colon	Female	Caucasian	44 years	MRP1,3,4 and BCRP
NCI-H146 [H146]	Lung	Male	Caucasian	55 years	-
Resistant cell lines					
Caco-2	Colon	Male	Caucasian	72 years	P-gp, MRP1,2,3,4,5,6 and BCRP
HCT-15	Colon	Male	-	-	P-gp and MRP-1
LS513	Colon	Male	Caucasian	63 years	P-gp, MRP2 and BCRP
H69AR	Lung	Male	Caucasian	55 years	MRP1

5.3. METHODS

5.3.1. Preparation of test materials

5.3.1.1. Preparation of the stock solution

Stock solutions of 20mg/ml of *C. sativa* L. crude extracts and 2mg/ml of control drugs were prepared. DMSO was used for reconstitution of each crude extract and control drug. This was prepared one day in advance. Multiple aliquots of each sample were stored for initial tests and retests, if necessary. Each stock solution was sterilized by filtration before preparing working solutions.

5.3.1.2. Preparation of the working solution

Working solutions were prepared in concentrations of 100µg/ml, 10µg/ml and 1µg/ml by a serial 10-fold dilution of the stock solution using complete growth medium.

5.3.1.3. Positive controls

The positive controls used were verapamil, doxorubicin and docetaxel.

5.3.2. Cell culture

Human lung carcinoma NCI-H146, colorectal adenocarcinoma HCT-15 and colorectal carcinoma LS513 cells were maintained in RPMI-1640 medium supplemented with 10% FBS. Human small cell lung cancer multidrug resistant H69AR cells were maintained in RPMI-1640 supplemented with 20% FBS. Human colorectal adenocarcinoma HT-29 cells were maintained in McCoy's 5a medium supplemented with 10% FBS. Human epithelial colorectal adenocarcinoma Caco-2 and normal colon CCD-18Co cells were maintained in EMEM supplemented with 20% and 10% FBS, respectively. All afore mentioned cell lines were cultured at 37°C in 5% CO_2 in a humidified atmosphere incubator until cells were ready to be sub cultured.

5.3.3. Cell harvesting and cell counting

The confluent cells were harvested through trypsinization, which involved aspiration of the media from the flask and washing the cells with DPBS to remove dead cell debris. 4ml of pre-warmed 0.25% trypsin-EDTA was added, followed by gentle swirling of the flask and incubation at 37°C for 5-10 minutes, in order to detach the cells from the flask surface. Thereafter, 10-15ml of complete medium was added to inactivate/neutralize the trypsin and the cells were centrifuged at 1500rpm for 5 minutes at 25°C.

Cells were counted using a cell counter. The cell suspension (10µl) was diluted by adding an equal volume of trypan blue dye. About 10µl of the coloured suspension was placed into the counting chamber. Here, viable cells remained uncoloured whereas the non-viable cells absorbed the blue dye. The viable cell density was adjusted to 10, 000 cells/100µl per well with a total plate density of 1 x 10^6. 100µl of the cell suspension together with a 100µl of the complete growth medium were added to all the wells in the plate, except for well A1-A6, which contained complete growth medium only and served as blank. Thereafter the plate was incubated and the cells were allowed to adhere for 24 hours. Following the 24 hour incubation, the cells were exposed to different test substances as described in Section 5.3.4.

5.3.4. Addition of *C. sativa* L. crude extracts and positive controls

After the 24 hour incubation, each cell line was exposed to 50µl/well of the test materials with concentrations of 1µg/ml, 10µg/ml and 100µg/ml as described below in Figure 5.1 below. The plate was further incubated at 37°C for 72 hours. Each experiment was carried out three times (n=3) and analysed in quadruplicates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	DMSO	DMSO	DMSO	CELLS	CELLS	CELLS
B	DOX 1µg/ml				DOX 10µg/ml				DOX 100µg/ml			
C	DOC 1µg/ml				DOC 10µg/ml				DOC 100µg/ml			
D	HEX 1µg/ml				HEX 10µg/ml				HEX 100µg/ml			
E	DCM 1µg/ml				DCM 10µg/ml				DCM 100µg/ml			
F	D:M 1µg/ml				D:M 10µg/ml				D:M 100µg/ml			
G	MEOH 1µg/ml				MEOH 10µg/ml				MEOH 100µg/ml			
H	H_2O 1µg/ml				H_2O 10µg/ml				H_2O 100µg/ml			

BLANK = Culture medium, CELLS= cells in media, DOX= Doxorubicin, DOC= Docetaxel, HEX= Hexane, DCM= Dichloromethane, D:M= Dichloromethane: Methanol (1:1; v/v), MEOH= Methanol and H_2O= Water

Figure 5.1: Presentation of the preparation of a 96-well plate for HT 29 cells

5.3.5. Antiproliferation assay

The MTT colorimetric assay was performed with slight modifications as previously described by Mosmann (1983), to determine the antiproliferative activity of doxorubicin, docetaxel, verapamil and the *C. sativa* L. crude extracts on the selected lung- and colon cancer cell lines. The assay is based on the capacity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow, water-soluble substrate, MTT, into an insoluble, coloured formazan product that can be measured spectrophotometrically (72).

5.3.5.1. Reagent preparation

5mg/ml of MTT solution was prepared by dissolving 25mg MTT in 5ml of DPBS. The solution was sterilized by filtration through a 0.2µM filter into a sterile light-protected container.

5.3.5.2. MTT assay

Following the 72 hour incubation, 20µl of MTT was added to each well and the plate was left to incubate for an additional 4 hours at 37°C in a CO_2 incubator. After incubation, the produced formazan salts produced appeared as dark crystals at the bottom of the wells. The plate was centrifuged for 10 minutes and the supernatant was carefully aspiration from each well to prevent disruption of the cell monolayer. Thereafter, DMSO was added to each well to solubilize the formed formazan crystals, producing a purple solution. The absorbance of each plate was measured using a microplate reader at a wavelength of 570nm.

The absorbance readings obtained showed the cell viability. Absorbance values greater than the cell control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation. The absorbance readings of the blank must be subtracted from all samples, and readings from test samples were divided by those of the control and multiplied by a 100 to give percentage cell viability.

The growth inhibition percentage was calculated using the formula below (Section 5.3.7). The half-maximal inhibitory concentration (IC_{50}) values were extrapolated from a dose-response graph (see Figure 5.2) to indicate the concentration of the test samples required to inhibit 50% of the cancer cells' growth, relative to the control.

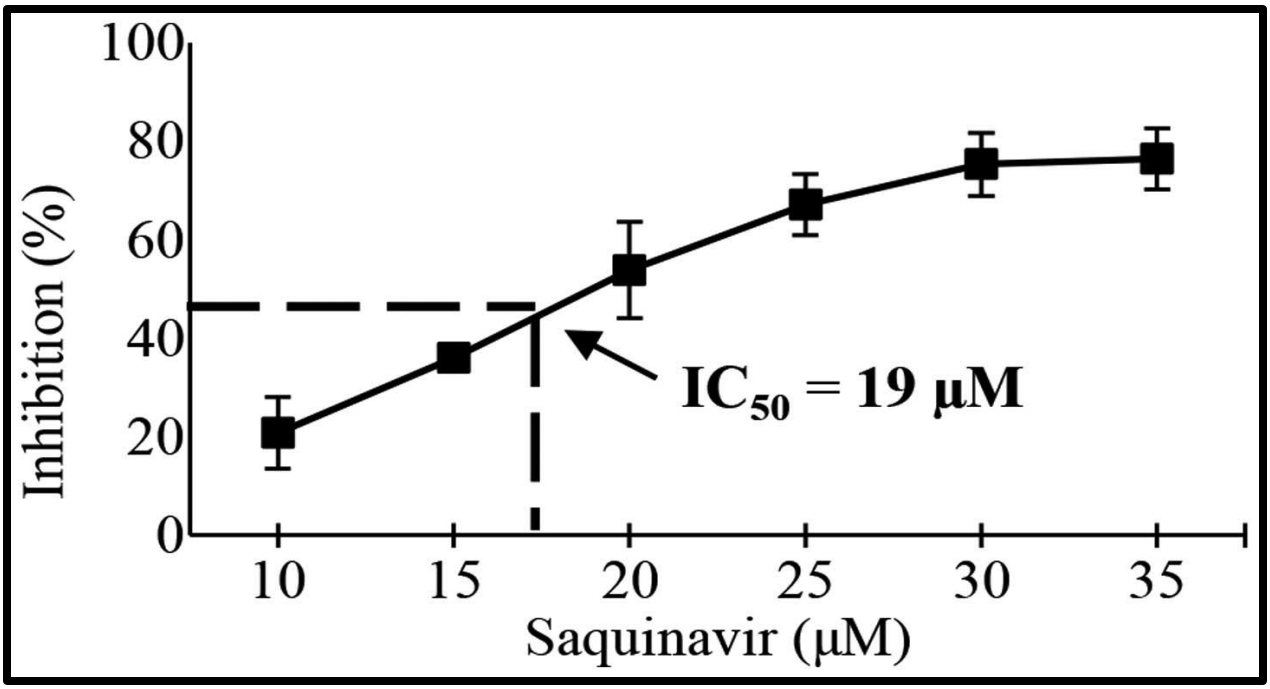

Figure 5.2: An illustration of the IC_{50} value extrapolated from a dose-response graph Available from: https://www.spandidos-publications.com/article_images/ol/12/4/ol-12-04-2493-g01.jpg.

5.3.6. Resistance reversal assay

Resistance reversal studies were performed on the MDR cell lines (Caco-2, HCT-15, LS513 and H69AR) by comparing the growth inhibition of the cells after exposure to a mixture of *C. sativa* L. and doxorubicin versus a mixture of verapamil and doxorubicin (control). Each cell line was co-treated with 25µl of doxorubicin at concentrations of 1-, 10- and 100µg/ml along with 25µl of a fixed IC_{50} (Caco-2), IC_{50} (HCT-15), IC_{50} (LS513) and IC_{50} (H69AR) concentrations of the crude extracts.

The effect of the combination therapy was analyzed using the fold-reversal factor and combination index assay as described in Sections 5.3.8 and 5.3.9. When the combination of two or more drugs result in an effect greater than the potency of each individual drug, it is regarded as synergistic. When only one drug is active alone, a greater combination effect is generally referred to as "*potentiation*." Lastly, when the

combination of two or more drugs result in an effect lower than the potency of each individual drug, it is regarded as antagonism (73).

5.3.7. Calculation of growth inhibition percentage

The percentage cell growth inhibition of each plate was calculated according to the following equation:

Percentage cell growth inhibition = $(1 - \frac{At-Ab}{Ac-Ab})x\ 100$ (74)

Where, At = absorbance value of the test compound, Ab = absorbance value of the blank and Ac = absorbance value of the control.

5.3.8. Calculation of the selectivity index (SI) and fold-reversal factor

The selectivity index was calculated to determine how selective the test samples were in producing the desired effects against cancer cell lines (75). Similarly, the fold-reversal factor or dose reduction index was calculated to determine the fold number, or ratio, between the extracts/drug alone and the reduced concentration of the extract/drug in combination, as it is an important factor in clinical levels. A higher fold-reversal factor results in better drug sensitization effect as this is an important issue in clinical levels (74,76,77). These were calculated using the following formulas:

Selectivity index (cancer cells) = $\frac{IC50\ (normal\ cells)}{IC50\ (cancer\ cells)}$ (75)

Fold-reversal factor = $\frac{IC50\ (MDR\ cells\ with\ extracts\ alone)}{IC50\ (MDR\ cells\ with\ extracts\ and\ doxorubicin)}$ (74,76,77)

5.3.9. Calculation of the combination index (CI)

The interaction between the extracts of *C. sativa* L. and doxorubicin/verapamil in Caco-2, HCT-15, LS513 and H69AR cell lines, respectively, was evaluated by the combination index method, respectively (73,76,77). The combination index ratio was calculated based on the two models mentioned below:

- The response additivity approach also referred to as linear interaction effect, where a positive drug combination effect occurs when the observed drug combination effect is greater than the effect produced by the sum of the effect of

the individual drugs. The combination index is calculated as: CI = $\frac{Ca+Cb}{Cab}$ (73,76,77).

- The bliss independence model, which is based on the principle that drug effects are outcomes of a probabilistic process and assumes that drugs act independently at different sites of action, but each contribute to a common result. The combination index is calculated as: CI = $\frac{Ca+Cb-CaCb}{Cab}$ (73,76,77).

Where, C_A and C_B = concentration of drug A and B required to produce 50 % growth inhibitory effect alone and C_{AB} = concentration of drug A and B in combination required to produce the same effect. The type of interaction was analyzed and defined as described in the table below (73,76,77).

Table 5.2: Range of combination index analysis

Range of combination index	Description
<0.1	Very strong synergism
0.1-0.3	Strong synergism
0.3-0.7	Synergism
0.7-0.85	Moderate synergism
0.85-0.90	Slight synergism
0.90-1.10	Nearly additive
1.10-1.20	Slight antagonism
1.20-1.45	Moderate antagonism
1.45-3.3	Antagonism
3.3-10	Strong antagonism
>10	Very strong antagonism

5.4. STATISTICAL ANALYSIS

The data is presented as mean ± standard deviation (SD) of three independent experiments performed in quadruplicates. Statistical analysis was evaluated by the Student's *t*-test. A statistically significant difference was considered to be present at $p<0.05$. Microsoft Excel 2010 software was used to generate graphs.

5.5. RESULTS

5.5.1. Effect of *C. sativa* L. extracts, doxorubicin and docetaxel in HT-29 cells

The results of the antiproliferative effect of the plant extracts, doxorubicin and docetaxel in HT-29 cells are tabulated in Table 5.3 and graphically illustrated in Figure 5.3. Exposure of *C. sativa* L. extracts, doxorubicin and docetaxel to HT-29 cells showed growth inhibition in a dose-dependent manner. The DCM- and H_2O extracts potently inhibited the cells with IC_{50} values of 7.52±0.51µg/ml and 10.06±1.67µg/ml, respectively, where the highest and lowest growth inhibition effect were observed at 1µg/ml and 100µg/ml, respectively. The hexane-, DCM: methanol- and methanol extracts exerted minimal growth inhibitory activity with IC_{50} values of >100µg/ml. Doxorubicin and docetaxel were used as positive control drugs. They both significantly reduced cell viability in HT-29 cells with IC_{50} values of <1µg/ml.

Table 5.3: Cell growth inhibition (n = 3) of *C. sativa* L. extracts, doxorubicin and docetaxel in HT-29 cells

Test samples	Concentrations			IC_{50} value (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Doxorubicin	86.88 ± 0.01	91.29 ± 0.05	92.95 ± 0.03	0.001 ± 0.03	0.9360
Docetaxel	82.31 ± 0.08	84.99 ± 0.19	93.74 ± 0.02	0.001 ± 0.09	0.9141
Hexane	11.24 ± 0.10	19.04 ± 0.14	25.85 ± 0.01	>100 ± 0.31	0.9982
DCM	62.6 ± 0.13	53.48 ± 0.10	26.74 ± 0.55	7.52 ± 0.51	0.9514
DCM: Methanol	17.97 ± 0.08	24.46 ± 0.01	28.37 ± 0.06	>100 ± 1.25	0.9799
Methanol	18.06 ± 0.21	20.33 ± 0.06	22.77 ± 0.03	>100 ± 2.36	0.9996
H_2O	67.73 ± 0.13	56.97 ± 0.23	23.81 ± 0.42	10.06 ± 1.67	0.9674

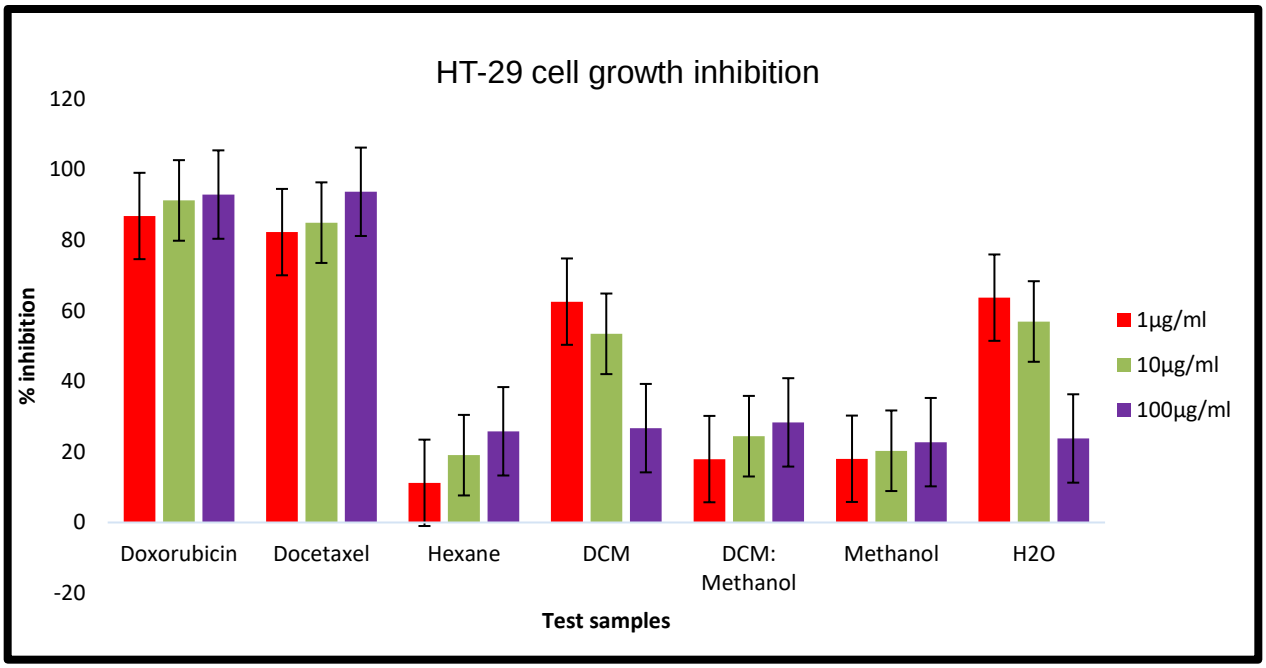

Figure 5.3: Antiproliferative effect of *C. sativa* L. extracts, doxorubicin and docetaxel in HT-29 cells (n = 3)

5.5.2. Effect of *C. sativa* L. extracts, doxorubicin and docetaxel in Caco-2 cells

The results of the antiproliferative effect of the plant extracts, doxorubicin and docetaxel in Caco-2 cells are tabulated in Table 5.4 and graphically illustrated in Figure 5.4. Exposure of the DCM- and hexane extracts to Caco-2 cells showed the highest growth inhibitory effect at concentrations of 1µg/ml followed by 10µg/ml and a significant stimulation in cell growth at the highest concentration of 100µg/ml. The H_2O and methanol extracts only demonstrated cell growth inhibition at 1µg/ml while cell growth stimulation was observed from 10µg/ml to 100µg/ml dose-dependently. Overall, all the extracts showed potent growth inhibition with IC_{50} values between 0.02-0.67µg/ml. Doxorubicin and verapamil were used as positive control drugs. Doxorubicin and verapamil exerted growth inhibition with IC_{50} values of 0.99±0.09- and 4.27±2.42µg/ml, respectively.

Table 5.4: Cell growth inhibition (n = 3) of *C. sativa* L. extracts, doxorubicin and docetaxel in Caco-2 cells

Test samples	Concentrations			IC_{50} value	R^2 value
	1µg/ml	10µg/ml	100µg/ml	(µg/ml)	
Verapamil	20.03 ±1.41	93.34 ± 3.78	101.85 ±2.08	4.27 ± 2.42	0.8271
Doxorubicin	50.37 ± 1.34	62.67 ± 1.71	77.56 ± 2.94	0.99 ± 0.09	0.997
Hexane	18.07 ± 0.77	14.34 ± 0.14	-6.4 ± 0.17	0.64 ± 0.36	0.8613
DCM	20.33 ± 0.12	16.56 ± 0.14	-0.86 ± 0.19	0.65 ± 0.15	0.8785
DCM: Methanol	21.64 ± 0.11	18.24 ± 0.16	3.03 ± 0.12	0.67 ± 0.13	0.8816
Methanol	10.42 ± 0.14	-3.81 ± 0.14	-11.94 ± 0.16	0.02 ± 0.15	0.9758
H_2O	3.86 ± 0.21	-8.64 ± 0.17	-13.95 ± 0.16	0.55 ± 0.18	0.9485

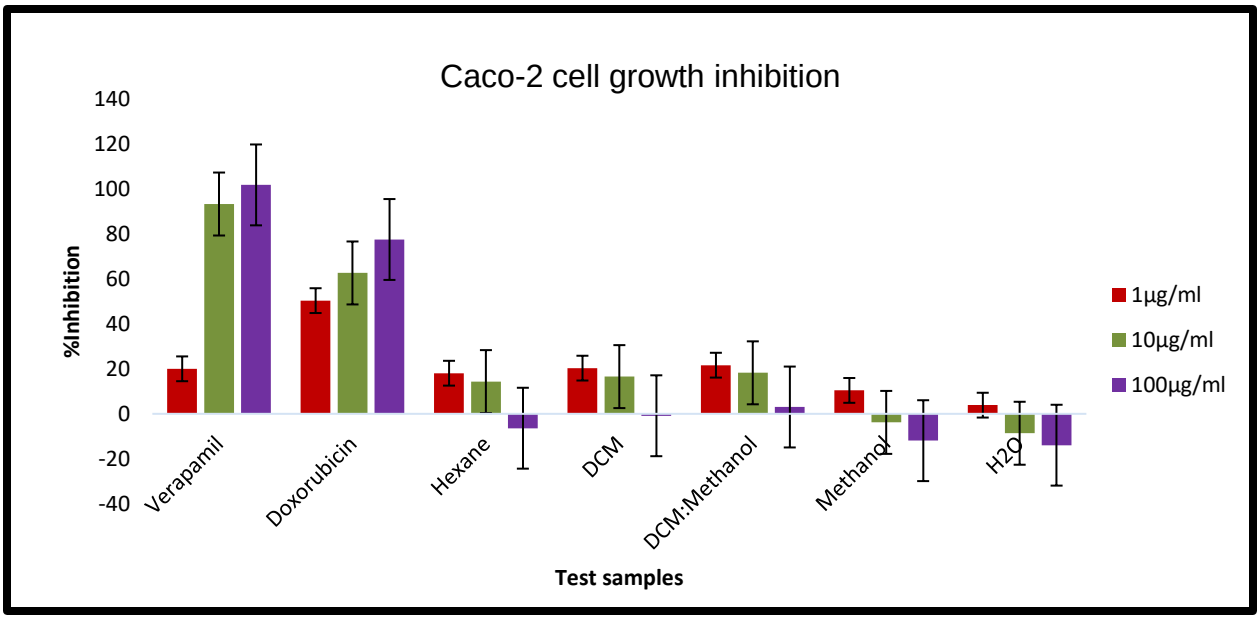

Figure 5.4: Antiproliferative effect of *C. sativa* L. extracts, doxorubicin and docetaxel in Caco-2 cells (n = 3)

5.5.3. Effect of *C. sativa* L. extracts, doxorubicin and verapamil in HCT-15 cells

The results of the antiproliferative effect of the plant extracts, doxorubicin and verapamil in HCT-15 cells are tabulated in Table 5.5 and graphically illustrated in Figure 5.5. The DCM: methanol- and H_2O extracts demonstrated moderate growth inhibition with IC_{50} values of 47.80±0.51- and 25.6±0.03µg/ml, respectively. In contrast, the hexane-, DCM- and methanol extracts showed minimal growth inhibition with IC_{50} values of >100µg/ml. Doxorubicin and verapamil were used as positive control drugs. Doxorubicin and verapamil exerted potent growth inhibition with IC_{50} values of 0.08±1.39- and 4.60±2.42µg/ml, respectively.

Table 5.5: Cell growth inhibition (n = 3) of *C. sativa* L. extracts, doxorubicin and verapamil in HCT-15 cells

Test samples	Concentrations			IC_{50} values (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Verapamil	33.69 ± 3.48	87.42 ± 1.68	95.77 ± 2.11	4.60 ± 2.42	0.8488
Doxorubicin	61.65 ± 0.68	72.12 ± 1.66	86.39 ± 1.84	0.08 ± 1.39	0.9922
Hexane	0.00 ± 0.14	0.00 ± 0.05	15.80 ± 0.07	180.5 ± 0.09	0.7974
DCM	3.76 ± 0.66	16.1 ± 0.17	21.59 ± 0.14	140.4 ± 0.32	0.9531
DCM: Methanol	22.36 ± 0.88	42.55 ± 0.12	55.89± 0.53	47.80 ± 0.51	0.9863
Methanol	8.77 ± 0.01	15.36 ± 1.02	29.31 ± 5.56	>100 ± 2.20	0.959
H_2O	32.8 ± 0.03	45.03 ± 0.03	57.74 ± 0.04	25.6 ± 0.03	0.9999

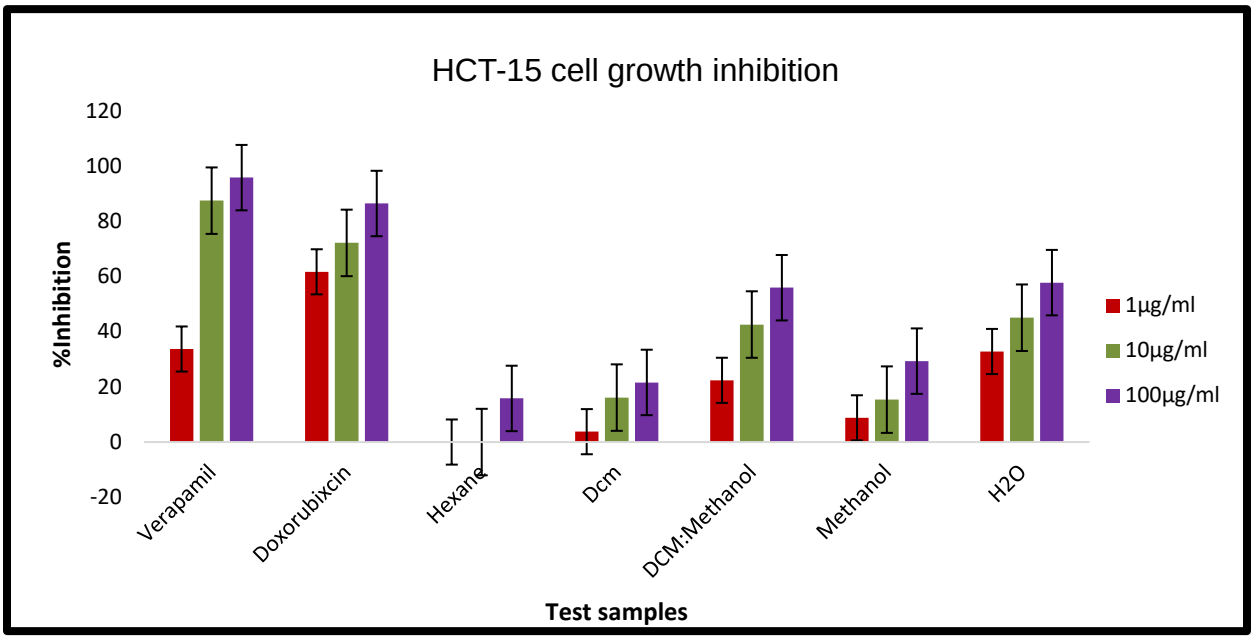

Figure 5.5: Antiproliferative effect of *C. sativa* L. extracts, doxorubicin and verapamil in HCT-15 cells (n = 3)

5.5.4. Effect of *C. sativa* L. extracts, doxorubicin and verapamil in LS513 cells

The results of the antiproliferative effect the plant extracts, doxorubicin and verapamil in LS513 cells are tabulated in Table 5.6 and graphically illustrated in Figure 5.6. The hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts exhibited moderate cytotoxic activity with IC_{50} values of 23.2±0.13-, 30.07±0.10-, 40.1±0.45-, 30.8±0.07- and 40.7±0.05µg/ml, respectively. Doxorubicin and verapamil were used as positive control drugs. Doxorubicin and verapamil demonstrated potent cytotoxic activity with IC_{50} values of 6.21±0.02µg/ml and 4.21±0.35µg/ml respectively.

Table 5.6: Cell growth inhibition (n = 3) of *C. sativa* L. extracts, doxorubicin and verapamil in LS513 cells

Test samples	Concentrations			IC_{50} values (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Verapamil	40.85 ± 0.04	59.2 ± 0.01	94.11 ± 0.01	6.21 ± 0.02	0.8643
Doxorubicin	44.41 ± 0.57	65.7 ± 0.14	95.12 ± 0.33	4.21 ± 0.35	0.9915
Hexane	39.82 ± 0.05	47.66 ± 0.04	63.99 ± 0.03	23.2 ± 0.13	0.9605
DCM	41.08 ± 0.01	44.89 ± 0.01	54.47 ± 0.27	30.07 ± 0.10	0.9417
DCM: Methanol	39.24 ± 0.51	44.11 ± 0.47	58.17 ± 0.37	40.1 ± 0.45	0.9272
Methanol	33.89 ± 0.05	42.52 ± 0.05	62.03 ± 0.11	30.8 ± 0.07	0.9525
H_2O	31.14 ± 0.01	39.01 ± 0.09	66.49 ± 0.05	40.7 ± 0.05	0.9070

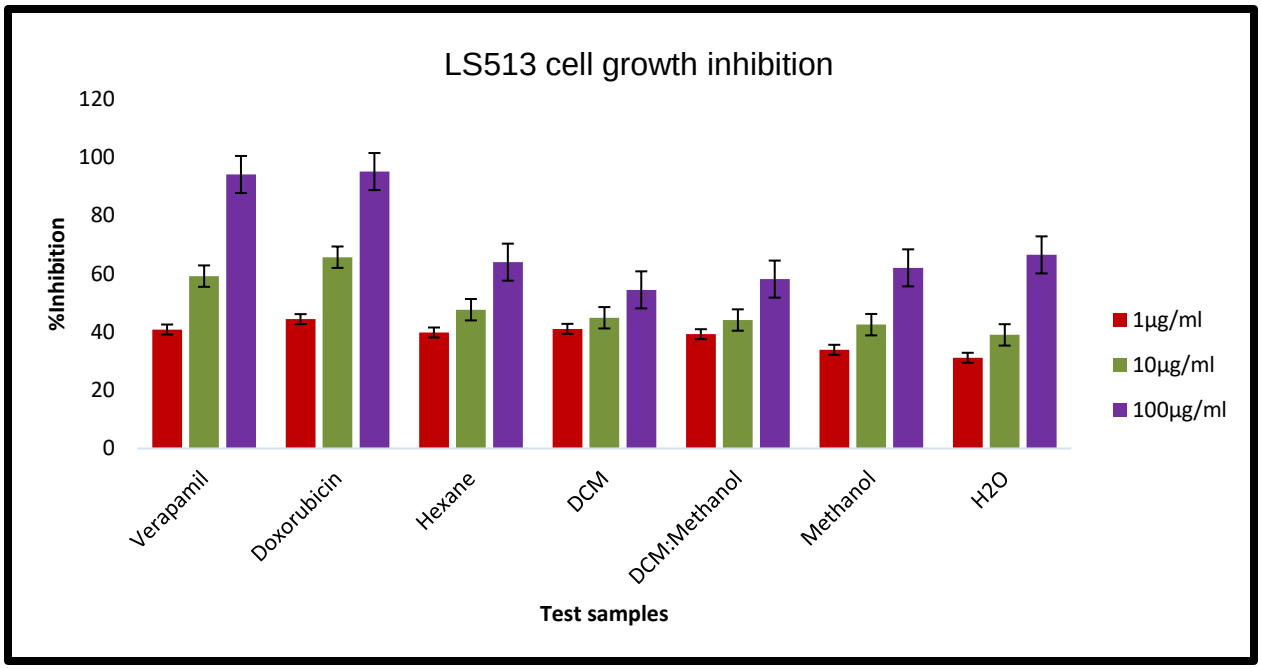

Figure 5.6: Antiproliferative effect of *C. sativa* L. extracts, doxorubicin and verapamil in LS513 cells (n = 3)

5.5.5. Effect of *C. sativa* L. extracts, verapamil, doxorubicin and docetaxel in CCD-18Co normal colon cells

The results of the antiproliferative effect of the plant extracts, verapamil, doxorubicin and docetaxel in CCD-18Co cells are tabulated in Table 5.7-5.8 and graphically illustrated in Figure 5.7-5.8. The hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts exerted growth inhibitory effects in normal colon cells, CCD-18Co, with IC_{50} values of >100µg/ml. Doxorubicin, verapamil and docetaxel inhibited cell growth with IC_{50} values of 6.78±1.16-, 1.48±0.03- and 15.72±0.10µg/ml, respectively. The SI data (Table 5.7) indicates that the hexane-, and DCM extracts were selective towards the Caco-2, HT-29 and LS513 cells whilst the DCM: methanol- and methanol extracts showed selectivity towards the Caco-2, HCT-15 and LS513 cells. The H_2O extract was selective towards the Caco-2, HT-29, HCT-15 and LS513 cells SI values >2. Docetaxel and verapamil showed non-selectivity to Caco-2, HT-29, HCT-15 and LS513 cells respectively. Doxorubicin exhibited non-selectivity to LS513 cells.

Table 5.7: Selectivity index of the cytotoxicity of *C. sativa* L. extracts verapamil, doxorubicin and docetaxel in CCD-18Co normal colon cells

Test samples	Selectivity index values			
	Caco-2	**HT-29**	**HCT-15**	**LS513**
Verapamil	0.35	-	0.01	0.24
Doxorubicin	6.85	6780	84.75	1.61
Docetaxel	-	0.5	-	-
Hexane	265.63	26.94	0.94	7.33
DCM	223.08	19.39	1.18	5.51
DCM: Methanol	244.78	1.37	3.43	4.09
Methanol	8900	1.62	1.78	5.81
H_2O	327.27	16.98	7.03	4.42

Selectivity index value > 2 indicates high selectivity

Table 5.8: Cell growth inhibition (n = 3) of *C. sativa* L. extracts, verapamil, doxorubicin and docetaxel in CCD-18Co cells

Test samples	Concentrations			IC_{50} value (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Verapamil	36.61 ± 0.02	93.88 ± 0.01	96.35 ± 0.05	1.48 ± 0.03	0.9124
Doxorubicin	31.47 ± 0.47	59.82 ± 2.26	70.93 ± 0.75	6.78 ± 1.16	0.9402
Docetaxel	23.61 ± 0.05	40.57 ± 0.19	73.54 ± 0.07	15.72 ± 0.10	0.9834
Hexane	13.36 ± 0.22	25.76 ± 0.22	27.12 ± 0.01	170 ± 0.15	0.9145
DCM	18.43 ± 0.46	22.20 ± 0.26	38.00 ± 1.74	165.8 ± 0.82	0.8881
DCM: Methanol	20.62 ± 0.04	24.56 ± 0.69	37.11 ± 3.12	164 ± 1.28	0.9166
Methanol	17.74 ± 3.15	31.42 ± 1.05	40.91 ± 0.04	178.9 ± 1.41	0.9892
H_2O	13.27 ± 0.25	25.78 ± 0.06	40.99 ± 0.16	180 ± 0.16	0.9968

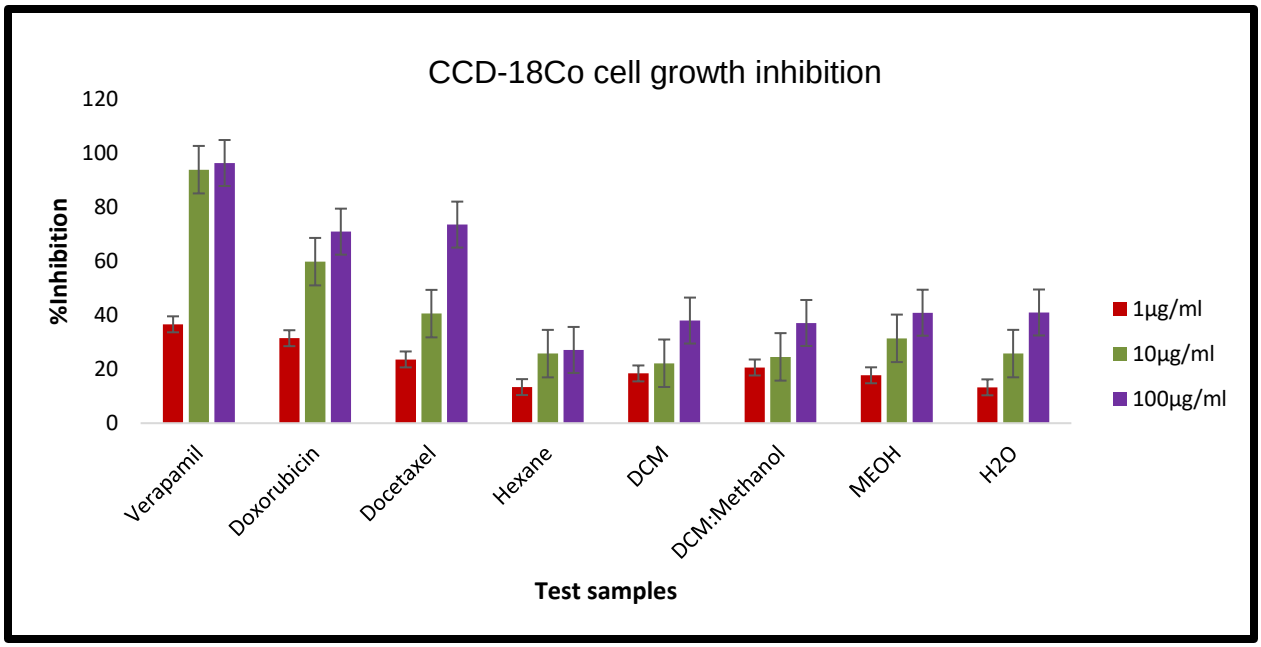

Figure 5.7: Antiproliferative effect of *C. sativa* L. extracts, verapamil, doxorubicin and docetaxel in CCD-18Co cells (n = 3)

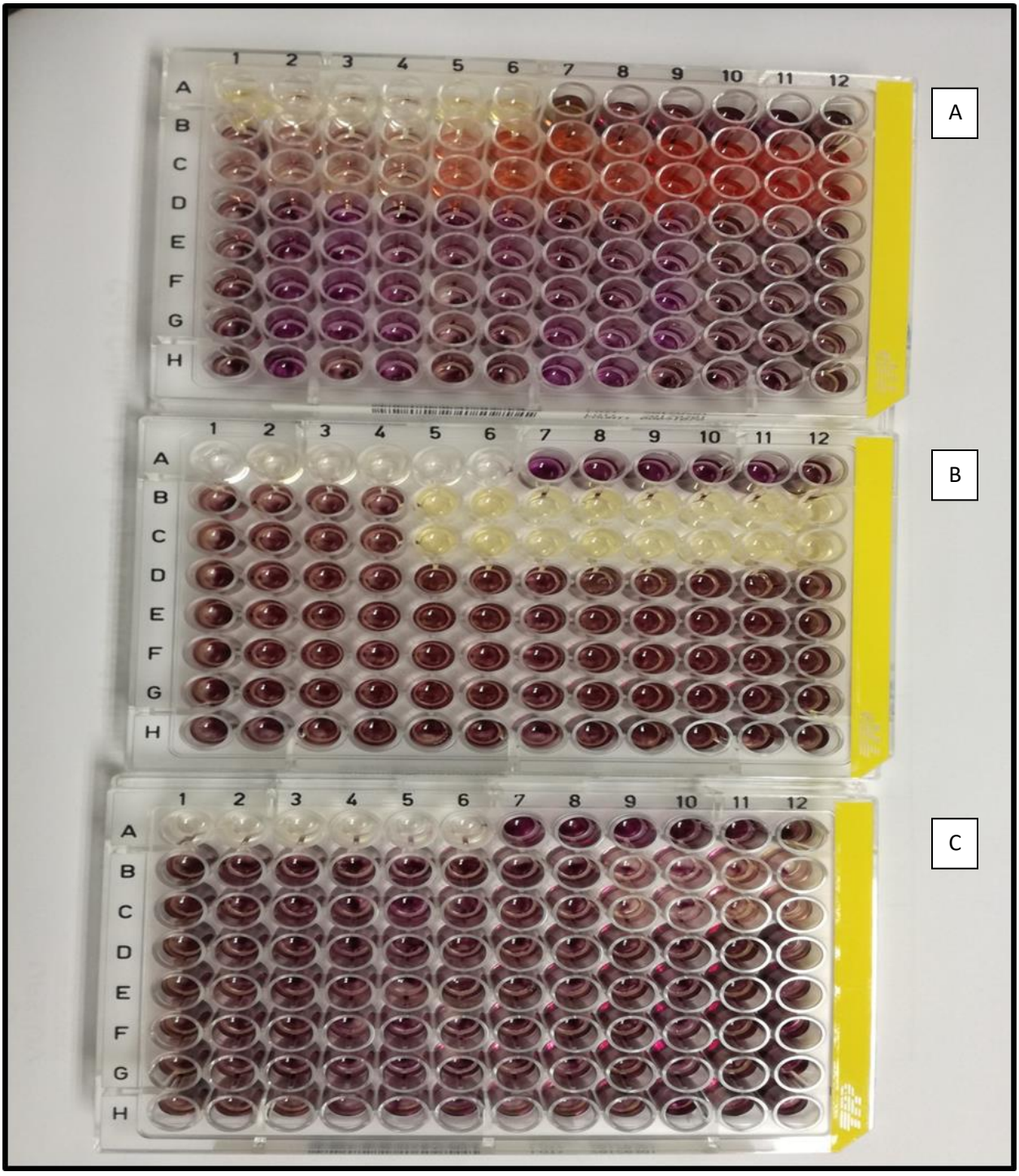

Figure 5.8: Photograph showing CCD-18Co cells after treatment with the *C. sativa* L. extracts, (A) doxorubicin, (B) verapamil and (C) docetaxel

5.5.6. Combination treatment of *C. sativa* L. extracts and doxorubicin in Caco-2 cells

The results of the resistance reversal effect of the plant extracts in Caco-2 cells are tabulated in Table 5.9-5.10 and graphically illustrated in Figure 5.9. Co-treatment of each extract with doxorubicin resulted in a normal to very strong synergistic interaction (CI <1), while co-treatment with verapamil (control) indicated a very strong synergistic interaction (CI <1) by 4.80-fold compared to the extracts. The hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts showed an increase in their IC_{50} values from 0.64±0.36-, 0.65±0.15-, 0.67±0.13-, 0.02±0.15- and 0.55±0.18µg/ml to 2.1±0.03-, 1.92±0.34-, 5.67±0.05-, 8.72±0.06- and 1.56±0.05µg/ml, respectively. These extracts were 0.32-, 0.34-, 0.12-, 0.002- and 0.35-fold less sensitive to doxorubicin compared to verapamil with a 4.80-fold reversal factor.

Table 5.9: Cell growth inhibition (n = 3) from the combination treatment of *C. sativa* L. extracts and doxorubicin in Caco-2 cells

Test samples	Concentrations			IC_{50} value (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Verapamil	52.92 ± 0.40	79.93 ± 2.48	86.69 ± 0.59	0.89 ± 1.16	0.893
Hexane	42.37 ± 0.03	72.78 ± 0.01	85.89 ± 0.05	2.1 ± 0.03	0.95
DCM	40.37 ± 0.18	65.63 ± 0.53	74.88 ± 0.30	1.97 ± 0.34	0.9331
DCM: Methanol	30.06 ± 0.06	63.78 ± 0.05	79.51 ± 0.05	5.67 ± 0.05	0.9683
Methanol	29.47 ± 0.06	53.5 ± 0.04	66.16 ± 0.07	8.72 ± 0.06	0.969
H_2O	45.7 ± 0.07	67.44 ± 0.07	73.58 ± 0.03	1.56 ± 0.05	0.9055

Table 5.10: Combination index and fold-reversal factor from the combination treatment of *C. sativa* L. extracts and doxorubicin in Caco-2 cells

Test samples	Fold-reversal factor	CI value	CI interaction
Verapamil	4.80	<0.1	Very strong synergism
Hexane	0.32	0.66	Synergism
DCM	0.34	0.68	Synergism
DCM: Methanol	0.12	0.24	Strong synergism
Methanol	0.002	<0.1	Very strong synergism
H_2O	0.35	<0.1	Very strong synergism

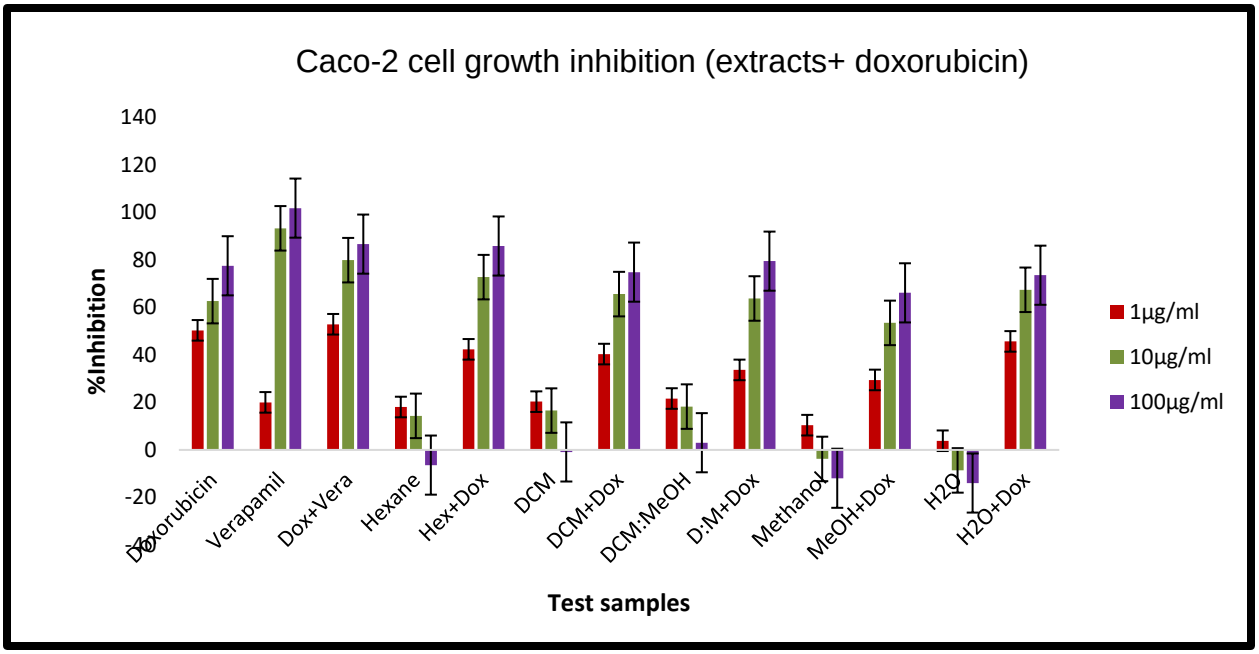

Figure 5.9: Antiproliferative effect of *C. sativa* L. extracts alone, doxorubicin alone, verapamil alone, combination of *C. sativa* L. extracts with doxorubicin, and combination of doxorubicin with verapamil in Caco-2 cells (n = 3)

5.5.7. Combination treatment of *C. sativa* L. extracts and doxorubicin in HCT-15 cells

The results of the resistance reversal effect of the plant extracts in HCT-15 cells are tabulated in Table 5.11–5.12 and graphically illustrated in Figure 5.10. Co-treatment of each extract with doxorubicin resulted in a slight to strong synergistic interaction (CI <1), while co-treatment with verapamil (control) indicated a moderate synergistic interaction (CI <1) by 1.41-fold compared to the extracts. The IC_{50} values showed that the hexane and DCM extracts moderately inhibited the cells growth whilst the DCM: methanol, methanol and H_2O extracts exerted potent cell growth inhibition. The hexane-, DCM-, DCM: methanol- methanol- and H_2O extracts showed a reduction in their IC_{50} values from 180.5±0.09-, 140.4±0.32-, 47.08±0.51-, 140±2.21- and 25.6±0.03µg/ml, respectively, to 39.33±0.04-, 40.13±0.07-, 1.45±0.06-, 1.89±0.04- and 12.3±0.05µg/ml, respectively, with higher fold-reversal factors than verapamil.

Table 5.11: Cell growth inhibition (n = 3) from the combination treatment of *C. sativa* L. extracts and doxorubicin in HCT-15 cells

Test samples	Concentrations			IC_{50} value (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Verapamil	48.83 ± 0.91	74.75 ± 1.09	85.34 ± 0.88	0.95 ± 0.96	0.9445
Hexane	28.56 ± 0.04	39.27 ± 0.07	57.01 ± 0.03	39.33 ± 0.04	0.9801
DCM	34.12 ± 0.07	45.72 ± 0.08	53.06 ± 0.06	40.13 ± 0.07	0.9835
DCM: Methanol	47.31 ± 0.03	56.76 ± 0.07	69.78 ± 0.07	1.45 ± 0.06	0.9587
Methanol	48.20 ± 0.03	54.80 ± 0.02	65.97 ± 0.07	1.89 ± 0.04	0.992
H_2O	36.57 ± 0.06	48.26 ± 0.05	56.81 ± 0.05	12.3 ± 0.05	0.9784

Table 5.12: Combination index and fold-reversal factor from the combination treatment of *C. sativa* L. extracts and doxorubicin in HCT-15 cells

Test samples	Fold-reversal factor	CI value	CI interaction
Verapamil	1.41	0.8	Moderate synergism
Hexane	4.59	0.9	Slight synergism
DCM	3.50	0.9	Slight synergism
DCM: Methanol	32.97	0.8	Moderate synergism
Methanol	74.07	0.54	Synergism
H_2O	2.08	0.3	Strong synergism

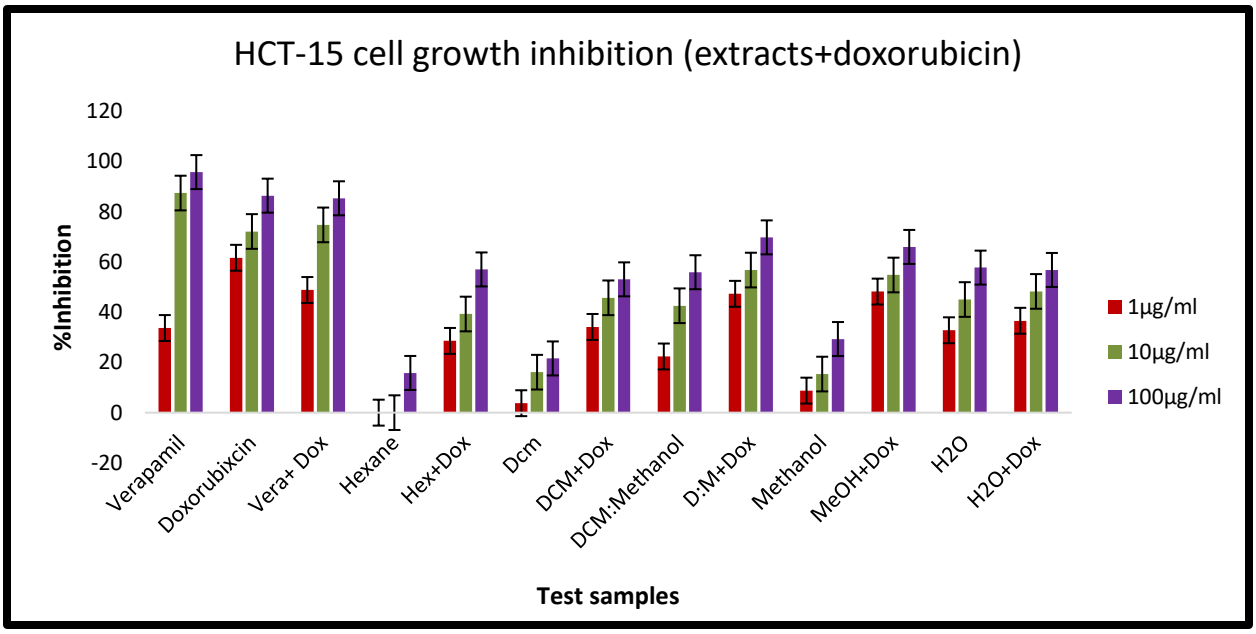

Figure 5.10: Antiproliferative effect of *C. sativa* L. extracts alone, doxorubicin alone, verapamil alone, combination of *C. sativa* L. extracts with doxorubicin, and combination of doxorubicin with verapamil in HCT-15 cells (n = 3)

5.5.8. Combination treatment of *C. sativa* L. extracts and doxorubicin in LS513 cells

The results of the resistance reversal effect of the plant extracts in LS513 cells are tabulated in Table 5.13-5.14 and graphically illustrated in Figure 5.11. Co-treatment of doxorubicin with each extract, and verapamil resulted in a normal to strong synergistic interaction (CI <1). The IC_{50} values demonstrated that combined effect with the extracts exerted potent growth inhibition compared to the combined effect with verapamil (control). The hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts showed MDR activity with IC_{50} values which decreased from 23.2±0.13-, 30.07±0.10-, 40.1±0.45-, 30.8±0.07- and 40.7±0.05µg/ml, respectively, to 10.52±0.08-, 0.1±0.02-, 4.4±0.02-, 4.27±0.06- and 3.7±0.07µg/ml, respectively. These extracts were 2.2-, 300.7-, 9.11-, 4.3- and 11-fold more sensitive to doxorubicin than verapamil with a 0.05-fold reversal factor.

Table 5.13: Cell growth inhibition (n = 3) from the combination treatment of *C. sativa* L. extracts and doxorubicin in LS513 cells

Test samples	Concentrations			IC_{50} value (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Verapamil	38.37 ± 0.07	45.79 ± 0.04	79.52 ± 0.06	26.7 ± 0.17	0.8801
Hexane	46.19 ± 0.07	49.55 ± 0.07	70.69 ± 0.10	10.52 ± 0.08	0.8507
DCM	74.67 ± 0.02	84.22 ± 0.02	87.92 ± 0.02	0.1 ± 0.02	0.939
DCM: Methanol	43.95 ± 0.02	56.7 ± 0.03	91.26 ± 0.01	4.4 ± 0.02	0.9338
Methanol	38.62 ± 0.07	56.89 ± 0.08	83.84 ± 0.05	7.2 ± 0.06	0.9897
H_2O	41.51 ± 0.07	59.61 ± 0.05	82.22 ± 0.09	3.7 ± 0.07	0.9959

Table 5.14: Combination index and fold-reversal factor from the combination treatment of *C. sativa* L. extracts and doxorubicin in LS513 cells

Test samples	Fold-reversal factor	CI value	CI interaction
Verapamil	0.05	0.6	Synergism
Hexane	2.21	0.8	Synergism
DCM	300.7	0.3	Strong synergism
DCM: Methanol	9.1	0.3	Strong synergism
Methanol	4.3	0.2	Strong synergism
H_2O	11	0.4	Synergism

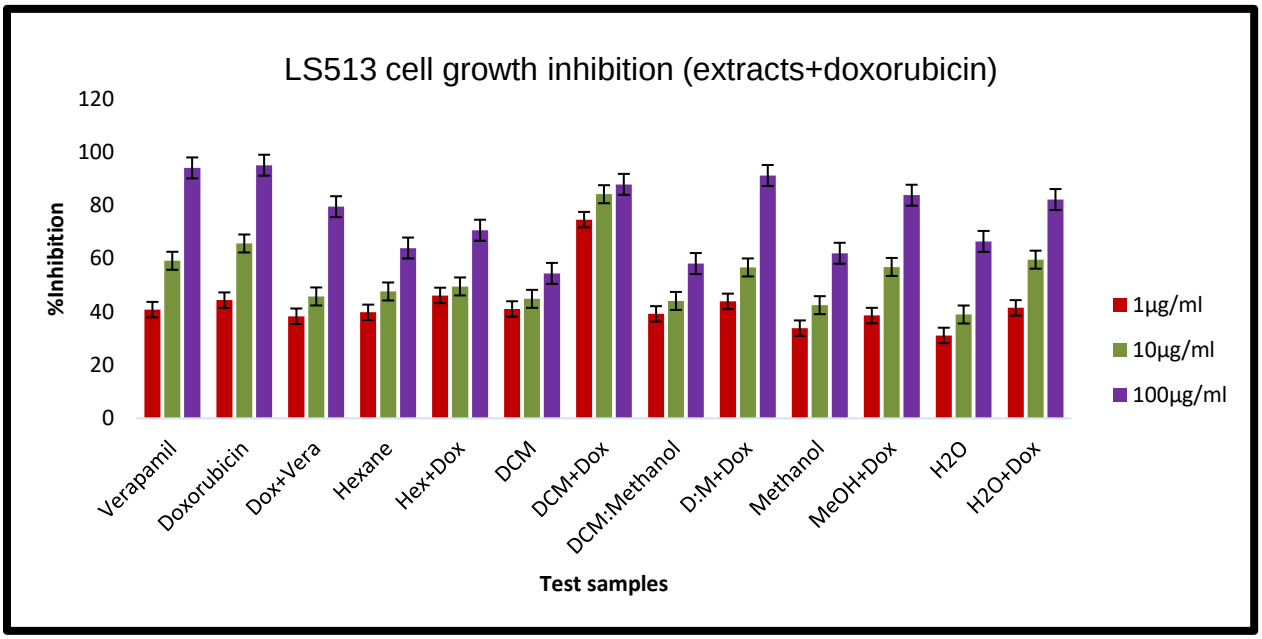

Figure 5.11: Antiproliferative effect of *C. sativa* L. extracts alone, doxorubicin alone, verapamil alone, combination of *C. sativa* L. extracts with doxorubicin, and combination of doxorubicin with verapamil in LS513 cells (n = 3)

5.5.9. Effect of *C. sativa* L. extracts, doxorubicin and docetaxel in NCI-H146 [H146] lung cancer cells

The results of the antiproliferative effect of the extracts, doxorubicin and docetaxel in NCI-H146 [H146] cells are tabulated in Table 5.15 and graphically illustrated in Figure 5.12. The hexane-, DCM-, DCM: methanol- and methanol extracts exerted moderate growth inhibition with IC_{50} values between 20-100µg/ml and showed significant cytotoxic activity at 10µg/ml and 100µg/ml. The H_2O extract had minimal growth inhibition when compared to the other extracts. Doxorubicin and docetaxel were used as positive control drugs. Doxorubicin showed potent growth inhibition at 10µg/ml only, while docetaxel demonstrated a significant dose dependent growth inhibition effect with an IC_{50} of 9.66±0.08µg/ml**.**

Table 5.15: Cell growth inhibition (n = 3) of *C. sativa* L. extracts, doxorubicin and docetaxel in NCI-H146 [H146] cells

Test samples	Concentrations			IC_{50} value (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Doxorubicin	14.64 ± 0.18	69.63 ± 0.06	8.86 ± 0.17	7.00 ± 0.14	0.0074
Docetaxel	15.82 ± 0.02	53.76 ± 0.12	79.49 ± 0.09	9.66 ± 0.08	0.9879
Hexane	3.06 ± 0.04	42.37 ± 0.26	55.07 ± 0.10	45.26 ± 0.13	0.9197
DCM	11.90 ± 0.10	35.45 ± 0.05	58.22 ± 0.03	69.32 ± 0.06	0.9879
DCM:Methanol	9.34 ± 0.13	25.99 ± 0.09	51.36 ± 0.11	88.14 ± 0.11	0.9858
Methanol	8.97 ± 0.22	19.64 ± 0.10	51.07 ± 0.01	96.42 ± 0.11	0.9993
H_2O	12.33 ± 0.15	19.34 ± 0.15	27.24 ± 0.18	> 100 ± 0.16	0.9988

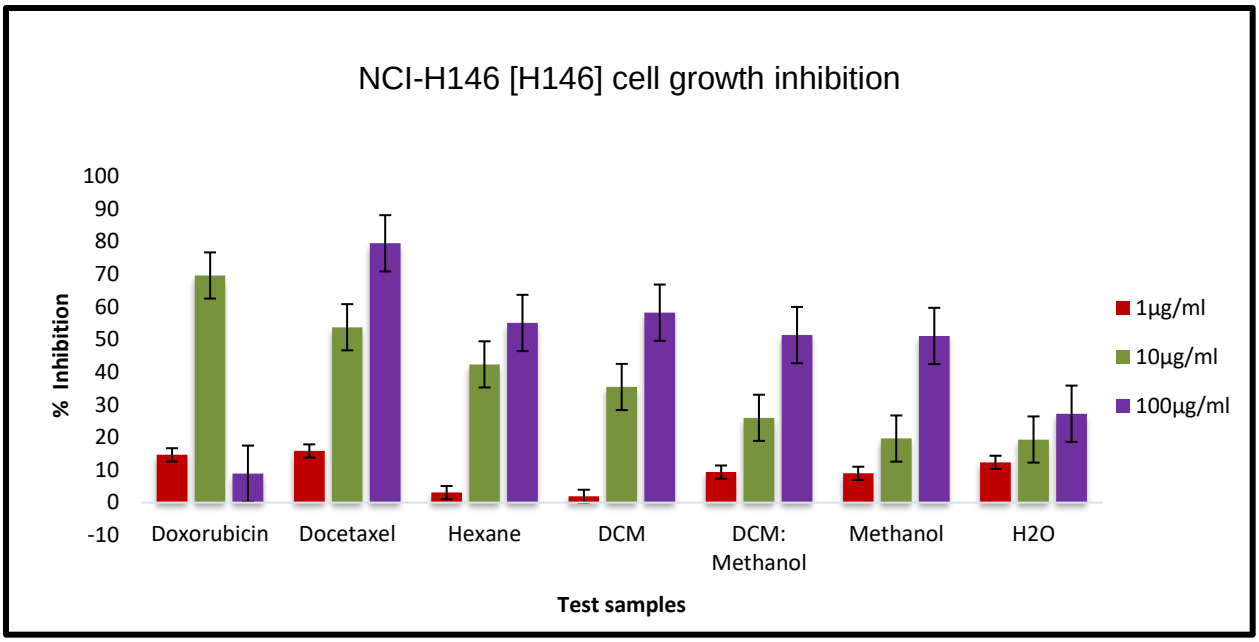

Figure 5.12: Antiproliferative effect of *C. sativa* L. extracts, doxorubicin and docetaxel in NCI-H146 [H146] cells (n = 3)

5.5.10. Effect of *C. sativa* L. extracts, doxorubicin and verapamil in H69AR lung cancer cells

The results of the antiproliferative effect of the extracts, doxorubicin and verapamil in H69AR cells are tabulated in Table 5.16 and graphically illustrated in Figure 5.13. The hexane-, DCM- and H_2O extracts moderately inhibited the growth of H69AR cells with IC_{50} values of 80±0.06µg/ml, 68.8±0.10µg/ml and 70.8±0.08µg/ml, respectively. A lack of cytotoxic activity was observed from the DCM: methanol- and methanol extracts both with IC_{50} values above 100µg/ml. Verapamil and doxorubicin were used as control drugs. Verapamil inhibited H69AR cell growth with an IC_{50} of 25±0.12µg/ml while doxorubicin presented with an IC_{50} of 7.8±0.09µg/ml.

Table 5.16: Cell growth inhibition (n = 3) of *C. sativa* L. extracts, doxorubicin and verapamil in H69AR cells

Test samples	Concentrations			IC_{50} value (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Verapamil	21.65 ± 0.11	39.15 ± 0.16	82.37 ± 0.08	25 ± 0.12	0.9435
Doxorubicin	23.56 ± 0.10	62.84 ± 0.07	80.75 ± 0.09	7.8 ± 0.09	0.9987
Hexane	6.32 ± 0.08	33.16 ± 0.03	51.74 ± 0.06	80. ± 0.06	0.9891
DCM	6.37 ± 0.12	28.66 ± 0.05	53.83 ± 0.14	68.8 ± 0.10	0.9988
DCM: Methanol	0.68 ± 0.03	30.35 ± 0.04	49.63 ± 0.15	110 ± 0.07	0.9852
Methanol	12.17 ± 0.06	13.98 ± 0.07	39.40 ± 0.22	160 ± 0.12	0.7995
H_2O	10.91 ± 0.01	30.35 ± 0.02	54.27 ± 0.21	70.8 ± 0.08	0.9965

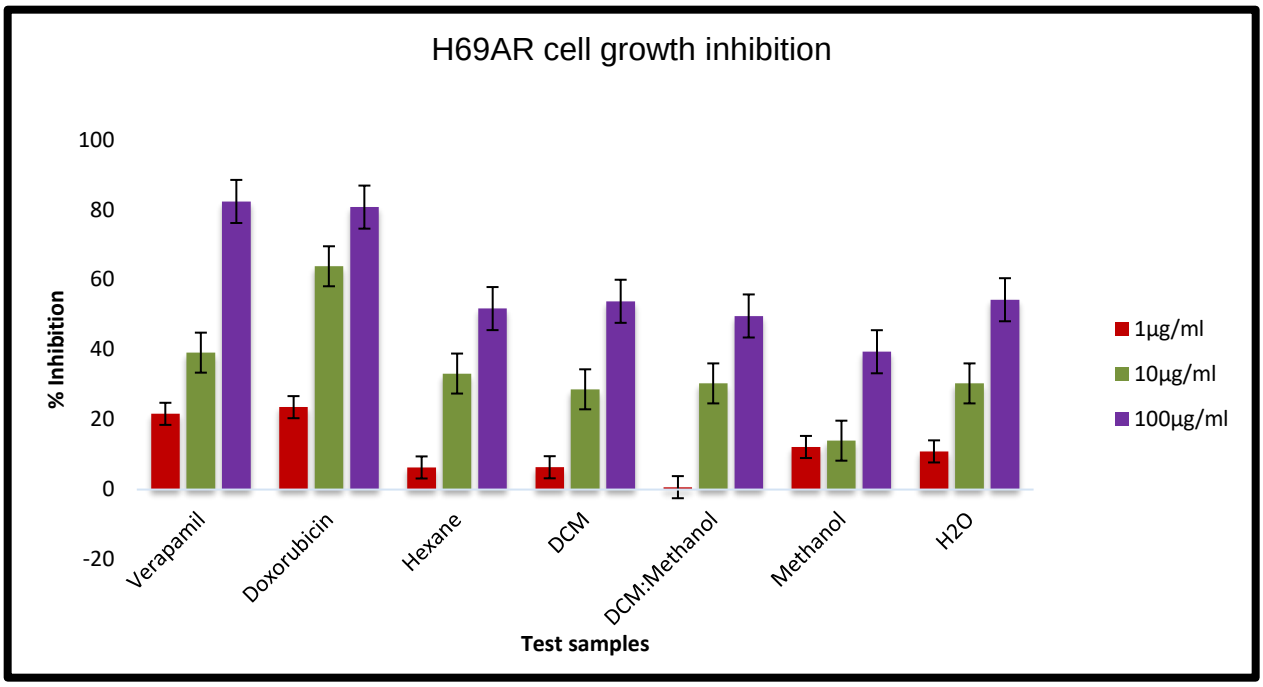

Figure 5.13: Antiproliferative effect of *C. sativa* L. extracts, doxorubicin and verapamil in H69AR cells (n = 3)

5.5.11. Combination treatment of *C. sativa* L. extracts and doxorubicin in H69AR cells

The results of the resistance reversal effect of the plant extracts in H68AR cells are tabulated in Table 5.17–5.18 and graphically illustrated in Figure 5.14. All the extracts were not cytotoxic at 1µg/ml and only demonstrated cytotoxicity towards H69AR cells at 10µg/ml. All the extracts showed good inhibitory effects in H69AR cells with IC_{50} values between 6.2 – 9.8µg/ml. The hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts portrayed doxorubicin resistant reversal effects with a decrease in the IC_{50} values from 80±0.06-, 68.8±0.10-, 110±0.07-, 160±0.12- and 70.8±0.08µg/ml to 9.3±0.01-, 9.7±0.06-, 9.8±0.05-, 7.8±0.01- and 6.2±0.03µg/ml, respectively. These extracts were 8.60-, 7.09-, 11.34-, 20.51- and 11.42-fold more sensitive to doxorubicin compared to verapamil with a 0.87-fold reversal factor**.** The combination of doxorubicin and verapamil was used as a positive control. Verapamil potently inhibited cell growth, although not in a dose dependent manner, and demonstrated its highest cytotoxic effect at 10µg/ml. The combination index analysis showed that verapamil and the DCM extract yielded a nearly additive interaction while the hexane-, DCM: methanol-, methanol- and H_2O extracts yielded slight to moderate antagonistic interactions (CI >1).

Table 5.17: Cell growth inhibition (n = 3) from the combination treatment of *C. sativa* L. extracts and doxorubicin in H69AR cells

Test samples	Concentrations			IC_{50} value (µg/ml)	R^2 value
	1µg/ml	10µg/ml	100µg/ml		
Verapamil	27.91 ± 0.07	57.46 ± 0.02	23.21 ± 0.02	8.8 ± 0.04	1.0000
Hexane	0.00 ± 0.00	60.69 ± 0.01	16.94 ± 0.02	9.3 ± 0.01	1.0000
DCM	0.00 ± 0.00	57.68 ± 0.01	32.38 ± 0.09	9.7 ± 0.06	1.0000
DCM: Methanol	0.00 ± 0.00	60.24 ± 0.06	32.84 ± 0.08	9.8 ± 0.05	1.0000
Methanol	0.00 ± 0.00	68.34 ± 0.01	32.93 ± 0.03	7.8 ± 0.01	1.0000
H_2O	0.00 ± 0.00	71.82 ± 0.06	28.58 ± 0.02	6.2 ± 0.03	1.0000

Table 5.18: Combination index and fold-reversal factor values from the co-treatment of *C. sativa* L. extracts and doxorubicin in H69AR cells

Test samples	Fold-reversal factor	CI value	CI interaction
Verapamil	0.87	1.10	Nearly additive
Hexane	8.60	1.13	Slight antagonism
DCM	7.09	1.10	Nearly additive
DCM: Methanol	11.34	1.12	Slight antagonism
Methanol	20.51	1.3	Moderate antagonism
H_2O	11.42	1.4	Moderate antagonism

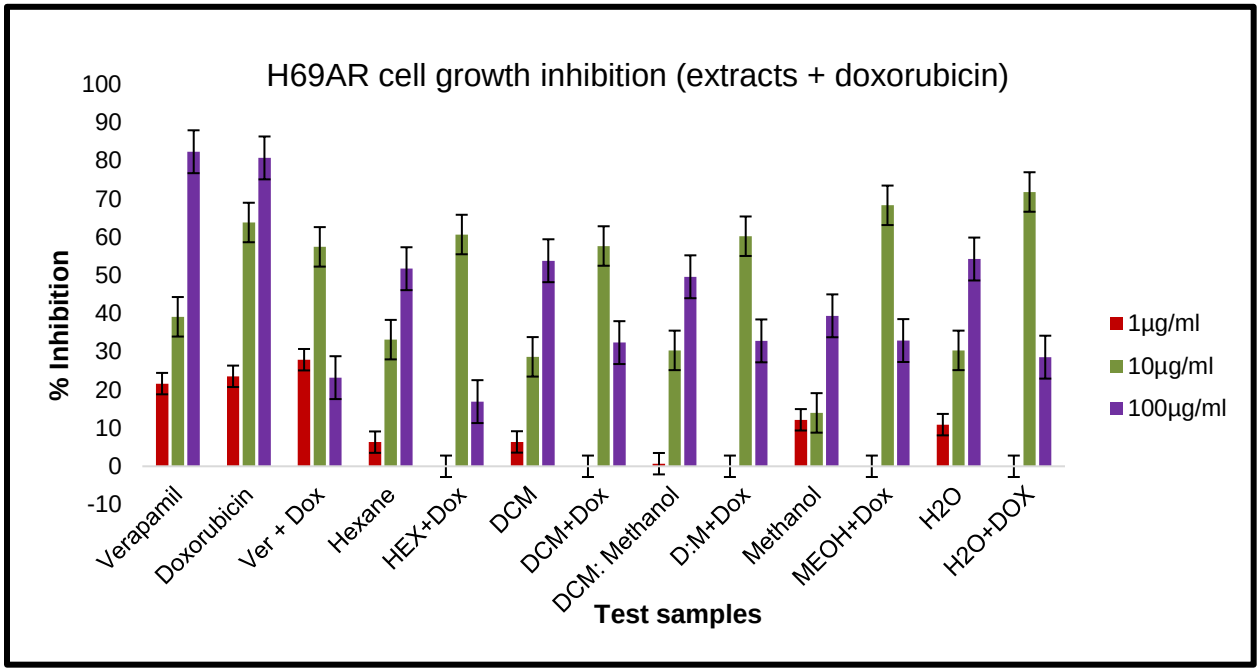

Figure 5.14: Antiproliferative effect of *C. sativa* L. extracts alone, doxorubicin alone, verapamil alone, combination of *C. sativa* L. extracts with doxorubicin, and combination of doxorubicin with verapamil in H69AR cells (n = 3)

5.6. DISCUSSION

The present study investigated the ability of *C. sativa* L. extracts to produce anti-cancer effects and to reverse doxorubicin resistance in colon cancer cells (HT-29), doxorubicin resistant colon cancer cells (Caco-2, HCT-15 and LS513), lung cancer cells (NCI-H146 [H146]) and doxorubicin resistant lung cancer cells (H69AR). Cytotoxicity of the test drugs and extracts was done using normal colon cells (CCD-18Co). Current research only reports on the anticancer effects of Cannabis but not resistant reversal properties of the plant. Extract-drug interactions were assessed by the combination index method (76,77). For the cytotoxicity results, according to the American National Cancer Institute (NCI), the IC_{50} values of plant extracts at less than 20µg/ml, between 20-100µg/ml and more than 100µg/ml are regarded as active, moderately active and inactive, respectively (78–80).

Based on the NCI criterion, HT-29 colon cancer cells were potently inhibited after exposure to the DCM- and H_2O extracts, with IC_{50} values of 7.52±0.51- and 10.06±1.67µg/ml, respectively. The hexane-, DCM: methanol- and methanol extracts exhibited the least inhibitory activity (IC_{50} of >100µg/ml) compared to the other extracts. Doxorubicin and docetaxel significantly reduced cell viability in HT-29 cells with IC_{50} values of <1µg/ml.

Kogan *et al*. (2004) studied the anti-cancer activity of three quinonoids synthesized from cannabinoids, and reported that all three quinones inhibited HT-29 cell growth with IC_{50} values of 3.125-, 12.5- and 25.0µg/ml. The most active quinone compound with an IC_{50} of 3.13µg/ml also displayed significant growth inhibition of the cells *in vivo*. Doxorubicin and daunorubicin are some of the widely used anti-cancer drugs that originated from anthracyclines, the largest group of quinonoid compounds (81). The IC_{50} values obtained from the mentioned study are within the same range as that of the *C. sativa* extracts, further suggesting their potential to inhibit cell growth in other mechanistic models both *in vitro* and *in vivo*.

Caco-2 is a human epithelial cell line originally derived from colorectal adenocarcinoma. This cell line is commonly used as a model for predicting intestinal absorption and

excretion in humans. One of its most advantageous properties, is its ability to mimic the enterocytes with brush borders found in the small intestine, and to express several efflux transporters involved in drug absorption and -excretion such as P-gp, BCRP and MRP2 (79,82,83).

The MTT results showed that the extracts did not have any growth inhibitory effect on the cells. In fact, the exposure of Caco-2 cancer cells to *C. sativa* L. extracts exhibited cell growth stimulation in a dose-depended manner. The hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts produced good inhibitory activity with IC_{50} values of 0.64±0.36-, 0.65±0.15-, 0.67±0.13-, 0.02±0.15- and 0.55±0.18µg/ml, respectively. Interestingly, the hexane-, DCM- and DCM: methanol extracts only exerted cytotoxic activity at concentrations of 1µg/ml and 10µg/ml, while the methanol- and H_2O extracts showed cytotoxic activity at 1µg/ml. This finding was unusual, as a higher cell growth inhibition is expected at the highest drug concentration. This anomaly can be due to colour intensity that interferes with absorbance readings. Several studies have shown that continuous exposure of Caco-2 cells to doxorubicin, a known P-gp substrate, strongly induce the expression of *P*-gp in a time- and dose-dependent manner (83,84).

The resistant reversal experiments portrayed that combination of the extracts with doxorubicin did not have any MDR reversal activity. The extracts demonstrated an increase in their IC_{50} values during the combination studies when compared to either agents alone. However, doxorubicin decreased the IC_{50} value of verapamil from 4.27±2.42- to 0.89±1.16µg/ml and was 4.80-fold more sensitive to verapamil than the extracts. The CI analysis yielded a synergistic to strong synergistic interaction (CI <1).

From the experiments performed on HCT-15 cells, the DCM: methanol- and H_2O extracts demonstrated moderate growth inhibition with IC_{50} values of 47.8±0.51- and 25.6±0.03µg/ml, respectively while the hexane-, DCM- and methanol extracts showed no cytotoxic activity with IC_{50} values of >100µg/ml. Doxorubicin and verapamil exerted potent growth inhibition with IC_{50} values of 0.08±1.39- and 4.6±2.42µg/ml. HCT-15 cells are known to be resistant to doxorubicin due to the overexpression of P-gp. Therefore, in this study, it was not surprising that doxorubicin inhibited the growth of HCT-15 cells

with an IC_{50} of 0.08µg/ml. Interestingly, present findings on the cytotoxic effect of doxorubicin in HCT-15 cells, were not consistent with results from a study conducted by Park *et al*. (2010), where growth inhibition was observed at an IC_{50} value of 0.0161µM (85). Another study showed that colon carcinoma (HCT-116) cells were moderately sensitive to doxorubicin with an IC_{50} of 0.027µg/ml (86).

The potential of *C. sativa* L. extracts to reverse drug resistance in HCT-15 was investigated by combining a fixed IC_{50} of each extract with increasing concentrations of doxorubicin. Combination studies showed that the hexane-, DCM-, DCM: methanol-methanol- and H_2O extracts reversed MDR in HCT-15 cells with a decrease in their IC_{50} values from 180.5±0.09-, 140.4±0.32-, 47.08±0.51-, 140±2.20- and 25.6±0.03µg/ml to 39.33±0.04-, 40.13±0.07-, 1.45±0.06-, 1.89±0.04- and 12.3±0.05µg/ml, respectively. These extracts increased the doxorubicin sensitivity in the HCT-15 cells by 4.59-, 3.50-, 32.97-, 74.07- and 2.08-fold, respectively, in comparison to verapamil, which showed a 1.41-fold resistance reversal factor. The CI analysis showed that all the extracts and doxorubicin yielded normal to moderate synergistic interaction (CI <1) in HCT-15 cells. Similarly, combined treatment of doxorubicin with verapamil (control) yielded a moderate synergistic interaction (CI <1), and reversed MDR with a decrease in IC_{50} values from 0.08- and 4.6µg/ml to 0.95µg/ml.

The human colorectal carcinoma LS513 cell line has been reported to express P-gp, BCRP and MRP2 efflux transporters, which can limit the intestinal absorption of numerous drugs when over-expressed in cancer cells (79). The antiproliferative effect of *C. sativa* L. extracts in LS513 colorectal cancer cells was exhibited in a dose-dependent manner. The hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts exhibited moderate cytotoxic activity with IC_{50} values of 23.2±0.13-, 30.07±0.10-, 40.1±0.45-, 30.8±0.07- and 40.7±0.05µg/ml, respectively. Doxorubicin and verapamil demonstrated cytotoxic activity with IC_{50} values of 6.21±0.32- and 4.21±0.02µg/ml, respectively. These IC_{50} values were higher when compared to IC_{50} values of the most active extracts.

C. sativa L. extracts were also tested to see if they would reverse drug resistance in LS513 cells by combining a fixed IC_{50} of each extract with increasing concentrations of doxorubicin. These results showed that each extract portrayed MDR reversal activities,

characterized by a decrease in the IC_{50} values of both doxorubicin and each extract when compared to the individual drug and extracts tested alone, and the combined effect of doxorubicin and verapamil (control). The extracts also had higher fold-reversal factors compared to the control, while the CI analysis showed that all the extracts and verapamil produced a moderate to strong synergistic interaction (CI <1) in LS513 cells. The hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts were found to be 23.2-, 300.7-, 9.11-, 4.27- and 11-fold more sensitive to doxorubicin than verapamil with a 0.05-fold reversal factor.

The present study's results on the effect of verapamil on colon MDR cells appear to be in support of other studies that reported on tumor growth inhibition by verapamil in colon- and pancreatic cancer. The exact mechanism by which verapamil exerts growth inhibition in colon cancerous- and MDR cells has not been demonstrated in this study. However, recent reports have shown that verapamil might inhibit tumour progression in resistant cancer cells by inducing apoptosis and inhibiting P-gp expression (87).

The main objective of cancer chemotherapy is to kill cancer cells with as little damage as possible to normal cells (88). *C. sativa* L. extracts were more cytotoxic in all the colon cancerous cell lines than the normal colon (CCD-18Co) cell line. This is indicative that the cytotoxicity of the extracts were more selective to cancerous cells than normal colon cells. In addition, higher cell growth inhibition (see Figure 5.7) was observed from verapamil, docetaxel and doxorubicin than the extracts in the normal colon cells. This highlights at least some specificity of the *C. sativa* L. extracts towards targeting malignant colon cells with little effect on normal colon cells.

The selectivity index (SI) was calculated to determine how the drugs and extracts behaved in the cytotoxicity tests. An SI value of less than 2 indicates general toxicity of the extracts (75). Based on this understanding, the SI data shown in Table 5.7 indicated that the hexane-, and DCM extracts were selective towards the Caco-2, HT-29 and LS513 cells whilst the DCM: methanol- and methanol extracts showed selectivity towards the Caco-2, HCT-15 and LS513 cells. The H_2O extract was selective towards the Caco-2, HT-29, HCT-15 and LS513 cells SI values >2. Similarly, docetaxel and verapamil were also selective to Caco-2, HT-29, HCT-15 and LS513 cell lines, while

doxorubicin exerted selectivity (SI >2) towards the LS513 cell lines. These drugs were indiscriminately toxic to both normal and cancerous cells.

In experiments where the NCI-H146 [H146] lung cancer cell line was used, the hexane-DCM-, DCM: methanol- and methanol extracts produced moderate growth inhibition with IC_{50} values between 30-100µg/ml. However, the H_2O extract was not active against this cell line and had an IC_{50} of >100µg/ml when compared to other extracts. Doxorubicin and docetaxel reported lower IC_{50} values of 10±0.14µg/ml and 8.9±0.08µg/ml compared to IC_{50} values generated by the extracts.

The hexane-, DCM- and H_2O extracts moderately inhibited H69AR MDR cells with IC_{50} values of 80±0.06µg/ml, 68.8±0.10µg/ml and 70.8±0.08µg/ml, respectively. A lack of cytotoxic activity was observed from the DCM: methanol- and methanol extracts, both with IC_{50} values above 100µg/ml. The control drugs, verapamil and doxorubicin, inhibited H69AR cell growth with IC_{50} values of 25±0.12µg/ml and 7.8±0.09µg/ml compared to all the extracts. A comparative study on the sensitivity of H69AR cells to doxorubicin reported a higher IC_{50} of 41.42µg/ml in comparison to the IC_{50} of 7.8±0.09µg/ml from this study (86).

Furthermore, *C. sativa* L. extracts showed MDR reversal activities in the H69AR cell line with a decrease in the IC_{50} values of the extracts and a higher fold-reversal factor compared to verapamil. Interestingly, the CI analysis demonstrated that both the control and extracts yielded an antagonistic interaction (CI >1) in this cell line. The results obtained in this study on the combined effect of verapamil and doxorubicin are consistent with those of Cole *et al*. (1989) (89). They were also unable to show a clear dose-dependent effect and reported a 1.82-fold factor in H69AR cells. A possible explanation for this could be because only drug resistance associated with P-gp is susceptible to reversal by verapamil as H69AR cells are known to not overexpress P-gp (87,89,90)

5.7. CONCLUSION

In conclusion, the present study provides evidence on the growth inhibitory effect of *C. sativa* L. extracts on Caco-2, HT-29, NCI-H146 [H146], HCT-15, LS513 and H69AR cell

lines. The study also made a comparative exploration on the potential application of *C. sativa* L. extracts in combination with doxorubicin, compensating for the deficiency of previous research in the field of *C. sativa* L. extracts-based combination therapy. Therefore, combination of *C. sativa* L. extracts with doxorubicin showed MDR reversal activities by enhancing the *in vitro* growth inhibitory effect of doxorubicin in HCT-15, LS513 and H69AR cells than either agents alone. This suggests that *C. sativa* L. extracts can be used in combination therapy with doxorubicin to reverse MDR during the treatment of colon- and lung cancer. Future studies are necessary for more extensive biological evaluation of the active ingredients and to possibly confirm the mechanism through which the extracts exert their resistance reversal effects. Purification of the most active extract could be done to isolate, if at all, the compounds responsible for the observed activity.

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CHAPTER SIX: PHYTOCHEMICAL ANALYSIS, FRACTIONATION AND CHEMICAL CHARACTERIZATION OF *CANNABIS SATIVA* L.

6.1. SUMMARY

Characterizations of the crude extracts and fractions of the aerial parts of *C. sativa* L. was established. It involved subjecting the pulverized aerial plant parts to phytochemical analysis in order to determine the presence, and/or absence of flavonoids, glycosides, tannins, saponins, tannins, terpenoids and phytosterols. Furthermore, the crude extracts were fractionated by solid phase extraction (SPE), using the following solvent concentrations: 100% H_2O, 25% acetonitrile (ACN), 50% ACN, 75% ACN and 100% ACN. The DCM- and methanol extracts were chemically fingerprinted using UPLC-MS while the DCM: methanol crude extract, SPE fractions and Cannabis standards were fingerprinted by HPLC-MS. Phytochemical screening revealed the presence of glycosides, tannins, saponins, tannins, terpenoids and phytosterols; and absence of flavonoids. SPE resulted in five fractions from each crude extract. UPLC-MS analysis showed the presence of similar peaks in both DCM- and methanol extracts, although it eluted at different times. HPLC-MS analysis showed similar compounds at different intensities as indicated by their different retention times (RT), peak area and m/z values. Ultimately, successful chemical fingerprinting of the crude extracts and SPE fractions was achieved for identification and quality control purposes.

6.2. MATERIALS AND REAGENTS

6.2.1. Apparatus

A magnetic hot plate stirrer (FMH Instruments, SA) was used for mixing. A vortex mixer (Scientific Industries Inc., USA) was used to mix small vials of liquid. A sonicator bath (Fisher Scientific, UK) was used to speed up the dissolution process. A balance (Scaltec Instruments, UK) was used to weigh the plant samples. Strata SDBL (100µm Styrene-divinylbenzene, 500mg / 6mL, Tube) SPE cartridges were used to separate the different components of the sample (Phenomenex, SA).

6.2.2. Chemicals and reagents

Acetic acid, chloroform, sulphuric acid, ferric chloride, methanol, ethyl acetate, formic acid and chloroform were all purchased from Merck (SA). The ammonia solution was purchased from NT Laboratory (SA). The magnesium chips were purchased from Sigma-Aldrich (Austria). Olive oil was purchased from a local supermarket. HPLC grade toluene and acetonitrile were purchased from Honeywell Burdick and Jackson (USA).

6.3. METHODS

SECTION ONE: PHYTOCHEMICAL ANALYSIS

Qualitative phytochemical screening was performed on powdered plant samples using standard procedures, to provide information about the nature of the phytochemical constituents present in *C. sativa* L. (50,57,91).

6.3.1. Qualitative phytochemical analysis

6.3.1.1. Determination of flavonoids

0.5g of powdered plant sample was heated with 10ml of ethyl acetate in boiling water for 3 minutes. The mixture was filtered and then shaken with 1ml of 1% ammonia solution. The layers were allowed to separate. Formation of a yellow precipitate indicated the presence of flavonoids.

6.3.1.2. Determination of glycosides

To 0.5g of powdered plant sample, 2ml of acetic acid, containing 1 drop of 0.1% ferric chloride, was added. Thereafter, the mixture was carefully added to 1ml of concentrated sulphuric acid. Appearance of a brown ring indicated the presence of glycosides.

6.3.1.3. Determination of saponins

20ml of distilled water was added to approximately 2g of powdered plant sample and boiled for 6 minutes. Whilst still hot, the mixture was filtered to obtain 10ml of filtrate, and this was combined with 5ml of distilled water. Thereafter, the solution was vigorously shaken until frothing, after which 3 drops of olive oil were added to the froth. Formation of an emulsion was indicative of the presence of saponins.

6.3.1.4. Determination of tannins

To approximately 0.5g of powdered plant sample, 20ml of distilled water was added and the mixture was boiled for 6 minutes. The mixture was filtered whilst still hot, and 3 drops of 0.1% of ferric chloride were added to the filtrate. A blue-black precipitate indicated the presence of tannins.

6.3.1.5. Determination of terpenoids

0.5g of powdered plant sample was treated with 2ml of chloroform, after which 3ml of concentrated sulfuric acid was carefully added to the mixture. The formation of an interface with a reddish brown coloration indicated the presence of terpenoids.

6.3.1.6. Determination of phytosterols

To 0.5g of powdered plant sample, 10 ml of chloroform was added. Thereafter, 1ml of concentrated sulfuric acid was carefully added from the side of the test tubes to 0.5ml of the chloroform extract. Appearance of a reddish brown colour in the chloroform layer was indicated the presence of phytosterols.

SECTION TWO: FRACTIONATION OF CRUDE EXTRACTS USING SOLID PHASE EXTRACTION

6.3.2. Solid phase extraction procedure

1g of the hexane, DCM and DCM: methanol (1:1, v/v) extracts, respectively, and 3g of the methanol extract was dissolved in 20ml of ACN, after which it was sonicated for 5 minutes. A SPE cartridge was conditioned with 200ml 100% methanol. Thereafter, the sample was loaded on the cartridge and allowed to elute with 200ml 100% H_2O, 200ml 25% ACN, 200ml 50% ACN, 200ml 75% ACN and 200ml 100% ACN, respectively. The resulting fractions were evaporated and freeze-dried as described in Section 6.3.5.

Finally, five fractions was produced for each extract, of which the percentage yield was determined as described in Section 6.3.6.

SECTION THREE: CHEMICAL FINGERPRINTING OF *C. SATIVA* L. CRUDE EXTRACTS AND SPE FRACTIONS

The UPLC-MS method used to quantify the *C. sativa* L. crude extracts and SPE fractions was adopted from Aizpurua-Olaizola *et al* (92)

6.3.3. Chromatographic system for DCM and methanol crude extracts

The UPLC-MS system used consisted of a Waters Acquity UPLC hyphenated with a Waters Synapt G2 Quadrupole time of flight (QTOF). The mass spectrometer was equipped with an electrospray ion source, quaternary pump, an auto sampler and a column oven. Data was accumulated by the software Masslynx V4.1.

6.3.4. Chromatographic system for DCM: methanol crude extract, SPE fractions and Cannabis standards

AB Sciex 4000: LC–MS/MS analysis was performed on a Shimadzu HPLC system composed of two LC-20AD XR pumps, degasser, column oven, solvent selection valve, temperature regulated auto sampler, and an external Valco divert valve installed between the LC and mass spectrometer. The LC system was coupled to an API 4000 linear ion trap triple quadrupole (QTRAP) tandem mass spectrometer operated with Analyst 1.5.2.

6.3.5. Sample preparation

Only the DCM and methanol crude extracts, respectively, were subjected to UPLC analysis. The DCM extract was reconstituted in ACN: H_2O (80:20, v/v) while the methanol extract was reconstituted in methanol: H_2O (80:20, v/v). The SPE fractions were reconstituted in ACN: H_2O (10:90, v/v).

6.3.6. Chromatographic conditions for DCM and methanol crude extracts

Chromatographic separation was achieved by running the mobile phase at flow rate of 0.400ml/min over a Waters Acquity UPLC™ BEH C_{18} column (2.1mm x 100mm, 1.7µm) with the column oven set at 30˚C. The mobile phase consisted of distilled H_2O with

0.1% formic acid (A): ACN (B). The gradient elution program started at 97:3 (A: B) and was changed to 0:100 over 14 minutes. This ratio was maintained from 14 – 16 minutes; after which it was changed back to 97:3 by 20 minutes. The injection volume was 10µl and the mass spectrometer with electrospray ionization (ESI) was operated in both negative and positive mode.

6.3.7. Chromatographic conditions for DCM: methanol crude extract, SPE fractions and Cannabis standards

Chromatographic separation was achieved by running the mobile phase at flow rate of 0.400ml/min over a reversed phase C_{18} column (2.1mm x 100mm, 1.7µm) with the column oven set at 30˚C. The mobile phase consisted of distilled H_2O with 0.1% formic acid (A): ACN (B). The gradient elution program started at 97:3 (A: B) and was changed to 0:100 over 14 minutes. This ratio was maintained from 14 – 16 minutes; after which it was changed back to 97:3 by 20 minutes. The injection volume was 20µl and the mass spectrometer with electrospray ionization (ESI) was operated in the positive mode for the SPE fractions and negative mode for the DCM: methanol crude extract. The capillary temperature and voltage were set at 400˚C and 4500V, respectively, while the fragmentor voltage was set at 160V.

6.4. RESULTS

6.4.1. Phytochemical analysis

Table 6.1 shows the results of the phytochemical analysis, while Figure 6.1 and 6.2 is that of the colour changes observed. Here, the powdered aerial parts *C. sativa* L. tested positive for the presence of glycosides, saponins, tannins, terpenoids and phytosterols; and negative for the presence of flavonoids.

Table 6.1: Results of the phytochemical analysis of powdered *C. sativa L*. aerial plant parts

Phytochemical constituent	Results
Flavonoids	-
Glycosides	+
Saponins	+
Tannins	+
Terpenoids	+
Phytosterols	+

(+): Indicates the presence of phytochemical constituent found in the plant sample, and
(-): Indicates the absence of phytochemical constituent in the plant sample

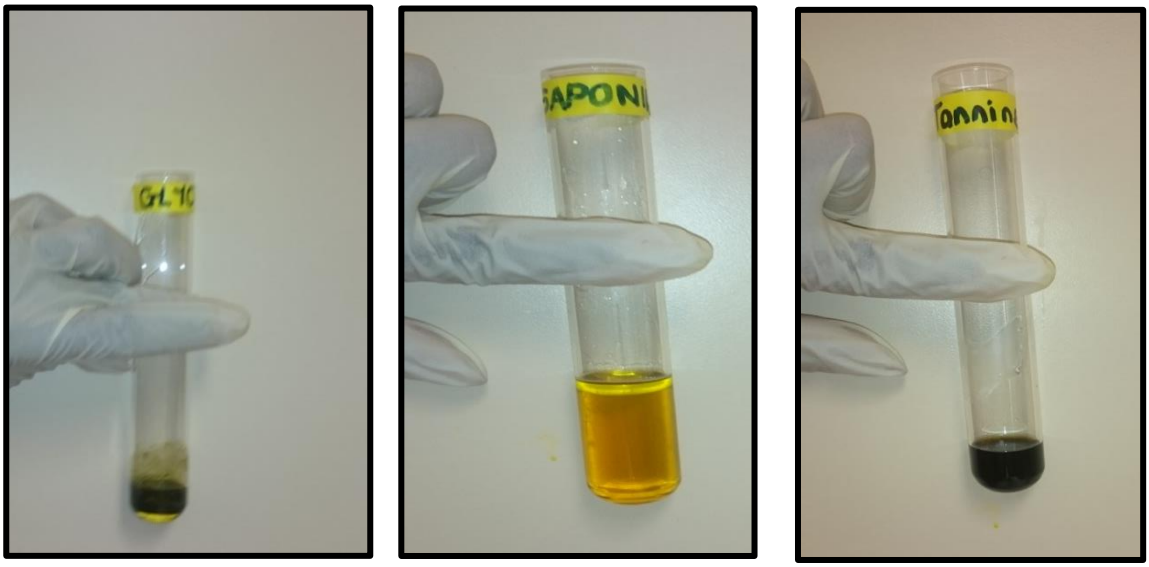


Figure 6.1: A photograph showing the colour change observed for (A) glycosides, (B) saponins and (C) tannins

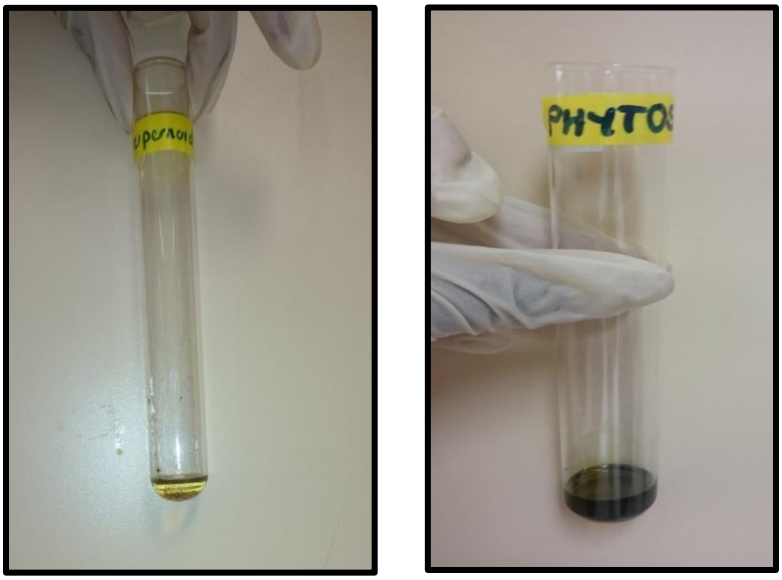


Figure 6.2: A photograph showing the color change observed for (D) terpenoids and (E) phytosterols

6.4.2. Solid phase extraction of *C. sativa* L. extracts

Table 6.2 shows the mass of fractions obtained from the respective solvents' crude extracts, as well as the percentage yield of each fraction. Each extract produced five fractions.

Table 6.2: Percentage yield of the respective *C. sativa* L. fractions

***C. sativa* L. extracts**	***C. sativa* L. fractions**									
	100% dH_2O		**25% ACN**		**50% ACN**		**75% ACN**		**100% ACN**	
	Mass (g)	**% yield**	**Mass (g)**	**% yield**	**Mass (g)**	**% yield**	**Mass (g)**	**% yield**	**Mass (g)**	**% yield**
Hexane	0.12	12	1.34	134	1.32	132	3.86	386	0.35	35
DCM	0.98	98	0.15	15	0.46	46	1.53	153	0.40	40
DCM: Methanol	0.50	50	0.15	15	0.19	19	0.82	82	0.57	57
Methanol	0.69	23	0.25	8.33	0.37	12.33	0.69	23	0.21	7

DCM: Dichloromethane; dH_2O: Distilled water; ACN: Acetonitrile

6.4.3. Chemical characterization of *C. sativa* L. DCM, DCM: methanol and methanol crude extracts and standards by LC-MS

Figure 6.3 (A) and 6.3 (B) shows the chromatograms of the *C. sativa* L. DCM- and methanol extracts, while their retention times and mass per ratio of major peaks in UPLC-MS ESI in negative mode are tabulated in Table 6.2 and 6.3. Figure 6.3 (C) and (D) shows the chromatograms of the DCM- and methanol extracts, while their retention times and mass per ratio major peaks in UPLC-MS ESI in positive mode are tabulated in Table 6.4 and 6.5. Figure 6.3 (E- G) shows the HPLC-MS chromatograms of the DCM: methanol extract and the standards, CBD and THC, in negative mode.

The negative mode detected more peaks compared to the positive mode. Interestingly, major peaks in the DCM extract with retention times of 10.38- (327.1967m/z), 11.31- (359.2227m/z), and 12.76 minutes (353.1766m/z) were also observed in the methanol extract, with only slight variation in the retention times at 10.37- (327.2158m/z), 13.68 (359.2227m/z), and 14.67 minutes (353.1758m/z). In the positive mode, only one peak in the DCM extract, retention time of 12.96 minutes (282.2805m/z), was similarly observed in the methanol extract at a retention time of 14.06 minutes (282.2798m/z). Analysis of the DCM: methanol extract, THC, and CBD revealed the presence of different compounds with different molecular weight. Some of the major peaks observed in both the DCM- and methanol chromatograms were also seen in the DCM: methanol chromatogram.

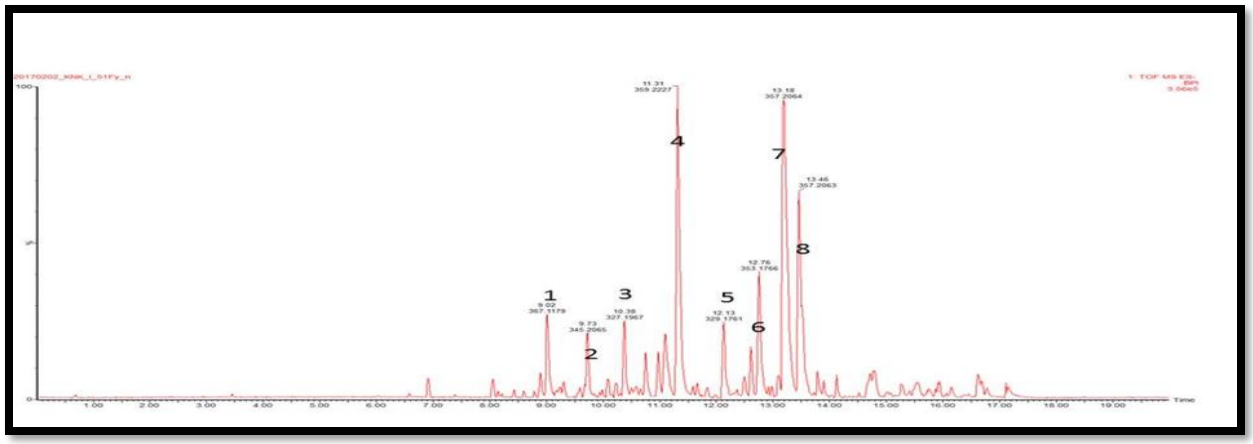


Figure 6.3 (A): UPLC-MS chromatogram of *C. sativa* L. DCM extract in negative mode

Table 6.3: Retention time and m/z of major peaks in UPLC-MS ESI negative mode of *C. sativa* L. DCM extract

Peak number	Retention time	m/z
1	9.02	367.1179
2	9.73	345.2065
3	10.38	327.1967
4	11.31	359.2227
5	12.13	329.1761
6	12.76	353.1766
7	13.18	367.2064
8	13.46	357.2063

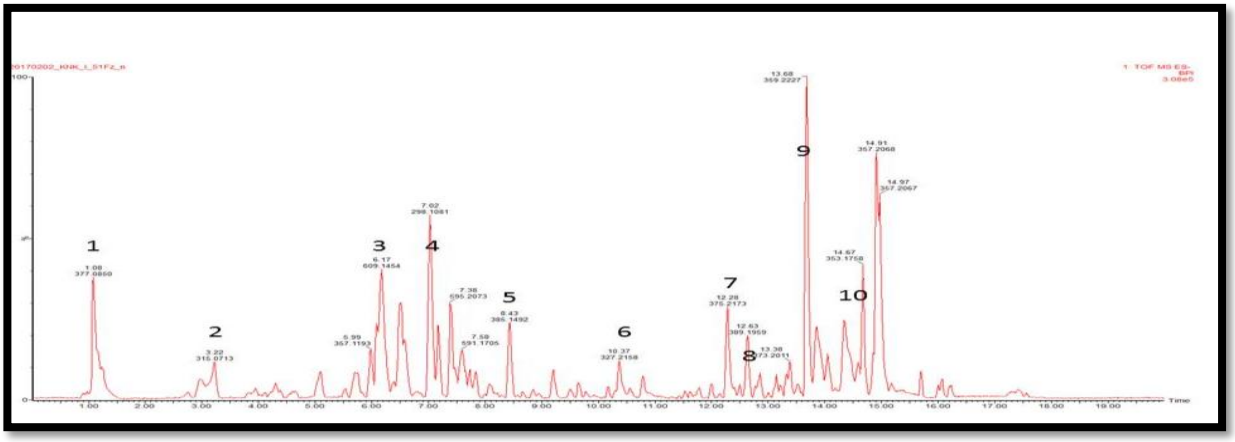


Figure 6.3 (B): UPLC-MS chromatogram of *C. sativa* L. methanol extract in negative mode

Table 6.4: Retention time and m/z of major peaks in UPLC-MS ESI negative mode of *C. sativa* L. methanol extracts

Peak number	Retention time	m/z
1	1.08	377.0850
2	3.22	315.0713
3	6.17	609.1454
4	7.02	298.1081
5	8.43	385.1492
6	10.37	327.2158
7	12.28	375.2173
8	12.63	389.1959
9	13.68	359.2227
10	14.67	353.1758

Table 6.5: Common peaks in both the *C. sativa* L. DCM and methanol extracts in negative mode

Extract	Retention time	m/z
DCM	10.38	327.1967
	11.31	353.2227
	12.76	353.1766
Methanol	10.37	327.2158
	13.68	359.2227
	14.67	353.1758

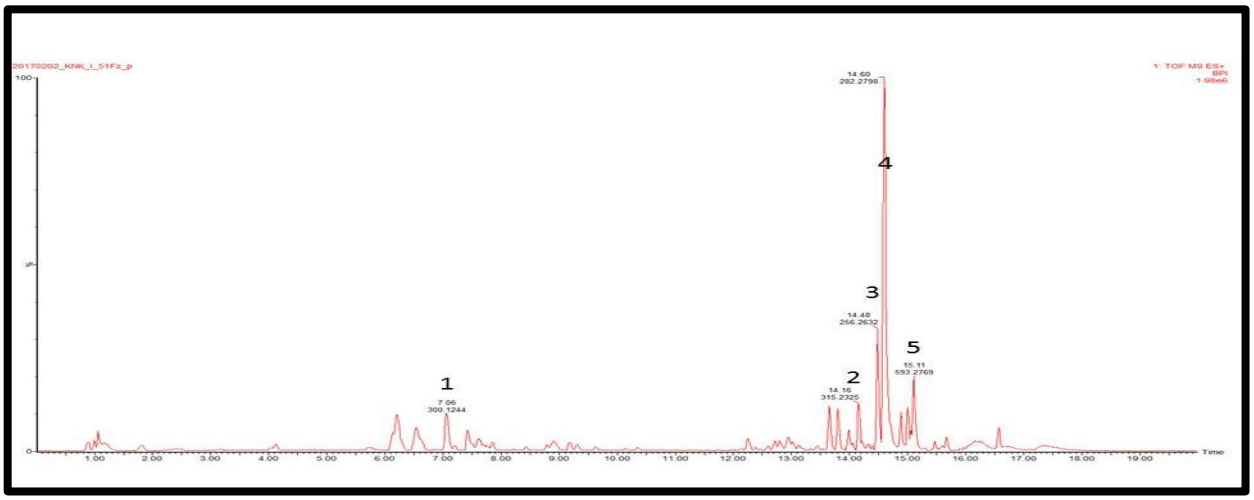


Figure 6.3 (C): UPLC-MS chromatogram of *C. sativa* L. methanol extract in positive mode

Table 6.6: Retention time and m/z of major peaks in UPLC-MS ESI positive mode of *C. sativa* L. methanol extract

Peak number	Retention time	m/z
1	7.06	300.1244
2	14.16	315.2325
3	14.48	256.2632
4	14.60	282.2798
5	15.11	593.2769

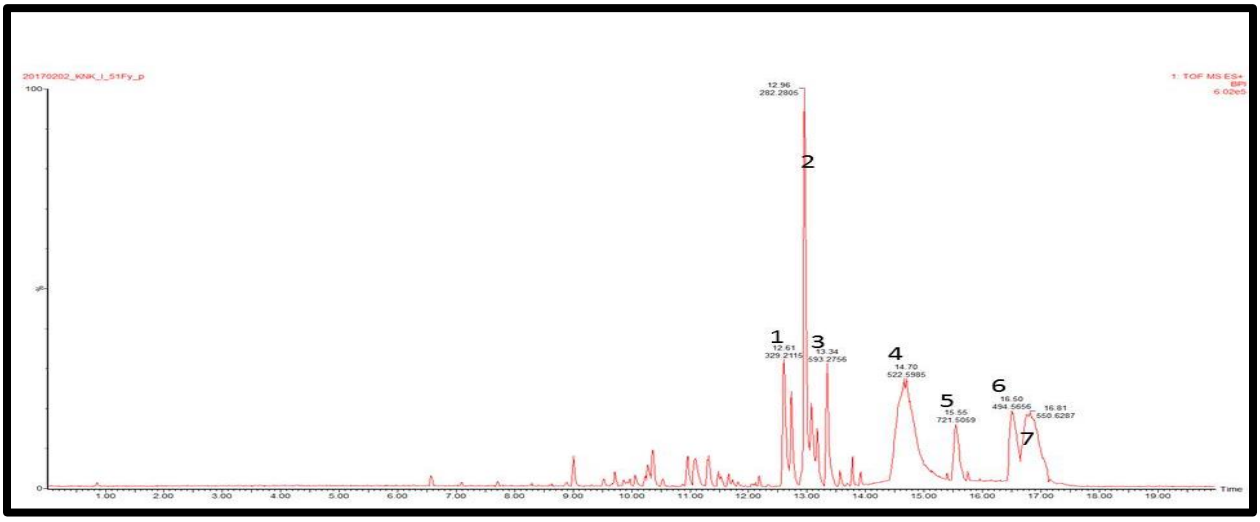


Figure 6.3 (D): UPLC-MS chromatogram of *C. sativa* L. DCM extract in positive mode

Table 6.7: Retention time and m/z of major peaks in UPLC-MS ESI positive mode of *C. sativa* L. DCM extract

Peak number	Retention time	m/z
1	12.61	329.2115
2	12.96	282.2805
3	13.34	593.2756
4	14.70	522.5985
5	15.55	721.5059
6	16.50	494.5656
7	16.81	550.6287

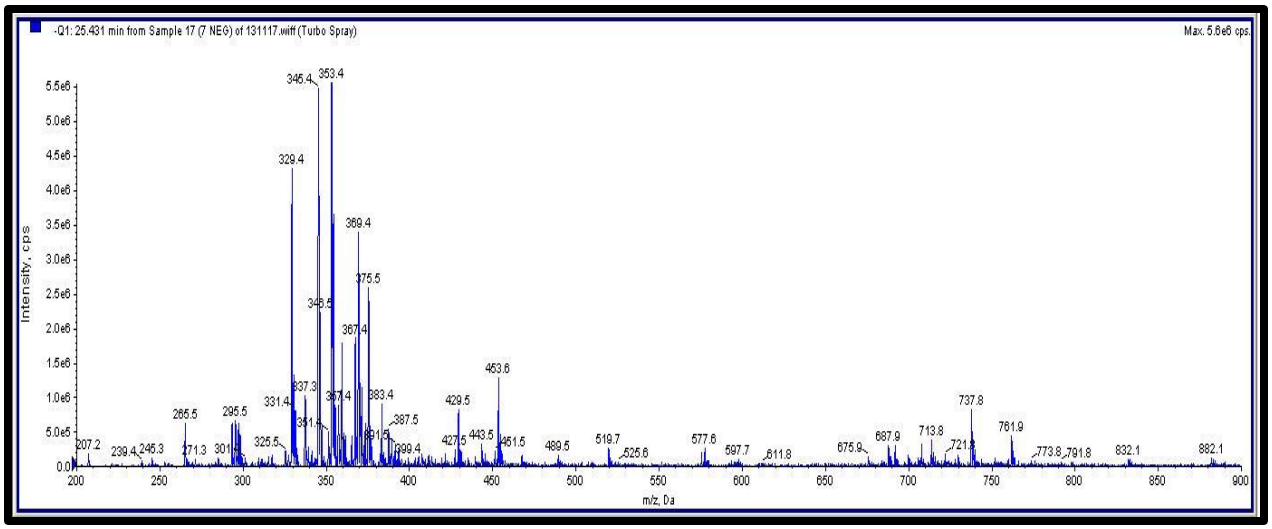


Figure 6.3 (E): HPLC-MS chromatogram of *C. sativa* L. DCM: methanol extract in negative mode

Table 6.8: m/z of major peaks in HPLC-MS ESI negative mode of *C. sativa* L. DCM: methanol extract

Peak number	m/z
1	329.4
2	346.5
3	345.4
4	353.4
5	367.4
6	369.4
7	375.5

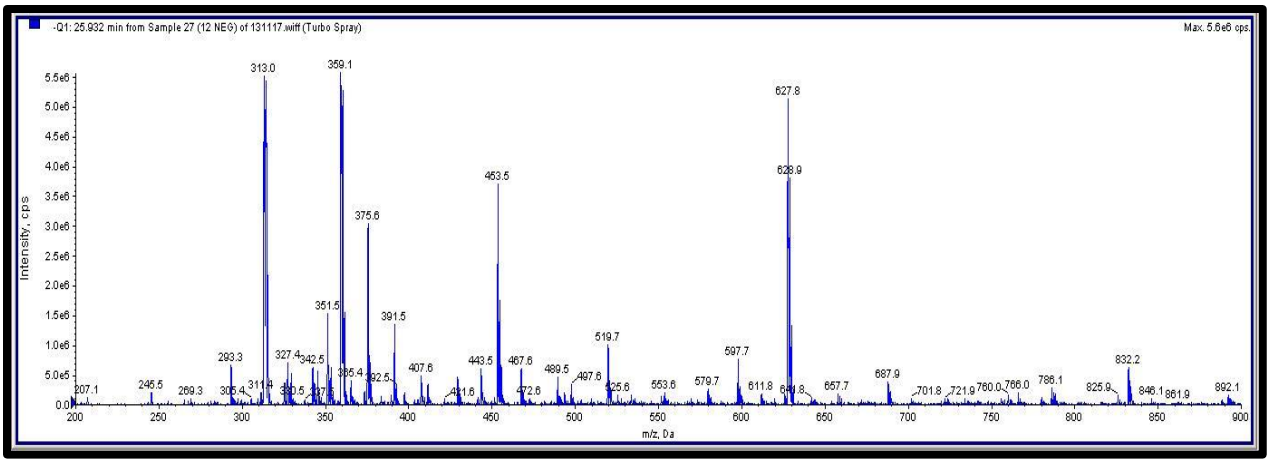


Figure 6.3 (F): HPLC-MS chromatogram of CBD in negative mode

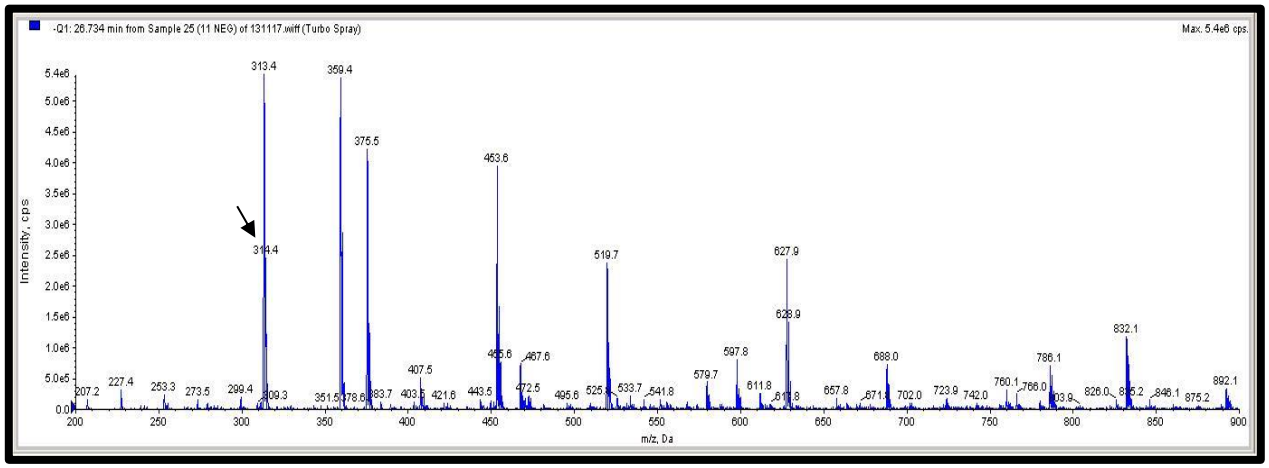
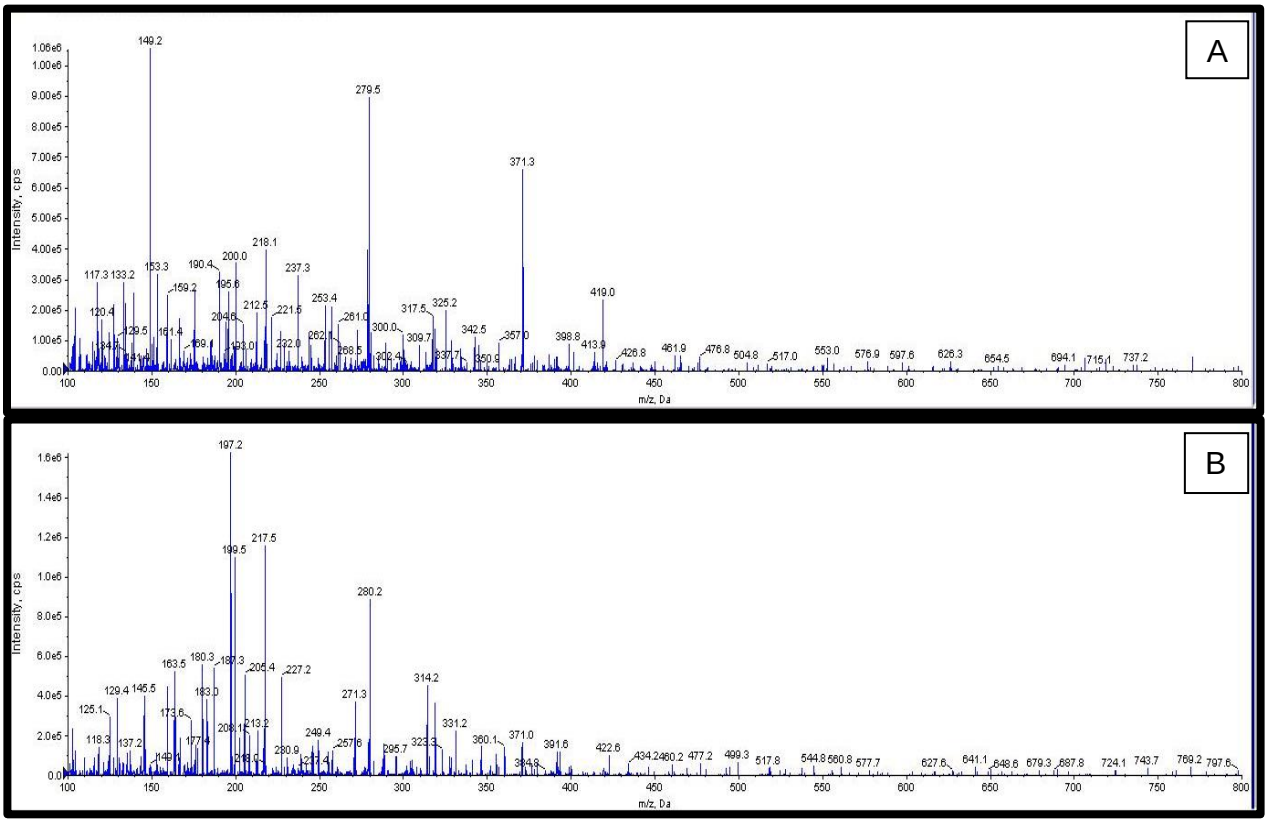


Figure 6.3 (G): HPLC-MS chromatogram of THC in negative mode

6.4.4. Characterization of *C. sativa* L. fractions by LC-MS

Figures 6.4-6.7 shows the HPLC-MS chromatograms of the *C. sativa* L. hexane, DCM, DCM: methanol and methanol fractions. HPLC-MS analysis revealed the presence of different compounds having different molecular weights for each fraction. Chromatograms of fractions isolated from the same extract also showed similar presence of compounds.



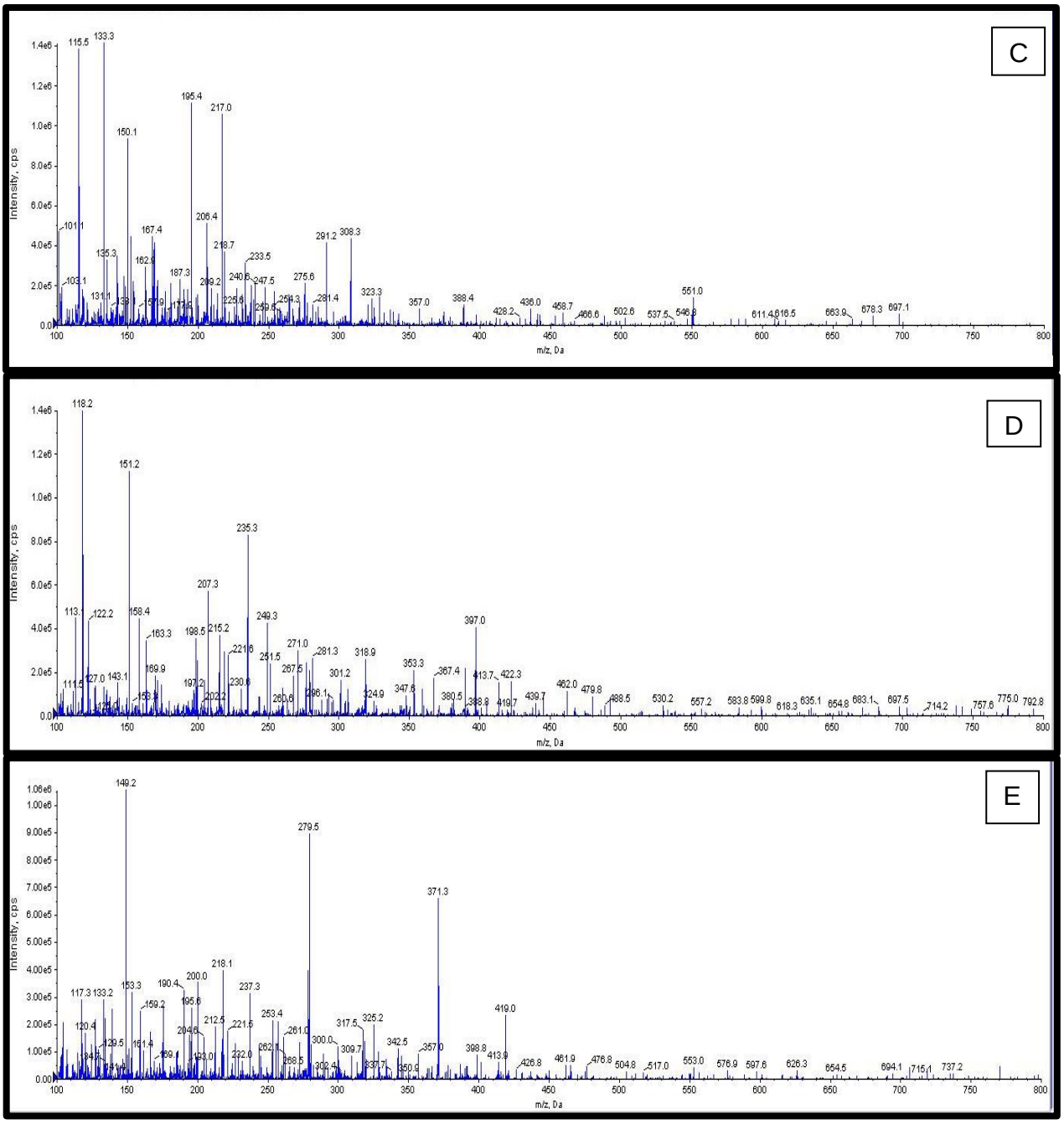
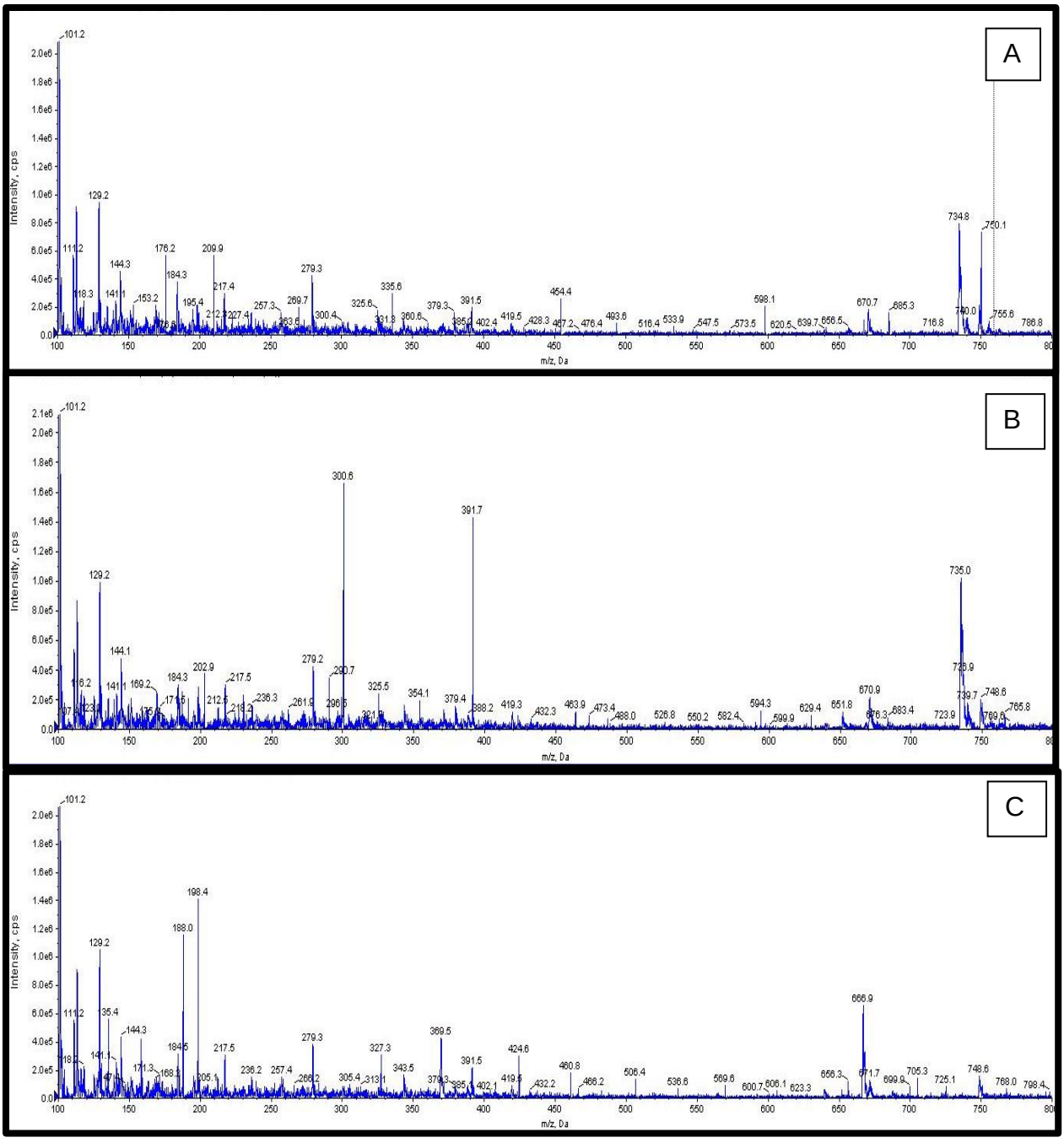


Figure 6.4: HPLC-MS chromatograms of *C. sativa* L. hexane fractions with (A) 100 % H_2O, (B) 25 % ACN, (C) 50% ACN, (D) 75 % ACN and (E) 100 % ACN


A
B
C
Intensity, cps
m/z, Da

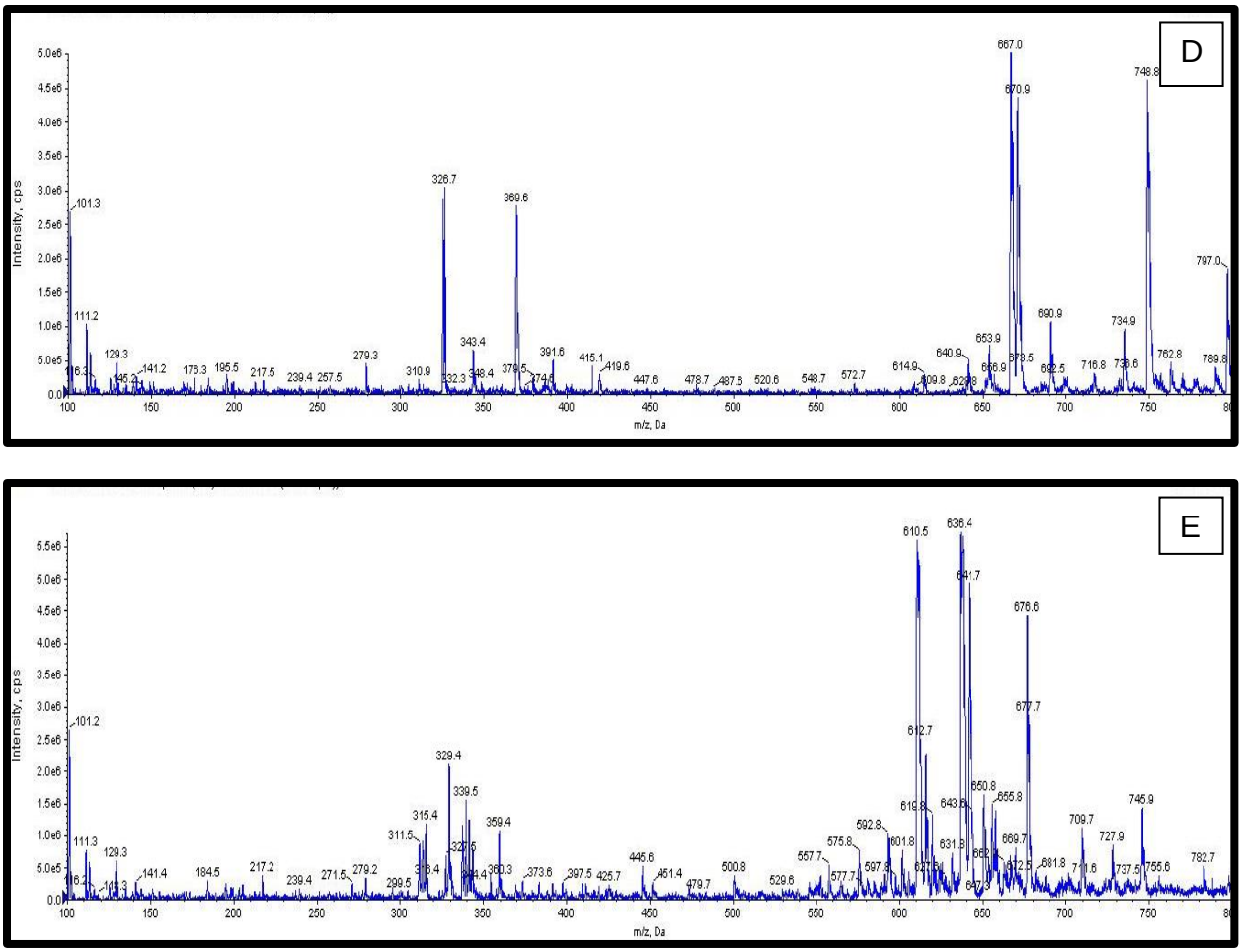


Figure 6.5: HPLC-MS chromatograms of *C. sativa* L. DCM fractions with (A) 100 % H_2O, (B) 25 % ACN, (C) 50% ACN, (D) 75 % ACN and (E) 100 % ACN

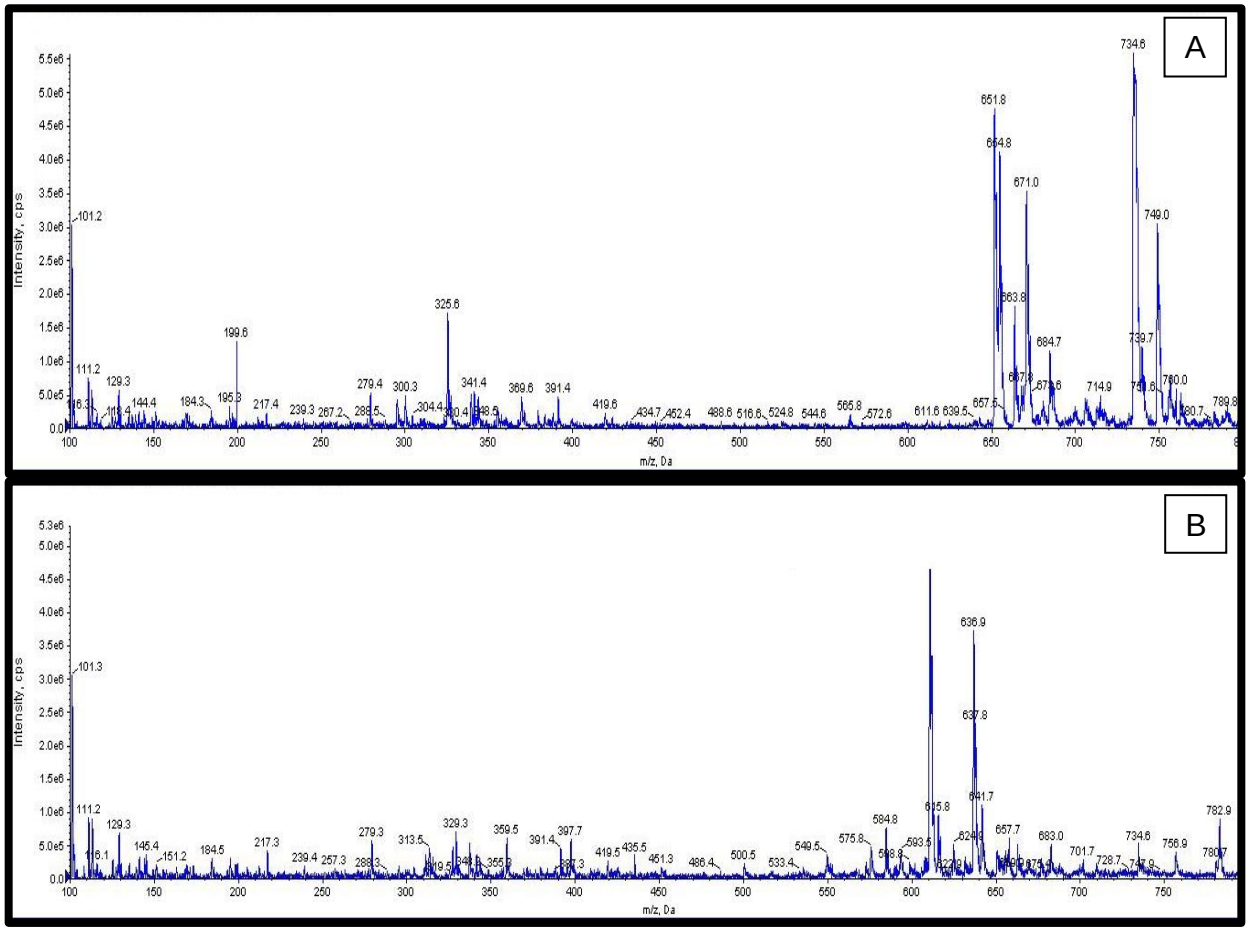
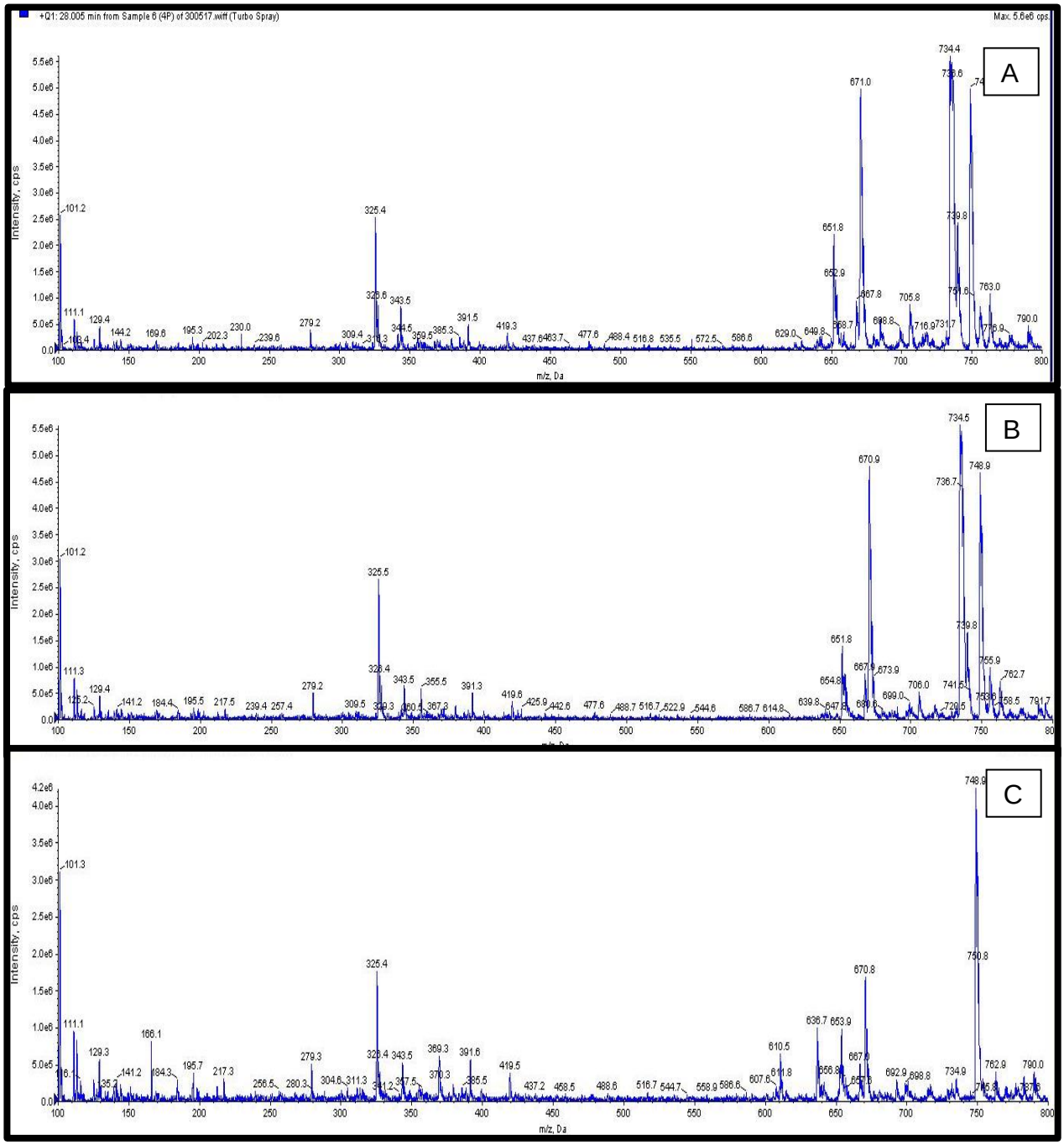


Figure 6.6: HPLC-MS chromatograms of *C. sativa* L. DCM: methanol fractions with (A) 100 % H_2O and (B) 100 % ACN


+Q1: 28.005 min from Sample 6 (4P) of 300517.wiff (Turbo Spray)
Max. 5.6e6 cps.
A
B
C
Intensity, cps
m/z, Da

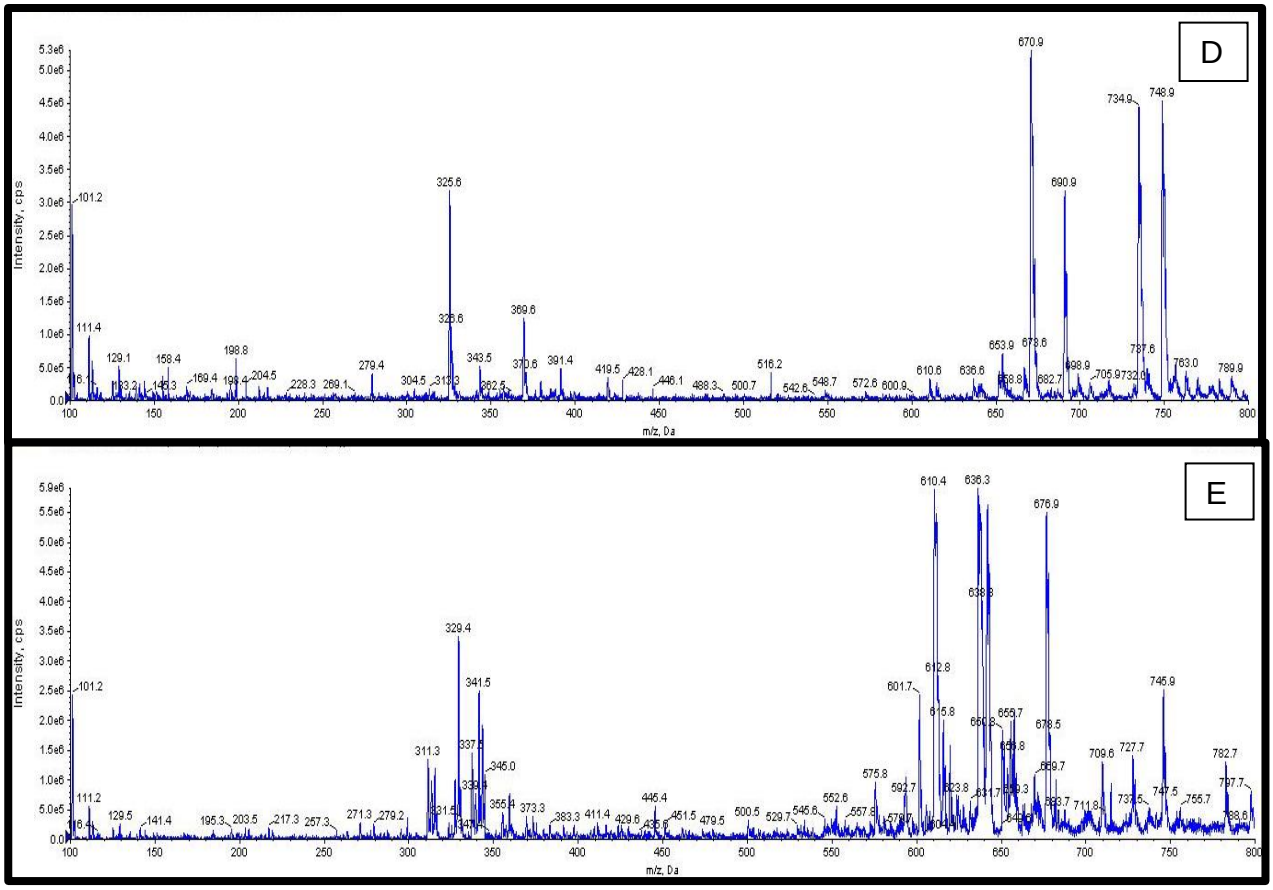


Figure 6.7: HPLC-MS chromatograms of *C. sativa* L. methanol fractions with (A) 100 % H_2O, (B) 25 % ACN, (C) 50% ACN, (D) 75 % ACN and (E) 100 % ACN

6.5. DISCUSSION

Phytochemical screening of the pulverized aerial parts of *C. sativa* L. revealed the presence of the most important secondary phytochemical constituents, which may actively play a role as therapeutic agents in the reversal of multidrug resistance in cancer. These phytochemical metabolites were not quantified in the extracts, as the purpose of the screening was to initially determine the presence or absence of each secondary metabolite. As tabulated in Table 6.1, the phytochemical compounds present included saponins, which have been reported to demonstrate significant anticancer activity against various tumors, including lung- and colon cancer. These activities were anti-proliferation, anti-metastasis, anti-angiogenesis and reversal of MDR effects through mechanisms such as induction of apoptosis and promotion of cell-differentiation.

Saponins are said to reinforce its anticancer effects in conjunction with docetaxel, gemcitabine, cisplatin, doxorubicin and verapamil (93). The availability of tannins, which are known to increase the efficacy of doxorubicin and enhance the doxorubicin concentrations when reversing ABCB1-mediated MDR, was a positive finding towards proving that *C. sativa* L. extracts could reverse drug resistance in chemotherapy (8,44). The presence of terpenoids, glycosides and phytosterols indicates that the aerial plant parts may exert cytotoxic properties against both colon- and lung cancer cells. This is relevant in potentiating the effect of current treatment of MDR in colon- and lung cancer, as these groups are reported to do (32,48,51). Therefore, the results demonstrated that the aerial parts of *C. sativa* L. could be further evaluated for their ability to reverse MDR in colon- and lung cancer cells.

SPE is a widely used sample purification technique that fractionates crude extracts into groups of compounds by eluting the extract with different solvents for chromatographic separation (67,94). In the present study, SPE was used to concentrate- and obtain fractions rich with certain chemical compounds for further analysis. SPE analysis yielded five fractions from each crude extract. The hexane and DCM extracts yielded some fractions with percentage yields of greater than a 100%. This can only be possible

if the fractions contain impurities that causes its mass to be greater than it would be if the product was pure.

For the LC-MS analysis of the crude extracts and fractions, only the non-volatile samples were analyzed given the nature of the operation of the technology. Only the DCM- and methanol extracts was submitted for UPLC-MS analysis, as the hexane extract is known to have non-polar compounds such as oils that can block the UPLC column. Although the DCM- and methanol extracts were characterized by MS analysis in both the negative and positive modes, it was found that more peaks were detected and resolved better in the negative mode of system operation. Therefore, the negative ionization mode was used for most of the sample analysis.

The UPLC-MS analysis results showed that the major peaks/compounds were more ionized in the negative mode, for both the DCM- and methanol extracts. Major peaks in the DCM extract with retention times of 10.38- (327.1967m/z), 11.31- (359.2227m/z) and 12.76 minutes (353.1766m/z), were also observed in the methanol extract with slightly different retention times at 10.37- (327.2158m/z), 13.68- (359.2227m/z) and 14.67 minutes (353.1758m/z), respectively.

The following cannabinoids: tetrahydrocannabinolic acid (THCA-C4) at 345.2065m/z (92), THC at 315.0713m/z (92), CBD at 315.0713m/z (92), cannabichromenic acid (CBCA) at 359.2227m/z (92), cannabigerolic acid monomethylether (CBGAM) at 375.2158m/z (92), CBV at 282.2798m/z (95) and ethyl-3,10-dimethyl-undecanoate (Palmitic acid) at 256.2632m/z (95); were identified and verified by means of a liquid chromatography coupled to a Q-TOF detector. Several more unknown compounds were seen in both DCM- and methanol crude extracts.

HPLC-MS analysis of the SPE fractions revealed the presence of different compounds having different molecular weight. It was also observed that the fractions isolated from the same extract produced chromatograms which showed similar compounds at different intensities, as indicated by their different RT, peak areas and m/z values.

HPLC-MS analysis of the DCM: methanol crude extract showed the presence of peaks with similar m/z values to that produced by UPLC-MS analysis of the DCM- and

methanol crude extracts ionized in the negative mode. Peaks with the following m/z values: 329.4, 345.4, 353.4, 367.4 and 375.5 were seen in both the DCM- and methanol chromatograms. The Cannabis standards were successfully analyzed using HPLC-MS for fingerprinting purposes. Due to poor separation, it became difficult to determine how the two Cannabis standards differed from each other, except for one peak at 314.4m/z that appeared only in the THC chromatogram.

HPLC and UPLC are widely used analytical techniques with a wide range of variations available mainly used as preparative tools and to obtain chemical fingerprints of plant material (96). Traditionally, HPLC fingerprinting is used in the quality control of herbal medicines but has been restricted by its low efficiency and longer separation time compared to UPLC with its unparalleled advantages of ultra-high column efficiency, separation capability and separation velocity (96–99).

The MS and Q-TOF, coupled to these techniques, allow for faster analysis time, being used for the identification and structure elucidation of the components therein. In the drug development process, MS has been used for lead compound discovery, structural analysis, synthetic development, combinatorial chemistry, pharmacokinetics and drug metabolism while QTOF allows for a more accurate mass analysis, which is based on principle that the velocities of two ions vary based on the mass of the two ions (100).

A BEH C_{18} column used in the UPLC-MS analysis was selected due to its ability to retain and separate polar, basic compounds (101). Although it is important that acid be added to the mobile phase in the positive ionization mode in order to promote ionization and enhance resolution of the separation, 0.1% formic acid was added to the mobile phase in both positive and ionization modes. Gradient separation was found to be a better system for maximum elution, peak resolution and to eliminate the matrix effect caused by endogenous substances (102).

The overall goal was to obtain a chemical fingerprint of the crude extracts and SPE fractions for identification- and authentication purposes. LC-MS analysis was used to develop the chromatographic fingerprints. These chromatographic chemical fingerprints can be helpful in explaining the variation in results obtained from this study to that of

other studies, and to ensure that there is reproducibility and consistency in the results. In case where a pharmaceutical product results from this study, the chemical fingerprint can be used for quality control purposes.

6.6. CONCLUSION

Chemical fingerprints of *Cannabis sativa* L. extracts, SPE fractions and standards were established using LC-MS for their identification, authentication and quality control purposes. Phytochemical analysis showed the presence of glycosides, saponins, tannins, terpenoids and phytosterols; and absence of flavonoids. SPE successfully yielded five fractions for each extract.

CHAPTER SEVEN: CONCLUSIONS AND FUTURE STUDIES

The objectives of this study were achieved as follows:

- Qualitative phytochemical analysis on the pulverized crude plant material indicated the presence of glycosides, saponins, tannins, terpenoids and phytosterols; and absence of flavonoids.

- Fractionation of the crude extracts by solid phase extraction yielded five fractions from each of the extracts that were fractionated.

- Chemical fingerprinting of the crude extracts, SPE fractions and cannabis standards was performed by LC-MS for their identification, authentication and quality control purposes.

- Anticancer assay results revealed that *C. sativa* L. extracts inhibited cell growth inhibition in the following cancer cell lines: HT-29, Caco-2, NCI-H146 [H146], HCT-15, LS513 and H69AR.
    - Cell growth stimulation was observed in Caco-2 cells when exposed to higher extract concentrations.
- Cytotoxicity results showed that *C. sativa* L. extracts appeared to be more cytotoxic in lung- and colon cancerous cells than in the normal cells.

- Doxorubicin resistant reversal assay was performed in Caco-2, HCT-15, LS513 and H69AR cell lines through combination studies, and revealed the following:
    - Verapamil increased cell sensitivity to doxorubicin by 4.8-fold compared to the extracts in Caco-2 cells.
    - Hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts increased doxorubicin sensitivity in HCT-15 cells by 4.59-, 3.50, 32.97-, 74.07- and

2.08-fold, compared to verapamil with a 1.41-fold resistance reversal factor.

- Hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts increased doxorubicin sensitivity in LS513 cells by 3.6-, 300.7-, 4.6-, 4.4- and 6.3-fold compared to verapamil with a 0.03-fold resistance reversal factor.
- Hexane-, DCM-, DCM: methanol-, methanol- and H_2O extracts increased doxorubicin sensitivity in H69AR cells by 8.60-, 7.09-, 11.34-, 20.51- and 11.42-fold compared to verapamil with a 0.87-fold resistance reversal factor.
- CI analysis demonstrated that both the control and extracts yielded a normal to very strong synergistic interaction (CI <1) in Caco-2 cells, normal to moderate synergistic interaction (CI <1) in HCT-15 cells, moderate to strong synergistic interaction in LS513 cells and nearly additive (CI=1) to antagonistic (CI >1) interaction in H69AR cells.

Study limitations:

- The study conducted a qualitative phytochemical analysis, wherein a quantitative analysis would have shown variable amounts of pharmacologically important phytochemical constituents.
- The study only made use of MTT assay to determine proliferation and cytotoxicity of the test samples. Other methods such as SulforhodamineB assay could have been used as it evaluates the effects of gene expression modulation and can be used to study the effects of miRNA replacement on cell proliferation.

Future studies:

The following are the proposed possible future studies that could be done as a follow up to this study.

- Biological evaluation of the most active extracts to confirm the mechanisms through which these extracts exert their resistance reversal effects.

- ❖ The purification of the most active extracts, to isolate if at all, the compounds responsible for the observed activity.

- ❖ Chemical characterizations of the active compounds and their quantification including scale up extraction or chemical synthesis for further pharmacokinetics to clinical studies.

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APPENDIX A: RESEARCH OUTPUTS

1. CONFERENCE PRESENTATIONS

I.M. Mangoato*, P.M. Chandrashekara and M.G. Matsabisa. The potential of *Cannabis sativa* L. aerial plant parts extracts to reverse drug resistance in colon cancer cell lines. **(Poster presentation)**. The South African 51st Annual Pharmacology Conference, University of the Free State, Bloemfontein, South Africa, 01st-04th October 2017.

2. ACCEPTED FOR PRESENTATION

- I.M. Mangoato*, P.M. Chandrashekara and M.G. Matsabisa. *Cannabis sativa* L. aerial plant parts extracts reverse drug resistance in LS513 MDR colon cancer cells *in vitro.* The 1st Conference of Biomedical and Natural Sciences and Therapeutics, Spier Estate, Stellenbosch, South Africa, 7th-10th October 2018.
- I.M. Mangoato*, P.M. Chandrashekara and M.G. Matsabisa. *Cannabis sativa* L. aerial plant parts extracts reverse drug resistance in LS513 MDR colon cancer cells *in vitro.* The 50th Faculty of Health Sciences Research Forum, University of the Free State, Bloemfontein, South Africa, 30th-31st August 2018.

3. MANUSCRIPT IN PREPARATION

- Mangoato M Innocensia*, Chandrashekara P Mahadevappa and Matsabisa M Gilbert: *Cannabis sativa* L. extracts can reverse drug resistance in colorectal carcinoma cells *in vitro.* Submitted: International Journal of Synergy and Research.

4. WORKSHOP PRESENTATIONS AND ATTENDANCE

- **Workshop:** South Africa-Jamaica research launching workshop **(Attended/Exchange student).**
  Time period: 13th-15th March 2018.
  Location: Kingston, Jamaica.
- **Workshop:** Scientific evaluation and validation of South Korean and South African medicinal plants for cancer and diabetes **(Oral presentation).**
  Time period: 23rd-26th April 2018.

Location: Bloemfontein, South Africa.

5. AWARDS

- South African Women in Science Award (2018)

Category: Natural (Physical and Life) and Engineering Sciences.

SUMMARY

Key terms: MDR, Cannabis, colon cancer, lung cancer, doxorubicin, verapamil, cytotoxicity

A major problem related to the successful application of chemotherapy in human cancer is anti-cancer drug resistance. Verapamil is one of the first drugs known to circumvent MDR, but is limited by lack efficacy in clinical trials, enhanced toxicity to normal cells and inhibition of cytochrome P450 enzymes resulting in pharmacokinetic interactions with increased host toxicity, thereby leading to severe adverse effects.

Thus, this study was designed to demonstrate the potential reversal of doxorubicin resistance by *Cannabis sativa* L. extracts using selected MDR expressing lung- and colon cancer cells in an *in vitro* test model. Firstly, the pulverized plant material was sequentially extracted with four organic solvents, in order of increasing polarity, starting with hexane, dichloromethane (DCM), DCM: methanol (1:1; v/v) and methanol, respectively. A water extract was prepared to simulate traditional preparation of the plant. Crude extracts were further fractionated by means of solid phase extraction (SPE) using the following eluting concentrations: 100% H_2O, 25% acetonitrile (ACN), 50% ACN, 75% ACN and 100% ACN. The SPE yielded five fractions from each of the extracts. Qualitative phytochemical analysis performed on the pulverized crude plant material indicated the presence of glycosides, saponins, terpenoids, tannins, phytosterols and the absence of flavonoids.

Chemical fingerprinting of the *C. sativa* L. crude extracts, SPE fractions and cannabis standards was determined by liquid chromatography tandem mass spectrometry (LC-MS). The DCM- and methanol extracts were subjected to UPLC-MS analysis while the DCM: methanol crude extract, SPE fractions, and cannabis standards (CBD and THC) were analysed using HPLC-MS. Compound separation was achieved with a gradient mobile phase of distilled H_2O with 0.1% formic acid (A): ACN (B) at a flow rate of 0.400 ml/min. The mass spectrometer with electrospray ionization was operated in both negative and positive mode for the DCM- and methanol extracts to avoid destroying any

liable compounds, negative mode for the DCM: methanol extract and positive mode for the SPE fractions.

UPLC-MS analysis showed that the negative mode detected more peaks compared to the positive mode. The major peaks in the DCM extract with retention times of 10.38- (327.1967m/z), 11.31- (359.2227m/z), and 12.76 minutes (353.1766m/z) were also observed in the methanol extract, with only slight variation in the retention times at 10.37- (327.2158m/z), 13.68 (359.2227m/z), and 14.67 minutes (353.1758m/z). In the positive mode, only one peak in the DCM extract, retention time of 12.96 minutes (282.2805m/z), was similarly observed in the methanol extract at a retention time of 14.06 minutes (282.2798m/z). Analysis of the DCM: methanol extract, SPE fractions, THC, and CBD revealed the presence of different compounds with different molecular weights. Some of the major peaks observed in both the DCM- and methanol extracts were also seen in the DCM: methanol extract.

Anticancer and cytotoxicity assays were conducted against a panel of human lung- and colon cancer cells, namely; HT-29, Caco-2, NCI-H146 [H146], HCT-15 MDR, LS513 MDR and H69AR MDR cells; and human normal colon (CCD-18Co) cells. According to the American National Cancer Institute (NCI) guidelines, plant extracts with IC_{50} values of less than 20µg/ml, between 20-100µg/ml and more than 100µg/ml are considered active, moderately active and inactive, respectively. Cytotoxicity results showed that DCM: methanol extract potently inhibited the growth of Caco-2, whilst moderately inhibiting the HCT-15, LS513 and NCI-H146 [H146] cells growth. The methanol extract showed moderate growth inhibition of LS513 and NCI-H146 [H146] cells, and potently inhibited the Caco-2 cells. The hexane extract showed good growth inhibition of Caco-2 cells; and moderately inhibited LS513, NCI-H146 [H146] and H69AR cells. Similarly, the DCM and H_2O extracts showed good growth inhibition of Caco-2 and HT-29 cells, whilst moderately inhibiting the growth of HCT-15, LS513, NCI-H146 [H146], and H69AR cells growth. All the extracts appeared to be more cytotoxic towards all the lung- and colon cancerous cell lines than the normal colon cells as indicated by their selectivity indices.

The resistant reversal effect of doxorubicin by *C. sativa* L. extracts was determined on Caco-2, HCT-15, LS513 and H69AR cells through combination of the extracts with

doxorubicin. *C. sativa* L. extracts showed MDR reversal activities in HCT-15, LS513 and H69AR cells characterized by decreased IC_{50} values of the extracts. In Caco-2 cells, the hexane-, DCM-, DCM: methanol- methanol- and H_2O extracts showed an increase in their IC_{50} values from 0.64-, 0.65-, 0.67-, 0.02- and 0.55µg/ml to 2.0-, 1.92-, 5.67-, 8.72- and 1.56µg/ml, respectively, and were 0.32-, 0.34-, 0.12-, 0.002- and 0.35-fold more sensitive to doxorubicin compared to verapamil with a 4.80-fold reversal factor. In contrast, the same extracts showed a reduction in their IC_{50} values from 180.5-, 140.4-, 47.08-, 140- and 25.6µg/ml to 39.33-, 40.13-, 1.45-, 1.89-and 12.3µg/ml and increased doxorubicin sensitivity in HCT-15 cells by 4.59-, 3.50-, 32.97-, 74.07- and 2.08-fold, respectively, compared to verapamil, which showed a 1.41-fold reversal factor. These extracts showed 2.2-, 300.7-, 9.1-, 4.3- and 11-fold more sensitivity to doxorubicin than verapamil with a 0.05-fold reversal factor in LS513 cells. These extracts were 0.32-, 0.34-, 0.12-, 0.002- and 0.35-fold sensitive to doxorubicin compared to verapamil with a 4.80-fold reversal factor. The same extracts also increased doxorubicin sensitivity in H69AR cells by 8.60-, 7.09-, 11.34-, 20.51- and 11.42-fold compared to verapamil that showed 0.87-fold reversal factor.

The combination index (CI) analysis demonstrated that both the control and extracts yielded a normal to very strong synergistic interaction (CI<1) in Caco-2 cells, normal to strong synergistic interaction (CI <1) in HCT-15 cells, moderate to strong synergistic interaction (CI <1) in LS513 cells and nearly additive (CI=1) to antagonistic interaction (CI >1) in H69AR cells. Based on this evidence, the extracts were successful in increasing the sensitivity of HCT-15, LS513 and H69AR cells to doxorubicin *in vitro*. Future research is warranted to purify the most active extract and study the biological mechanisms involved in reversing doxorubicin resistance both *in vitro* and *in vivo*.