CHAPTER 4

DISCUSSION

4.1 ESSENTIAL ROLE OF MEDICINAL PLANTS IN DRUG DEVELOPMENT

Medicinal plants have been widely used for the treatment of diseases in traditional medicine for several generations. By establishing an interaction between this medicine and modern biotechnological tools we can exploit the vast knowledge towards new drug development. Many of our present medicines are derived directly or indirectly from higher plants. In addition, a number of novel plant derived substances have entered into the western drug market. Plant based research has made rewarding progress in the important fields of anti-cancer (ex: taxoids and camptothecin) and anti-malarial (ex: artemisinin compounds) therapies. Natural products research continues to provide tremendous variety of lead structures, which are used as templates for the development of new drugs by the pharmaceutical industry. Sensitivity of chemical and biological techniques have greatly improved prospects for finding new drug entities and for investigating traditional medicines. Biological tests are useful for bioassay-guided fractionation of plant extracts. Breakthrough in molecular biology, cell biology and genetic engineering has given access to understanding at the molecular or the gene level. Subsequently, constructing novel target directed screening assay systems of promising therapeutic significance, automation and miniaturization resulting in high throughput screening systems will lead to target elucidation and lead finding.
4.1.1 Identification of active principle from *P. urinaria* and *A. marmelos*

A considerable proportion of people in developing countries depend on traditional medicine for their primary healthcare needs (Neto et al., 2002; Ahmad et al., 1998). Accordingly, medicinal plants are used in prenatal care, in obstetrics, in gynaecology, in respiratory disorders, in skin disorders, in cardiac diseases, in nervous and muscular disorders, and in mental health. Since little information is available on the anti-tumour activity of plant extracts, analysing variety of plants for their ability to inhibit *in vitro* cell growth of human tumour cell lines will provide information on the plants that are capable to inhibit growth. The recent approach to validate different medicinal plants using high throughput screening has resulted in identifying many new molecules. The new techniques that are constantly developed may change the face of drug discovery in the future. Interfacing traditional medicine and new assays have provided an opportune environment to pharmaceutical industries. These leads may help to develop new molecules for therapeutic purposes. In this study detailed investigations on the anti-proliferative effects of medicinal plants *Phyllanthus urinaria* which belongs to *Euphorbiaceae* and *Aegle marmelos* which belongs to *Rutaceae* obtained in South India was carried out. Several species of *Phyllanthus* have been identified and have been used in traditional practice in tropical and subtropical regions of Central and South America countries and India widely used for the treatment of jaundice, asthma, urolitic diseases (Mulchandani and Hassarajani, 1984). These plants have also been examined in detail for their ability to inhibit DNA polymerase and reverse transcriptase from Hepatitis B virus (Unander 1991). Several leads have been obtained namely Phyllanthin (Mangalan et al., 1989), Phyllanthol (Hnatyszyn and Ferraro 1985) and Nirurin (Gupta and Ahemed 1984) that have been used mainly for hepato protective activities. The small thorny tree known as beal (*Aegle marmelos*) yields a fruit that is a panacea for digestive disorders. *Aegle marmelos* belongs to *Rutaceae* family.
Bioactivity based *in vitro* screens are simple and rapid, enable to target specific molecules more rapidly than conventional animal model based studies (Workman 1997). This thesis elucidates the use of bioactivity-based screens in monitoring and identifying anti-proliferative principles from medicinal plant. The bioactivity-based screens used in this study involves measuring proliferation of cells using $[^3H]$ Thymidine (Krishnamoorthy et al., 2000) and monitoring known index of apoptosis, namely *c-myc c-H-ras* at a second level, with a view to postulate the possible mechanism by which these extracts induce cell death. Signal transduction therapeutics is new dominant theme of drug discovery and has a major impact in cancer therapeutics (Verweij 1996). It is also clear that for both growth and cell death, multiple signalling pathways are involved and hence it is essential to understand the possible cascades that are either over expressed or inhibited by a drug leading to the ultimate result that is death. (Huang et al., 1999).

The different solvent fractions of *Phyllanthus urinaria* and *Aegle marmelos* were tested on HEp-2 cells to find out their anti-proliferative potentials. In case of both the plants maximum anti-proliferative activity was found in ethyl acetate extract when compared to the other solvent extracts (Figure 3.3A and Figure 3.3B). Earlier studies in isolating anticancer principles in plants show that in most cases, fraction from dichloromethane, methanol, ethyl acetate and chloroform extractions showed anti-proliferative principles (Ren and Tang 1999). The anticancer effect of the genus Phyllanthus has only been reported in few papers. *Phyllanthus amarus* could protect the liver from hepatocarcinogenesis induced by N-nitrosodiethylamine in animal model (Jeena et al., 1999; Rajeshkumar and Kuttan 2000). Glycosides isolated from *Phyllanthus acuminatus* have been shown to exert anti-tumour activity on murine P-388 lymphocytic leukemia and B-16 melanoma cell lines.
The approach has been to exploit the knowledge of traditional medicine practiced in South India to screen plants that have been used for the treatment of cancers. The approach is to integrate chemical extracts and purification of the active molecule by determining its bioactivity on known cellular targets that induce apoptosis at different levels of purity (Figure 3.6A). By this approach we have been able to isolate and structural elucidate a lignan, 7'-hydroxy-3' ,4',5,9,9'-pentamethoxy-3,4-methylene dioxy lignan from *Phyllanthus urinaria* (Figure 3.8A) (Giridharan et al., 2002). In case of *Aegle marmelos* fractionation of ethyl acetate extract followed by column purification has enabled us to pin point on specific fractions that still exhibited anti proliferative activities (Figure 3.6B). This was isolated and analysed for further purification before analyzing the structure by mass and NMR. The structure was elucidated as 9-hydroxy-decahydro-furo [3,2-g] chromen-7-one (Figure 3.8A). The anti-proliferative activity of both these crude ethyl acetate extract and pure compound was also tested on other different tumour derived cell lines, like MCF-7, EL-1 monocytes and HeLa cells (Figure 3.9 A and 3.9 B). Many classes of naturally occurring secondary metabolites have been isolated and characterised in the plants of the genus *Phyllanthus* such as flavonoids, alkaloids, terpenes, lignans, tannins and phenols (Ueno et al., 1988; Bachmann et al., 1993).

4.2 MITOTIC ARREST INDUCED IN HEp-2 CELLS RELATING TO ENHANCED TUBULIN POLYMERIZATION AND THEIR ROLE IN ANTI-CANCER DRUG DEVELOPMENT

The essential cellular functions associated with microtubules have led to the wide use of microtubule-interfering agents in cancer chemotherapy with promising results. Microtubule-disrupting agents are thought to arrest cells in
mitosis by triggering the mitotic checkpoint, a series of biochemical reactions that ensure proper attachment of chromosomes to the mitotic spindle before cells enter anaphase (Rudner and Murray 1996), (Amon 1999) and (Shah and Cleveland 2000). Microtubules serve as an intracellular scaffold, and their unique polymerisation dynamics are critical for many cellular functions (McNally 1996).

In the present work, the effects of both crude extract and purified compounds on tubulin polymerisation was examined and the accumulation of tubulin around the periphery of the HEp-2 cells at 12 hours after the treatment was noted (Figure 3.12). This may direct the rapidly dividing cell in to mitotic arrest. It is conceivable that cytoskeletal dysfunction, manifested as either a disrupted microtubule network or a stabilised, “rigid” microtubule cytoskeleton, is an intracellular stress. In another study, they report that disruption of the equilibrium between tubulin monomer/dimers and microtubule polymers with microtubule stabilizing (paclitaxel, docetaxel) or destabilizing (vinblastine, vincristine, nocodazole, colchicine) agents activated the stress-activated protein kinase (JNK/SAPK) signalling cascade. They found that in both BR and MCF-7 cells, JNK/SAPK remained activated for up to 8 hours after treatment with Microtubule Inhibitory Agents (Tzu-Hao Wang et al., 1999). Since induction of JNK/SAPK in T-cell activation and apoptosis can occur in a transient or persistent pattern, respectively (Chen et al., 1996), the sustained activation of JNK/ SAPK following Microtubule Inhibitory Agents treatment may reflect the apoptosis-inducing nature of these drugs. A great variety of natural products are used as anticancer agents, their antimitotic activity being due to their interaction with microtubular protein. Plant-derived antimitotic drugs that interfere with the normal formation of mitotic spindles and cytoplasmic microtubules include cornigerine (Hamel et al., 1988), alkaloids such as colchicine (Margolis and
Wilson 1978), vincristine and vinblastine (Johnson et al., 1963). In the preliminary studies it was found that both the crude extract and pure compound were capable of enhancing tubulin polymerisation (Figure 3.12).

4.3 TARGETING APOPTOTIC SIGNAL SWITCHES FOR POTENTIAL ANTI-CANCER DRUG DEVELOPMENT

Apoptosis or programmed cell death (PCD), an evolutionary conserved intracellular pathway functions to remove unwanted or damaged cells involved in embryonic development, homeostasis, tumourigenesis and as a defense mechanism against pathogens. Defects in the programmed cell death mechanisms can extend cell life-span, contributing to neoplastic cell expansion independently of cell division and contribute to the development of several pathological conditions including cancer, auto-immunity and AIDS.

The majority of chemotherapeutic agents as well as radiation utilises the apoptotic pathway to induce cancer cell death. Resistance to standard chemotherapies also seems to be determined by alterations in the apoptotic pathways of cancer cells. Understanding the signals and mechanisms leading to apoptosis or resistance to apoptosis may allow the development of better chemo- or radio therapeutic regimens for the treatment of cancer and potentially the development of therapeutic agents for other diseases in which de-regulation of apoptosis is implicated.

Apoptosis is characterised by maintenance of intact cell membranes during the suicide process so as to allow adjacent cells to engulf the dying cell so that it does not release its contents and trigger a local inflammatory reaction. Cells undergoing apoptosis usually exhibit a characteristic morphology,
including fragmentation of the cell into membrane-bound apoptotic bodies, nuclear and cytoplasmic condensation and endolytic cleavage of the DNA into small oligonucleosomal fragments (Steller 1995). The cells or fragments are then phagocytosed by macrophages. In propidium iodide staining of nuclear localisation, HEp-2 cells were treated with both P. urinaria and A. marmelos crude ethyl acetate extract and pure compound for 72 hours. The treated monolayer of HEp-2 cells contained more apoptotic cells than untreated monolayer. Figure 3.13 showed characteristic nuclear fragmentation of nuclei in treated HEp-2 cells whereas the untreated control cells did not show any nuclear fragmentation. Subsequently externalisation of Phosphatidyl serine in HEp-2 cells treated with both Phyllanthus urinaria and Aegle marmelos crude extract and purified compound for 72 hours which are bound with Annexin V and they are viewed under fluorescent microscope (Vanengleand et al., 1998) (Figure 3.14). Flow cytometry analysis of the appearance of distinct sub-G1 peak was seen in cells treated either with the crude ethyl acetate extract or with the pure compound as early as 72 hours, more than 70% of cells undergo apoptosis (Figure 3.15). Since apoptotic programme can be manipulated to produce massive changes in cell death, the genes and proteins controlling apoptosis are potential targets (Giridharan et al., 2002).

4.3.1 Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death

Most immortal cell lines derived from human cancers or transformed in vitro maintain telomeres by endogenous expression of telomerase. Stable telomere length in many cancer and immortal cell lines is maintained by the endogenous expression of telomerase. Telomerase is not expressed in most normal tissues but is present in 85–90% of all human tumours (Shay and
Bacchetti 1997). Using a PCR-based telomerase assay (the telomerase repeat amplification protocol [TRAP] assay), people have found that approximately 85 to 90% of over 1,000 primary human tumours examined and 100% of tumour-derived cell lines display telomerase activity (Kim et al., 1994). Telomerase activity is specifically associated with immortal cells including cancer cells. A highly sensitive in vitro assay known as the Telomeric Repeat Amplification Protocol (TRAP) has been developed for detecting telomerase activity in cells and tissue. In the TRAP assay, viable or freshly frozen cells/tissues (with enzymatically active telomerase) are lysed and the telomerase activity in the cell extract is determined through its ability to synthesize telomeric repeats onto an oligonucleotide substrate in vitro upon the addition of the appropriate buffer conditions and dNTPs. Telomerase from the cell extract adds telomeric repeats onto a substrate oligonucleotide and the resultant extended products are subsequently amplified by the polymerase chain reaction (PCR).

Inhibition of telomerase by both the crude extract and pure compound (Figure 3.16 A and Figure 3.16 B) may be an indication by which the cells are driven to apoptotic cascade. However, whether this is due to direct effect on telomerase will need to be confirmed further, but the data on other targets of apoptosis may indicate that there could be a possible correlation. Overall, these findings would suggest that similar level of complexity is also likely to be found in human tumours and that simple screen of tumours to measure the telomerase activity alone may not be necessarily informative (Rachid et al., 2001). In other studies they report that several proteins such as p53, retinoblastoma (Rb), c-Myc, Bcl-2, protein kinase C, and protein phosphatase 2A have been shown to potentially regulate telomerase activity with transcriptional and posttranscriptional mechanisms (Wright et al., 1989). Therefore, identifying intracellular factors and their signalling pathways involved in the control of telomerase activity is extremely important for both cancer research and therapy.
4.3.2 Potential mechanisms of oncoproteins like c-myc and c-H-ras in apoptotic signalling

Apoptotic programme can be manipulated to produce massive changes in cell death; the genes and proteins controlling apoptosis are potential targets. In instances where apoptosis is disabled by protooncogenes, agents that disturb their anti-apoptotic function can produce remarkable increase in cell death. The role of c-myc in apoptosis is well known (Thompson 1995). Replicating cells are known to maintain high level of c-myc expression correlating with growth and onset of apoptosis (Evan et al, 1992). These results indicate that the both the crude ethyl acetate extract and pure compound were capable of increasing the levels of expression of c-myc (Figure 3.17), suggesting a possibility of induction of apoptosis. The induction of apoptosis in HEp2 cells after treatment with crude ethyl acetate extract and pure compound was also confirmed by PI and annexin V staining at 72 hours (Figure 3.13 and Figure 3.14). It has been shown earlier that c-myc enhance apoptosis in low concentration of survival factors or oxygen and following treatment with diverse cytotoxic agents (Graeber et al., 1996; Evan et al., 1992). On the other hand, an increased expression of c-myc can lead cells into the apoptotic direction. This has been shown in several cell lines including CHO (Wurm et al., 1986), fibroblasts (Evan et al., 1992; Wyllie et al., 1995). Also, apoptosis in T-cells can be prevented by the administration of c-Myc antisense oligonucleotides (Shi et al., 1992). Although the induction of apoptosis was studied in detail on HEp2 cells, results with other cell lines tested suggest that both crude ethyl acetate fraction and the pure compound induce cell death as early as 24 hours after treatment (Figure 3.9) suggesting that inducing apoptotic cascade may be a useful target in cancer cells for effective therapeutic measures.
Ras gene products function as binary switches by cycling between the active state, GTP bound, and the inactive state, GDP bound and are positioned at a critical check point in the cell signalling cascade. Ras plays a direct role in the control of normal and transformed cell growth is supported by several lines of evidence. First, expression of activated ras or high levels of wild type ras results in mitogenic stimulation of a variety of tumour cell lines (Stacey and Kung 1984). Second, cellular proliferation is inhibited following inactivation of ras. In these studies the suppression of c-H-ras mRNA expression was seen in HEp-2 cells treated with both crude ethyl acetate extract and pure compound of P. urinaria for 12 hours when compared to that of control. HEp2 untreated cells showed prominent levels of c-H-ras mRNA expression at 12 hours. In case of cells treated with A. marmelos no significant changes on c-H-ras mRNA expression was noted. For example, inactivation of c-K-ras by homologous recombination (Shirasawa et al., 1993), inhibition of activated c-H-ras (Monia et al., 1992, 1993; Gray et al., 1993; Schwab et al., 1994; Duroux et al., 1995; Godard et al., 1995; Liao et al., 1997) and c-K-ras by antisense strategies (Mukhopadhyay et al., 1991; Georges et al., 1993; Zhang et al., 1993; Aoki et al., 1995), inhibition of post-translational modification by farnesyl protein transferase inhibitors (FPTase) (Kohl et al., 1994) and microinjection of neutralizing antibody (Mulcahy et al., 1985; Stacey et al., 1987) all result in inhibition of cellular proliferation.

4.4 SUPPRESSION OF ANTI-APOPTOTIC bcl2 AND ACTIVATION OF PRO-APOPTOTIC BAX PROTEINS IN HEp-2 CELLS, DIRECT THE CELL TOWARDS APOPTOSIS

Apoptosis is regulated and executed by different interplay of many genes responsive to various stimuli. Lung carcinoma cells have been induced by
a variety of chemical reagents to undergo apoptosis through different pathways such as p53-dependent pathway or Bcl-2 family-related pathway (Adachi et al., 1998; Sirzen et al., 1998). To clarify the molecular mechanism of apoptosis mediated by *P. urinaria*, the expression of genes including p53, Bax and Bcl-2 were examined. Results indicated that *P. urinaria* induced apoptosis in HEp-2 cells accompanied by the down-regulation of Bcl-2 gene expression (Figure 3.25 A), while *A. marmelos* did not have any significant on Bcl-2 gene expression. It has been demonstrated that Bcl-2 family members, such as Bcl-2 itself and Bax, are mediators of apoptosis. The balance of proapoptotic Bax and antiapoptotic Bcl-2 is known to be important in determining whether cells die or survive. Bax/Bcl-2 ratio in a cell acts to regulate its own susceptibility to apoptosis (Korsmeyer et al., 1993). In the present study, the relative increase of apoptotic Bax/Bcl-2 ratio correlated well with *P. urinaria*-induced apoptosis in HEp-2 cells.

Bcl-2 proteins predominantly localise on the outer mitochondrial membrane, and mediate anti-apoptotic effect by stabilising the mitochondrial membrane, inhibiting permeability transition pore ability and the release of cytochrome c (Egan et al., 1999). In contrast, Bax proteins predominantly localise in the cytosol, and upon activation, translocate to the mitochondria and trigger the loss of mitochondria membrane potential and mediate the release of cytochrome c (Priault et al., 1999). The over-expression of bax in HEp-2 cells on treatment with both *P. urinaria* crude extract and pure compound (Figure 3.23), signifies that *P. urinaria*-induced apoptosis might, at least in part, be correlated with the loss of mitochondrial transmembrane potential. It is likely that both the mitochondria-dependent intrinsic pathway and mitochondria-independent extrinsic pathway are involved in the apoptotic
process of HEp-2 cells induced by *P. urinaria* extract. The similar results were observed with *P. urinaria* on LLC cells (Sheng et al., 2003).

### 4.4.1 Suppression of *bcl2* expression with respect to inhibition of telomerase activity in HEp-2 cells

The view that apoptosis is a tightly regulated cell self-destruction mechanism is widely accepted. Presently, many studies suggested that telomerase might be an important factor in suppressing apoptotic signalling cascades. Peptide nucleic acid and 2'O-me RNA oligomers inhibited the activity of telomerase, leading to progressive telomere shortening and inducing immortal human breast epithelial cell to undergo apoptosis with increasing frequency until no cell remains (Herbert et al., 1999). *P. urinaria* crude ethyl acetate extract and pure compound on telomerase activity in HEp-2 and EL-1 monocyte cells showed inhibition of telomerase activity (Figure 3.16A) and subsequently the downregulation of *bcl-2*. Treatment of phenochromocytoma cell with the telomerase inhibitors, such as oligodeoxynucleotide TTAGGG or 3,3'-diethyleoxadcarbocynine, enhanced mitochondria dysfunction and apoptosis induced by staurosporin, Fe^{2+}, and amyloid -B peptide was reported (Fu et al., 1999). Furthermore, inhibition of telomerase with an antisense telomerase expression vector not only decreased the telomerase activity but also increased susceptibility to cisplatin -induced apoptotic cell death in a human malignant glioblastoma cell line (Kondo et al., 1998). In addition, the stable expression of *bcl-2* was demonstrated to the result in an increased telomerase activity and decreased apoptotic cell death (Mandal and Kumar 1997). An enhanced apoptosis was associated with a decreased telomerase activity in quiescent and terminal differential cells (Bestilny et al., 1996), (Savoyksky et al., 1996). The present results also indicate that an increased apoptotic
population caused by berbamine was accompanied by a significant inhibition of telomerase activity. The results of this study is consistent with these previous reports and that telomerase may play an important role in modulation of apoptotic cell death, but whether or not a causal relationship exists between the reduction of telomerase activity and apoptosis induction in yet to be ascertained.

Apoptosis is a complex process and involves a number of gene product including the survival factor bcl2, which expression is frequently altered in human cancers. It has been reported that stable over-expression of bcl2 resulted in up-regulation of both the telomerase activity and resistance to apoptosis, indicating a link between bcl2 expression and the telomerase activity (Mandal et al., 1997). It appears that the diminished telomerase activity was accompanied by down regulation of bcl-2 gene expression. In summary, *P. urinaria* both crude extract and pure compound was capable of exhibiting both growth arrest and apoptotic cell death in HEp-2 cells. These effects may possibly associate with inhibition of the telomerase activity and down regulation of bcl2 gene expression during apoptotic process.

4.5 UPREGULATION OF p53 ACTIVITY

p53 is a tumour suppressor gene as well as a transcriptional factor that controls cell growth by the induction of apoptosis and G1 arrest. This is mainly achieved by p53-induced induction of bax and p21 (Gottlieb and Oren 1996), which then activates the caspase cascade. Immunoblot analysis of p53 in HEp-2 cells on exposure to both *P. urinaria* and *A.marmelos* crude ethyl acetate extracts and pure compound for 24 hours showed up-regulation of p53 in cells treated with *A.marmelos* crude ethyl acetate extract and pure compound.
Activation of p53 occurs in response to various stresses (Gottlieb and Oren 1996) including damage to DNA by chemical and physical agents (genotoxic stress) (Maltzman and Czyzyk 1984; Kastan et al 1991), disorders in the regulation of assembly/disassembly of microtubules (Tishler et al., 1995), the activation of oncogenes (Serrano et al., 1997) hypoxia (Graeber et al., 1996), hyperthermia (Valenzuela et al., 1997) etc. The activation of p53 results either in the arrest of the cell cycle at one of its "check points" or in the induction of apoptosis. The choice between these alternatives depends on the cell type and the nature of the stress signal. For example, while thymocytes die from rapid apoptosis caused by damage to DNA (Clarke et al., 1993; Lowe et al., 1993). The stabilisation of p53 provides for its accumulation in the nucleus where it binds to specific sequences of DNA and modulates the transcription (strengthens or weakens) of various p53-regulated genes. The activity of these genes at least partially explains the cell response to stress. Thus, the p53-dependent induction of an inhibitor of cyclin-dependent kinases p21/Waf-1 (El-Deiry et al., 1993) causes the arrest at the G1 phase of the cell cycle. Another p53-dependent factor, the nuclear protein 14-3-3-sigma (Hermeking et al., 1997), is involved in arrest of the cell cycle at the G2 phase. The p53-regulated gene bax is involved in the activation of p53-dependent apoptosis (Miyashita and Reed 1995). The mechanism by which p53 promotes apoptosis is obscure although many studies indicate that it involves induction of specific target genes such as Bax, CD95, DR5, the insulin-like growth factor-I (IGF-I) receptor and the binding protein IGF-BP3, components of the renin-angiotensin system and protein regulating angiogenesis. More recently, the NOXA, PIDD (having similarity to TNF and Fas) and P53AIPI genes have
been found to be directly activated by p53. Like Bax, the NOXA and the P53AIPI are located in the mitochondria. Finally, p53 may induce death by directly stimulating mitochondria to produce excess of highly toxic reactive oxygen species.

4.6 ACTIVATION OF INTERFERON-γ AND iNOS IN HEp-2 CELLS

Inducible NOS (iNOS) is a Ca2+-independent isoform that is expressed, upon induction by cytokines, in several cell types including macrophages, endothelial cells, and hepatocytes. Expression of the inducible nitric oxide synthase (iNOS) gene results in profound physiologic consequences. Therefore, the regulation of iNOS expression is tightly controlled. In humans, synergistic combinations of cytokines (IL-1β + TNFα + INF-γ) are important for the transcriptional regulation of iNOS gene expression (Burke et al., 1997). Together, these cytokines activate their respective signal transduction pathways, and through functional NF-κB, AP-1, and STAT1 elements located upstream of 4.7 kb, initiate the transcriptional machinery required for human iNOS expression. In addition, post-transcriptional mechanisms of regulation that alter mRNA stability and translational efficiency are important regulatory points for iNOS gene expression (Xu et al., 1998). Furthermore, numerous mechanisms within this pathway have evolved to down-regulate iNOS expression and subsequent NO production. Nitric oxide (NO) is an important signalling molecule that acts in many tissues to regulate a diverse range of physiological processes including vasodilation, neuronal function, inflammation and immune function. Nitric oxide has also been demonstrated to be involved in the regulation of apoptosis. The effects of apoptosis vary depending upon the dose of NO and the type of cell used and has
been shown to be able to both induce apoptosis and to protect from apoptosis in different cell types.

Both \textit{P. urinaria} and \textit{A. marmelos} with crude ethyl acetate extract and pure compound shown major augmentation in the expression level of IFN-\(\gamma\) (Figure 3.19 A) and subsequently the iNOS mRNA expression was greatly augmented in cells treated with both \textit{P. urinaria} and \textit{A. marmelos} crude ethyl acetate extract and pure compound. In the same time point, the untreated control HEp-2 cells does not show any expression of iNOS (Figure 3.20 A).

Interferon-\(\gamma\) (IFN-\(\gamma\)) induces the expression of the inducible nitric-oxide synthase (iNOS) and the generation of nitric oxide (NO). NO inhibits both NF-\(\kappa\)B DNA-binding property and its activation by interacting with \(O_2^*\) (superoxide) and reducing the generation of \(H_2O_2\), a potent NF-\(\kappa\)B activator and thereby interferes with TNF-\(\alpha\) dependent NF-\(\kappa\)B activation. Inhibition of NF-\(\kappa\)B nuclear translocation by NO donors directly correlates with the intracellular concentration of \(H_2O_2\) and is reversed by the addition of exogenous \(H_2O_2\). Therefore, NO-mediated disruption of NF-\(\kappa\)B activation results in the removal of anti-apoptotic/resistance signals and sensitises tumour cells to cytotoxic cytokines like TNF-\(\alpha\). In other reports they have found that induction of Inducible Nitric Oxide Synthase is an essential part of TNF-\(\alpha\) induced apoptosis in MCF-7 and other epithelial tumour cells. TNF-\(\alpha\) induced cytotoxicity is mediated by the intracellular "death domain" of the 55-kDa TNF-\(\alpha\) receptor and has been demonstrated to be coupled with induction of inducible nitric oxide synthase (iNOS), leading to generation of nitric oxide radicals (Shyu et al., 2000).
4.7 DEATH RECEPTORS INDUCED APOPTOSIS IN HEp-2 CELLS

Programmed cell death or apoptosis is a genetically controlled mechanism essential for the maintenance of tissue homeostasis, proper development and the elimination of unwanted cells. In mammalian cells, two major apoptosis pathways are proposed: the first one involves signals transduced through death receptors; the second relies on a signal from the mitochondria (Steller 1995). Both pathways are involved in an ordered activation of a set of cysteine proteases called caspases, which in turn cleave cellular substrates and result in the morphological and biochemical changes characteristic of apoptosis (Steller 1995; Wyllie et al., 1980).

In the death receptor pathway, apoptosis induction involves the engagement of a set of ligands and their corresponding receptors and then transmission of the apoptotic signal in the cytoplasm by a number of caspases (Hansen and Braithwaite 1996). There are a number of ligand-receptor systems and target caspases involved in apoptosis. These death-inducing receptors belong to the tumour necrosis factor (TNF) superfamily including Fas/APO1, TNFR, and TRAIL receptors, all of which are characterised by a cysteine-rich extracellular domain and an intracellular “death domain”. When a ligand binds to the receptor, the receptor becomes trimerized and activated. The activated receptor recruits a death domain containing adaptor and a pro-caspase 8 molecule through protein-protein interactions to form a death-inducing signalling complex (DISC) (Boldin et al., 1996). FADD is directly recruited into the DISC during Fas-mediated apoptosis, while TNF-induced apoptosis involves both FADD and TRADD (Huang et al., 1996). Recruitment of pro caspase 8 to the DISC activates caspase 8 by oligomerization-induced
autocatalytic processing (Chinnaiyan et al., 1995), which results in activation of downstream caspases, such as caspases 3, 6, and 7, and induction of apoptosis (Huang et al., 1996). TRAIL, a recently identified member of the TNF family, has been shown to induce apoptosis in a variety of transformed or tumour cells but not normal cells (Hsu et al., 1995). In this study with HEp2 cells treatment with *A. marmelos* crude ethyl acetate extract and pure compound showed elevated levels of TNFRp55 (TNFRI) and TRADD, Fas and caspase 8 (Figure 3.24). These results indicate that *A. marmelos* was capable of inducing apoptosis by elevating TNF-α expression, which in turn activate TNFRI, and recruit TRADD and activate the Caspase 8. Further in the immunoblot analysis the activation of TRADD at protein level was also confirmed. Death Receptors—cell surface receptors that transmit apoptosis signals initiated by specific “death ligands”—play a central role in apoptosis. These receptors can activate death caspases within seconds of ligand binding, causing an apoptotic demise of the cell within hours. Death receptors belong to the tumour necrosis factor (TNF) receptor gene superfamily, TNF trimerises TNFRI upon binding, inducing association of the receptors’ death domains. Subsequently an adapter termed TRADD (TNF-associated death domain) binds through its own death domain to the clustered receptors death domains, TRADD functions as a platform adapter that recruits several signalling molecules to the activated receptor (Wallach et al., 1999).

### 4.7.1 Role of NF-κB in TNF-α induced apoptosis

Apoptosis or programmed cell death is applied to a group of characteristic structural and molecular events that separate this type of cell deletion from necrosis TNF-α is one of the prime signals that induces apoptosis
in a host of cells. TNF-α also activates the transcription factor NF-κB. It is not clear whether the TNF-α induced apoptosis and activation of NF-κB are linked. In 1996 issue of Science, three groups report that these effects of TNF-α are linked. NF-κB plays a protective role in the apoptosis induced by TNF-α (Wang et al., 1996, Antwerp et al., 1996, Beg and Baltimore, 1996). The activation of the transcription factor NF-κB (nuclear factor-kappa B) by TNF (tumour necrosis factor), ionizing radiation, or certain chemotherapeutic compounds has been found to protect cells from apoptosis. In addition, when NF-κB is inhibited, apoptotic killing by these reagents is enhanced; this, however, does not occur with apoptotic stimuli that do not activate NF-κB (Wang et al., 1996).

The most common forms of the NF-κB is a 50 kDa heterodimer protein (p50) and a 65 kDa protein which is also called RelA or p65. These investigators showed that the protective effect of the NF-κB is mediated by NF-κB subunits. Treatment of the RelA deficient mouse fibroblasts (Rel A−/−) and macrophages with TNF-alpha resulted in apoptosis of a significant number of cells whereas the RelA (+/+ ) cells were spared from this effect of TNF-α (Wang et al., 1998). In our studies on HEp-2 cells treatment with A. marmelos crude extract and pure compound, suppressed the LPS induced NF-kappa B activation (Figure 3.21). Reintroduction of the RelA into the Rel A−/− promoted survival of the cells after treatment with TNF-alpha (Beg and Baltimore, 1996). Expression of p65 and p55 also conferred protection against apoptosis induced by TNF-α (Wang et al., 1996). The apoptosis inducing effect of TNF-α was also enhanced by a proteasome inhibitor, MG132 (Z-Leu-Leu-Leu-H), that inhibits the NF-κB (Wang et al., 1996) and by dominant-negative I-κBα (Antwerp et al., 1996). On the other hand, the apoptosis induced by a
combination of TNF-α and cyclohexamide was inhibited by IL-1 that is a strong activator of NF-κB (Wang et al, 1996). Taken together, the findings show that NF-κB protects against the apoptosis induced by TNF-α. This insight would be useful for devising strategies in the treatment of cancer and inflammatory responses that involve TNF-α.

Resistance to cancer therapies appears to be mediated by resistance to apoptosis, and thus stimuli are less effective due to the simultaneous activation of NF-κB. Therefore, new approaches to cancer therapy that inhibit nuclear translocation of NF-κB may prove to be highly effective in the treatment of tumours. Such new approaches include: gene therapy that includes a super-repressor vector and use of agents that block NF-κB function; glucocorticoids (which act to inhibit NF-κB) in combination with other chemotherapeutic agents to reduce the anti-apoptopic threshold of tumours. The inhibition of NF-κB in conjunction with TNF treatment may aid cytokine function in fighting cancer.

4.8 ACTIVATION OF CASPASE 8 AND CASPASE 3 IN HEp-2 CELLS

Two main starting points of apoptosis are known; these are controlled by death receptors, mitochondria and, as published recently, the endoplasmic reticulum (Nagata 2000). Although much effort has been made concerning death receptor signalling as well as initiation of the apoptotic cascade by mitochondria, still more investigation has to be made to elucidate the precise mode of action of these different pathways to death. Furthermore, there is an ongoing debate about potential cross-talk of different pathways. Nevertheless,
Nevertheless, parallel mechanisms of induction, execution and regulation of differentially triggered pathways are likely to exist.

Although the initiating events in apoptosis are diverse and might involve cell specific characteristics, it is likely that all signals converge into a common final pathway (King and Cidlowski 1995). These final execution events in apoptosis are performed through a cascade of proteases, which proteolytically cleave proteins involved in the maintenance of cellular integrity and homeostasis (Patel et al., 1996). These proteases, named caspases, belong to the family of cysteine proteases (Solary et al., 1998). The caspases can be divided into two categories according to their role in apoptosis: the initiator caspases, for example caspase-8, and executor caspases, for example, caspase-3, and −6 (Patel et al., 1996).

Caspases are synthesised as proenzymes and remain inactive in most healthy cells. Upon activation by different death signals, the single-chain procaspases are cleaved, and the resulting activated subunits assemble into a heterotetramer to form the active protease (Thornberry and Lazebnik 1998, Song and Steller 1999). Caspases are very specific proteases cleaving only after a specific aspartic acid residue (Thornberry and Lazebnik 1998). The targets of apoptotic proteolysis include for example poly (ADPribose) polymerase (PARP), laminin B1, α-fodrin, β-actin, I CAD (inhibitor of caspase-activated deoxyribonuclease), and proteins involved in DNA repair, mRNA splicing and DNA replication. Their proteolysis contribute to the characteristic apoptotic morphology and DNA fragmentation, which is further culminated in the formation and engulfment of apoptotic bodies containing the remainders of the apoptotic cell (Thornberry and Lazebnik 1998, Kinloch et al., 1999). Immunoblot analysis of Caspase-8 and Caspase-3 in HEp-2 cells treated with...
both *P. urinaria* and *A. marmelos* crude ethyl acetate extract and pure compound for 36 hours showed activation of Caspase-8 in cells treated with both *P. urinaria* and *A. marmelos* crude ethyl acetate extract and pure compound (Figure 3.26 A) which in turn cleave the pro caspase-3 (32kDa) into active Caspase-3 (17 kDa) (Figure 3.26 B). These data which is also further supported by our RPA analysis where the activation of Caspase-8 at message (mRNA) level was noted. Caspase-8 is the key initiator caspase in the death-receptor pathway (Martamuzio et al., 1998). Upon ligand binding death receptors such as CD95, TNFR1 form membrane complexes that recruit, through adapter proteins, several molecules of procaspase-8, resulting in a high local concentration of the zymogen. Under these crowded conditions, the low intrinsic protease activity of procaspase-8 is sufficient to allow the various proenzyme molecules to mutually cleave and activate each other (Medema et al., 1997). It is likely that caspases 8 and 10 are the apical protease in TNF and anti-fas induced apoptosis, which activate the downstream caspases (Stadheim et al., 2002). It is clear from these results that the above compound induces apoptosis by *bcl2* suppression and activation of caspases (Figure 3.25). Earlier studies have suggested that suppression of *bcl2* activity in cells and activation of caspases 3 and 8 is strictly associated with apoptosis (Kang et al., 2001; Stegh and Peter 2001).

4.9 CONCLUSION

The main objective of this project is to exploit the knowledge that is available in traditional medicine, especially in the use of natural products, leading to the development of value added new drug entities. In this study the aim was to investigate the biological properties of traditional Indian medicinal plants leading to the mechanism of their action, identification of active components and
eventually development of new drugs which can be promoted either as an alternate or complementary medicines.

Bioassay-guided fractionation of *P. urinaria* has enabled to obtain a pure compound with proven anti-cancer activity. Its molecular structure was elucidated as 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylenedioxy lignan. There studies highlight the ability of integrating ethanobotanical leads aided with chemical isolation techniques, and studying various aspects of apoptotic cell signalling cascades to isolate molecules with potent anti-cancer activity. This study also indicates that the pure compound and crude ethyl acetate extract induces apoptosis in a broad spectrum of cancerous cell lines and block known anti-apoptotic (*bcl-2*) and stimulate proapoptotic (*bax*) cascades. Inhibition of telomerase activity, enhanced polymerisation of tubulin, activation of IFN-γ and iNOS were observed. The activation of pro caspase 8 which in turn activates Caspase 3, whose significance in apoptosis is well known was also monitored.

From *A. marmelos*, an anti-proliferative agent, 9-hydroxyl-7H-furo-[3,2,9] [1]-benzopyran-7-one was isolated and by using simple *in vitro* screens, it was found to exhibit anti-proliferative activity in a wide range of cancerous cell lines. The schematic events in apoptosis were analysed in detail, using a HEp-2 cell line as a model. In this study both pure compound and crude ethyl acetate extract were capable of inducing apoptosis through inhibition of telomerase, enhanced polymerization of tubulin, p53 activation, activation of IFN-γ and iNOS, altogether ensues in the suppression of NF-κB. Activation of TNFα-TNFR1, in turn activates TRADD, caspase 8 and caspase 3. These communal actions drive the cell in concert, into the apoptotic phase.
Apoptosis or programmed cell death is a highly organized physiological mechanism to destroy injured or abnormal cells. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The life span of both normal and cancer cells is significantly affected by the rate of apoptosis. Thus modulating apoptosis may be useful in the management and therapy or prevention of cancer. The bioactivity based screens used in this study involves measuring proliferation of cells using $[^3H]$ Thymidine and monitoring known index of apoptosis, namely $P53$, $Bcl2$ and Caspase 3 at a secondary level, with a view to postulate the possible mechanism by which these extracts induce cell death. These studies highlight the efficiency of blending old and new knowledge, such that both the forms of medicine are benefited by the new developments of cell signalling therapeutics. The integration of chemistry and signal biology will help to not only isolate novel molecules, but also understood the mechanistic operations of these molecules in specific cell signalling targets. Signal transduction therapeutics is a new dominant theme of drug discovery and has a major impact in cancer therapeutics and has heralded a new way of approach in the cancer chemotherapy.