EVALUATION OF MOLECULAR MECHANISM(S) ASSOCIATED WITH THE ANTI-PROLIFERATIVE AND APOPTOSIS-INDUCING PROPERTIES OF THE CRUDE METHANOLIC AND SEMI-PURIFIED EXTRACTS OF *DICEROCARYUM* SPECIES IN JURKAT T CELLS

BY

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Submitted in fulfilment of the requirements for the degree of

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DECLARATION

I, Raetahle Paulina Mphahlele, declare that the dissertation hereby submitted to the University of Limpopo for the degree Master of Science in Biochemistry has not been previously submitted by me for a degree at this or any University, that it is my own work in design and in execution and that all materials contained therein have been duly acknowledged.

_____ _____

Signature Date

DEDICATIONS

I would like to dedicate this work to my siblings Mamphunye Gladys Mofomme and Ramakgodu Benjamin Mphahlele.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APAF-1	Apoptotic protease activating factor-1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bad	Bcl-2 antagonist of cell death
Bax	Bcl-2 associated protein-X
BCA	Bicinichonic acid
Bcl-2	B-cell leukaemia-2
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CARD	Caspase recruitment domain
cDNA	complementary deoxyribonucleic acid
CD-95L	Human fas ligand
CDKI1	Cyclin dependent kinase inhibitor 1
CDKN1	Cyclin dependent kinase inhibitor 1
CED-3	Cell death abnormality gene
CK1	Cyclin kinase 1
CO_2	Carbon dioxide
^oC	Degrees centigrade (Celsius)
DDT	Dichlorodiphenyltrihloroethane
DEPC-H_2O	Diethylpyrocarbonate water
DFF	DNA fragmentation factor
DISC	Death inducing signaling complex
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DR-3	Death receptor-3
DR-6	Death receptor-6
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FADD	Fas associated death domain
FAS	Fas-1 induced apoptosis signal

FBS	Foetal bovine serum
H	Hour
HMG-CoA	3-Hydroxymethylglutaryl-Coenzyme A
HRP	Horseradish peroxidase
IC_{50}	Half maximal inhibitory concentration
IgG	Immunoglobulin G
KCl	Potassium chloride
kDA	kiloDalton
MgCl	Magnesium chloride
Min	Minute
ml	Millilitres
mM	Millimolar
MOPS	4-Morpholinopropanesulfonic acid
mRNA	messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide
MuLV	Murine leukaemia virus
NaCl	Sodium chloride
$NaHPO_4$	Sodium hydrogen phosphate
$NaVO_3$	Sodium orthovanadate
NP-40	Non-ionic detergent P-40
PARP	poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PSN	Penicillin, streptomycin, neomycin
qacgg	(Glutamine- alanine- cysteine- glycine –glycine)
qacrg	(Glutamine- alanine- cysteine - arginine –glycine)
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	ribosomal RNA
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
Sec	Seconds
SEM	Standard error of the mean
TBS-Tween	Tris-buffered saline-Tween
TEMED	N, N, N', N'–Tetramethylenethylenediamine
TNF	Tumour necrosis factor
TNF-R1	Tumour necrosis factor receptor-1
TRAIL	Tumour necrosis factor alpha- related apoptosis inducing ligand
TRIS	Trihydroxymethylaminomethane
UV	Ultraviolet
µg	Microgram
µl	Microliter

ABSTRACT

Cancer is one of the largest single causes of death in both men and women worldwide. In the last decade, basic cancer research has produced remarkable advances in the understanding of cancer biology and genetics. Its control may benefit the lives of many individuals; therefore, there is a need to utilize different approaches in the prevention and cure of this killer disease. To date, the focus is on natural products that are implicated in cancer prevention and can also promote health without recognizable side-effects.

Dicerocaryum species is an indigenous African plant used as a shampoo for hair due to its ability to remove dandruff from the scalp and its leaves are edible as a vegetable. The plant is also purported to possess some anti-inflammatory and antioxidant activities. The current study was performed to evaluate the anticancer and anti apoptosis-inducing properties of the crude methanolic and semi-purified extracts of *Dicerocaryum* species in Jurkat T cells. The leaves of *Dicerocaryum* species were collected from the grounds of University of Limpopo, dried and ground into a powder. Different solvents, namely: methanol, n-hexane, dichloromethane, n-butanol and water were used for the extraction of putative bioactive compounds with potential anticancer activities. The cells were routinely cultured in the presence of various concentrations of the extracts after which the cell proliferation and cytotoxicity effects were determined using 3-(4, 5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and trypan blue dye exclusion method, respectively. The results showed that the crude methanolic and dichloromethane/ethanol extracts exhibit significant growth inhibitory effects against Jurkat T cells in a dose- and time-dependent manner. Based on the IC_{50} values, dichloromethane/ethanol extract was found to be more potent than the crude methanolic, n-hexane, n-butanol and water extracts.

In order to explore the mode of cell death elicidated by the crude methanolic and dichloromethane/ethanol extracts, cellular and nuclear morphological changes and the expression levels of major apoptotic genes and proteins

were investigated in the treated Jurkat T cells. Subsequently, nuclear shrinkage and chromatin condensation were observed in cells treated with crude methanolic and dichloromethane/ethanol extracts. The results obtained suggested that both the crude methanolic and dichloromethane/ethanol extracts induce cell death through the process of apoptosis as characterized by the afore mentioned features. In addition, the results obtained from the analysis of DNA fragmentation demonstrated a concentration-dependent DNA laddering in Jurkat T cells treated with the crude methanolic and dichloromethane/ethanol after 48 h.

RT-PCR results indicated that the expression levels of major apoptotic genes such as *bcl-2* were down-regulated whereas those of *bax* were up-regulated in a dose- and time-dependent manner, after treatment. The results obtained from the Western blot analyses were similar to that of RT-PCR whereby a dose-dependent decrease in Bcl-2 protein expression levels in Jurkat T cells treated with crude methanolic and dichloromethane/ethanol extracts was observed. Cells treated with 400 µg/ml and 600 µg/ml of crude methanolic extract showed a decrease in Bcl-2 protein expression after 24 and 48 h. Dichloromethane/ethanol extract also demonstrated down-regulation in expression levels of Bcl-2 protein after 24 and 48 h. No data were obtained from Bax protein expression in cells treated with both crude methanolic and dichloromethane/ethanol extracts after 12, 24 and 48 h, which suggested that Bax protein might have been a target of the ubiquitin/proteasome degradation pathway.

Therefore, the results alluded to the fact that crude methanolic and dichloromethane/ethanol extracts of *Dicerocaryum* species exert their antiproliferative effects in Jurkat T cells through the dysregulation of apoptosis responsive genes. Thus, the effects that are elicited by the crude methanolic and dichloromethane/ethanol extracts could be due to up/down-regulation of the expression levels of major apoptotic genes and proteins. The findings also suggest that crude methanolic and dichloromethane/ethanol extracts from *Dicerocaryum* species are capable of reducing cell growth, cell survival and inducing apoptosis in Jurkat T cells. Thus, *Dicerocaryum* species could be

used as a valuable source for the isolation of novel and effective anticancer drugs.

CHAPTER 1

INTRODUCTION

1. CANCER

Cancer is one of the major causes of death in both men and women claiming many lives each year worldwide (Yi *et al*., 2003). The disease is still a serious clinical problem and has a significant social and economic impact on the human health care system (Cheng *et al*., 2005). Its control may benefit and restore the lives of many individuals; therefore, there is a need to utilize different scientific approaches to come up with solutions that can assist in the prevention and possibly a cure for this deadly disease.

Cancerous cells result due to a disorder that occurs in the normal processes of cell division controlled by the genetic material (DNA) of the cell. Various factors like viruses, chemical carcinogens, chromosomal rearrangement and tumour suppressor genes have been implicated in the cause of cancer (Reddy *et al.*, 2003). The above mentioned factors are triggered by incorrect diet, genetic predisposition and environment factors. It is estimated that at least 35% of all cancers worldwide are caused by incorrect diet. On the other hand, genetic predisposition leads to approximately 20% of cancer cases while the majority of cancers are associated with environmental carcinogens (Reddy *et al.*, 2003).

1.1. Carcinogens

Human cancers may result from exposure to environmental carcinogens which include the natural and man-made chemicals, radiation and viruses. These carcinogens may be divided into different classes namely: genotoxic carcinogens, pro-carcinogens and epigenetic carcinogens. Genotoxic carcinogens are those that react with nucleic acids so as to directly affect the cellular constituents. Pro-carcinogens require the metabolic activation in order to induce carcinogenesis whereas epigenetic carcinogens are cancer initiating compounds like metals and complex organic chemicals. On the other

hand, the epigenetic carcinogens are carbon based compounds and mixtures that include industrial chemicals such as polychlorobiphenyls, pesticides such as DDT and combustion by-products including dioxins. These primary products and by-products can travel through air and water from place of origin to far where they can accumulate and bioconcentrate (Corsolin *et al*., 2004).

1.2. Diet

The changing profiles in the incidence of certain cancers have been attributed to urbanization with increase in consumption of meat, refined carbohydrates, alcohol and smoking. When humans consume meat they ingest a highly concentrated load of dioxin which is linked to several cancers. Colon cancer is caused by high intake of animal protein and fat. The fat content of many types of meat brings about the alterations of the cell membrane and so favour the entry of carcinogens. The cell membrane alterations due to an increase in cholesterol facilitate the entry of carcinogens in the cells (Dosil-Diaz *et al*., 2006). The cooking methods exert an influence in the production of carcinogens. High temperatures of cooking meat also lead to the formation of heterocyclic aromatic amines through pyrolysis of amino acids. These heterocyclic aromatic amines are highly mutagenic and induce tumours in animal species. The polycyclic aromatic hydrocarbons formed during frying, grilling and barbecuing of meat lead to mutation and cancer. The main factors that influence the production of these products are cooking time and temperature (Le-Marchand *et al*., 2002).

Isaacson (2004) reported that the staple diet of black South Africans from sorghum to maize is the cause of epidemic of squamous carcinoma of the oesophagus. The maize is considered as a favourable habitat for microbes like *Fusarium* fungi which produce fumonisins that reduce nitrates to nitrites and synthesis of cancer producing nitrosamines. The higher incidence of oesophageal cancer in Black males is due to their high consumption of traditional beer produced by fermenting maize. Consumption of alcoholic beverages is another factor that increases the risk of cancers of the oral cavity and the pharynx. The risks from this are due to the ethanol content found in the

beverages. The risks tend to increase with the increase in the amount of ethanol drunk (Levi, 1999).

Several attempts in cancer research have led to different approaches in the treatment of the disease. Of late, the focus has been in the area of indigenous medicinal plant research. For the past decades some of the basic research done has produced advances in the understanding of cancer biology and genetics. The generated knowledge led to many anticancer drugs being discovered, developed and applied by clinicians. Although most anticancer drugs are clinically used everyday, the resistance to and the drug side-effects have been a major problem (Yang *et al*., 2000). However, chemotherapy is still used as one of the primary treatment for malignancies in different forms of cancer despite the fact that there are disadvantages associated with it (Oliver and Vallette, 2005).

Chemoprevention *via* non-toxic agents could be one of the approaches for decreasing the incidence of cancer. An effective and acceptable chemopreventive or anticancer agent should have the following properties:

- Non-toxic effects in normal and healthy cells
- High efficacy against different cancers
- Capability of oral consumption
- Known mechanism of action
- Low toxicity and should be accepted by human population

2. NATURAL PRODUCTS AGAINST CANCER

Throughout medical history, plants have formed the basis for treatment of diseases in traditional medicine system and continue to play a major role in primary health care. *Dicerocaryum* species is an indigenous and creeping plant that grows widely in sandy soils of south and south-east of Africa. Its leaves have specialized mucilaginous hairs which when boiled or simply steeped in cold water they give a mucilaginous preparation. The plant is traditionally used as a remedy to aid the expulsion of retained placenta in women (abortion), for treating gonorrhoea, measles, as a shampoo for hair

and its leaves are edible as a vegetable when boiled with water (Benhura and Marume, 1993; Barone *et al*., 1996).

One example of Chinese herbal medicine which has been widely used as an anti-inflammatory, anti-viral, anti-bacterial and anti-cancer compound is *Scutellaria baicalensis*. Recent studies have shown that the plant extracts inhibit growth of breast, hepacellular, pancreatic and colon cancer cells *in vitro* (Ikemoto *et al*., 2000; Ye *et al*., 2002).

The high cost, side-effects and therapeutic limitations of conventional medications are key factors driving the revival of herbal remedies. People can easily obtain herbal medicines and feel comfortable consuming these products. Dietary consumption of foods and herbal medicines is a convenient method of administering potentially beneficial phytochemicals in a cost effective manner. In the area of cancer treatment, plant products have showed to be valuable sources of novel anticancer drugs. The non-nutritive compounds, which are phytochemicals, exert important effects on the progression, initiation, promotion and remission of cancer in cell-, tissue-culture and humans (Dennis *et al.,* 2000). Therefore, the roles of phytochemicals in cancer prevention and treatment are receiving increasing attention as the focus of modern therapy shifts from avoiding deficiency to prolonging life.

2.1. Dietary phytochemicals

Phytochemicals are natural bioactive compounds found in plant foods and work with nutrients and dietary fibre to protect against diseases. These compounds have complementary mechanisms of action in the body that include antioxidant effects, modulation of detoxification enzymes, stimulation of the immune system, modulation of hormone metabolism and antibacterial and antiviral effects. The major groups of these products are antioxidants, others are phenolic in nature and the remainder include reactive groups that confer protective properties. These natural products are found in fruits, vegetables, plant extracts and herbs. Phytochemicals are associated with decreased risk of cancer and increase in survival of cancer patients (Ho *et al*.,

2002; Setzer and Setzer, 2003; Weiss and Landauer, 2003). The mechanism of the protective effect of the above products is unclear but the fact that consumption of fruits and vegetables effectively lowers the risk of cancer is broadely supported.

2.1.1. Antioxidants

Antioxidants are found in a variety of fruits and vegetables, plant extracts, beverages, herbs and spices. They have been found to inhibit various types of diseases including cancer and cardiovascular diseases through their ability to react with free radicals. Antioxidants neutralize, interact and terminate their chain reaction before vital molecules like DNA are damaged. Free radicals are chemical compounds that contain one or more unpaired electrons. These can be produced by exposure to energy such as radiation or may be the product of incomplete reactions in the cells that produce electrons. From antioxidants detected in plant foods, the antioxidants vitamins C, A, E and pro-vitamin β-carotene have received the most attention.

Vitamin C, also known as ascorbic acid, is a water soluble vitamin that is present in citrus fruits and juices, green peppers, cabbage, spinach and strawberries. Vitamin E is a fat soluble vitamin found in nuts, seeds, vegetable and fish oils, whole grains, fortified cereals and apricots. It is a lipid phase scavenger of free nitrites and reduces cancer. β-carotene is an antioxidant found in orange vegetables and can inhibit further cell proliferation in cancer cells and induce increased cell to cell communication lacking in cancer cells (Reddy *et al*., 2003). An example of common fruit that contains dietary phenolics with antioxidants is an apple. Eberhardt *et al.* (2000) determined the phytochemical content of 100 g of apples and found that flavonoids and phenolics were more abundant in apples retaining their skins. In addition to that, the antioxidant capacity of apples relative to vitamin C is higher in apples with skin than without.

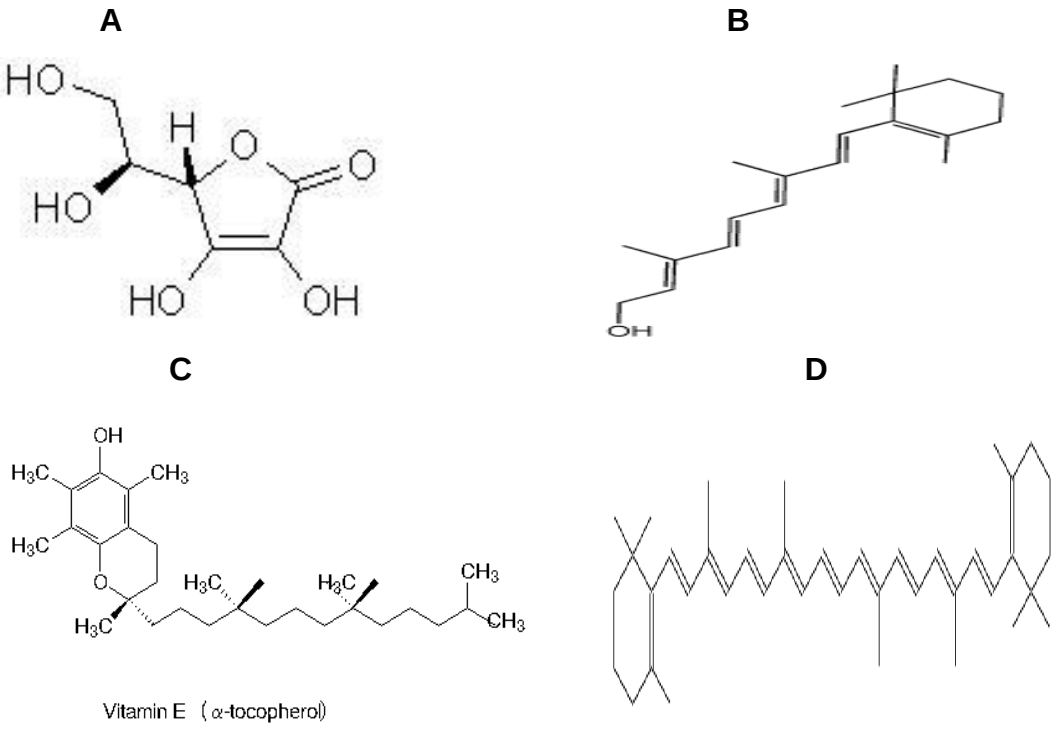

Figure 1.1: Molecular structures of vitamin C (A), vitamin A (B), vitamin E (C) and β-carotene (D) (http://www.chm.bris.ac.uk)

2.1.2. Amino acids and related compounds

Amino acids and other related compounds found in blood act as a sort of passive defense system against the development of tumours. Cancer cells are harmed by these compounds because their uptake is unregulated while normal cells regulate their uptake of nutrients and are not affected by them. Liu *et al*. (2000) presented evidence that orally administered glutamine inhibits tumour growth in animals. In this case, administration of 300 mg/kg of glutamine induced the growth of liver cancer cells injected in mice. The equivalent human dose is approximately 2.9 g/day. Bounous (2000) also confirmed that whey proteins concentrate has also been found to produce anticancer effects in humans with prostate cancer.

2.1.3. Flavonoids

Flavonoids are another large family of protective phytochemicals found in fruits and vegetables. These are water soluble, polyphenolic pigments/compounds naturally found in fruits, vegetables, flowers, leaves, bark and beverages such as tea and wine.

These compounds can prevent or inhibit cancer development by scavenging superoxides, hydroxyl and proxyradicals breaking the lipid peroxide chain reactions. They have also been shown to protect cells from X-ray damage and block the progress of the cell cycle. Flavonoids have been categorized into flavones, flavonols, flavon-3-ols and isoflavones. They all consist of a benzene ring fused with a pyrone ring in position 2 or 3 and carry a phenyl ring B substituent (Casagrande and Darbon, 2001).The structure of flavonoids is represented below:

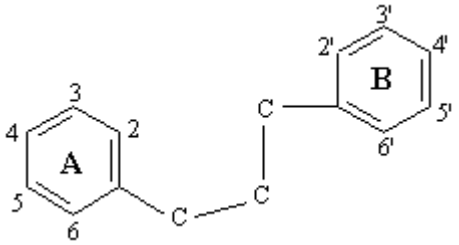

Figure 1.2: The basic structure of a flavonoid (Http://en.wikipedia/org/wiki/flavonoid)

Over 4,000 flavonoids have been identified, many of which occur in fruits, vegetables and beverages such as tea, coffee, beer, wine and fruit drinks. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health and have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumour and antioxidant activities (Martens, 2001). The beneficial effect of flavonoids in cancer therapy is due to their ability to act as antioxidants which include their reducing capabilities and their ROS scavenging capabilities. Flavonoids may also exert modulatory actions at the protein kinase and lipid kinase signaling pathways. In these regard, flavonoids and their metabolites act as antioxidants and affect cellular function (Williams *et al*., 2004). Some of the known flavonoids include resveratrol, anthocyanins, quercetins and tangeritin. Resveratrol was found to reduce the risk of heart disease, cancer, blood clots and stroke. These biocompound is found in red grapes. Anthocyanins are found in high concentrations in blueberries and have shown to protect against the signs of aging and prevent urinary tract infections. Tangeritin is found in citrus fruits and their juices prevent against cancers of the head and neck. The following figure illustrates the structures of anthocyanins, quercetins and tangeritin:

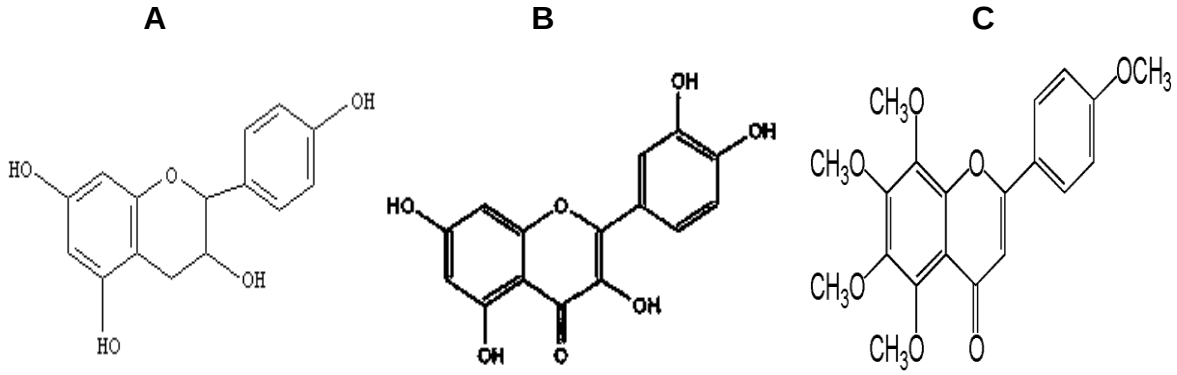

Figure 1.3: Molecular structures of anthocyanin (A), quercetin (B) and tangeritin (C) (http://www.astaxanthin.org/carotenoids.htm,
http://www.geocities.com/agnihotrimed/paper,htm,
http://upload.wikimedia.org/wikimedia/commons/a/a4/Tangeritin_structure.png)

2.1.4. Isoprenoids

Another class of phytochemicals broadly defined is pure and mixed isoprenoids which are isoprene derivatives of little function and structural common ground. Isoprenoids are products of secondary plant metabolites terpenes that function as defense in physiological and pathological processes (Gabrielsen *et al*., 2004). Their name is from an isoprene, which is a branched 5-C molecule that can be further classified on the basis of the number of isoprene units present. These polymeric derivatives result in a large family of compounds of little functional and structural common ground like steroids, carotenoids and gibberellic acids. Members of these plant products differ in size, complexity and function. Pure and mixed isoprenoids play regulatory roles in germination, growth, differentiation and flowering, provide antioxidant activity, attract pollinating insects and provide defence against insects and fungi (Mo and Elson, 2006).

Steroids are triterpenes/triterpenoids present in most eukaryotes and function in the maintenance of membrane fluidity, act as hormones and bile acids, as electron transport carriers and plant hormones involved in biochemical functions of membrane systems. They are found in green leafy vegetables and can protect against cancer by their ability to block and scavenge free radicals. Other known isoprenoids have

valuable commercial interest as food colourants and antioxidants (carotenoids), aroma and flavour enhancers (terpenes) and antiparasitic and anticarcinogenic compounds (taxol). Despite all structural and functional differences, all isoprenoids are synthesized in the mevalonate pathway from isopentenyl diphosphate (Chemler *et al*., 2006). The chemical structures of the above-mentioned isoprenoids are illustrated in Figure 1.4.

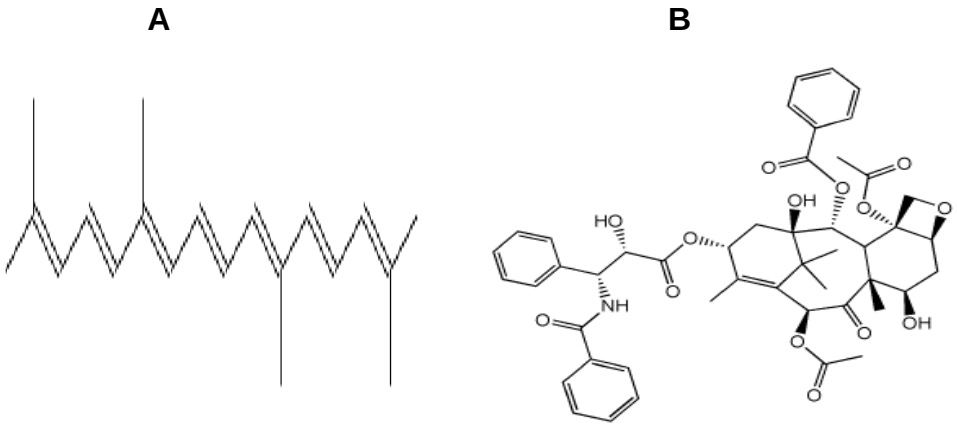

Figure 1.4: The chemical structure of carotenoid (A) and taxol (B).

3. MEVALONATE PATHWAY

The biosynthesis of non-sterol isoprenoid compounds involved in cell proliferation and cholesterol result from the mevalonate pathway which involves a sequence of enzymatic reactions leading to farnesyl pyrophosphate, a common substrate. Most of the compounds produced in the pathway are rate-limiting for cellular growth and have regulatory function in cell proliferation (Tatman and Mo, 2002). The inhibitors of 3-hydroxymethylglutaryl CoA (HMG-CoA) reductase involved in the pathway as a limiting step in synthesis of farnesyl pyrophosphate suppress cell division and induce apoptosis in a variety of cells (Mo *et al*., 2000).

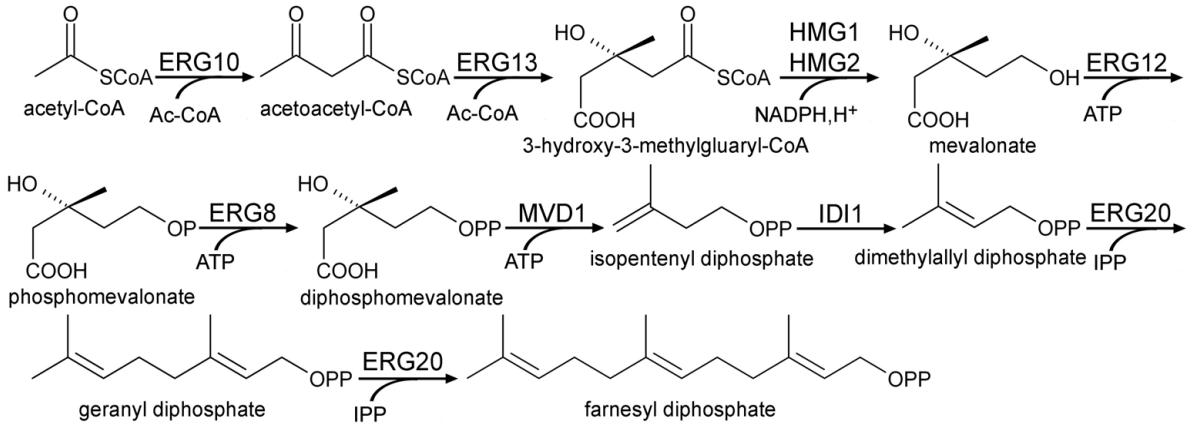

Figure.1.5: Mevalonate pathway and isoprenoid synthesis. IPP:Isopentenyl diphosphate, ERG10:acetoacetyl-CoA ligase, ERG13:2-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase), HMG-1 and HMG-2:2-hydroxy-3-methylglutaryl-CoA reductase, ERG12:mevalonate kinase, ERG8:phosphomevalonate kinase, MVD1: diphosphomevalonate decarboxylase, IDI1:isopentenyl pyrophosphate-dimethylallyl diphosphate isomerase, ERG20:farnesyl diphosphate synthase (Chemler *et al*., 2006)

The structural diversity of isoprenoids which results in the difference in number of residues, double bond configuration, cyclization and polarity makes the isoprenoids to differ in potency against tumours. Therefore, different tumour cells differ in sensitivity to individual isoprenoids and cell analysis studies demonstrated that isoprenoids have an impact on cell division and cell death (Mo *et al*., 2000; Crowell and Elson, 2001; Tatman and Mo, 2002). Cyclic monoterpenes such as farnesol and geraniol arrest cells at the G_1 phase through decreased expression of *c-myc*, *cyclin* D (Park *et al*., 2001; Bardon *et al*., 2002; Shi and Gould, 2002). On the other hand, cyclic and acyclic caretenoids, cyclic monoterpenes (Burke *et al.*, 2002; Li *et al*., 2002) and tocotrienols (Agarwal *et al*., 2004) suppress the expression of anti-apoptotic Bcl-2 protein. Farnesol has been known to initiate apoptotic cell death (Rioja *et al*., 2000).

4. ANTI-PROLIFERATIVE MECHANISMS

The search for and development of drugs which are safe and that limit resistance is necessary in cancer therapy. An ideal suitable chemotherapeutic agent or drug should induce cancer cell death without showing any cytotoxic effects on the normal cells or triggering any inflammatory reactions (Yi *et al*., 2003). There must be a clean removal of unwanted and damaged cells or tissues triggered by the drug in question. Cell death may occur by the process of apoptosis or necrosis, depending on the stimuli. Thus, induction of apoptosis in cancer cells is an ideal strategy for anticancer drug development.

4.1. Apoptosis

The induction of apoptosis is a desirable mode of cell death and an often preferred therapeutic strategy for cancer control (Hong *et al*., 2003). The *in vitro* study of apoptosis through its regulation at the molecular level will provide useful information on cancer therapy. Recently, the focus is on the utilization of compounds derived from natural indigenous plants towards the treatment of cancer. Screening of bioactive compounds from plants and the subsequent evaluation of their biomolecular activities on cancer cells will help in our understanding of disease progression and the development of anticancer drugs.

Apoptosis is a genetically regulated biological process caused by a variety of physiological and pathological stimuli (Hu and Kavanagh, 2003). It is a continuous physiological process for non-inflammatory, programmed cell death and is one of today's most active fields of biomedical research. Cell death is characterized by specific morphological patterns including cell shrinkage, plasma membrane blebbing, nuclear condensation, chromatin condensation, endonucleolytic degradation of DNA and formation of apoptotic bodies (Hengartner, 2000; Cummings and Schnellmann, 2002; Hu and Kavanagh, 2003). The membrane packaged cell components after cleavage of the chromosomal DNA are then removed by phagocytic cells preventing induction of inflammatory response. The two major cells for clearing and removal of apoptotic bodies are the dendritic cells and the macrophages.

Apoptosis generally has three major phases, which involve and are regulated by specific molecules:

- *Induction phase* – death-inducing signals stimulate the transduction of pro-apoptotic signals in this phase. The examples of death-inducing signals include: reactive oxygen and nitrogen intermediates, TNF-α, ceramide, calcium pathways over-activation and Bcl-2 family of proteins.
- *Effector phase* - key regulators commit cells to die in this phase. Examples of key regulators are: cell surface death domains, nuclear activators, endoplasmic reticulum pathways and mitochondrial-induced pathways.
- *Degradation phase* - in this phase, activation of cytoplasmic caspases (cysteine proteases) and nuclear endonucleases lead to the cleavage of cellular components and DNA fragmentation, respectively.

4.2. Necrosis

Necrosis occurs in response to pathological change like hyperthermia and viral invasion initiated outside the cell and it is characterized by mitochondrial swelling, dysfunction of the plasma membrane with loss of homeostasis, cell swelling and rapture. The loss of membrane integrity leads to the release of cell contents including proteases and lysozyme which induce inflammatory response. The DNA on agarose gel is characterized by a smudge patterns confirming degradation. In contrast to apoptosis, cleanup of cell debris by phagocytes is generally more difficult, as this disorderly death generally does not send cell signals which tell nearby phagocytes to engulf the dying cell. This lack of signaling makes it harder for the immune system to locate and recycle dead cells which have died through necrosis than if the cell had undergone apoptosis (Chautan *et al*., 1999; Kitanaka and Kuchino, 1999). Unlike in apoptosis, cells that die by necrosis may release harmful chemicals that damage other cells. Therefore, induction of apoptosis is more preferred than necrosis in drug discoveries (Vercammen *et al*., 1997). The figure below illustrates the different features of apoptosis and necrosis processes.

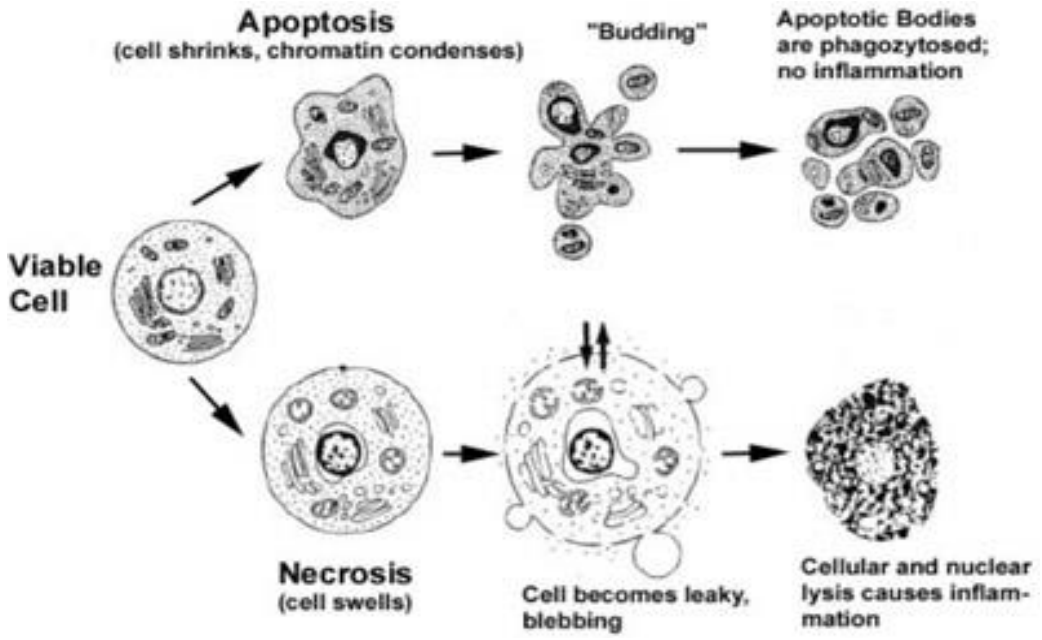

Figure1.6: The morphological features of apoptosis and necrosis processes (http://www.celldeath.de./encyclo/aporev/revfigs/revfg_2.htm)

5. MOLECULAR BASIS OF APOPTOSIS

The apoptotic pathway plays an integral part in many biological events including morphogenesis and the removal of harmful cells. Balanced apoptosis is crucial in ensuring good health. The pathway is characterized by the activation of a family of cell death proteases called the caspases which are activated by catalytic cleavage, thereby setting up a cascade of proteolytic cleavage that disrupts the function of regulatory proteins and commit the cell to the suicide pathway (Jaattela, 2002). Caspases are important in cells for apoptosis, in development and in stages of adult life. Some caspases are required in the immune system for maturation of cytokines (Iwata *et al*, 2002; Iwata *et al*., 2003; Agarwal *et al.*, 2004).

Caspases are novel cysteine protease produced as pro-enzymes and became activated by proteolytic cleavage at internal aspartate residues upon stimulation (Qin *et al.*, 2001). The caspase family members differ in their primary sequence and their substrate specificity but have other common features. All caspases contain a highly

homologous protease domain and are synthesized as inactive zymogens. Their domain contain an N-terminal pro-domain, a large subunit of approximately 20 kDa and a small subunit of approximately 10 kDa (Chang and Yang, 2000; Prior and Salvesen, 2004).

The two categories of caspases that are important during apoptosis are: the initiators and the executioners or effectors. The initiator caspases include caspase -2, -8, -9 and -10 which are activated in the earlier phase of apoptosis. The executioner caspases including caspase -3, -6 and -7 activated by the initiator caspases are responsible for dismantling the cell. These caspases are responsible for cleaving apoptotic substrates such as poly(ADP-ribose) polymerase (PARP) and DNA fragmentation factor 45, DFF-45 (Sadowski, 2002; Fischer *et al*., 2003).

Caspase mediated events regulate pro-apoptotic and anti-apoptotic proteins that are triggered by the two major pathways: the death receptor-induced extrinsic pathway and the mitochondrial apoptosome-mediated apoptotic intrinsic pathway. Both pathways lead to caspase activation and cleavage of specific cellular substrates (Hu and Kavanagh, 2003). The following figure illustrates the initiator and executioner caspases involved in apoptotic process:

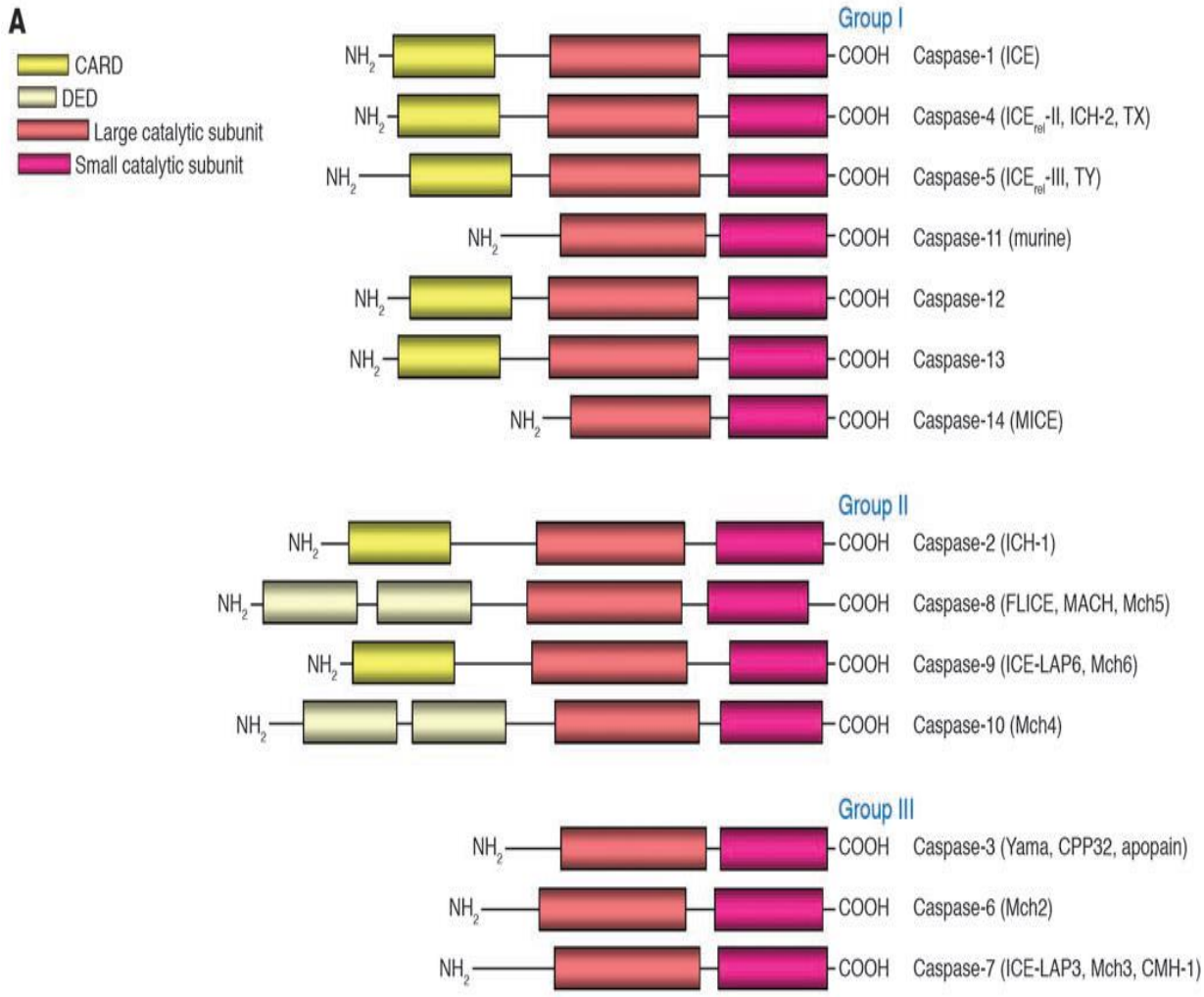

Figure 1.7: Schematic diagram of caspases involved in apoptosis (Lavrik *et al*. 2005, http:www. wikipedia.org/wiki)

5.1. Activation of initiator caspases

Caspase activation proceeds through autoactivation *via* oligomerization, transactivation of death receptor or mitochondrial pathways and proteinases evoked proteolysis (Shi, 2002; Slovister, 2002). Pro-apoptotic signal leads to activation of initiator caspases which in turn activate the effector caspases resulting in cellular disassembly. The activated caspase initiates cell death programme by destroying key components of cellular infrastructure and activates factors which damage the cell.

Different initiator caspases mediate distinct sets of signals. Caspase-2 is an enzyme involved in the programmed cell death process. Over-expression of caspase-2 results in cellular death. Using agents that remove caspase-2 from cells prevent these cells from dying from a variety of death inducing agents. While various researches show that caspase-2 is important in the cell death pathway, its role and under what circumstances it is activated remains unclear. In future works, maybe research will concentrate on the biochemical activation of caspase-2 and the physiological significance of this. Critical for the regulation of caspase-2 activity are the proteins that it interacts with (Baliga and Kumar, 2002; Kumar and Vaux, 2002; Read *et a*l., 2002; Baliga and Kumar, 2003). Caspase-8 is associated with apoptosis involving death receptors. In contrast, caspase-9 is involved in death induced by cytotoxic agents and responds to changes in mitochondrial potential (Mac-Lachlan and El-Diery, 2002). Caspase-10 has two death effector domains (DEDs) that bind to the DED in the adaptor molecule Fas associated death domain (FADD) and recruits both tumour necrosis factor receptor 1 (TNFR1) and CD95 to form complexes with these receptors. Caspase-10 cleaves and activates caspase-3, -4, -6, -7, -8 and -9 which cause the proteolytic cleavage of many key proteins such as poly(ADP-ribose) polymerase (PARP). Based on gene expression studies, caspase-10 may be crucial in embryonic development.

5.2. Activation of effector caspases

Activation of the effector caspases requires binding to specific cofactors in a mechanism commonly observed with proteases. The initiator caspases cleave the pro-enzyme forms of effector caspases-3 and -7 allowing digestion of essential targets that affect cell viability (Mac-Lachlan and El- Deiry, 2002). The caspase cascade can be activated by Granzyme B released by cytotoxic T lymphocytes which is known to activate caspases-3 and -7; death receptors (e.g., Fas 1 induced apoptosis signal (FAS), TNF alpha related apoptosis inducing ligand (TRAIL) and tumour necrosis factor (TNF). These can activate caspases-8 and -10 and the apoptosome, regulated by cytochrome c and the Bcl-2 family, which activates caspase-9. Once this cascade is started, a positive feedback ensures that the cell will inevitably undergo apoptosis.

For an example, apoptosome-activated caspase-9 cleaves and activates caspase-3. Caspase-3, besides cleaving its target proteins, will also cleave more of caspase-9, which in turn will activate more of caspase-3 (Danial and Korsmeyer, 2004; Gregersen, 2007).

Caspase-3 activation plays a major role in apoptosis and is triggered by a variety of stimuli. It cleaves many cytoskeletal protein such as fodrin and proteins involved in DNA repair and fragmentation such as poly (ADP-ribose) polymerase and DNA factor 45 (DFF-45). Caspase-3 activation results from two pathways through the release of cytochrome c and cleavage of pro-caspase-9 and ligation of death receptors by tumour necrosis factor (TNF) and Fas ligand (Chang and Yang, 2000; Link and Harrison, 2001). Figure 1.7. shows the schematic summary of caspases involved during the process of apoptosis.

Caspases mediated events occur through several routes including the apoptotic mitochondrial independent and dependent pathways which are the extrinsic and intrinsic pathways, respectively.

5.3. The extrinsic pathway

Activation of apoptotic extrinsic pathway is initiated by the binding of specific protein ligand to a cell surface receptor. The extrinsic pathway begins outside the cell when the condition in the extracellular determines that a cell must die. It involves engagement of particular “death receptor” that belongs to the tumour necrosis factor (TNF-R) family with members characterized by a conserved extracellular cysteine rich motif. Six different death receptors commonly known are: Fas, TNF-R1, DR-3, and TNF which relates to apoptosis inducing ligand (TRAIL) and DR-6 (Schultz and Harrington, 2003). Fas is a member of receptor family which include the TNF receptor, and binding of CD-95L to Fas-L at the receptor initiates the pathway. The Fas-Fas-L causes receptor oligomerization whereby the death domain of the receptor then recruits the adaptor protein which also has the death domains. An example of such protein is the Fas-associated death domain (FADD) protein. The protein has a death

domain at its C-terminus and a second protein-protein interaction domain called a death effector domain at its N-terminus. The death effector domain of the adaptor binds to the death effector domain or pro-domain of caspase-8 to form a complex called the death-inducing signaling complex (DISC). This leads to a cascade of activation of caspases including caspases -8 and -3 which in turn induce apoptosis (Haupt *et al.*, 2003).

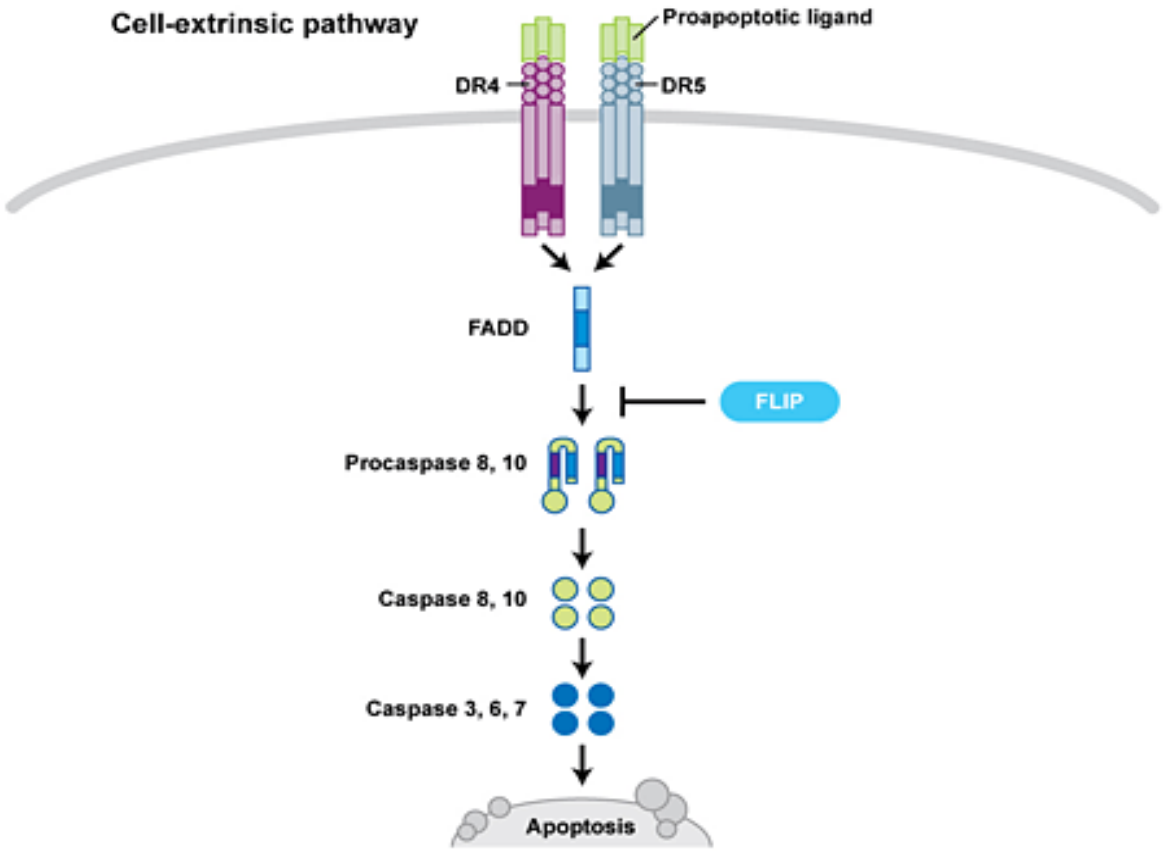

Figure 1.8: The extrinsic apoptosis pathway (Ashkenazi, 2002)

5.4. The intrinsic pathway

Mitochondrion plays an important role in the execution of death program and activation of cellular organelles critical in the pathway. The intrinsic pathway begins when an injury occurs within the cell. Therefore, it is apoptotic and involves inflammatory response but the apoptotic machinery in the damaged cell is packaged and removed in order to prevent inflammatory response. The pathway is triggered in response to damage and is associated with mitochondrial depolarization and release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm. At the mitochondrion, cytochrome c, apoptotic protease activating factor 1 (APAF-1) and pro-caspace-9 form a complex termed apoptosome in which caspase-9 is activated and promotes activation of caspases-3 and -7 involved in apoptosis (Nicholson and Thornberry, 2003). Cytochrome c is released from the mitochondria of pre-apoptotic cells and binds to Apaf-1 in the presence of dATP/ATP. The interaction results in a conformational change in Apaf-1 allowing the molecules of Apaf-1 to associate with each other. This formation results in a wheel-like structure that contains seven molecules each of Apaf-1, cytochrome c and ATP. Pro-caspase-9 contains a caspase recruitment domain (CARD) that is used to mediate specific interactions with Apaf-1, which becomes exposed on the apoptosome during assembly. Pro-caspase-9 is autoactivated and after cleavage, mature caspase-9 remains bound to the apoptosome where it is able to activate executioner caspases such as caspase-3 and caspase-7. Caspase-9 (ICE-LAP6, Mch6) is a member of the CED-3 subfamily and bears similarity to caspase-3, though there is a main difference in the active-site pentapeptide whereby caspase-9 shows the sequence Glu-ala-cys-gly-gly (Qacgg) instead of the more usual Glu-ala-cys-arg-gly (Qacrg). Compared to caspase-3, caspase-9 possesses a longer N-terminal pro-domain with high similarity to the pro-domains of CED-3 and caspase-2, which contain CARDs. Caspase-9 is ubiquitously expressed, with high levels of mRNA expression in the heart, testis and ovary. Multiple mRNA species are found, due to alternative splicing. Activated caspase-9 is able to cleave caspase-3 *in vitro* (Kuida, 2000).

In addition to caspases involvement in apoptosis, the process is a highly regulated and genetically encoded self destruction process in the cells. A variety of genes and their associated proteins involved in the process have been discovered and studied. Several characteristics which include up-regulation and down-regulation make apoptotic genes and proteins targets for cancer treatment.

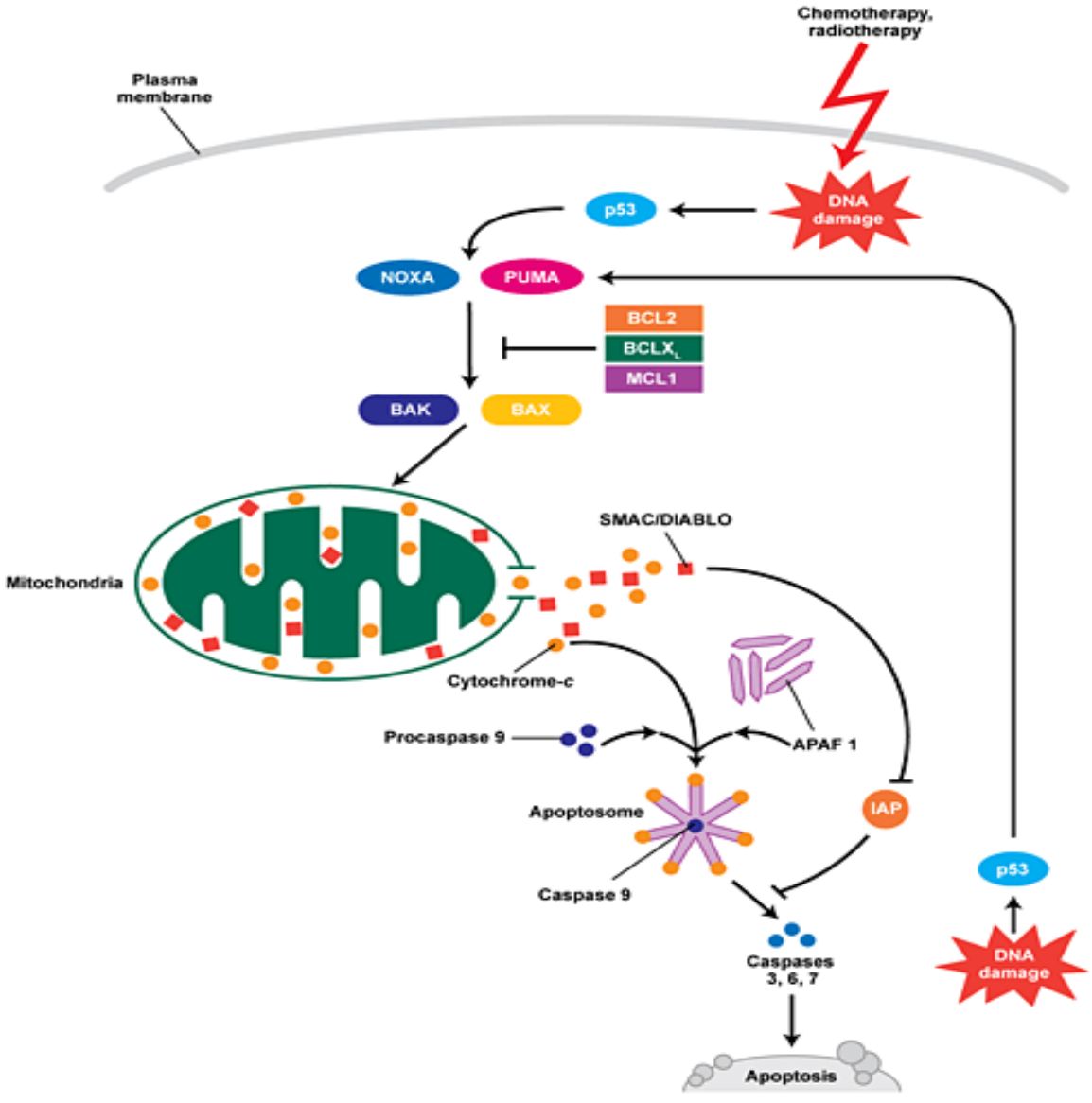

Figure 1.9: The intrinsic apoptosis pathway (Ashkenazi, 2002).

6. REGULATORY GENES AND PROTEINS INVOLVED IN APOPTOSIS

6.1. *p*53

The *p*53 gene is mutated in about half of all human cancers of different types. It is a transcription factor whose activity gives rise to a variety of cellular outcomes like cell cycle arrest and apoptosis thus eliminating cancer prone cells from the replicative pool. It activates vital damage procedures in order to restrict cell growth in response to DNA damage, oncogene activation, hypoxia and loss of normal cell contacts (Lohrum and Vousden, 1999). p53 restricts cellular growth by inducing cell cycle arrest at G1 and/or G2 phase or apoptosis (Jin and Levine, 2001).

Several factors that influence the cell to be arrested or undergo apoptosis include the *p*53 expression level, the cell type and cellular context at the time of exposure to stress (Vogt-Sionor and Haupt, 1999; Balint and Vousden, 2001). *p*53 can induce apoptosis through several mechanisms both by regulating the expression of the genes which can participate in the apoptotic response and through transcriptionally independent manner. Its activation involves the stabilization and enhancement of its DNA binding and transcriptional activity. These changes are mediated by extensive post-translational modification of p53 and protein-protein interactions with co-operating factors (Bates and Vousden, 1999; Haupt *et al*., 2003).

p53 is a 53 kDa protein consisting of 393 amino acids and undergoes numerous post translational modifications including phosphorylation, acetylation, ubiquitination, sumoylation and neddylation. At the N-terminal and C-terminal, the protein consists of a transactivation domain, a proline rich domain, a specific DNA binding domain, three nuclear localization signals and a tetramerization domain that includes a nuclear export signal and a negative regulatory domain. The protein has a short half-life of approximately 30 min and when activated, it leads to two outcomes. The major outcomes include: cell cycle arrest due to an increase in *p21* expression; another is when *p53* activation mediates up-regulation of *bax* while down-regulating *bcl*-2 expression (Tweddle *et al*., 2002).

6.2. *p21*

*p*21, also known as cyclin-dependent kinase inhibitor 1A (CKI1A) or CDKN1A, is a human gene that encodes a cyclin-dependent kinase inhibitor that directly inhibits the activity of cyclin-CDK2 and cyclin-CDK4 complexes. *p21* functions as a regulator of cell cycle progression at G1 phase (Gartel and Radhakrishnan, 2005). The expression of *p21* is controlled by the tumour suppressor protein *p*53.The function of this gene relates in part to stress response (Rodriguez and Meuth, 2006). Gartel and Tyner (2002) reported that *p*21 may also play an important role in the regulation of apoptosis by either mediating cell cycle arrest causing resistance to apoptosis or blocking apoptosis directly. Whereas the mechanisms of p53-dependent apoptosis are not well understood, p53-dependent cell cycle arrest is primarily mediated by the CDK inhibitor p21. There is evidence that p21 is a major inhibitor of p53-dependent apoptosis. It is not entirely clear how a cell chooses between apoptosis and p21-dependent cell cycle arrest after DNA damage and stabilization of p53, but often high levels of p21 expression mediate cell cycle arrest and protect from p53-dependent apoptosis (Gartel, 2002).

6.3. The Bcl-2 family

The Bcl-2 family plays a major role in determining whether a cell undergoes apoptosis. It is composed of three categories, the pro-apoptotic, anti-apoptotic and BH3 family members (Huang and Strasser, 2000). The BH domains are crucial for function as deletion of these domains *via* molecular cloning affects survival or apoptosis rates. The anti-apoptotic members include *Bcl*-2 and *Bcl-x*$_L$, whereas the pro-apoptotic members include *Bax* and *Bad* (Gross *et al*., 1999). Both family members dimerize to form heterodimers and homodimers. The family has also been proposed to have a role in regulating permeability of the mitochondrial membrane which is important during the induction of apoptosis (Danial and Korsmeyer, 2004; Gregersen, 2007).

6.3.1. *Bcl*-2

Human *bcl*-2 was the first proto-oncogene identified to function by protecting cells from programmed cell death. It is an anti-apoptotic gene which is a regulator of apoptosis and promotes cell survival by inhibiting factors needed for the activation of caspases that dismantle the cells (Schultz and Harrington, 2003). There are a number of theories concerning how the *bcl*-2 gene exerts its anti-apoptotic effect. This is achieved by activation or inactivation of an inner mitochondrial permeability transition pore involved in the regulation of matrix Ca^{2+}, pH and voltage. The *bcl*-2 gene codes for a 25 kDa protein. The protein Bcl-2 derives its name from B-lymphoma-2 as it is the second member of a range of proteins initially described as reciprocal gene translocation in chromosomes 14 and 18 in follicular lymphoma (Reed and Pallecchia, 2005). Some basic features of the protein include the presence of 21 amino acids, its place of residency on the outer mitochondrial membrane, the nuclear envelope and the endoplasmic reticulum.

The site of action for the Bcl-2 proteins is mostly on the outer mitochondrial membrane. Within the mitochondria, there are apoptogenic factors such as cytochrome c that activates the caspases if released. Depending on their function once activated, Bcl-2 proteins either promote the release of cytochrome c or keep it sequested in the mitochondria. Bcl-2 over-expression has been shown to prevent chemotherapeutic agents from apoptosis associated with altered mitochondrial transmembrane. It is an integral protein located in mitochondria. The protein prevents loss of membrane potential and the efflux of cytochrome c resulting in the initiation of apoptosis. Thus, Bcl-2 has a role in regulating permeability of the mitochondrial membrane which is important during the induction of apoptosis (Oltersdorf *et al*., 2005).

6.3.2. *Bax*

Bax is a pro-apoptotic gene and as a protein it heterodimerizes with Bcl-2 to block Bcl-2 enhanced cell survival dependent on the ratio of Bcl-2 to Bax. When Bcl-2 is in excess, Bax/Bcl-2 heterodimers are formed and cell survival results. When Bax predominates, homodimers are formed resulting in cells susceptible to programmed cell death. Bax as a protein is situated in the cytosol and removed into the mitochondria during apoptosis (Bedner *et al*, 2000; Gao *et al*., 2001; Jia *et al*., 2001). Bax is a known target of p53 and its transcriptional activation occur *via* the p53 response elements (Sheikh and Fornace, 2000). Its activation is considered as a key regulatory step in the mitochondrial apoptotic pathway initiation. In apoptotic cells, its translocation from the cytosol targets the mitochondrial intermembrane contact sites and accelerates the opening of the mitochondrial channel. The channel which is one components of the permeability transition allows the release of cytochrome c. The release of cytochrome c from the mitochondria cleaves and activates caspases-3 and -9 resulting in the sequence of apoptotic process (Shimuzu *et al*., 1999; Kashkar, 2005).

7. RATIONALE

Cancer control and prevention may be through alternative therapies that utilise natural products obtained from plants and herbs. Different studies have shown that some plants and herbs contain bioactive compounds that have the potential to treat different cancers and promote human health without recognisable side effects. Most of these bioactive compounds exert their anticancer activities by blocking the cell cycle progression and triggering apoptosis. Apoptosis is a genetically controlled process therefore it provides opportunities to manipulate genes and proteins that lead to increased cell death.

Dicerocaryum species is an indigenous and creeping plant that grows widely in sandy soils of south and south-east of Africa. Its leaves have specialized mucilaginous hairs which when boiled or simply steeped in cold water they give a mucilaginous (sticky and viscous) preparation (Benhura and Marume, 1993). The plant is traditionally used

as a remedy to aid the expulsion of retained placenta in women (abortion), for treating gonorrhoea, measles, as a shampoo for hair and its leaves are edible as a vegetable when boiled with water (Barone *et al*., 1996; Benhura and Marume, 1993). The preliminary and unpublished findings from our laboratory suggested that the crude methanolic extract from *Dicerocaryum* species contain bioactive compounds that possess anti-proliferative activities against a human cancer line, Jurkat T cells.

The current study was aimed to determine the regulatory effects of crude methanolic and semi-purified extracts on the expression of apoptotic genes and their associated protein products and to evaluate the extent of growth inhibitory effects on Jurkat T cells. The Jurkat T cell line (originally called JM) was established in the late 1970s from the peripheral blood of a 14 year old boy with T cell leukaemia. These cells are an immortalized line of T lymphocyte cells that are used to study acute T cell leukemia, T cell signaling and the expression of various chemokine receptors susceptible to viral entry. Their primary use is to determine the mechanism of differential susceptibility of cancers to drugs and radiation.

8. HYPOTHESIS

Extracts of *Dicerocaryum* species contain putative phytochemicals that possess antiproliferative activities against Jurkat T cells, thus resulting in the induction of apoptosis, up-regulation and down-regulation of apoptotic regulatory genes and their corresponding proteins.

9. AIMS AND OBJECTIVES

The specific objectives of the study were to:

- Prepare crude methanolic and semi-purified extracts (n-hexane, dichloromethane/ethanol, n-butanol and water) of *Dicerocaryum* species in order to obtain bioactive fractions.
- Test the crude methanolic and semi-purified (n-hexane, dichloromethane/ ethanol, n-butanol and water) extracts on Jurkat T cells in order to determine their potential anti-proliferative/cytotoxic activities.

- Determine the cellular and nuclear morphological changes in Jurkat T cells treated with various concentrations of crude methanolic and dichloromethane/ethanol extracts.
- Determine the induction of DNA fragmentation in Jurkat T cells treated with various concentrations of crude methanolic and dichloromethane/ethanol extracts.
- Determine the expression levels of apoptosis regulatory genes by semi-quantitative RT-PCR after the treatment of Jurkat T cells with various concentrations of crude methanolic and dichloromethane/ethanol extracts.
- Analyze the expression levels of the corresponding apoptotic proteins, under treatment conditions, using Western blot.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Equipment

- Büchi Rotary evaporator R-205 (Büchi Labortechnik AG, Switzerland)
- CO_2 Incubator (NAPCO model, Instrulab cc, RSA)
- Centrifuges (Models GS-6R and GS-15R, Beckmann Instruments Inc., USA)
- EC 120 mini vertical gel system (E-C apparatus Corporation, UK)
- Easy-cast electrophoresis system (Owl Scientific, Inc., UK)
- Hybaid Onigene thermal cycler (Hybaid Limited, UK)
- Hypercassettes (Amersham, UK)
- Light and fluorescent microscope (Zeiss, Germany)
- Microtiter plate reader (Model 550, Bio-Rad Laboratories, CA, USA)
- Millex Filter Unit 0.22 µm (Millipore Corporation, MA, USA)
- Powerpack (Bio-Rad Laboratories, CA, USA)
- Spectronic Genesys 5 spectrophotometer (Milton Roy Company, USA)
- Syngene image analyser (Vacutec, RSA)

2.1.2. Cells, culture media and biochemicals

- Acetic acid, bromophenol blue, coomassie blue R200, dichloromethane, dimethyl sulfoxide, n-butanol, n-hexane, ponceau S, sodium chloride, ethanol, methanol, $NaHPO_4$, (Saarchem (Pty) Ltd. RSA.)
- Acrylamide and bisacrylamide, Nonidet P-40 (Fluka Biochemica, Switzerland)
- BCA protein assay kit (Pierce, USA)
- Blotting Grade blocker, non-fat milk (Bio-Rad Laboratories, USA)
- CL-X Posure film (Pierce, USA)
- EDTA (BDH, RSA)

- Foetal bovine serum (FBS), RPMI-1640 media (Gibco, New Zealand)
- Glycerol (NT Laboratory Suppliers (Pty) Ltd, RSA)
- High pure RNA isolation kit, PCR kit (Perkin-Elmer, Roche Diagnostics, Boehringer-Mannheim, Germany)
- Hoechst 33528 staining powder (Sigma Chemicals, MO, USA)
- Immunobilon-P transfer membrane (Millipore Corporation, MA, USA)
- Jurkat T cells (American Type Culture Collection, USA), normal monkey Vero cells (University of Pretoria)
- (2-Mercaptoethanol, Tween-20, DMSO, KCl (Merck Laboratory Suppliers, Ltd, RSA)
- 3-(Morpholino) propane-sulfonic acid (MOPS) (Research Organics, Inc., OH, USA)
- Monoclonal IgG primary antibodies, goat anti-mouse IgG-HRP conjugated secondary antibodies, western blotting luminol reagent (Santa Cruz Biotechnology, Inc, CA, USA)
- PCR primer pairs (Invitrogen Life Technologies, UK)
- Penicillin, streptomycin and neomycin (PSN) mixture (Gibco, New Zealand)
- Sodium dodecyl sulphate (BDH Laboratory suppliers, England)
- Trypan blue, CAPS [3-(cyclohexylamino)-1-propanesulphonic acid], ammonium persulphate, TEMED, sodium orthovanadate, Tris, aprotinin (Sigma Chemicals, MO, USA)

2.2. METHODS

2.2.1. Preparation of the extract

The leaves of *Dicerocaryum* species were collected from the grounds of the University of Limpopo and dried for three days in an oven at 40°C. After drying, the leaves were crushed into a powder using a pestle and mortar and extracted with absolute methanol using a shaker for overnight. The crude methanolic extracts were further fractionated using n-hexane, dichloromethane, n-butanol and water. A flow chart for the extraction procedure is represented in Figure 2.1.

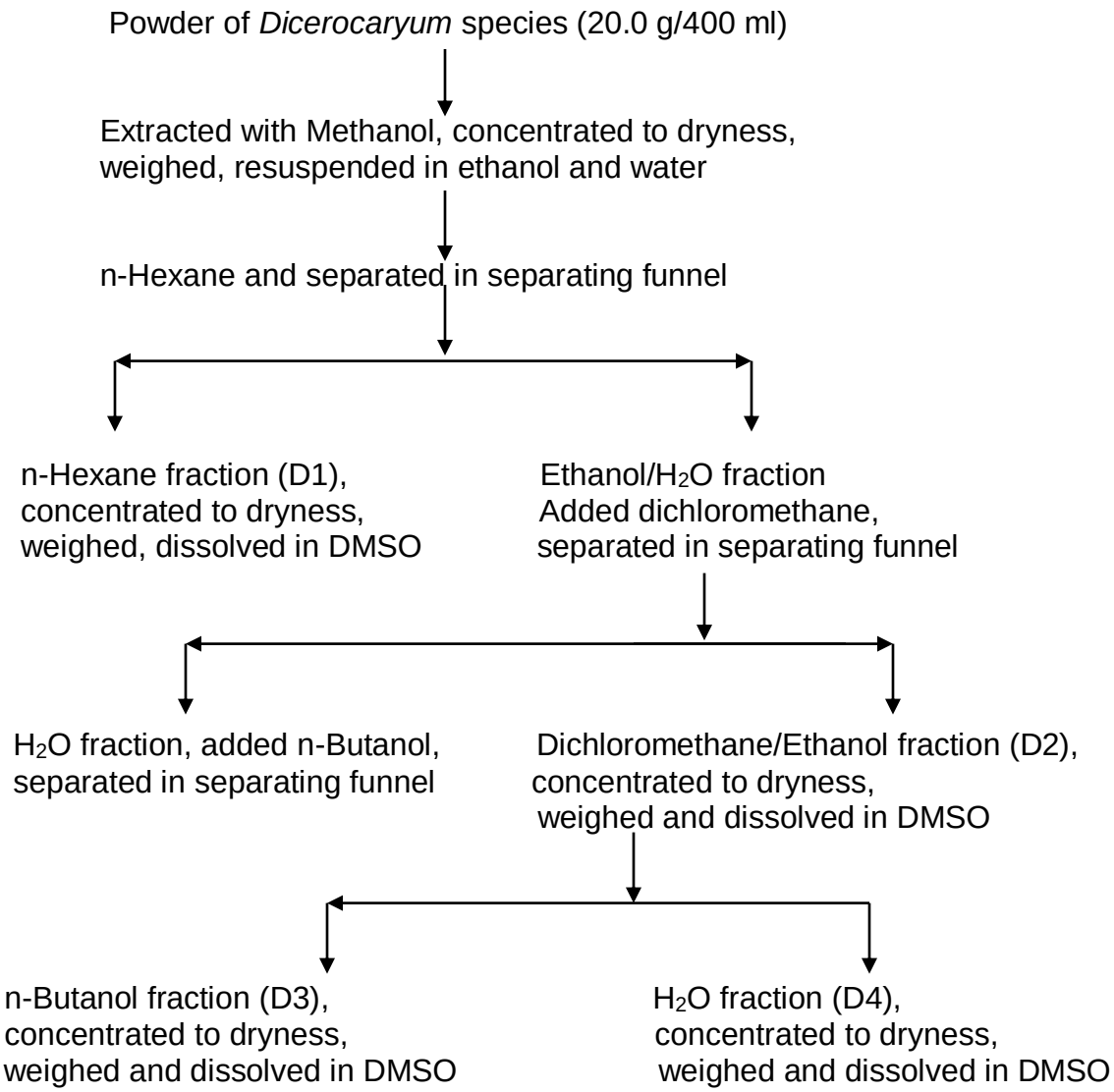

Figure 2.1: Flow chart diagram for solvent extraction of bioactive compounds from *Dicerocaryum* species

2.2.2. Cell culture conditions and treatment

Jurkat T cells (ATCC) and normal monkey Vero cells (University of Pretoria) were maintained at 37°C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 10% heat inactivated foetal bovine serum (FBS) and 1% antibiotic cocktail of penicillin, streptomycin and neomycin (PSN). Various concentrations of crude methanolic and semi-purified (n-hexane, dichloromethane/ethanol, n-butanol and water) extracts used for cell treatment were prepared by diluting with RPMI-1640 supplemented with 10% FBS. The extracts were filtered to sterility with a 0.22 µm filter paper (Millex Filter Unit, Millipore Corporation, USA) and used for cell treatment to evaluate the cytotoxic and anti-proliferative activities, at 24 h intervals, for three days.

2.2.3. Cell proliferation and viability assay

The effect of crude methanolic and semi-purified (n-hexane, dichloromethane/ethanol, n-butanol and water) extracts on the cell growth of normal monkey Vero cells and Jurkat T cells were assessed using hemocytometer viewed under the light microscope. After 24 h, cells treated with the extracts were stained with trypan blue and assessed for viability using the hemocytometer.

2.2.4. MTT Assay

3-[4, 5-Dimethylthiazol-2-yl]-2-5 diphenyltetrazolium bromide (MTT) assay was used to determine the IC_{50} of the cells treated with crude methanolic and semi-purified (n-hexane, dichloromethane/ethanol, n-butanol and water) extracts. The cells were seeded in 96-well culture plates at 2 x 10^5 cell/ml and exposed to various concentrations (0 µg/ml, 10 µg/ml, 25 µg/ml, 40 µg/ml, 55 µg/ml, 70 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml and 1000 µg/ml) of crude methanolic and semi-purified extracts (D1, D2, D3 and D4) for 24, 48 and 72 h. The cell survival fraction was determined with the MTT dye reduction assay described by Cheng *et al.* (2005). Briefly, after incubation with the extracts, MTT solution (5 mg/ml) was added (50 µl/well) to the cells. The cells were incubated for 4 h at 37ºC, the formazan formed was centrifuged at a speed of 143 x g for 10 min and the

medium and MTT removed. The cell pellets were dissolved in 200 µl of DMSO and 25 µl of glycine buffer (0.1M Glycine, 0.1M NaCl, pH 10.5). The amount of purple formazan formed, representing the amount of viable cells, was determined by measuring the absorbance at 570 nm using a microplate ELISA reader (Model 550, Bio-Rad Laboratories). The absorbance of untreated cells was considered as 100%.

The percentage survival was calculated as follows:

$$\% \text{ cell survival} = \frac{\text{Experimental absorbance values - Blank value}}{\text{Control (untreated cells) values - Blank value}} \times 100$$

Where:

Blank = medium and DMSO

Control = cells, DMSO and medium

Experimental = cells, extract, medium and DMSO

2.2.5. Morphological analysis

Jurkat T cells were treated with crude methanolic and dichloromethane/ethanol extracts of *Dicerocaryum* species for 24 h. The cells were washed with 1 x PBS, pH 7.4 and centrifuged at 277 x g for 5 min. The pelleted cells were stained with Hoechst 33258 (1 mg/ml) for 10 min, viewed and documented under fluorescent microscope (Zeiss, Germany) using the 40x magnification.

2.2.6. Isolation of fragmented DNA

Jurkat T cells were treated with crude methanolic and dichloromethane/ethanol extracts of *Dicerocaryum* species for 24 h. The cells were washed with 1 x PBS, pH 7.4 and centrifuged at 277 x g for 5 min. The pelleted cells were lysed with lysis buffer (5mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 % Triton-X). The cells were incubated at 37°C for 10 min and centrifuged at 15 994 x g for 10 min. Low molecular weight (supernatants) DNA and high molecular weight (pellets) DNA samples were separated and incubated with 0.5 mg/ml of RNase at 37°C for 1 h followed by treatment with 0.5 mg/ml of proteinase K at 50°C for 30 min. The lysates were cooled at room temperature and extracted twice with phenol:chloroform (1:1

v/v). The aqueous phase was precipitated with 1/10th of 3 M sodium acetate, pH 5.2 and 800 µl ice cold isopropanol for 24 h at -20°C. DNA samples were centrifuged at 15 994 x g for 10 min and then washed with 70% ethanol. The samples were air dried and the loading buffer (0.25% bromophenol blue, 30% glycerol) was added to each sample at a ratio of 1:5. The samples were heated at 70°C for 5 min before loading on a 1.2% agarose gel stained with 0.5 µg/ml ethidium bromide run at 70 V for 3 h and visualized under ultraviolet light.

2.2.7. Analysis of expression levels of apoptosis genes and proteins

2.2.7.1. RNA isolation

Total cellular RNA was isolated using a high pure RNA kit (Roche Diagnostics) following the manufacturer's protocol. For quantitation, 5 µl of RNA was diluted 100-fold and measured spectrophotometrically (Spectronic Genesys 5) at λ_{260} nm. The purity of RNA was assessed by the ratio of A_{260}/A_{280}. The quality of RNA products was analyzed on a 2% formaldehyde agarose gel stained with 0.5 µg/ml ethidium bromide and visualized under ultraviolet light.

2.2.7.2. RT-PCR

One microgram (1 µg) of total RNA was reverse transcribed into cDNA by incubating in a reaction mixture containing reverse transcriptase (2.5 units), 1.5 mM $MgCl_2$, 2.5 µM oligod$(T)_{16}$, 2.0 µM primers (sense and antisense), RNase inhibitor (1 unit), diethyl-pyrocarbonate (DEPC)-treated water and 0.5 mM deoxynucleotide triphosphates (dNTPs) at 42°C for 15 min. Two microliter (2 µl) of cDNA was used for PCR reaction as a template. PCR reaction was performed in a reaction mixture containing 10x PCR buffer, each primer set and Taq polymerase (1.25 units). The PCR reaction mixture was amplified for 34 cycles using amplification profile of denaturation at 95°C for 1 min, annealing at either 60°C (for *bax, bcl*-2 and *p53*) or 58°C (for β-actin and p21) for 1 min, extension at 72°C for 1 min and final extension for 7 min in a Hybaid Omnigene thermal cycler (Hybaid Limited, UK). The resulting PCR products were analyzed on 2% agarose gel containing 0.5 µg/ml ethidium bromide run at 70 V for 2 h and visualized under ultraviolet light. The gels were

photographed using the SynGene Image Analyser (Vacutec, RSA.). The oligonucleotide sequences of the primers were obtained from a publication (Huang *et al*., 2003) and synthesized by Invitrogen Life Technologies, UK. (Table 2.1)

Table 2.1: Oligonucleotide sequences used in PCR (Huang *et al*., 2003)

bax	Sense:	5'ACCAAGAAGCTGGCGAGTGTC3'
	Antisense:	5'ACAAAGATGGTCACGGTCGCC3'
bcl-2	Sense:	5'TGCACCTGACGCCCTTCAC3'
	Antisense:	5'AGACAGCCAGGAGAAATCAAACAG3'
*p*21	Sense:	5'CTCAGAGGAGGCGCCATG3'
	Antisense:	5'GGGCGGATTAGGGCTTCC3'
*p*53	Sense:	5'CCACCACAGCGACAGGGT3'
	Antisense:	5'CGTGCACATAACAGACTTGG3'
β-actin	Sense:	5'TTCATTGACCTCAACACAT3'
	Antisense:	5'GAGGGGCCATCCACAGTCTT3'

2.2.7.3. Western blot analysis

Western blot was used to determine the expression levels of proteins involved in the apoptotic pathway. The cells were collected after treatment with various concentrations (0, 10, 40, 70, 100, 200, 400 and 600 µg/ml) of crude methanolic and dichloromethane/ethanol extracts, washed with ice cold 1x PBS, pH 7.5 and lysed in 1 ml lysis buffer [2 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate ($NaVO_3$), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml aprotinin] for 20 min on ice. The lysates were centrifuged at 19 283 x g at 4°C for 15 min and aliquots of the supernatants were used to determine protein concentrations using the BCA assay (Pierce).

Aliquots containing equal amounts of proteins (20-30 µg/ml) were boiled in 2x sodium dodecyl sulphate (SDS-PAGE) sample loading buffer (125 mM Tris-HCl, 4%

SDS, 20% glycerol, 10 µM 2-mercaptoethanol) before being resolved on a 12% SDS-PAGE. Proteins on the gel were electroblotted onto Immunobilon-P transfer membrane (Millipore Corporation) using a blotting buffer (10% methanol, 10 mM CAPS, pH 11.0) at 200 mA for 2 h at 4°C. Following electro-blotting, the membranes were blocked with 0.05% non-fat dry milk for 1 h at room temperature.

After blocking, the membranes were washed three times for 10 min each with wash buffer (0.05% TBS–Tween without milk) and then incubated with specific primary goat monoclonal anti-mouse Bcl-2 antibody (1:1000), goat monoclonal anti-mouse Bax antibody (1:500) and goat monoclonal anti-mouse p53 antibody (1:1000) at 4°C for overnight. After washing three times with washing buffer for 10 min each, the membranes were further incubated for 1 h in the presence of a peroxidase-conjugated goat IgG secondary antibody (1:10 000) diluted with blocking buffer. The membranes were washed again as described above and immunoreactive proteins were then detected using the Western blotting luminol reagent (Santa-Cruz Biotechnology Inc., USA) following the manufacturer's protocol.

2.2.6. Statistical analysis

The results of each series of experiments are expressed as the mean values ± standard error of the mean (SEM). Levels of the statistical significance were calculated using the paired Student t-test when comparing two groups or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. P values of ≤ 0.05 were considered significant.

CHAPTER 3

RESULTS

3.1. THE EFFECT OF CRUDE METHANOLIC AND SEMI-PURIFIED EXTRACTS OF *DICEROCARYUM* SPECIES ON THE PROLIFERATION AND VIABILITY OF JURKAT T CELLS

The cytotoxic effects of crude methanolic and semi-purified extracts of *Dicerocaryum* species were assessed by seeding the cells at an initial concentration of 2 x 10^5 cells/ml. The normal monkey Vero cells were used as control and incubated for 48 h and Jurkat T cells were incubated (for 24, 48 and 72 h) in the presence of increasing concentrations of the extracts. The cells were counted using hemocytometer after staining with trypan blue. The results from two independent experiments done in duplicates indicated that the crude methanolic and dichloromethane/ethanol extracts inhibited the growth of Jurkat T cells in a dose- and time-dependent manner (Figures 3.1 and 3.7). Jurkat T cells treated with n-hexane, n-butanol and water extracts (Figures 3.4, 3.10 and 3.13, respectively) did not show any significant growth inhibition for each time interval.

After 24 h interval, the cell viability of Jurkat T cells was assessed by counting cells which excluded trypan blue in comparison which the ones which did not exclude the dye. Cells which excluded the dye were considered viable and those that did not were taken as non-viable. The crude methanolic extract showed a significant decrease in cell viability when compared with n-hexane, n-butanol and water extracts. After 48 and 72 h, 200-600 µg/ml of the crude methanolic extract (Figure 3.2) resulted in decreased cell viability. Cells treated with dichloromethane/ethanol extract showed a significant decrease in cell viability when treated with concentrations of 70 µg/ml and 100 µg/ml after 48 and 72 h (Figure 3.8). Thus, the viability of Jurkat T cells significantly decreased with an increase in the concentration of crude methanolic and dichloromethane/ethanol extracts after 48 and 72 h (Figures

3.2 and 3.8). On the other hand, n-hexane, n-butanol and water extracts did not show any significant decrease in cell viability of Jurkat T cells treated with 100-1000 µg/ml after each time interval (Figures 3.5, 3.11 and 3.14, respectively). Normal monkey Vero cells were used as control cells and their viability was not affected when treated with 0-400 µg/ml of crude methanolic and dichloromethane/ethanol extracts (Data not shown) thus demonstrating that the extracts were not cytotoxic to normal cells and their inhibitory effect was specific to Jurkat T cells.

The MTT assay was used to confirm the cytotoxic effects of the crude methanolic and semi-purified extracts of *Dicerocaryum* species. The assay is based on the conversion of the orange 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide salt to purple formazan crystals by the mitochondrial dehydrogenase. This gives the estimation of the number of viable cells. The data collected from two independent experiments done in duplicates gave different IC_{50} values at 72 h (Table 3.1). Jurkat T cells were more sensitive when treated with dichloromethane/ethanol extract than when treated with other extracts as illustrated in Table 3.1.

Table 3.1. IC_{50} values of crude methanolic and semi-purified extracts of *Dicerocaryum* species. The values are representatives of two sets of independent experiments done in duplicates.

Extract	**IC_{50} value**
Crude	150 µg/ml
n-Hexane	160 µg/ml
Dichloromethane/ethanol	70 µg/ml
n-Butanol	680 µg/ml
Water	880 µg/ml

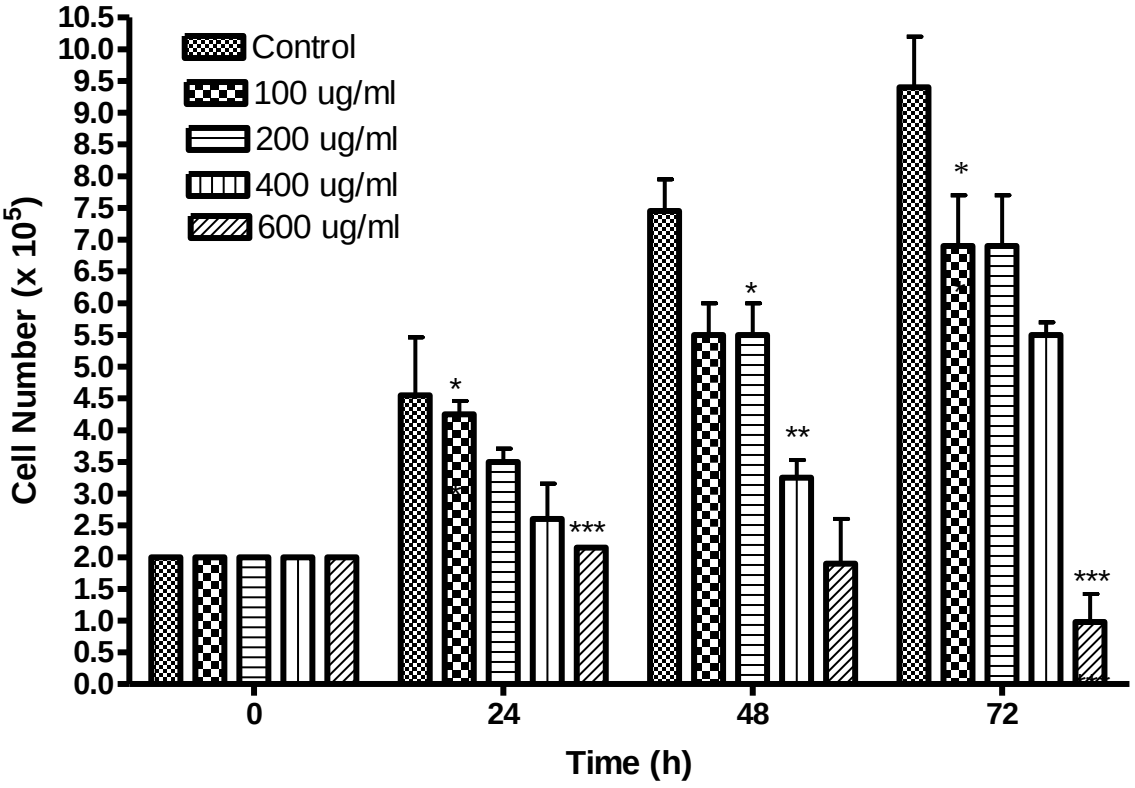

Figure 3.1. Effect of various concentrations of crude methanolic extract of *Dicerocaryum* species on the proliferation of Jurkat T cells. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. *p< 0.05, **p< 0.01, ***p< 0.001.

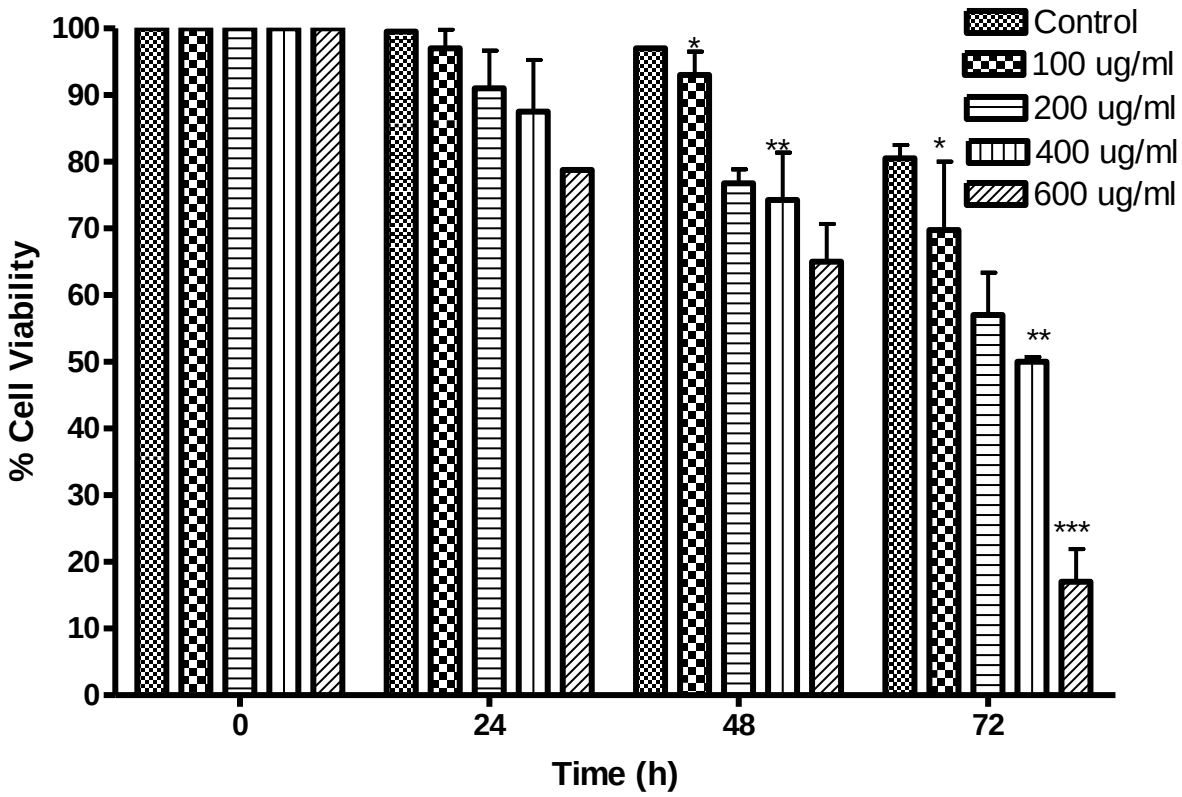

Figure 3.2. Effect of various concentrations of crude methanolic extract of *Dicerocaryum* species on the viability of Jurkat T cells. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. *p< 0.05, **p<0.01 and ***p<0.001.

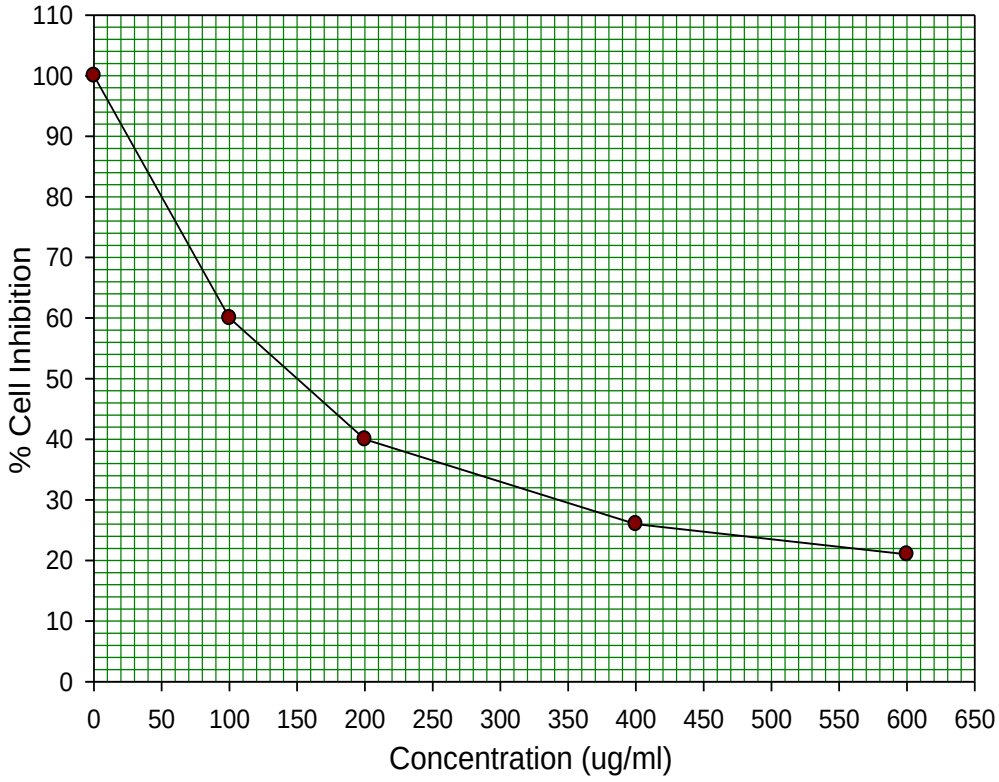

Figure 3.3. Determination of the IC_{50} value of crude methanolic extract of *Dicerocaryum* species on Jurkat T treated with various concentrations after 72 h. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control.

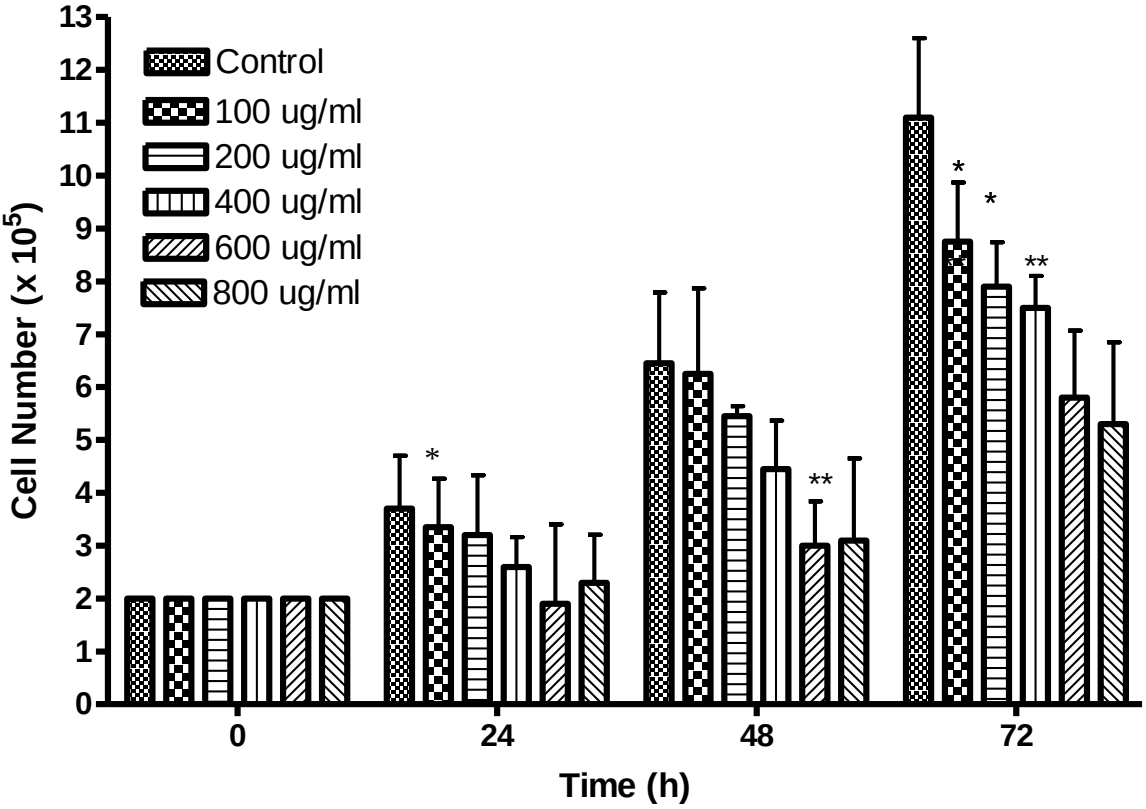

Figure 3.4. Effect of various concentrations of n-hexane extract of *Dicerocaryum* species on the proliferation of Jurkat T cells. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. *p< 0.05 and **p<0.01.

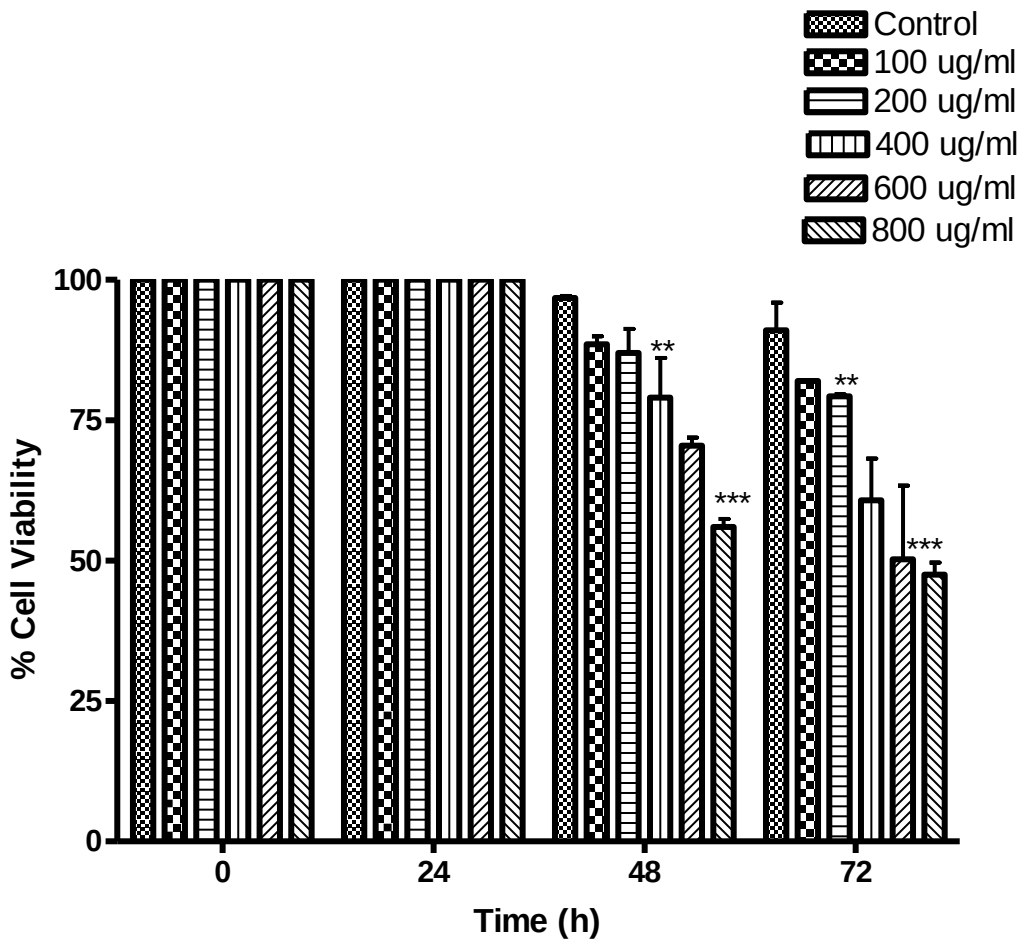

Figure 3.5. Effect of various concentrations of n-hexane extract of *Dicerocaryum* species on the viability of Jurkat T cells. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. **p<0.01 and ***p<0.001.

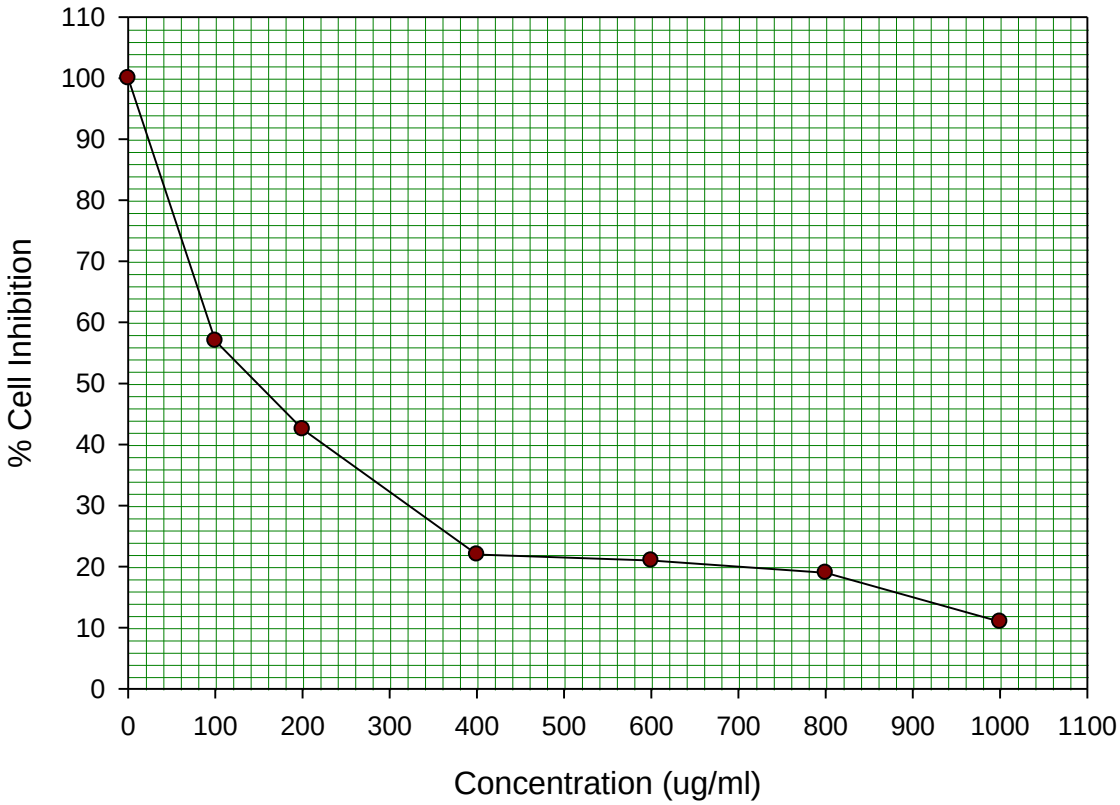

Figure 3.6. Determination of the IC_{50} value of n-hexane extract on Jurkat T cells treated with various concentrations of *Dicerocaryum* species after 72 h. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments. DMSO was used as control.

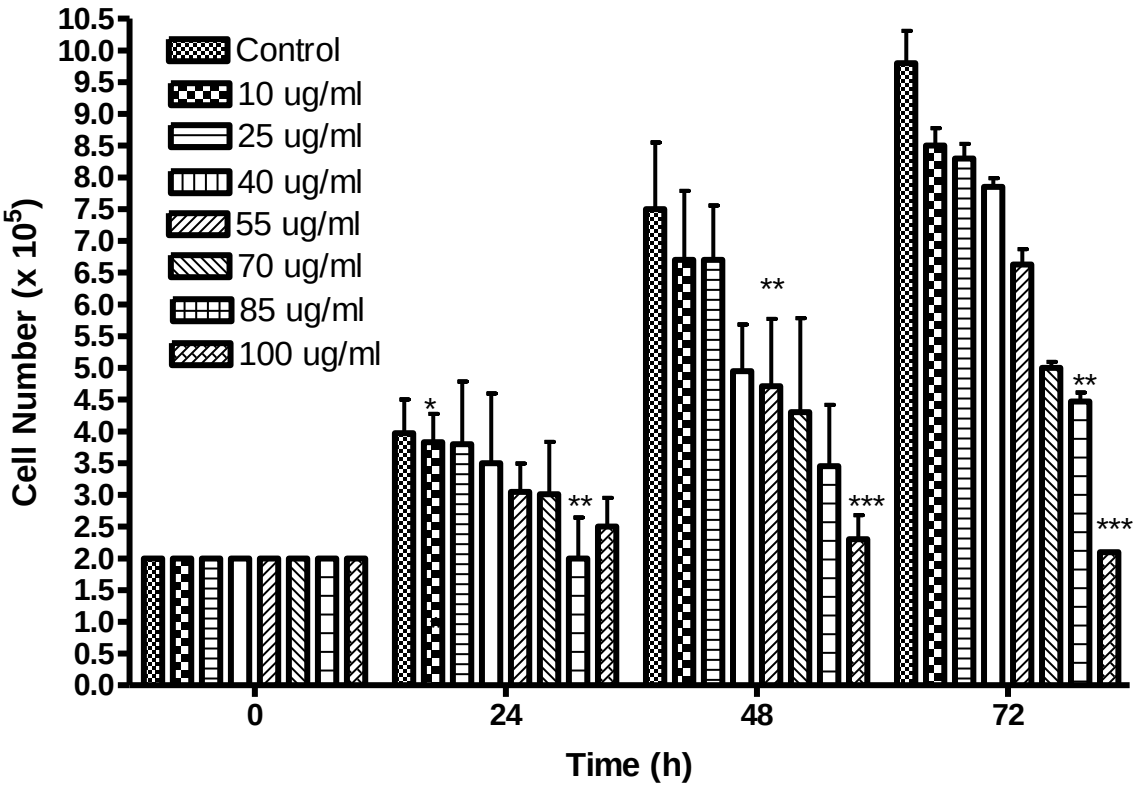

Figure 3.7. Effect of various concentrations of dichloromethane/ethanol extract of *Dicerocaryum* species on the proliferation of Jurkat T cells. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. *$p < 0.05$, **$p< 0.01$ and ***$p<0.001$.

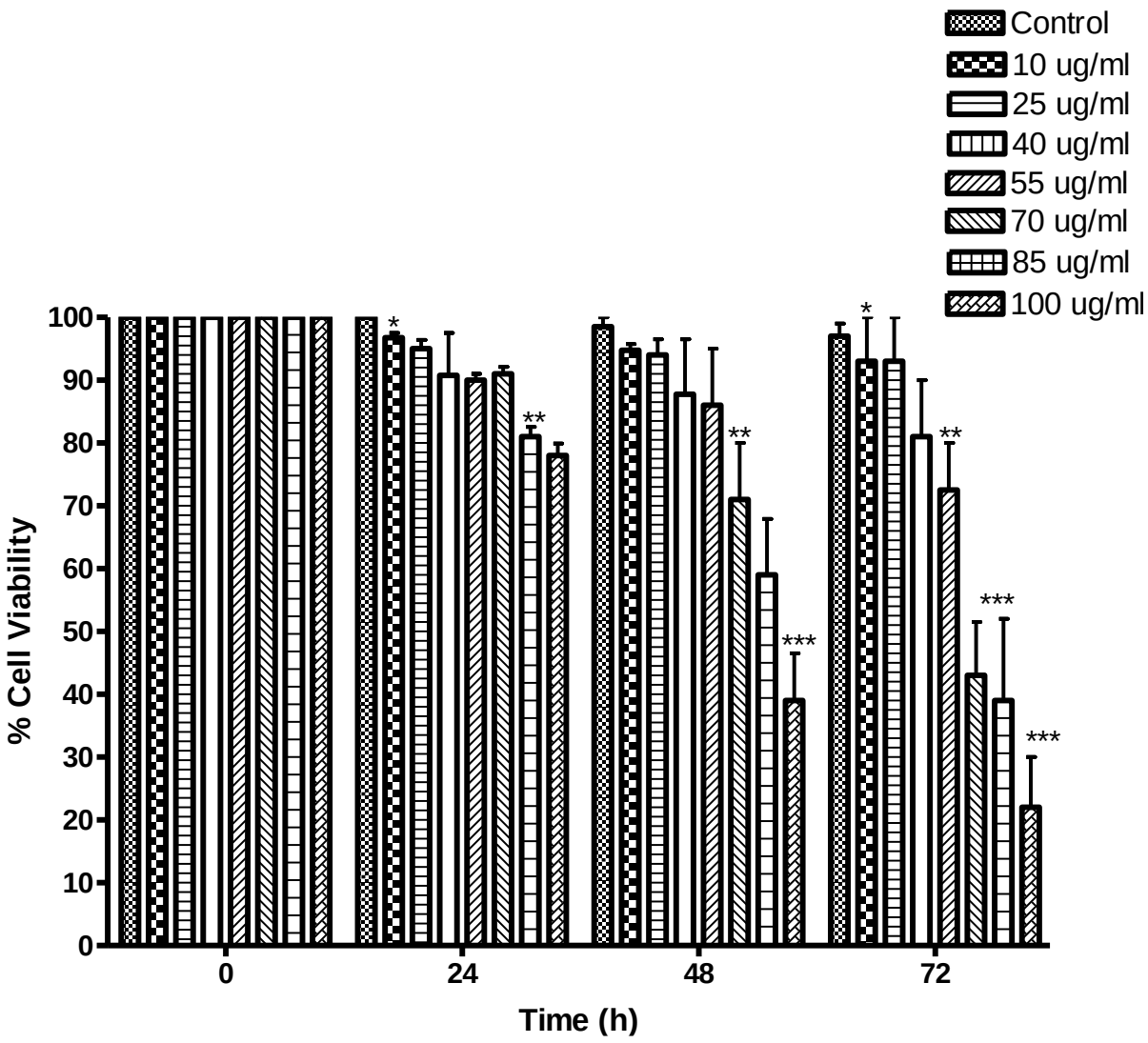

Figure 3.8. Effect of various concentrations of dichloromethane/ethanol extract of *Dicerocaryum* species on the viability of Jurkat T cells. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. *p<0.05; **p<0.01 and ***p<0.01.

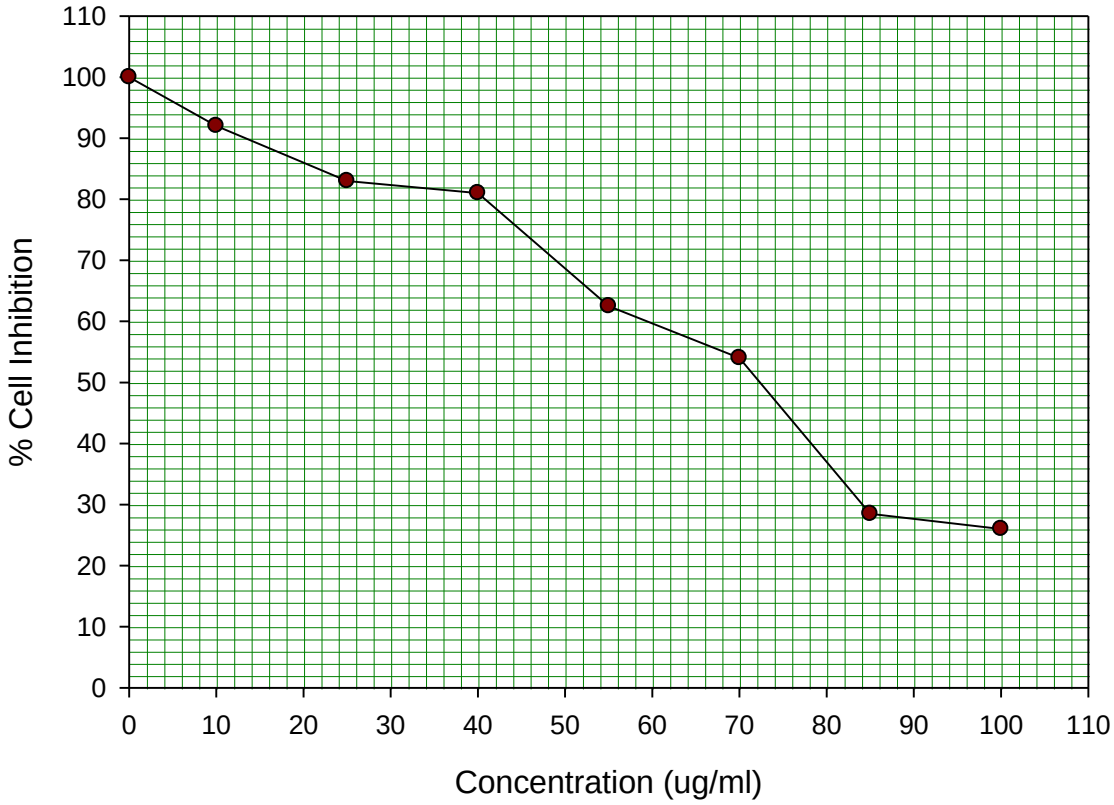

Figure 3.9. Determination of the IC_{50} value of dichloromethane/ethanol extract on Jurkat T cells treated with various concentrations of *Dicerocaryum* species after 72 h. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control.

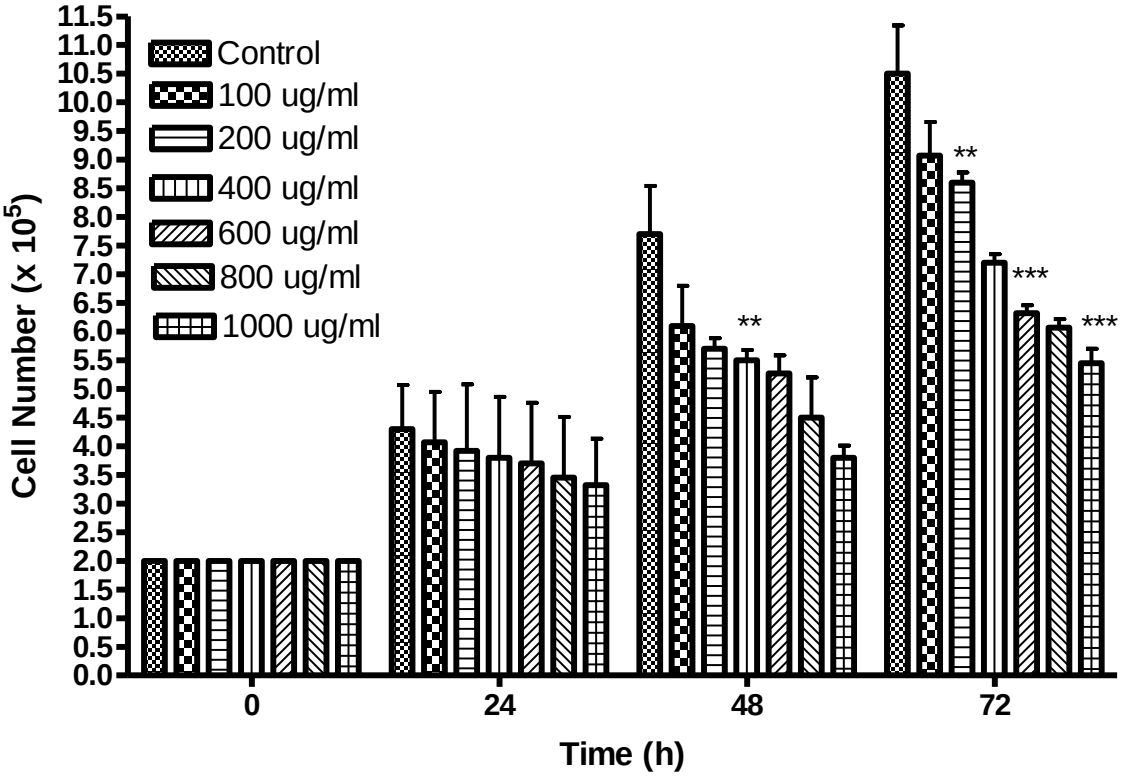

Figure 3.10. Effect of various concentrations of n-butanol extracts of *Dicerocaryum* species on the cell proliferation of Jurkat T cells. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. **p<0.01 and ***p<0.001.

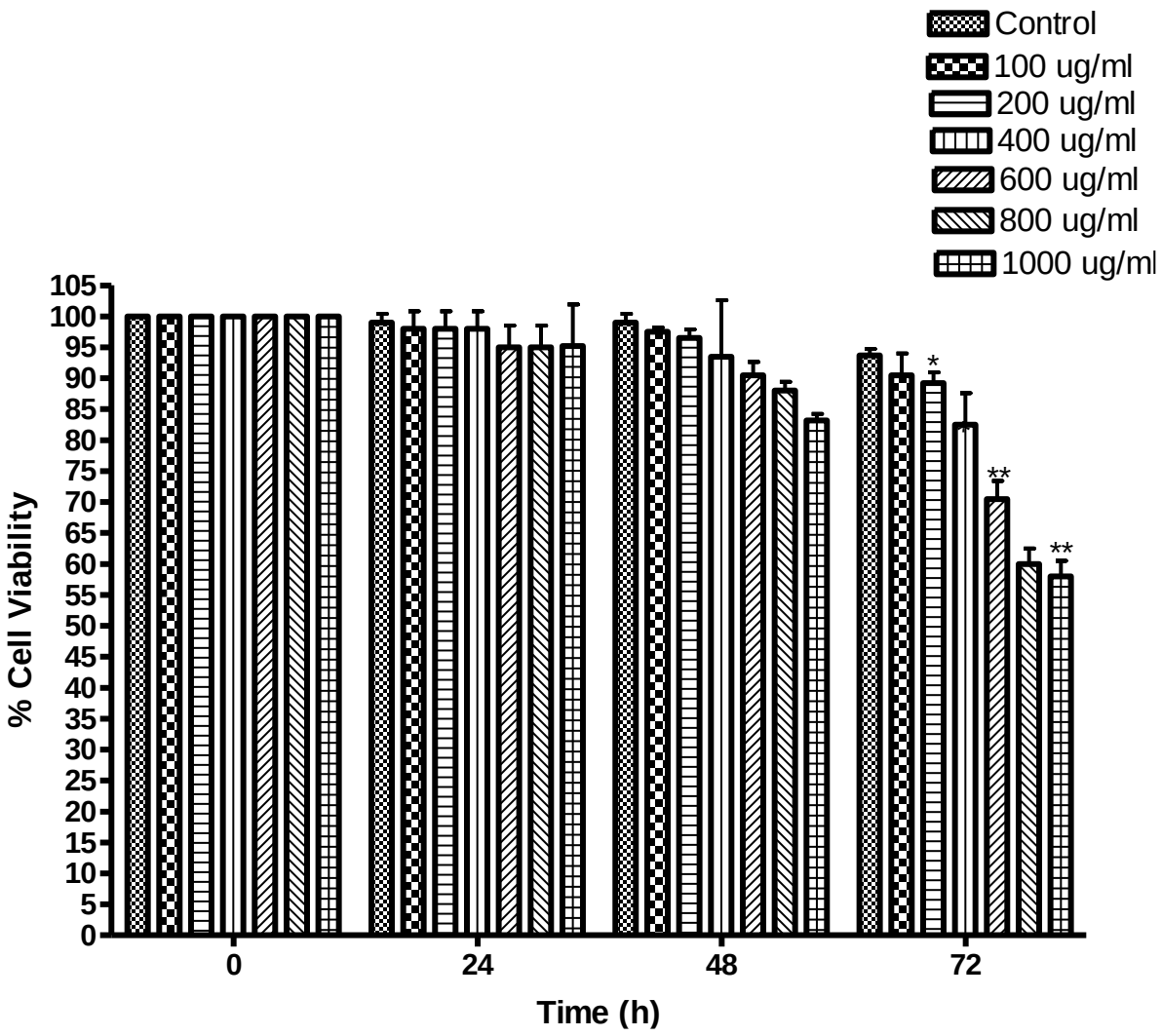

Figure 3.11. Effect of various concentrations of n-butanol extracts of *Dicerocaryum* species on the viability of Jurkat T cells. Each data point represents the mean and standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. *$p<0.05$ and **$p<0.01$.

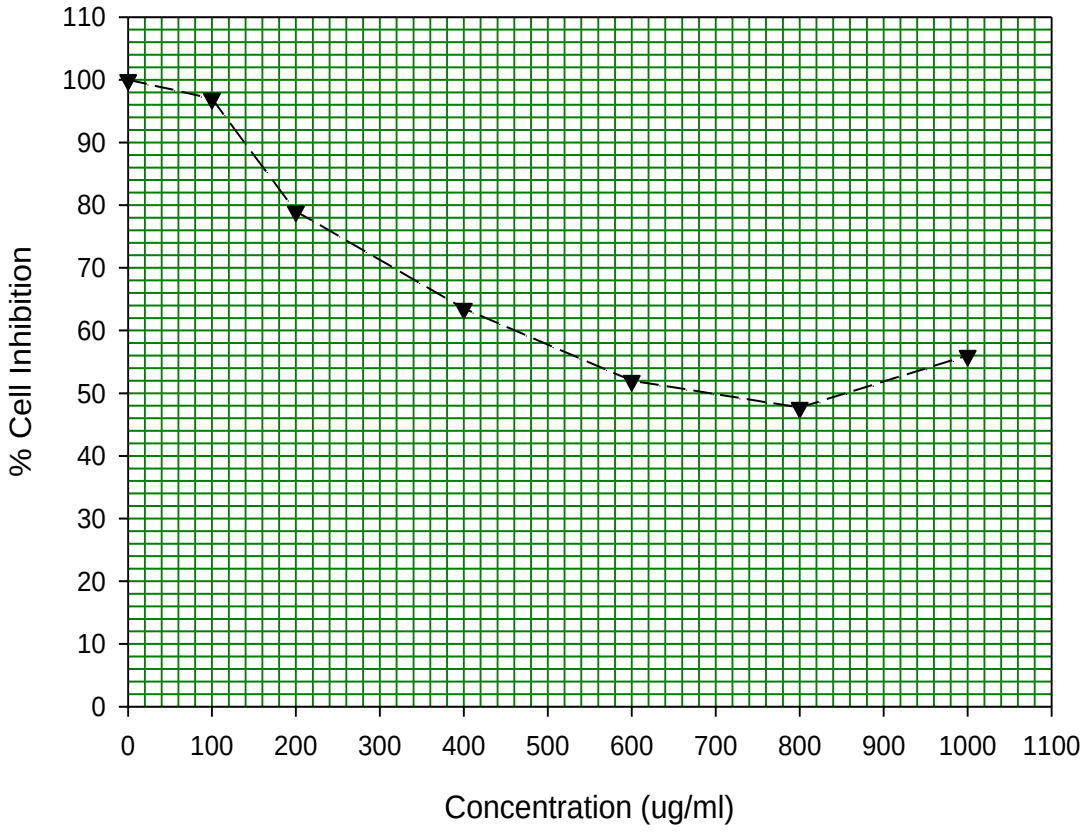

Figure 3.12. Determination of the IC_{50} value of n-butanol extract on Jurkat T cells treated with various concentrations of *Dicerocaryum* species after 72 h. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control.

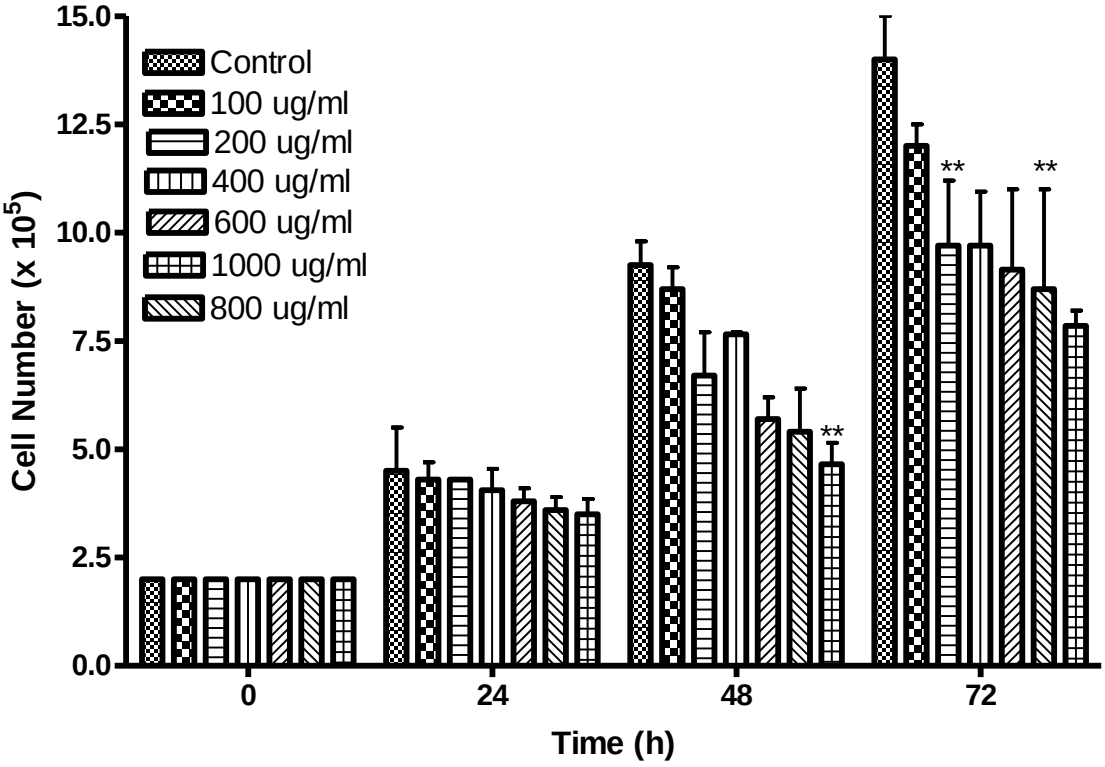

Figure 3.13. Effect of various concentrations of water extracts of *Dicerocaryum* species on the proliferation of Jurkat T cells. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. **p<0.01.

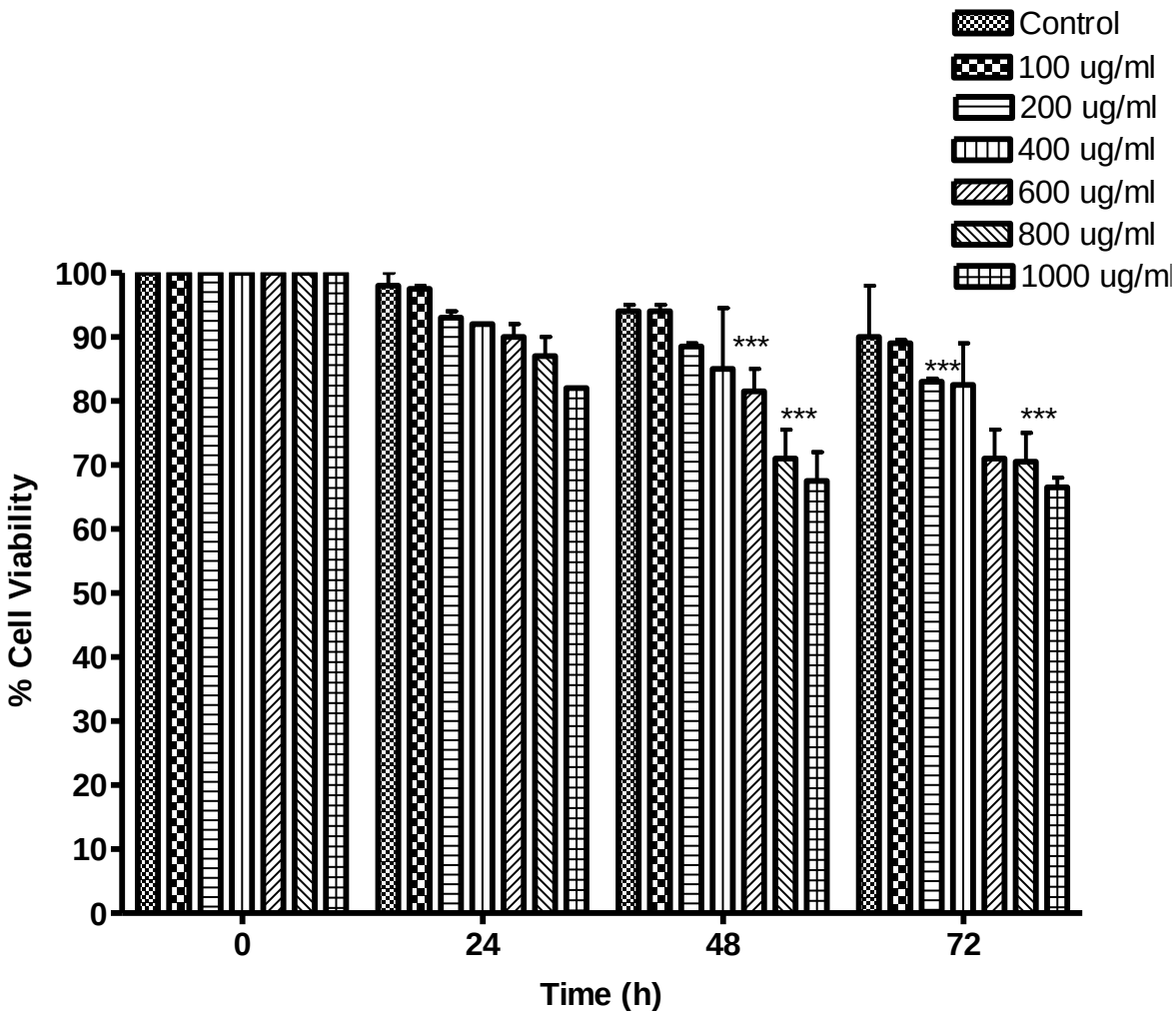

Figure 3.14. Effect of various concentrations of water extracts of *Dicerocaryum* species on viability of Jurkat T cells. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control. ***$p < 0.001$.

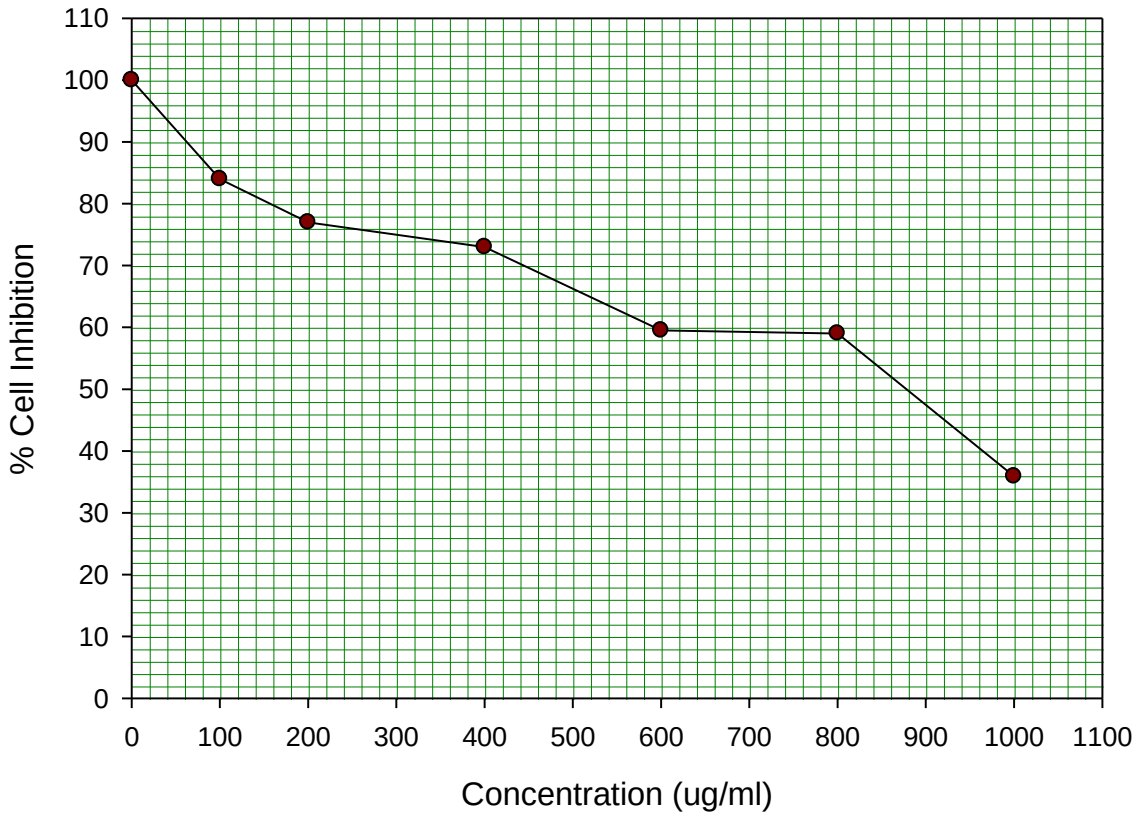

Figure 3.15. Determination of IC_{50} value of water extract on Jurkat T cells treated with various concentrations of *Dicerocaryum* species after 72 h. Each data point represents the mean ± standard error of the mean (SEM) values taken from two sets of independent experiments done in duplicates. DMSO was used as control.

3.2 NUCLEAR MORPHOLOGICAL CHANGES INDUCED BY CRUDE METHANOLIC AND DICHLOROMETHANE/ETHANOL EXTRACTS OF *DICEROCARYUM* SPECIES IN JURKAT T CELLS

The nuclear morphology of cells treated with crude methanolic and dichloromethane/ethanol extracts were evaluated after treatment of cells with 10, 70, 100, 150 and 600 µg/ml, to determine the mode of cell death. Treated cells displayed nuclear shrinkage and chromatin condensation after 24 h of treatment with 150 µg/ml and 600 µg/ml (Figure 3.16). Cells treated with 70 µg/ml and 100 µg/ml of dichloromethane/ethanol extract also demonstrated nuclear condensation (Figure 3.17). Subsequently, a dose-dependent appearance of DNA ladders were observed in Jurkat T cells treated with crude methanolic and dichloromethane/ethanol extracts (Figures 3.18 and 3.19, respectively). The data implied that both crude methanolic and dichloromethane/ethanol extracts induce apoptotic cell death in Jurkat T cells.

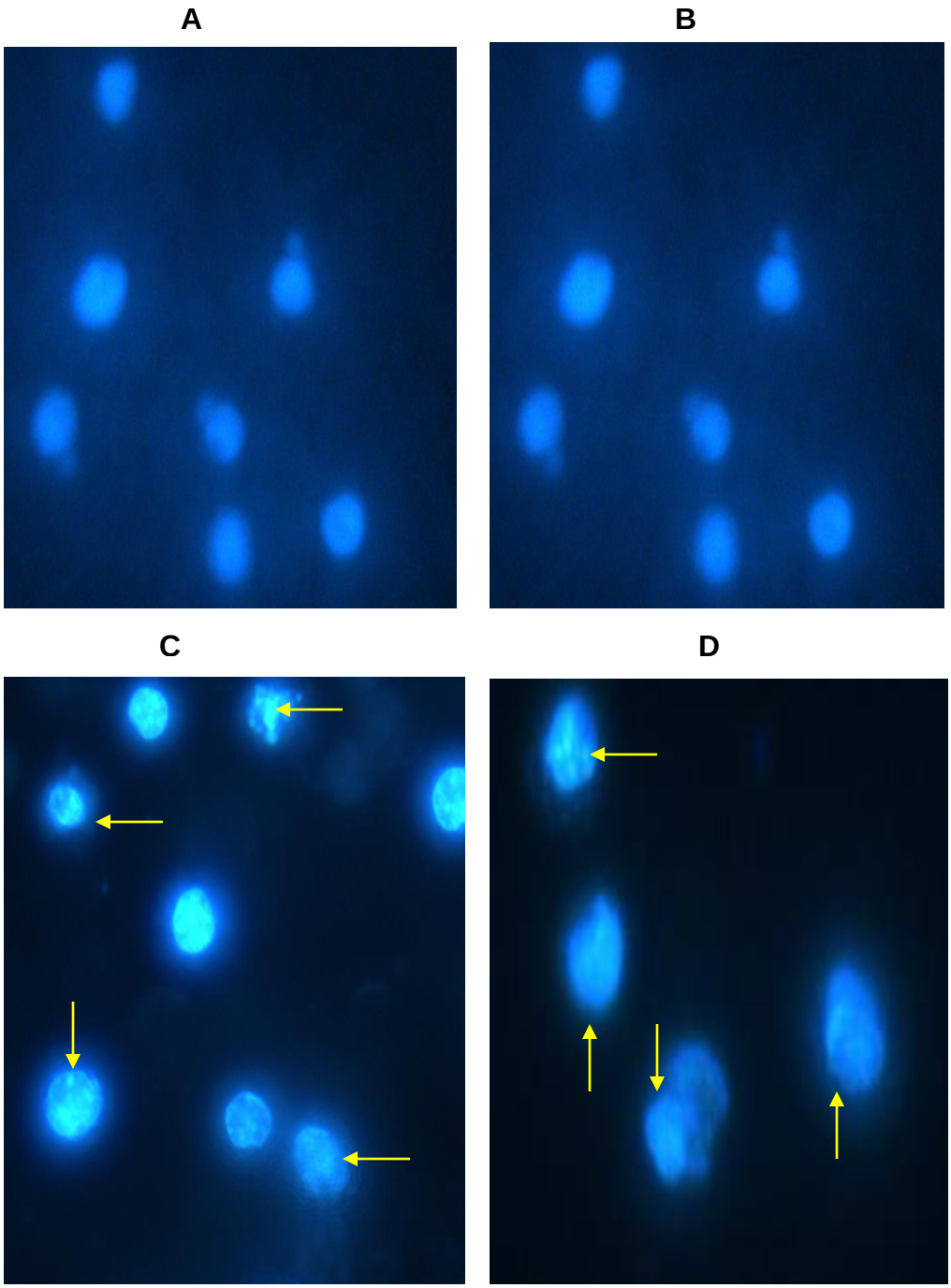

Figure 3.16. Effect of crude methanolic extract of *Dicerocaryum* species on the nuclei morphological changes of Jurkat T cells at 24 h. **A** = DMSO as control, **B** = 100 µg/ml, **C** = 150 µg/ml and **D** = 600 µg/ml. The cells were photographed under fluorescence microscope. Arrows highlight nuclear shrinkage and chromatin condensation.

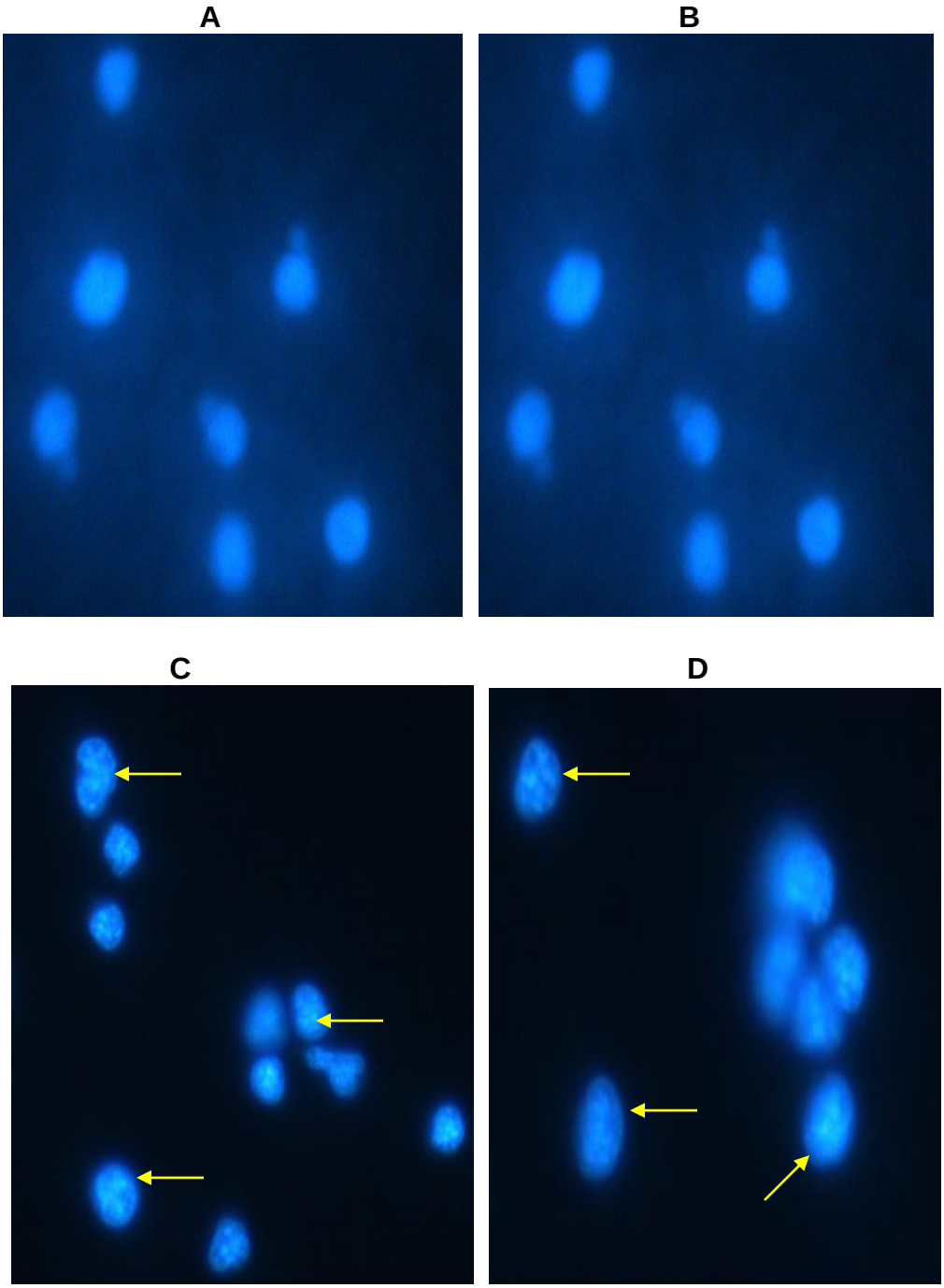

Figure 3.17. Effect of dichloromethane/ethanol extract of *Dicerocaryum* species on the nuclei morphological changes of Jurkat T cells at 24 h. **A** = DMSO as control, **B** = 10 µg/ml, **C** = 70 µg/ml and **D** = 100 µg/ml. The cells were photographed under fluorescence microscope. Arrows highlight nuclear shrinkage and chromatin condensation.

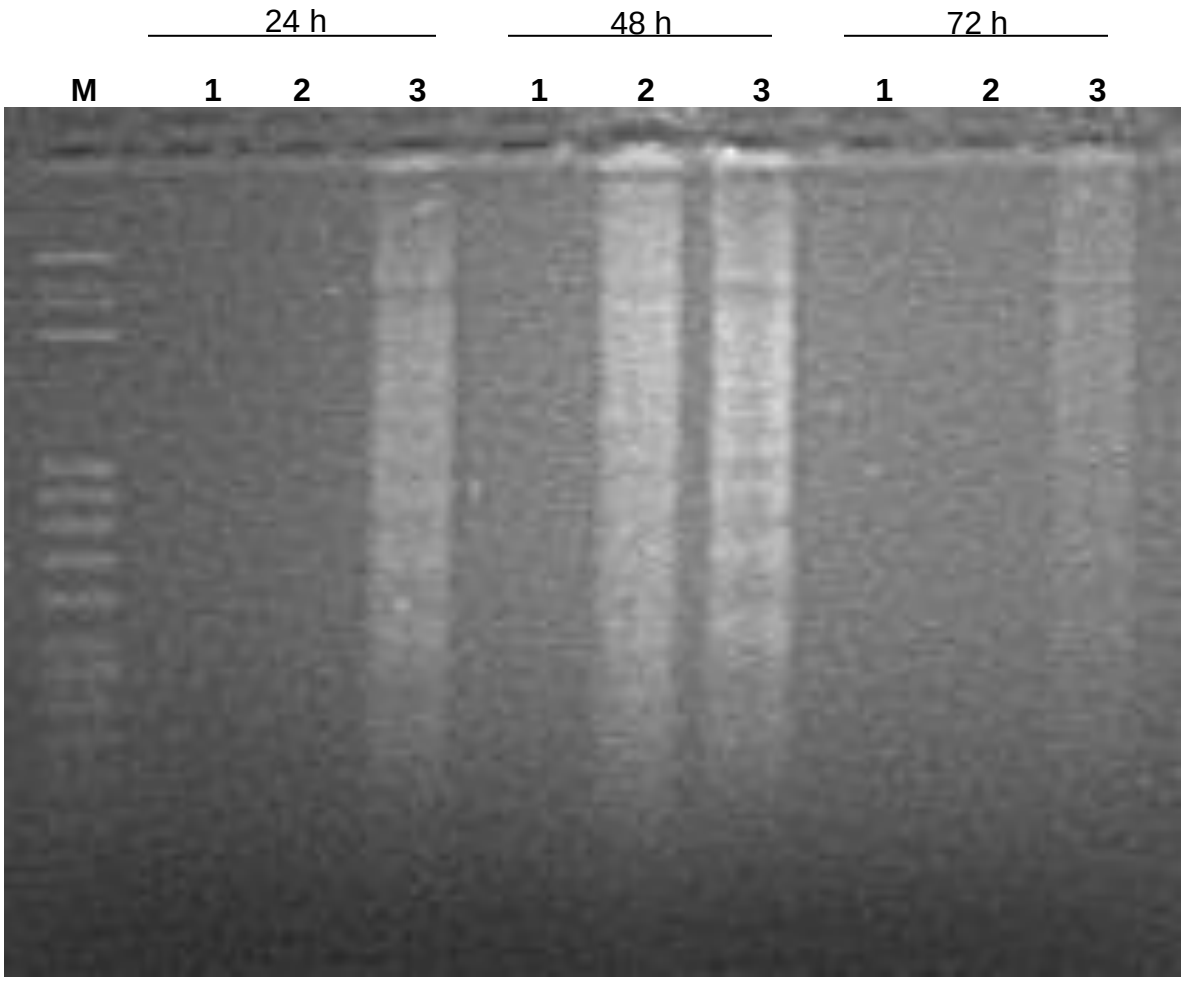

Figure 3.18: The induction of DNA fragmentation in Jurkat T cells treated with various concentrations of crude methanolic extract of *Dicerocaryum* species. The DNA was isolated and run on a 1.2% agarose gel. Lanes: **M** = molecular weight marker,
1 = DMSO as internal control, **2** = 150 µg/ml and **3** = 600 µg/ml.

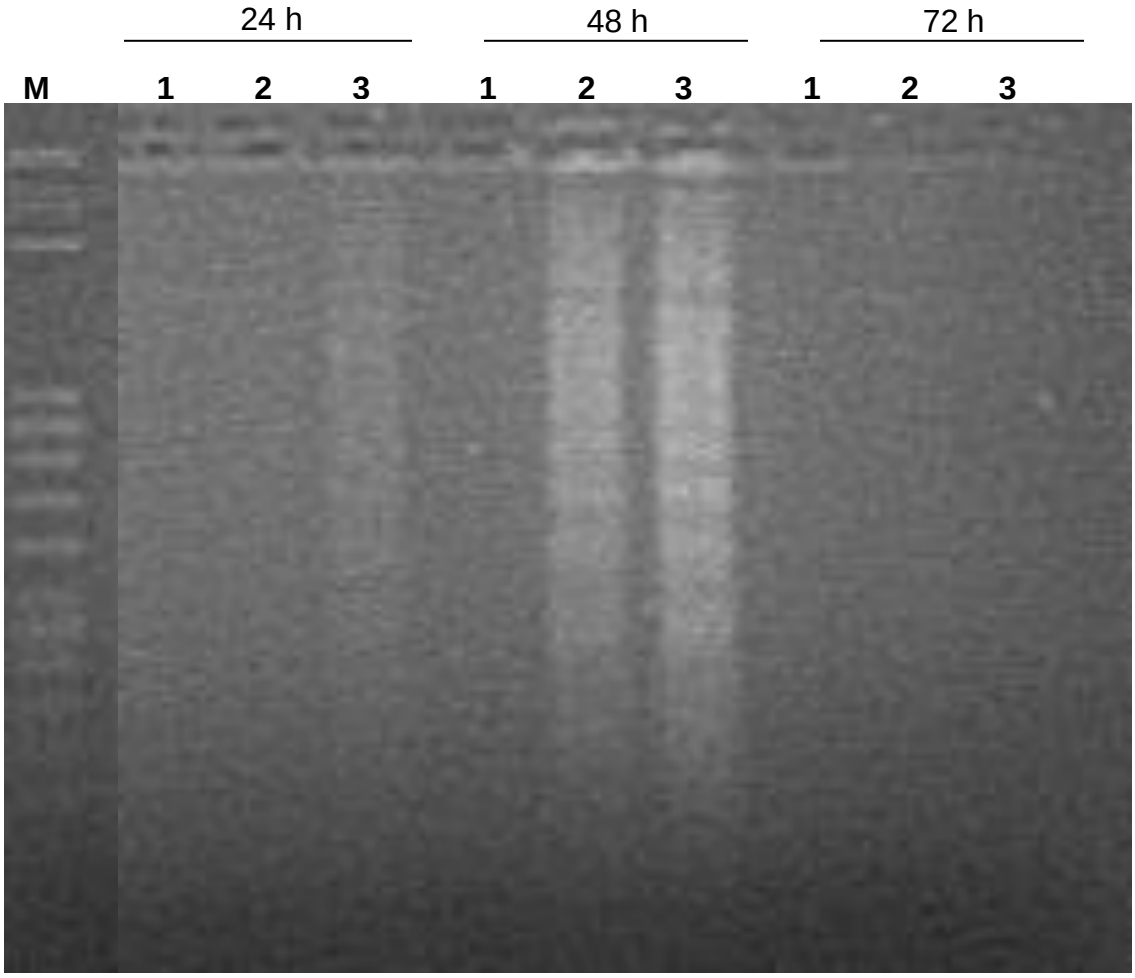

Figure 3.19: The induction of DNA fragmentation in Jurkat T cells treated with various concentrations of dichloromethane/ethanol extract of *Dicerocaryum* species. The DNA was isolated and run on a 1.2% agarose gel. Lanes: **M** = molecular weight marker,
1 = DMSO as internal control, **2** = 70 µg/ml and **3** = 100 µg/ml.

3.3 THE EFFECT OF CRUDE METHANOLIC AND DICHLOROMETHANE/ETHANOL EXTRACTS ON THE EXPRESSION LEVELS OF *Bax* and *Bcl-2* genes

To investigate the regulatory effect of crude methanolic and dichloromethane/ethanol extracts on apoptotic genes, total RNA was isolated from treated and non-treated cells. The RNA samples were reverse transcribed using a kit (Perkin-Elmer, Roche Diagnostics, Germany) following the manufacturer's protocol. RT-PCR analysis revealed that *bax* was up-regulated whereas *bcl*-2 was down-regulated after 48 h in cells treated with 400 µg/ml and 600 µg/ml of crude methanolic extract. Cells treated with 70 µg/ml and 100 µg/ml of dichloromethane/ethanol extract showed a down-regulation of *bcl*-2 after 24 and 48 h. The regulatory effects of the crude methanolic and dichloromethane/ethanol extracts on both genes were in a dose- and time-dependent manner (Figures 3.21 and 3.22, respectively).

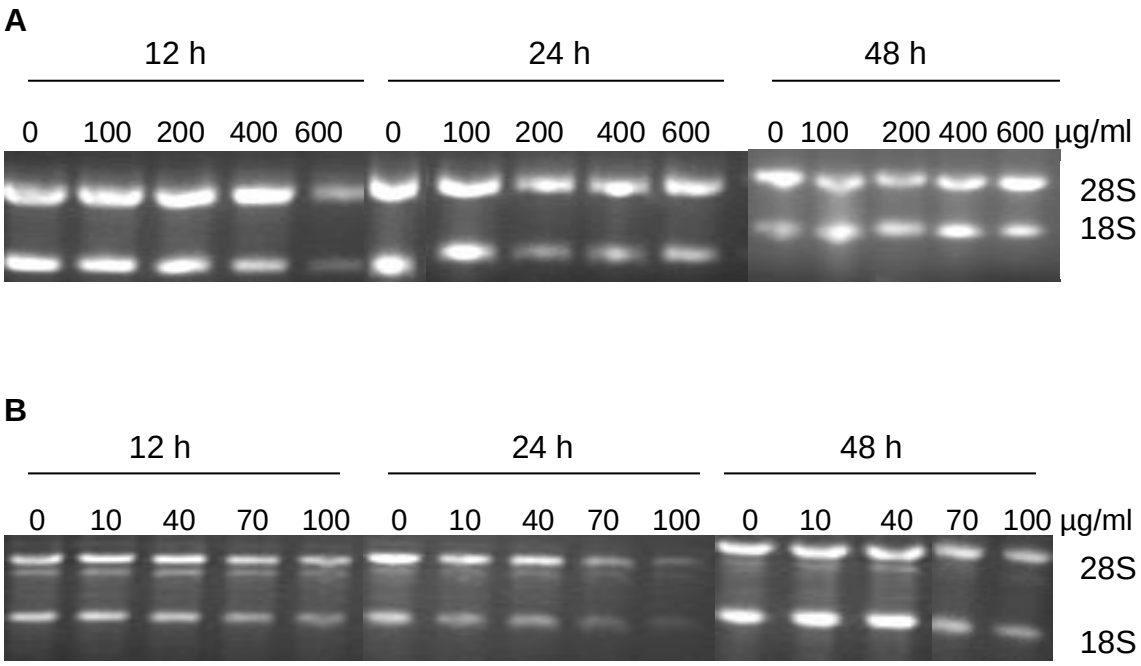

Figure 3.20: Formaldehyde gel analysis of total RNA isolated from Jurkat T cells treated with various concentrations of crude methanolic (**A**) and dichloromethane/ethanol (**B**) extracts for 12, 24 and 48 h. Note the quality of the 28S and 18S rRNA species.

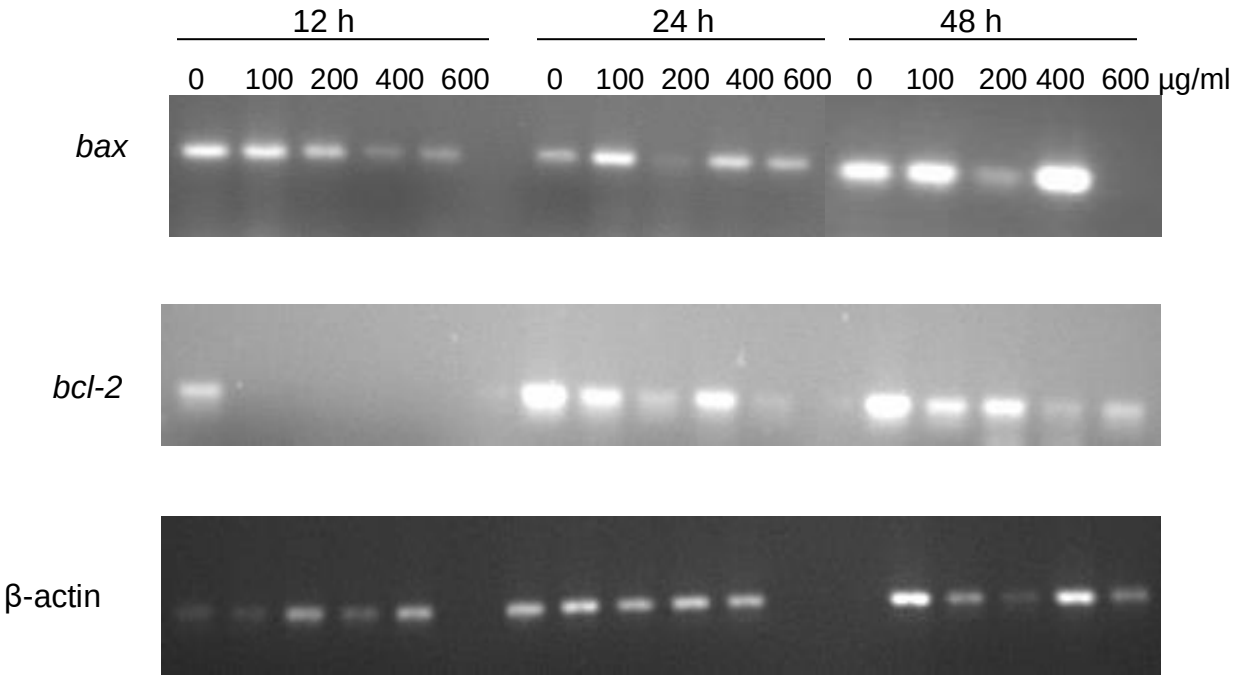

Figure 3.21. RT-PCR analysis of *bax* and *bcl*-2 genes in Jurkat T cells treated with various concentrations of crude methanolic extract of *Dicerocaryum* species. β-actin was used as an internal loading control.

Table 3.2: The ratio of *bax*/*bcl*-2 in Jurkat T cells treated with various concentrations of crude methanolic extract of *Dicerocaryum* species.

Concentration (µg/ml)	*bax*/*bcl*-2 ratio 12 h	*bax*/*bcl*-2 ratio 24 h	*bax*/*bcl*-2 ratio 48 h
Control	1.00	1.00	1.00
100 µg/ml	0.00	1.17	1.35
200 µg/ml	0.00	0.84	0.28
400 µg/ml	0.00	1.28	7.57
600 µg/ml	0.00	5.60	0.00

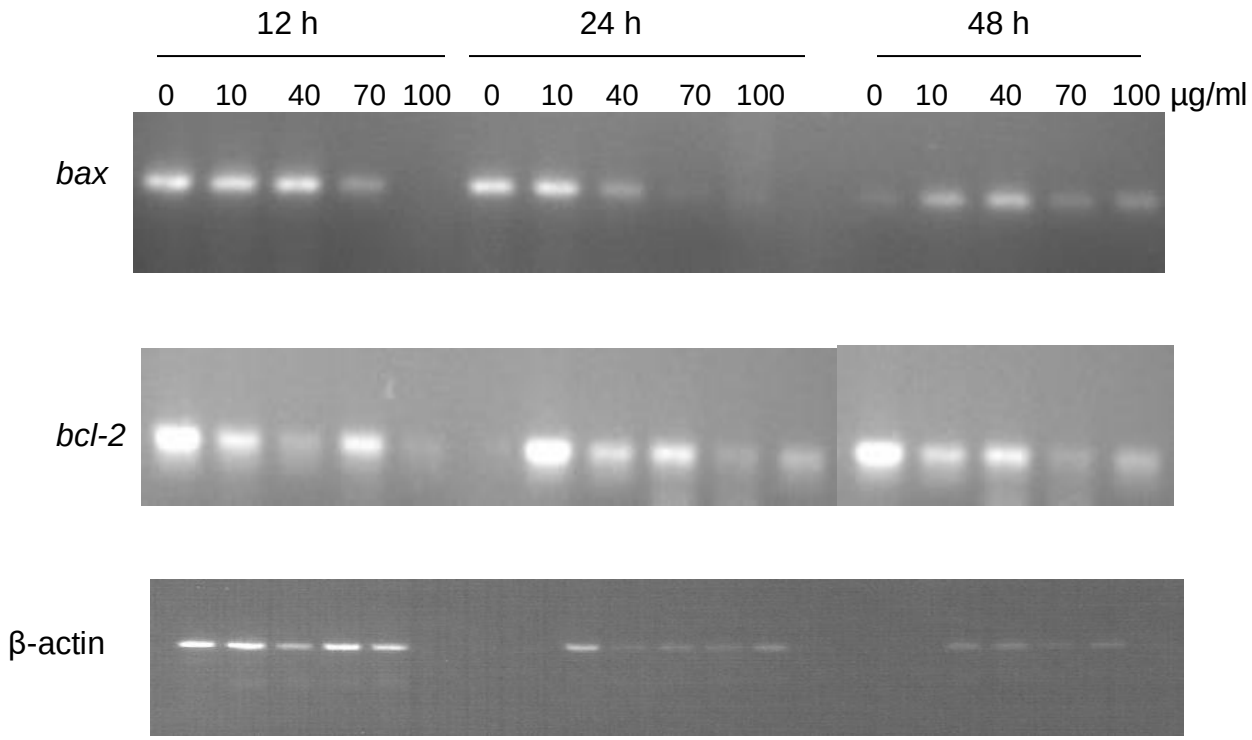

Figure 3.22. RT-PCR analysis of *bax* and *bcl*-2 genes in Jurkat T cells treated with various concentrations of dichloromethane/ethanol extract of *Dicerocaryum* species. β-actin was used as an internal loading control.

Table 3.3: The ratio of *bax*/*bcl*-2 in Jurkat T cells treated with various concentrations of dichloromethane/ethanol extract of *Dicerocaryum* species.

Concentration (µg/ml)	*bax*/*bcl*-2 ratio 12 h	*bax*/*bcl*-2 ratio 24 h	*bax*/*bcl*-2 ratio 48 h
Control	1.00	1.00	1.00
10 µg/ml	0.96	1.25	0.77
40 µg/ml	4.24	0.41	0.74
70 µg/ml	0.54	0.32	0.48
100 µg/ml	0.00	0.18	0.19

3.4 THE REGULATORY EFFECT OF CRUDE METHANOLIC AND DICHLOROMETHANE/ETHANOL EXTRACTS ON THE EXPRESSION LEVELS OF APOPTOTIC PROTEINS IN JURKAT T CELLS

To investigate the regulatory effect of crude methanolic and dichloromethane /ethanol extracts on the apoptotic gene products, proteins were extracted from treated and non-treated cells. Western blot analysis demonstrated a dose-dependent down-regulation of Bcl-2 in Jurkat T cells treated with crude methanolic and dichloromethane/ethanol extracts after 48 h (Figures 3.24 and 3.25, respectively). The p53 expression level was not significant in cells treated with high concentrations (400 µg/ml and 600 µg/ml) of crude methanolic extract for each time point than when treated with low concentrations (100 µg/ml and 200 µg/ml) of the crude methanolic extract (Figure 3.23). The p53 did not display any detectable levels in cells treated with dichloromethane/ethanol extract (data not shown) and this may be due to its short half life of 30 min. Bax protein did not give any detectable levels (data not shown) for both the crude methanolic and dichloromethane/ethanol extracts. The reason for these observations may be due to the fact that Bax might have been a target for proteasome-mediated degradation under the experimental conditions used.

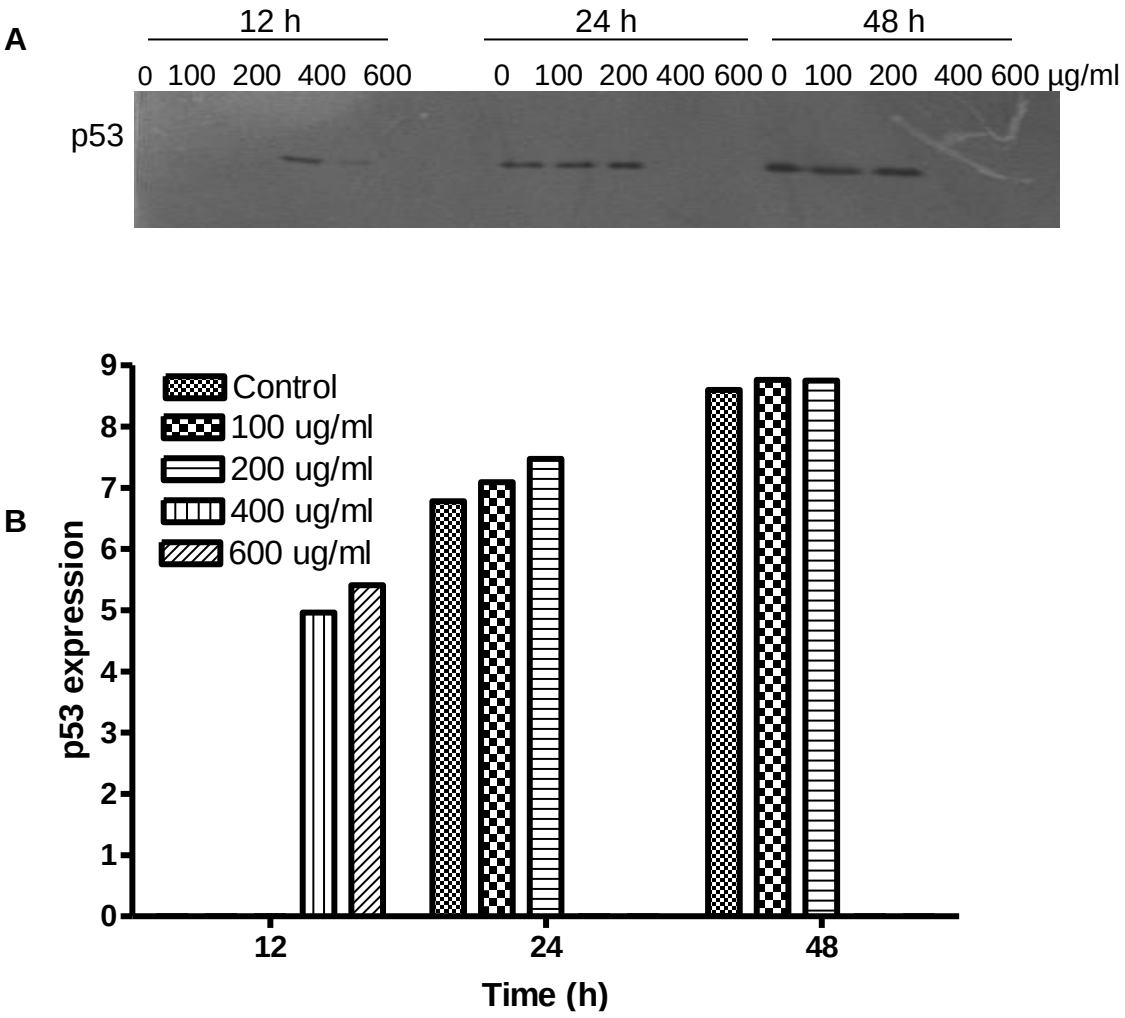

Figure 3.23. p53 expression levels of Jurkat T cells treated with various concentrations of crude methanolic extract of *Dicerocaryum* species . **A** = Immunoblots incubated with antibody specific for p53 and **B** = densitometric quantification of p53 expression.

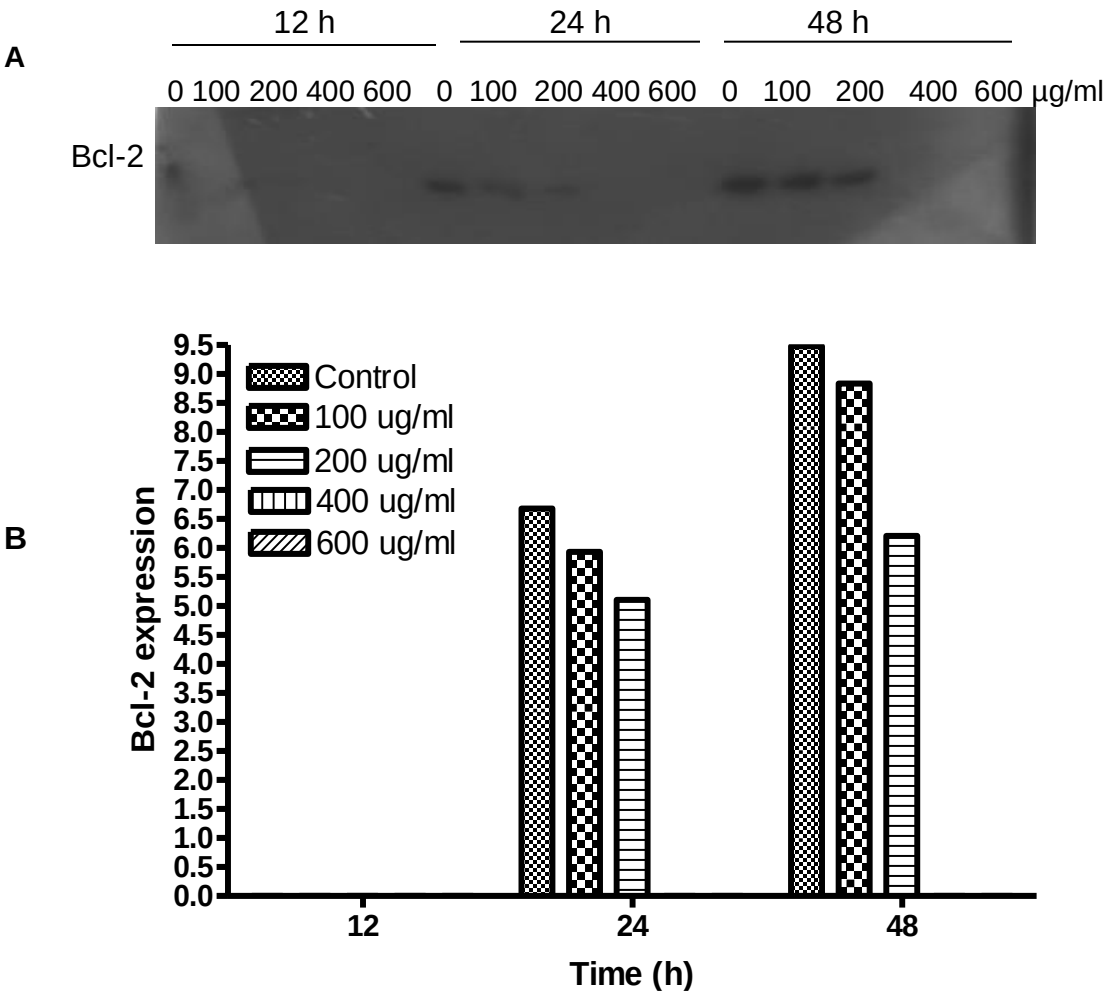

Figure 3.24. Bcl-2 expression levels of Jurkat T cells treated with various concentrations of crude methanolic extract of *Dicerocaryum* species. **A =** Immunoblots incubated with antibody specific for Bcl-2 and **B** = densitometric quantification of Bcl-2 expression.

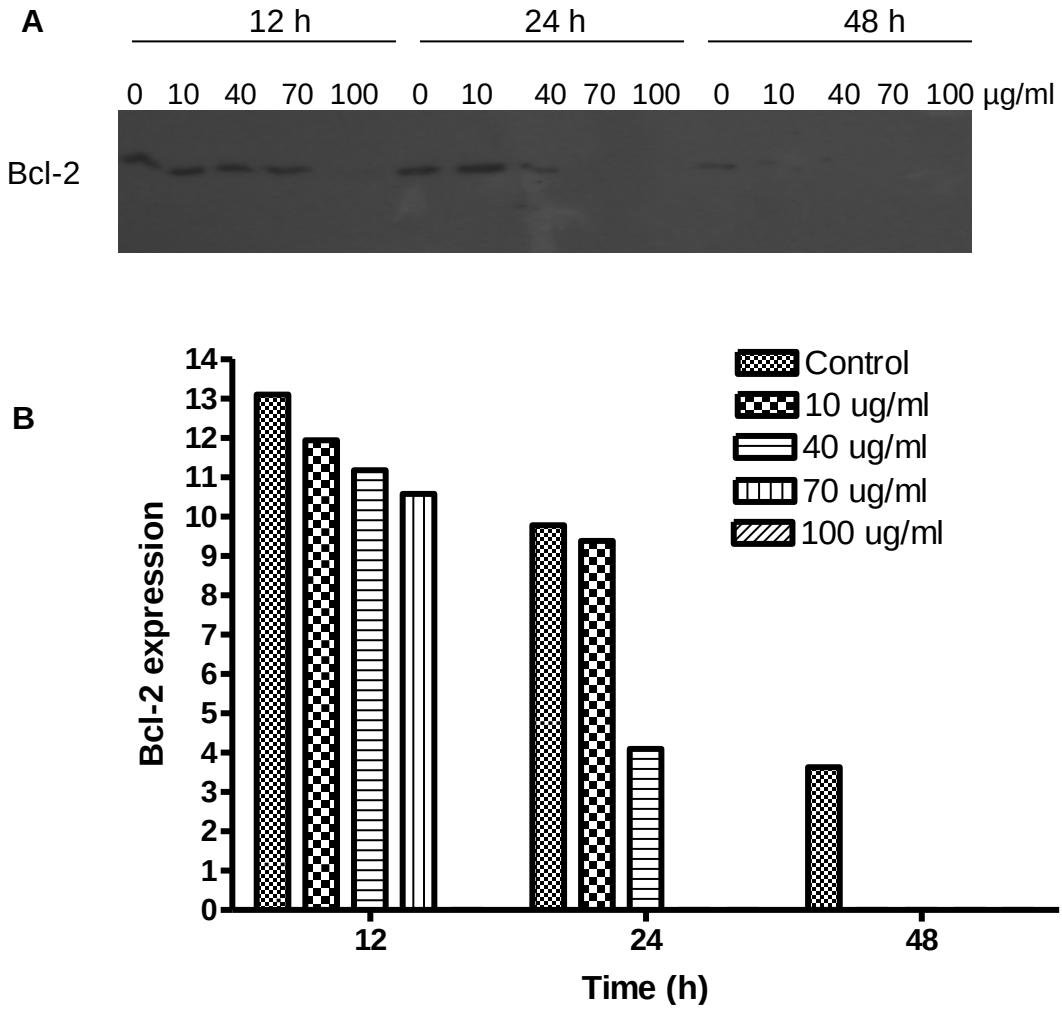

Figure 3.25. Bcl-2 expression levels of Jurkat T cells treated with various concentrations of dichloromethane/ethanol extract of *Dicerocaryum* species. **A** = Immunoblots incubated with antibody specific for Bcl-2 and **B** = densitometric quantification of Bcl-2 expression.

CHAPTER 4

DISCUSSION

Despite recent advances in the understanding of the molecular biology of cancer cells, the usage of different drugs and chemotherapy is still inefficient in the treatment of cancer. Plant-derived compounds/extracts are presently used as an alternative treatment in cancer therapy. *Dicerocaryum* species has been widely used indigenously in many parts of the African continent not only as food but also to treat various diseases like gonorrhoea, measles, as a hair shampoo and to aid in human and animal births. The plant has also been shown to possess anti-inflammatory and antioxidative activities (Madiga *et al*., 2008). Consequently, unpublished information from our laboratory showed that the plant also demonstrates anticancer properties in tissue cultures. The present study was thus aimed at using different solvents such as methanol, n-hexane, dichloromethane/ethanol, n-butanol and water to extract potential antiproliferative and apoptosis-inducing bioactive compounds from *Dicerocaryum* species for screening in Jurkat T cells. Jurkat T cells demonstrated varying degree of sensitivity to different extracts of *Dicerocaryum* species. The anti-proliferative studies using these extracts demonstrated that only the crude methanolic and dichloromethane/ethanol extracts demonstrated growth inhibitory properties against Jurkat T cells in a dose- and time-dependent manner. However, Jurkat T cells treated with n-hexane, n-butanol and water extracts showed less sensitivity to these extracts at different concentrations used. Low concentrations (10-100 µg/ml) of dichloromethane/ethanol extract significantly affected cell proliferation of Jurkat T cells; this shows that dichloromethane/ethanol extract was more potent than the other extracts.

Trypan blue dye exclusion method was used to evaluate the cytotoxic effects of crude methanolic and semi-purified (n-hexane, dichloromethane/ethanol, n-butanol and water) extracts of *Dicerocaryum* species on the viability of normal monkey Vero cells after 48 h and Jurkat T cells after 24, 48 and 72 h. Cells that excluded the dye were taken as viable when counted, using hemocytometer under the light

microscope and those that did not exclude the dye were taken as non-viable. The crude methanolic and semi-purified (n-hexane, dichloromethane/ethanol, n-butanol and water) extracts demonstrated toxicity effects in normal monkey Vero cells only at high concentrations (more than 400 µg/ml) after 48 h, thus indicating that the extracts were less toxic towards normal cells (Data not shown). The non-toxic nature of *Dicerocaryum* extracts were also demonstrated in normal human lymphocytes (Madiga *et al*., 2008). However, the crude methanolic and dichloromethane/ethanol extracts significantly decreased cell viability of Jurkat T cells in a dose-dependent manner. The viability of cells treated with n-hexane, n-butanol and water extracts were less significantly affected by the extracts even at high concentrations (Figures 3.5, 3.11 and 3.14, respectively). These observations indicated that the above extracts were less effective as they could not significantly affect the viability of Jurkat T cells even after 72 h of treatment. MTT assay was used to confirm the above cytotoxic effects and to obtain the IC_{50} values for each extract. The IC_{50} values showed that the crude methanolic and dichloromethane/ethanol extracts remarkably inhibited the viability of Jurkat T cells (Table 3.1). Dichloromethane/ethanol extract showed low IC_{50} value compared to the other extracts (Table 3.1), thus confirming that cells treated with this extract were more sensitive as compared to those treated with the crude methanolic, n-hexane, n-butanol and water extracts. Since the results obtained from cell proliferation and viability studies suggested that crude methanolic and dichloromethane/ethanol extracts were the only potent extracts, further experiments were performed to investigate their effects on cellular morphology and regulation on the expression levels of apoptotic genes and proteins.

The observed cellular cytotoxicity of Jurkat T cells in this study could have been through the induction of apoptosis or necrosis. Thus, in order to investigate the type of cell death involved, the morphological and biochemical features indicative of apoptosis were assessed. Apoptosis is a genetically regulated biological process characterized by cell shrinkage, plasma membrane blebbing, nuclear condensation, chromatin aggregation and DNA degradation (Hu and Kavanagh, 2003). The study demonstrated that Jurkat T cells treated with various concentrations of crude

methanolic and dichloromethane/ethanol extracts displayed nuclear shrinkage and chromatin condensation; all these features are indicative of cells undergoing apoptosis (Figures 3.16 and 3.17). A concentration- dependent DNA laddering was also observed in cells treated with both crude methanolic and dichloromethane/ethanol extracts. DNA ladders in cells treated with crude methanolic extract (150 µg/ml and 600 µg/ml) were observed after 24 h and increased after 48 h (Figure 3.18). Cells treated with dichloromethane/ethanol extract demonstrated DNA ladders after 24 and 48 h when treated with 70 µg/ml and 100 µg/ml of the extract (Figure 3.19). No DNA fragments were observed after 72 h and this may be due to the fact that most of the cells have died at this stage and thus no sufficient cells could be collected for analysis. Alternatively, the cells may have turned into a necrotic mode of cell death after a prolonged exposure to this toxic extract.

In order to evaluate the type of apoptotic pathway involved in Jurkat T cells treated with crude methanolic and dichloromethane/ethanol extracts, the expression levels of major apoptotic genes and proteins were investigated. The process of apoptosis is regulated by a number of genes and proteins which are targets for anticancer therapy. The p53 functions as a transcription factor regulating downstream genes and proteins involved in apoptosis. Activated p53 positively regulates the *bcl*-2 family of genes and their associated proteins. The *bcl*-2 family is classified into two groups: anti-apoptotic and pro-apoptotic. The *bcl*-2 gene encodes a 26 kDA protein that prolongs cell survival by blocking apoptosis. On, the other hand, *bax* is a pro-apoptotic gene that promotes cell death. The anti-apoptotic activity of *bcl*-2 is determined by its intracellular ratio to *bax*. RT-PCR analysis revealed a down-regulation of *bcl*-2 after 48 h for crude methanolic and dichloromethane/ethanol extracts (Figures 3.21 and 3.22, respectively). Concentrations (70 µg/ml, 100 µg/ml, 400 µg/ml and 600 µg/ml) of crude methanolic and dichloromethane/ethanol extracts resulted in a significant decrease of *bcl*-2 expression (Figures 3.21 and 3.22, respectively). The expression level of *bcl*-2 decreased in a dose- and time-dependent manner. The expression of *bax* was significantly up-regulated in Jurkat T

cells treated with both crude methanolic and dichloromethane/ethanol extracts after 48 h (Figures 3.21 and 3.22, respectively), where *bax* expression was remarkably increased with an increase in concentration for both extracts. Since the results obtained from RT-PCR analyses displayed different expression levels of *bax* and *bcl-2* genes, these observations suggest that the crude methanolic and dichloromethane/ethanol extracts exert a regulatory effect on apoptosis at a molecular level.

To further explore the regulatory effects of crude methanolic and dichloromethane/ethanol extracts on the apoptotic proteins, western blot analysis was performed on Jurkat T cells treated with various concentrations of the extracts. It is widely documented that members of the Bcl-2 family of proteins interact to regulate apoptosis. Homodimers and heterodimers of these proteins either promote or inhibit apoptosis. Indeed, these proteins may also function independently without forming heterodimers. In addition, ubiquitin/proteasome system plays an important role in the degradation of proteins involved in cellular processes such as apoptosis. Bax is mostly a target protein of the ubiquitin/proteasome pathway when compared to Bcl-2. Therefore, the ubiquitin/proteasome pathway may have a negative impact on p53-dependent apoptosis pathway through Bax degradation. In this attempt, Bax did not show any detectable levels under the current experimental conditions and this might have been due to it having been a direct target of the ubiquitin/proteasome pathway (results not shown for both extracts). The discrepancy, observed for the western blot and RT-PCR data, may be explained in terms of the *bax* gene being regulated at the level of mRNA expression rather than at the protein level. Another reason for this contradictory observation may be that there are other mechanisms such as post-translational modifications, apart from Bcl-2-to-Bax ratio, that are critical in the regulation of apoptosis in Jurkat T cells. The information presented above suggests that Jurkat T cells treated with both crude methanolic and dichloromethane/ethanol extracts undergo apoptosis independent of Bax expression.

Furthermore, examination of the expression levels of Bcl-2 protein using western blot showed a dose-dependent decrease of Bcl-2 expression in Jurkat T cells treated with crude methanolic and dichloromethane/ethanol when compared to the untreated cells (Figures 3.24 and 3.25, respectively). Cells treated with high concentrations (400 µg/ml and 600 µg/ml) of the crude methanolic extract showed a decrease in protein expression after 24 and 48 h (Figure 3.24). This clearly supports the evidence obtained from cell toxicity and morphological studies. Dichloromethane/ethanol extract also demonstrated a dose-dependent decrease in the expression levels of Bcl-2 protein after 24 and 48 h (Figure 3.25).

Several studies have suggested that inactivation of the anti-apoptotic function of Bcl-2 by phosphorylation occurs after treatment with microtubule-stabilizing anti-cancer drug paclitaxel or the phosphatase inhibitor okadaic acid (Ito et *al*., 1997). Therefore, Bcl-2 phosphorylation may be required but is not sufficient for Bcl-2's full and potent anti-apoptotic function (Ito *et al*., 1997). Post-translational modification of Bcl-2 family members may be important in the regulation of apoptosis (Hu *et al*., 1998). Our data did not give an indication of the phosphorylation of Bcl-2 protein in Jurkat T cells treated with either the crude methanolic or dichloromethane/ethanol extracts. Therefore, it can be suggested that Bcl-2 phosphorylation is not involved in the induction of apoptosis in Jurkat T cells.

In response to stress, p53 protein plays a role in the regulation of cell growth, DNA damage repair and apoptosis. p53 is a short lived protein regulated through post-translational modification including phosphorylation, acetylation and ubiquitination (Lee *et al*., 2006). Our data demonstrated that the level of p53 expression increased in cells treated with 100 µg/ml and 200 µg/ml of crude methanolic extract after 24 and 48 h (Figure 3.23) and later decreased to low undetectable levels. This might have been due to its degradation through ubiquitin/proteasome pathway. No results were obtained, under similar conditions, for cells treated with dichloromethane/ethanol extract and this might have been due to the fact that the

protein either has a short half-life under the current treatment condition or has been a target for early degradation by ubiquitin/proteasome pathway.

In conclusion, the study has demonstrated that crude methanolic and dichloromethane/ethanol extracts were cytotoxic than the other extracts and also induced apoptosis in Jurkat T cells. However, the dichloromethane/ethanol extract displayed more potency than the crude methanolic extract. Induction of apoptosis by these extracts involved a regulatory mechanism in the expression levels of apoptotic genes and proteins. The crude methanolic extract remarkably up-regulated pro-apoptotic *bax* gene and down-regulated the anti-apoptotic *bcl-2* gene. Homo- or heterodimerisation of these pro- and anti-apoptotic genes, in favour of apoptosis, is one of the suggested mechanism(s) by which the crude methanolic- and dichloromethane/ethanol extracts induce apoptosis in Jurkat T cells. In addition, Jurkat T cells treated with both extracts did not show any Bcl-2 phosphorylation, thus suggesting that Bcl-2 phosphorylation does not play a critical role in the crude methanolic and dichloromethane/ethanol-induced apoptosis in Jurkat T cells. The results obtained for p53 expression suggests that both Bax and Bcl-2 expression acted independently of p53. Taken together, these data suggest that the molecular mechanism(s) involved in the growth inhibition of Jurkat T cells involve(s) the modulation of Bcl-2 family proteins. Future studies will be aimed at determining the effect of each potent extract on the cell cycle regulatory proteins and to identify the molecular structures of putative bioactive compounds inherent in the crude methanolic and dichloromethane/ethanol extracts of *Dicerocaryum* species.

CHAPTER 5

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