CHAPTER 4

DISCUSSION

4.1  NATURAL PRODUCTS AS LEADS TO ANTICANCER DRUGS

Cancer like many other chronic afflictions is an ancient disease, which, despite all the progress in treatment and prevention, remains a powerful threat. Furthermore once cancers become metastasis, it is difficult to cure even the most common tumors, such as those of epithelial origin. Treating cancer remains one of medicine’s most difficult challenges. After a century of pill-and-scalpel medicine, researchers and physicians have discovered that fighting disease and restoring health are not the same. (Weinberg 1996, Vats and Emami 1993, Nebert 2002).

Currently, the therapies applied to treat cancer include surgery, radiation, chemotherapy, hormone therapy, biological therapy, and targeted therapy. However each therapy has its own disadvantages like reoccurrence, adverse side effects, pain, killing of adjacent normal cells etc (Lenhard et al 2001, DeVita et al 2005). Although surgery and radiation treatment is commonly used for a wide range of cancer types, chemotherapy is rapidly gaining momentum as an alternative in treating few cancer types and as combined therapy in most cases. Till date, in many cases, chemotherapy is choice of treatment given in combination with surgery or irradiation.

Chemotherapeutic agents generally interact with a specific target, causing dysfunction and injury, which is then interpreted by susceptible
cancer cells as an instruction to undergo apoptosis. An ideal chemotherapeutic agent should selectively target and kill the cancer cells, leaving the normal cells intact. Despite significant progress has been made in cancer chemotherapy, current drugs are ineffective against many common cancers and are often very toxic and hence there is a need for search of potential drug to cure majority of cancer types without any clinical limitations. The discovery of new drugs effective against resistant solid tumors, such as breast and prostate cancers, is also an important and necessary strategy in improving chemotherapy. With the advent of molecular targeted therapies, there has been a concomitant revolution in the process of anticancer drug development. The identification of novel targets and the development of drugs with greater selectivity towards cancer cells represent the primary goals of cancer therapy research.

Natural products have been used in traditional and folk medicine for therapeutic purposes. They provide one of the most important sources of promising leads for the development of novel therapeutics. Natural products include thousands of compounds that exist in fruits, vegetables, plants, and marine organisms, and several clinical antitumor drugs, such as Taxol, Vinblastine have been derived from such sources (Rates 2001; Simmonds 2003; Lee et al 2003; Smith-warner et al 2003). Within the sphere of cancer, a number of important new commercialised drugs have been obtained from natural sources particularly plants, by structural modification of natural compounds, or by the synthesis of new compounds, designed following a natural compound as model. This is further substantiated from the fact that over 50% of the anticancer drugs commercially available at present are derived from plants. It is thus considered important to screen apoptotic inducers from vast natural sources, especially plants, either in the form of crude extracts or as components isolated from them using various separation techniques (Dahanakur et al 2000; Takur Et al 1989). Most chemotherapeutic
agents isolated from natural sources till date comprise a diverse group of compounds with different mechanisms of action, but their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention (fulda 2005; Huang et al 2004; John Stone et al 2002; Reddy et al 2003, Souza Fagudes et al 2003). Understanding the modes of action of these compounds should provide useful information for their possible application in cancer therapy.

4.2 IDENTIFICATION OF ACTIVE EXTRACT AND LEAD MOLECULES FROM MEDICINAL PLANTS USING IN VITRO BIOASSAY GUIDED FRACTIONATION

Previously, anticancer drug candidates were identified by random screening and preclinical testing that relied heavily on murine tumours and the establishment of a maximum tolerated dose (MTD) is considered as the end point. With the advent of the era of molecularly targeted therapies in the last decade, the anticancer drug development process has undergone a steady and concomitant revolution (Rehman et al 2001). In contemporary anticancer drug development, the molecular target is first validated using a battery of molecular biology techniques and whole system approaches and then high-throughput screening is exploited to identify lead molecules and pharmacology is incorporated early on in the drug discovery cycle (Sawyers 2002).

Recent scientific research in the fields of medicinal chemistry and pharmacology has been increasingly addressed to the isolation and characterization of the active principle from plant extract for the production of chemotherapeutic agents comprised of single compound with multiple targets. Many compounds with well-defined biological and pharmacological activities have been isolated and structurally characterized from known medicinal
plants. In order to achieve the task, a multi disciplinary approach to drug discovery is needed, which is accomplished by a combination of ethnobotanical method and optimized modern methods and techniques from molecular biology, cell biology, molecular pharmacology and combinatorial (medicinal and analytical) chemistry. Recently, the state of the art technologies involving molecular genetics, computer modeling, and structural analysis have been leveraged to optimize the chances of success and minimize the time required.

The drug discovery approach followed in the present study is based upon the most current tenets of drug discovery used by major pharmaceutical companies in the screening of plant material and subsequent fractionation (Harvey 2000). This is commonly referred as bioassay-directed fractionation which primarily employs in vitro cell-based assays and can be divided into two groups: cellular assays and mechanism-based assays.

Cellular assays can also be divided into cytotoxicity assays and other assay types that include morphological assays. The simplest cytotoxicity assay is to measure the concentration of sample needed to inhibit cell growth by 50% against a single cell line. The major advantage of cytotoxicity assay is that all potential mechanisms concerning cellular proliferation are monitored simultaneously. In the present study, radiolabeled thymidine incorporation assay was carried out for measuring the cytotoxicity in cells in response to plant extracts. Apoptosis can be detected morphologically, by fluorescence microscopy using fluorochrome stains such as Propidium iodide and Hoechst to demonstrate condensed DNA apoptotic features in dying cells.

Mechanism-based assays look for activity using isolated systems such as enzymes, receptors, etc. or simply the molecular markers that play a significant role in the signaling cascade of tumor induction and progression.
Due to their selectivity and sensitivity combined with good reproducibility and high sample throughput, these assays are preferred over other assays and animal models. Utilization of these assay systems and monitoring the aftermath effects should help in the identification of unique natural products that can be compared with known standard clinical drugs currently available for treating tumors. Since most of the clinically effective antitumor drugs belong to a small number of mechanistic classes, this approach seems reliable, reproducible, time saving, safe, and appropriate.

The premise of the present study is to identify novel derivatives with potential anti-cancer property from various plants obtained from South Indian traditional healers. In order to evaluate the anti-proliferative effect of solvent extracts of ethyl acetate extract and MDPD from *Piper longum, Cissus quadrangularis* and *Ficus racemosa*, a simple, reproducible and non-complicated bioactivity based *in vitro* screening assay using thymidine incorporation into the cells was carried out. Previously it was shown that in most cases, plant extracts of ethyl acetate and methanol were used to isolate anti-cancer principles (Giridharan et al 2002; Ren and Tang 1999). Thymidine incorporation assay is considered as one of the best method to evaluate the proliferation of cells (Krishnamoorthy et al 2000). This assay utilises the incorporation of $[^3]H$-labelled thymidine into newly synthesized DNA. Radioactivity is used as an index of cells progressing through the S phase of the cell, and thus an index of cell growth. Reduction in the uptake of thymidine in the presence of plant extract can be directly correlated to the cytotoxic/ antiproliferative nature of corresponding plant (Giridharan et al 2002).

The results of $3H$ thymidine incorporation assay with solvent extracts of three plant varieties clearly indicated that ethyl acetate extract of *Piper longum* possess more inhibitory effect on MDA-MB-231 cells and PC-3
cell than the extracts of other plants (Figures 3.2, 3.3 and 3.4). However the difference in inhibition between ethyl acetate extract of *Piper longum* and methanolic extract of *Piper longum* and extracts of *Cissus quadrangularis* was marginal. The significant reduction in viability of cells upon treatment with extracts from *Piper longum* can be well correlated with previous studies, where inhibition of angiogenesis, the process which is essentially important in cancer growth and progression, by *Piper longum* methanolic extract was reported and ethyl acetate extract of *Piper longum* exhibiting antiproliferative activity in cells. Though no direct studies on the plant *Cissus quadrangularis* for anti cancer activity have been reported, one of its major constituent, B-sitosterol has been shown to inhibit cancer cell growth and induce apoptosis. Interestingly, *Ficus racemosa*, which inhibited the MDA-MB-231 and PC-3 cells at a higher dose, induced cell death in highly resistant leukemic K 562 cells at a lower concentration when compared to the extracts of other plants taken up for the study. Further studies regarding this may reveal the reason behind this property.

Time course and dose response study showed that 25 µg/ml of *Piper longum* ethyl acetate extract is good enough to inhibit around 50% of the breast adenocarcinoma and prostate adenocarcinoma cells *in vitro* (Figure 3.7).

*Piper longum* Linn, an important medicinal plant, is used in traditional Indian medicine and as a component of an ayurvedic mixture, Trikatukam. *Piper longum* is also an ingredient of medicines reported as good remedy for treating tuberculosis, respiratory tract infections, chronic gut related pain, and arthritic conditions. Piperine was the first amide isolated from *Piper* species and was reported to display anti-inflammatory activity.
Although the pharmacological role of *Piper longum* in the prevention and treatment of cancer has been a subject of increasing attention, little is known concerning the molecular mechanisms of action of *Piper longum*. In the present study, the effect of ethyl acetate extract and active molecule from *Piper longum* fruit on the viability and induction of apoptosis in highly invasive, ER negative breast adenocarcinoma and invasive, hormone refractory prostate adenocarcinoma cells were investigated.

As mentioned earlier, *Piper longum* ethyl acetate extract showed maximum antiproliferative activity based on the Thymidine incorporation assay, and was chosen for elution of components and purification studies, a process followed generally in drug discovery system (Giridharan et al 2002). The TLC profile of ethyl acetate extract of *Piper longum* demonstrated that the extract contain a mixture of compounds highlighted using UV rays and detection system. Fraction 3 of *Piper longum* showed maximum anti-proliferative effect on MDA-MB-231 cells with the concentration of 25 µg/ml at 36 hours. Therefore, Fraction 3 was further purified to get single active molecule. Thymidine incorporation assay revealed that 25 µg/ml of purified compound from *Piper longum* was capable of inhibiting more than 50% of the MDA-MB-231 cells at 36 hours incubation (Figure 3.11).

With the concomitant exploitation of structural chemistry using NMR spectroscopy and Mass Spectroscopy, the active molecule optimized was found to have the commonly seen skeletal structure of *Piper species* constituents (Methylenedioxy ring) except for the side chain moiety and the chemical name of pure molecule was 5-(3,4-methylenedioxy phenyl) do-1-deca-1-one (MDPD) and shown in Figure 3.7. Piperic acid, another constituent from *Piper longum* with a resembling structure has already been reported to have anti-inflammatory and anti-cancer properties. Anti-proliferative study performed in MDA-MB-231 cells and PC-3 cells with
crude extract and pure compound of *Piper longum* showed that these treatments were able to inhibit the growth of these cell lines but at different time intervals (Figure 3.11). One of the explanations of the process is that a particular active molecule can disturb the cell growth in different cell lines at different time intervals (Giridharan et al. 2002).

### 4.3 LACTATE DEHYDROGENASE RELEASE ASSAY IN MDA-MB-231 AND PC-3 CELLS

Evidence has emerged from various studies that cell death can either be the consequence of a passive, degenerative process, or the consequence of an active process. The former type of cell death is termed necrosis, the latter apoptosis. It was previously reported that lactate dehydrogenase (LDH), a cytosolic protein, is most likely to be released on cell lysis e.g. during necrosis (Renz et al. 2001). The present study has clearly shown that the ethyl acetate crude extract and pure compound (MDPD) of *Piper longum* are non-toxic on MDA-MB-231 and PC-3 cells (Figure 3.12). About 10% cytotoxicity at 36 hours and about 16% at 24 hours indicated that the maximum cell death assessed by anti-proliferative study in MDA-MB-231 at 36 hours and PC-3 at 24 hours with *Piper longum* were due to apoptosis and not due to necrosis. Previously it has been reported that 20% cytotoxicity can be considered as non-toxic to the cells (Renz et al. 2001).

### 4.4 INDUCTION OF APOPTOTIC FEATURES IN MDA-MB-231 CELLS AND PC-3 CELLS BY *Piper longum*

In malignant tumors, the balance between proliferation and cell death is lost, and defects in apoptosis mechanisms allow neoplastic cells to survive beyond normal levels of stress. Several studies proved the defect in apoptosis as one of the main reasons in the initiation and progression of tumor
In recent years, several strategies targeting pro or anti-apoptotic factors have shown promise in sensitizing tumor cells to the cytotoxic actions of traditional cancer chemotherapeutic drugs (Kaufmann and Earnshaw 2000). Moreover, compelling evidence suggests that the tumorigenic growth of the breast and prostate depends on the evasion of normal homeostatic control mechanisms, because of an increase in cell proliferation and a decrease in apoptotic death. Physiological cell death is characterized by typical apoptotic morphology, including chromatin condensation, membrane blebbing, internucleosomal degradation of DNA, and characteristic apoptotic body formation (Rodriguez and Schaper 2005). Treatment of MDA-MB-231 cells and PC-3 cells demonstrated that crude extracts and MDPD from *Piper longum* induced endonucleolytic DNA fragmentation and hence the characteristic “ladder” pattern, in a time-dependent manner in both cells, analyzed using agarose gel electrophoresis.

Being apoptosis a very common phenomenon during the response of cells to chemotherapeutic drugs in the course of cancer therapy, its quantitative evaluation represents an issue of considerable relevance. Flow cytometry is the choice technique for quantitation of apoptosis, particularly in cell culture experiments. Fixation of cells with precipitating fixatives (such as ethanol or acetone) causes the leakage of the cleaved low molecular weight DNA fragments that are produced during apoptosis. As a consequence, apoptotic cells can be identified as a hypodiploid peak, while healthy cells generate a typical cell cycle histogram. Flowcytometric analysis of *Piper longum* treated cells stained with the fluorescent dye Propidium iodide showed an increased accumulation of cells in the sub-G1 phase (hypodiploid cells), a substantiation of apoptotic cell death. Moreover, treatment of crude extract and MDPD from *Piper longum* in MDA-MB-231 cells at early hours but not in PC-3 cells, exhibited an augmentation of cells in the G2/M phase. Cell cycle arrest is one of the targets of many anticancer drugs, such as
doxorubicin, cisplatin, 5-fluorouracil, and paclitaxel. It has been shown that the ability of cells to arrest cell cycle in G2/M or S phase was related to their drug sensitivity.

There is no clear cut parameter that allows the separation by flow cytometry of necrotic from apoptotic cells, particularly at their late stages. On the contrary, such a distinction is immediate by morphological techniques. The intact membrane of living cells excludes cationic dyes, such as Propidium iodide or trypan blue. Due to their extensive membrane damage, cells in the late apoptotic stage are quickly stained by short incubations with propidium iodide. The most recognizable morphological feature of an apoptotic cell when viewed by light or fluorescent or electron microscopy is the condensation and aggregation of the nuclear chromatin into dense masses beneath the nuclear membrane. MDA-MB-231 cells and PC-3 cells, incubated with ethyl acetate extract and MDPD from *Piper longum* ethyl acetate crude extract and active lead for 36 h and 24 h respectively, exhibited significant morphological changes and chromosomal condensation, which is an indicative of apoptotic cell death.

Taken together, the results obtained from this experimental setup clearly reveals the breast and prostate cancer cells undergoes apoptosis upon treatment with *Piper longum* and its constituent.

4.5 DECIPHERING THE APOPTOTIC SIGNALLING CASCADE INDUCED BY *Piper longum*

Apoptosis is frequently impaired in many human tumours and modulation of apoptosis by targeting various proteins involved in the machinery is proved to be an ideal way of treating cancer. Several inhibitors of intracellular antiapoptotic proteins or activators of proapoptotic proteins
have attracted efforts to develop them for pharmaceutical application considering the fact that these proteins play a key role in cell survival and cell death by modulating signaling pathways.

4.5.1 Interaction between Apoptotic Regulatory Proteins Bcl-2 and Bax aids in Instigating Apoptotic Cascade in Breast and Prostate Cancer Cells in Response to Piper longum

Bcl-2 family proteins are key regulators of programmed cell death or apoptosis that is implicated in cancer. Bcl-2 family includes both death antagonists such as Bcl-2, which suppress cell death, and death agonists such as Bax, which promote cell death. Bcl-2 function has been extensively studied in relation to its regulation of permeability transition in mitochondria. The inhibition of mitochondrial membrane permeability (MMP), which avoids cytochrome c release, contributes to the anti-apoptotic functions of the Bcl-2 protein localized in the mitochondrial external membrane (Kluck et al 1997). In hormone refractory prostate cancer cell lines, over-expression of Bcl-2 induces resistance to androgen depletion, as well as many other stimuli of apoptosis. Similarly, in breast malignancies, bcl-2 confers broad-spectrum resistance to cancer chemotherapeutics by preventing the drug from reaching meaningful concentrations at its target or blocking the death signal that occurs following an effective drug target interaction. Down-regulation of Bcl-2 expression with gene-specific antisense oligonucleotides, abolish the resistance conferred by Bcl-2 to the induction of apoptosis by etoposide or androgen depletion in PC-3 cells. Hence any stimuli which have a suppression effect on Bcl-2 expression would contribute to the chemotherapeutic cure in cancer cells.

In light of these trends and developments, results from the present study, clearly demonstrate that Bcl-2 gene and protein expression was down
regulated in cells treated with ethyl acetate extract and MDPD from *Piper longum*. RT-PCR analysis done at various time intervals revealed that Bcl-2 mRNA expression was down regulated in MDA-MB-231 cells (at 6, 12 and 24 hours) and PC-3 cells (at 6 and 12 hours) incubated with ethyl acetate crude extract and MDPD from *Piper longum*. Also a similar decline in the Bcl-2 protein level (at 18 and 24 hours) was observed in Western blot analysis when the breast cancer cells and prostate cancer cells were treated with ethyl acetate extract and MDPD from *Piper longum*. Many natural products have been suggested to antagonize the anti-apoptotic function of Bcl-2 or Bcl-xL directly or indirectly. Moreover independent studies reveal that loss of bcl-2 expression has been related to high rates of apoptosis in invasive breast cancer and prostate cancer.

Previous studies have shown that pro-apoptotic Bax deficiency facilitates tumorigenesis, thus highlighting the importance of pro-apoptotic family of proteins in maintaining tissue homeostasis and resistance to oncogenic transformation. In addition, Bax also represent attractive target for anticancer therapy, as elevated expression in several experimental conditions has been demonstrated to increase sensitivity to both chemotherapy and radiotherapy as well as trigger spontaneous apoptosis (Deng et al 2006; Zhang et al 2005). Upon stimulation by various death insults, Bax undergoes conformational changes and subsequently translocate from the cytoplasm to the outer mitochondrial membrane, where they oligomerize to form a porelike structure, before releasing cytochrome c. Recent reports persistently assert that overexpression of Bax alone is sufficient to induce apoptosis in cultured androgen-independent prostate cancer cells, and to reduce prostate tumor size in animal models. Similarly in metastatic breast cancers, radiation and DNA damaging agents are capable of activating the Bax.

Hence the role of Bax in regulating the apoptotic induction was investigated and the results suggest that there was an undisputed increase in
the Bax expression at gene as well as protein level p53 mutated MDA-MB-231 cells and p53 null PC-3 cells in response to ethyl acetate crude extract and MDPD from Piper longum. In mitochondria mediated apoptosis, antitumor isolates from natural compounds initiates apoptotic machinery through the activation of p53, which then stimulate Bax. But in p53 null cells, antineoplastic drugs exert their action by directly targeting Bax or Bcl-2 and induce apoptosis in p53 independent manner.

It has been suggested that the protein - protein interactions involving Bcl-2 family proteins are critical for their biological functions. Imbalances in the ratio of antiapoptotic and proapoptotic Bcl-2 proteins may tip the balance in favor of tumor cell survival instead of cell death and have been shown to drastically alter apoptosis in response to several stimuli in a number of experimental systems. The above observations from the present study reveal that crude ethyl acetate extract and MDPD from Piper longum induced apoptosis in MDA-MB-231 cells and PC-3 cells correlated with the increased expression of Bax simultaneously inhibitng Bcl-2 expression suggesting a positive Bax: Bcl-2 ratio in favour of apoptosis induction.

4.5.2 Involvement of Cytochrome c as Mediators in the Induction of Apoptosis in Piper longum Treated Cells

Many conventional anticancer drugs, such as etoposide, doxorubicin and cisplatin, directly or indirectly exploit mitochondria, to exert their cytotoxic action, via multiple activation pathways. Mitochondria generally play a proapoptotic role in most models systems, evoking different mechanisms including ROS production, and through permeability transition pore opening which leads to collapse of mitochondrial membrane potential, resulting in the rapid release of cytochrome c into the cytoplasm. Among the postulated mechanisms, the cytochrome c release is most prominent, which then binds to Apaf-1 and ATP to form a high–molecular mass cytoplasmic
complex referred to as the apoptosome (Kim et al 2002; Sancho et al 2003). This complex then activates caspase -3 through caspase-9, culminating in cell death. Accumulating evidences demonstrate that mitochondria now appear as reservoirs of potential targets for anti-cancer therapy and various approaches to interfere with the vital mitochondrial functions in cancer cells have been proposed.

Western blot analysis clearly indicates increase in the protein levels of cytosolic cytochrome c in MDA-MB-231 cells and PC-3 cells upon treatment with ethyl acetate extract and MDPD from *Piper longum* extracts suggesting the involvement of cytochrome c as a consequence of increased Bax: Bcl-2 ratio, that was witnessed earlier, in the ethyl acetate extract and MDPD from *Piper longum* induced cell death. Generally, in mitochondria, cytochrome c plays an essential role in generation of mitochondrial transmembrane potential (ΔΨm).

### 4.5.3 Drug-induced Variation of the Mitochondrial Membrane Potential

Mitochondrial membrane potentials (ΔΨm) reflect the functional status of mitochondria within cells. Flow cytometry was used to measure mitochondrial membrane potential after staining the cells with cationic lipophilic green fluorochrome dye, rhodamine-123. In drug treated cells, disruption of ΔΨm is associated with a lack of rhodamine123 retention and a decrease in fluorescence (Chen 1989; Loew et al 1993; Plasek and Sigler 1996). Flow cytometric studies with Rhodamine 123 was done to focus on the relationship between the mitochondrial function in MDA-MB-231 cells and PC-3 cells, the release of cytochrome c, and induction of apoptosis on treatment. Earlier studies have reported that treatment of cells with drugs that
alter the mitochondrial membrane potential can alter the staining intensity (Gross et al 1999). The present study evaluated the mitochondrial role in the apoptotic process suggest the uptake of the potential sensitive dye Rh123 in MDA-MB-231 cells and PC-3 cells decreased considerably with the exposure to the ethyl acetate extract and purified compound, MDPD, from *Piper longum* as shown in Figures 3.24 and 3.25 indicating a loss in mitochondrial membrane potential leading to cytochrome c release. The release of cytochrome c to mitochondria was found to be the reason behind the loss of mitochondrial membrane potential. The changes in the mitochondrial membrane potential after treatment show that mitochondria are involved in the course of ethyl acetate extract and MDPD from *Piper longum* induced apoptosis.

Within the current paradigm of chemotherapy-induced apoptosis, mitochondria are key participants and the above observations from this study also falling in the category. A comprehensive analysis of the results of these cumulative experiments in response to crude extract and MDPD from *Piper longum* suggests that cell death induced by crude extract and pure compound (MDPD) was accompanied by decrease in Bcl-2 level with concomitant increase in Bax expression. This further lead to the translocation of Bax to mitochondria which disrupted the mitochondrial potential and consequently resulted in the release of cytochrome c, thus warranting the involvement of mitochondria mediated signalling pathways during the apoptotic process in breast adenocarcinoma cells and prostate cancer cells. All these observations in the present study well correlated with recent findings that reported the induction of mitochondrial mediated apoptosis by natural compounds in cancer cells.
4.5.4 Activation of Initiator Caspases, Caspase 9 and Caspase 8 Mediate the Apoptotic Cell Death in MDA-MB-231 Cells and PC-3 Cells

Understanding how a tumour cell's environment makes it more or less susceptible to certain drugs may also add to therapeutic strategies. Since tumours accumulate mutations which increase their resistance to certain environmental apoptotic triggers like hypoxia as well as to classical anti-neoplastic therapies, it is crucial to learn how to manipulate the downstream apoptosis machinery in new, perhaps more direct, ways. In addition, agents that directly induce apoptosis may reduce the risk of toxicity and reduce the opportunity for acquired drug resistance.

Caspases are a family of cysteiny1 aspartate–specific proteases which are synthesized as zymogens. Mammalian caspases include both initiators (e.g., 2, 8, and 9), which proteolytically activate the other set of caspases, effector caspases (caspases 3, 6, and 7). In the intrinsic pathway, upon activation within the apoptosome, caspase-9 can propagate the death signal by activating downstream effector caspases, thereby initiating the death cascade (Waxman and Schwartz 2003).

Because of its central role in the apoptosis, caspases are now considered as potential targets for pharmacological modulation of cell death. In prostate cancer, inhibition of the caspases by a viral inhibitor, such as CrmA, prevented androgen withdrawal-induced apoptosis in vitro. Similarly, in clinical studies it is presumed that the continued growth of androgen insensitive tumours after androgen withdrawal could be the result of altered apoptotic signalling mediated by caspase inhibition. Several compounds from natural sources such as Resveratrol, acts via caspase activation and drives the tumor cells to apoptosis.
Since cytochrome c release in MDA-MB-231 cells and PC-3 cells was confirmed, this warranted further investigation to determine the role of initiator caspases in the induction of apoptosis in response to ethyl acetate extract and purified compound of ethyl acetate extract and MDPD from *Piper longum* in both cell lines. From the western blot analysis (Figures 3.22E and 3.23E) it was evident that the exposure of MDA-MB-231 cells and PC-3 cells to ethyl acetate extract and MDPD from *Piper longum* revealed activation of caspase-9 from 18 hours onwards, suggesting that there could be a formation of a complex known as, apoptosome, downstream to the cytochrome c expression and confirming the involvement of caspase-9 in the cell death, apparently apoptosis.

To understand the involvement of the death receptor signalling in the apoptotic process in response to crude extract and MDPD from *Piper longum*, the study investigated the expression levels of caspase 8 in MDA-MB-231 cells and PC-3 cells on treatment. In death receptor pathway, initiator caspase, caspase-8, plays a major role in triggering the apoptotic cascade (Vier et al 2004; Chen and Wang 2002). This pathway is initiated by the ligation of death receptors belonging to the tumor necrosis factor receptor (TNF-R) superfamily. Binding of ligands promotes transmembrane oligomerization of the death receptors. Death receptors and their intracellular death domains then recruit FADD and TRADD adaptor proteins leading to the formation of a death-inducing signaling complex (DISC). FADD then causes the sequestration of the proenzyme forms of caspase-8, followed by activation of caspase-8. Activated caspase-8 in these apoptotic systems either directly activates the executioner caspase, caspase 3, or cleaves Bid, generating the t-Bid. Truncated t-Bid then translocates to the outer mitochondrial membrane promotes oligomerization of Bax to facilitate release of cytochrome c, thereby making the mitochondria a converging point to extrinsic and intrinsic pathways. The results in this study revealed that caspase-8 is not activated in MDA-MB-231 cells at 18 hrs and a marginal
increase in the expression level at 36 hrs suggested that the late induction of caspase-8 may not play any role in the apoptotic induction by ethyl acetate crude extract and MDPD from *Piper longum* as shown in the Figure 3.22D.

In contrast, ethyl acetate extract and MDPD from *Piper longum* treatment in PC-3 cells increased the caspase 8 levels after 12 hours demonstrating the availability of caspase 8 for the cell death induced in PC-3 cells. However further studies on other proteins involved in the death receptor pathway such as TNF-α, and Bid would reveal the actual involvement of caspase-8 in apoptotic cell death induced by crude extract and MDPD from *Piper longum*.

This accumulated observation of caspase 8 activation along with caspase 9 activation, reduction in Bcl-2 levels, up regulation of Bax levels, loss in mitochondrial membrane potential, cytochrome c release, in PC-3 cells on treatment revealed a possible involvement of the both the death receptor pathway and the mitochondria mediated pathway in the cell death induced in the prostate cells by the ethyl acetate extract and MDPD from *Piper longum*. All these observations stands in agreement with previous studies that demonstrated the contribution of both the apoptotic routes in the inhibition of prostate cancer cells (Hou et al 2005, Liu et al 2004, Wan et al 2005). The findings in this study further reveal a possible existence of a cross talk (interlinkage) between the two apoptotic pathways, via activated caspase 8 mediated truncation of Bid leading to its translocation to mitochondria activating the release of cytochrome c and sequential activation of caspase 9 (Kischkel et al 1995, Sun et al 1999).

Thus the time dependent activation of the initiator caspases, caspase 9 and caspase 8 may play a major role in triggering the cleavage of the effector caspase 3 needed for the execution of apoptosis induced by the
ethyl acetate extracts and purified compounds of ethyl acetate extract and MDPD from *Piper longum* in MDA-MB-231 cells and PC-3 cells.

### 4.6 ROLE OF CELL CYCLE PROTEINS IN THE G2/M PHASE ACCUMULATION OF MDA-MB-231 CELLS

Cell cycle control is the major regulatory mechanism of cell growth. Initially, cell cycle checkpoints may function to ensure that cells have time for DNA repair, but when the repair mechanism fails, cells undergo apoptotic cell death to eliminate unrepaired damaged cells. In many cancer cells, mutations occur within cell cycle regulating proteins, such as the tumor suppressor p53, which may impair function, lead to an accumulation of cells in different phases depending on the cell line. Many anti-cancer agents and DNA-damaging agents arrest the cell cycle at the G0/G1, S, or G2/M phase and then induce apoptotic cell death.

In the present study, the regulation of cell-cycle progression by ethyl acetate crude extract and MDPD from *Piper longum* in MDA-MB-231 cells is distinct from that of PC-3 prostate cancer cells. In MDA-MB-231 cells, ethyl acetate extract and MDPD from *Piper longum* induced G2/M checkpoint arrest before apoptosis, whereas in PC-3 cells, treatment of ethyl acetate extract and MDPD from *Piper longum* did not cause any such checkpoint arrest of the cell cycle. As shown in Figure 3.27A, accumulation of cells in the G2/M phase of the cell cycle at an early time point prior to apoptosis was observed in MDA-MB-231 cells treated with crude extract and MDPD from *Piper longum*. Reports reveal that G2/M phase accumulation has also been observed in cells exposed to DNA damaging agents such as c-irradiation (Hyun et al 2002), microtubule-stabilizing agents and topoisomerase inhibitors. Several natural compounds have been reported to inhibit proliferation of cancer cell lines by inducing cell cycle arrest and
apoptosis. One classical example is the Genistein, a phytochemical, which has been shown to arrest the cell cycle at the G2/M phases in *in vitro* studies involving cancer cells of different origin such as breast, prostate and lung cancer cells.

The targets on checkpoint pathways are potential anticancer strategies because abrogation of checkpoint function drive tumor cells towards apoptosis and enhances the efficacy of oncotherapy. In recent years, considerable advances have been made in understanding the roles of cyclins, cyclin-dependent kinases (Cdks) in cell cycle progression. A number of cyclin-dependent kinases have been isolated and shown to regulate the cell cycle event in mammalian/vertebrate cells in an orchestrated way in association with their specific regulatory cyclin proteins. Like other phases in cell cycle progression, G2 to M phase progression is also regulated by a number of the Cdks (mainly Cdk1 and Cdk2 kinases) and cyclin family (such as cyclin A and B1). In accordance with the previous reports, ethyl acetate extract and MDPD from *Piper longum* treated MDA-MB-231 undergone G2/M arrest before the apoptotic cell death which was concluded from the FACS analysis. The protein expression studies of various cyclins and cdks also suggest the cell cycle arrest in G2/M phase (Figure 3.27B).

### 4.7 GENERATION OF REACTIVE OXYGEN SPECIES LEVEL IN THE APOPTOTIC MECHANISM INDUCED BY *Piper longum* IN PC-3 CELLS

Exposure to cytotoxic therapies like hormone withdrawal or chemotherapy leads to the overexpression of many stress-induced proteins, leading to recovery of cells and developing resistance. There are now numerous reports which suggest that oxidative stress can be a common mediator of apoptotic cell death. The antiapoptotic protein Bcl-2 was shown
to prevent apoptosis by decreasing the generation of Reactive Oxygen Species (ROS) and various antioxidants can substitute for Bcl-2 expression in preventing apoptosis. The JNK and p38-mediated phosphorylation of the proapoptotic protein Bax has been shown most recently to lead to Bax activation and translocation to the mitochondria. Moreover, the use of specific phosphorylation site mutants has suggested that Bax Thr167 is the target of JNK-mediated phosphorylation.

The antiapoptotic Bcl2 protein, has been shown to be phosphorylated following cell exposure to a variety of stimuli, and JNK has been implicated as the protein kinase in these events. The phosphorylation of Ser70 was suggested to inactivate the antiapoptotic function of Bcl2. However, this contradicts earlier suggestions that phosphorylation of this Bcl2 site would enhance its antiapoptotic functions or subsequent studies that showed that Bcl2 phosphorylation at Ser70 was associated with increased cell survival. It remains to be evaluated whether this reflects the differences in cell types examined, the type or level of the stimulus, or differences in function upon Bcl2 phosphorylation at sites in addition to Ser70.

In this study, a dramatic ROS burst was observed in the early period of ethyl acetate extract and MDPD from Piper longum treated PC-3 cells when compared to untreated cells. However, MDPD treatment significantly produced more intracellular ROS than ethyl acetate extract. PC-3 cells coincubated with antioxidants NAC and catalase and then treated with MDPD, showed comparatively less fragments suggesting that induction of apoptosis was not fully dependent on ROS. In the model of ROS-mediated apoptosis, the generation of ROS has been suggested as a primary regulatory component followed by the activation of caspases. But in general, ROS generation occurs in response to various factors like TNF-α, or cytotoxic drugd and induces cell death in coordination with mitochondria. There are reports that ROS can directly cause cell death in a caspase independent
mechanism, riveting the role of ROS in apoptosis. Consequently, this form of cell death could be prevented by antioxidants. A number of drugs used in cancer chemotherapy such as doxorubicin, daunorubicin, mitoxantrone, bleomycin, and cisplatin induce oxidative stress by generation of oxygen free radicals.

Schematic diagram of ethyl acetate extract and MDPD from *Piper longum* on MDA-MB-231 and PC-3 cells is illustrated below.