With a growing worldwide population of men over the age of 50, prostatic diseases have become an important health concern. Prostatic cancer is the most frequently diagnosed malignancy and second leading cause of cancer death amongst men (Jemal et al., 2008). The incidence has increased rapidly in the recent years (Klein and Thompson, 2004) even in developing countries such as in India. Though there are numerous treatment for the management of prostate cancer such as androgen deprivation therapy, orchidectomy and still there is a relapse of prostate cancer. Moreover, prostate cancer cells are only modestly responsive or even unresponsive to the cytotoxic effects of chemotherapeutic agents or radiotherapy. Increased concentrations of cytotoxic drugs and higher dosages of irradiation fail to improve the response to therapy and it leads to resistance to apoptosis in prostate cancer cells. Radiation or chemotherapy both have potentially distressing side-effects including the possibility of impotence, incontinence or both. Thus, it is imperative to identify anticancer agents that are non-toxic or with less side effects and highly effective in inducing apoptosis preferentially in tumor cells.

The World Health Organization (WHO) has estimated that approximately 80% of the world’s population depends on traditional medicines for meeting their primary health care needs. Specifically, *Azadirachta indica* (family name: *Meliaceae*, common names: lilac, margosa tree, neem and neem chal) has been used successfully for centuries to reduce
tumors by herbalists throughout Southeast Asia. Researchers in India, Europe and Japan have found that polysaccharides and limonoids found in neem bark, leaves and seed oil reduced tumors and cancers (Arivazhagan et al., 2000; Akudugu et al., 2001; Subapriya and Nagini, 2003). Therefore two different prostate cancer cell lines LNCaP (androgen-dependent) and PC-3 (androgen-independent) have been chosen for the current study. These cell lines have been extensively used as cell models to study the progression of this disease. LNCaP cells are considered to represent early stages of prostate cancer development (Horoszewicz et al., 1983; Nunlist et al., 2004) that progress to AR-independent growth (Lin et al., 2003). In contrast, PC-3 cells are considered to represent advanced disease based on their source of origin (Kaighn et al., 1979). It is further reported that LNCaP and PC-3 cells express different levels of angiogenic factors (Tesan et al., 2008) and pro-inflammatory cytokines (Araki et al., 2007). PC-3 cells have acquired a more aggressive phenotype with unique expressions of VEGF C, TGFβ2, and TGFβ1. More over, IL-8, MMP-9 and TGFα are expressed at higher levels in PC-3 cells than in LNCaP cells (Aalinkeel et al., 2004). Therefore, the present study was aimed at investigating the effect of ENLE on signaling molecules involved in proliferation, metastasis and apoptosis of both PC-3 and LNCaP prostate cancer cells.
ENLE regulates PI3K/Akt pathway

Phosphatidylinositol-3 kinase (PI3K) is a heterodimeric enzyme which catalyzes the production of the lipid secondary messenger phosphatidylinositol-3,4,5-triphosphate (Cantley, 2002), which in turn activates a wide range of downstream targets, including the serine/threonine kinase Akt (Luo et al., 2003). Akt is an important regulator of cell survival and cell proliferation that significantly contribute to tumor growth and progression by promoting cell invasiveness and angiogenesis. Overexpression of Akt has been reported in a variety of human cancers including prostate cancer (Datta et al., 1999; Hill and Hemmings, 2002). PI3K/Akt survival pathway is constitutively upregulated either due to the loss of function and/or mutations of the tumor suppressor PTEN, which functions as a negative regulator of PI3K through its lipid phosphatase activity. It is reported that loss of PTEN in cancer cells such as in PC-3 cells (Teng et al., 1997) leads to constitutive activation of the PI3K/Akt signal transduction pathway (Datta et al., 1999). There are evidences suggesting that quercetin a dietary flavonoids is one of the major constituent of neem leaf extract (Manikandan et al. 2008; Vinothini et al., 2009) has been reported to have broad range of pharmacological effects (Block et al., 1992). The anticancer effect of quercetin on androgen- independent prostate cancer cells (PC-3) was assessed earlier in our laboratory. Quercetin along with PI3K inhibitor synergistically inhibited the phosphorylation of Akt and thus prevented cell survival,
proliferation and further induced apoptosis in PC-3 cells (Senthilkumar et al., 2010). Therefore in the present study, it is evident that the ENLE has significantly decreased the Akt phosphorylation at Ser-473 probably due to the presence of quercetin in it. A significant decrease in the total Akt was also observed at 100 µg/ml concentration of ENLE in both PC-3 and LNCaP cell lines. The protein expression of PI3K, an up-stream regulator of Akt, was also significantly decreased upon ENLE treatment in both PC-3 and LNCaP cell lines. This is further supported by a significant increase in the mRNA expression of PTEN upon ENLE treatment in LNCaP cell line. In PC-3 cell line, the PTEN is frequently mutated or deleted as in advanced prostate cancer cells.

Progression through the cell cycle is controlled by the activity of protein kinase complexes consisting of cyclins and cyclin-dependent kinases (cdks) and associated regulatory proteins (Malumbres and Barbacid, 2001). Cyclin D1 is known as a proto-oncogene whose gene amplification and protein over expression are frequently observed in tumor cells. Cyclin-dependent kinases (cdks) 4 and 6 are cyclin D1 binding partners. Activated cyclin D1/cdk4 and cyclin D1/cdk6 complex phosphorylate the retinoblastoma protein to induce the expression of target genes essential for S phase entry, resulting in facilitation of the progression from G1 to S phase (Takahashi-Yanaga and Sasaguri, 2008). Studies have demonstrated that Akt regulates cyclin/cdk activity and cell cycle progression through several
mechanisms. During G1/S transition, Akt regulates the level of cyclin D, c-myc, p27\text{kip1} and p21\text{cip1/waf1} by preventing their proteosomal degradation. GSK-3\text{β} phosphorylates cyclin D1 at Thr 286 which promotes its degradation via ubiquitin-mediated pathway (Diehl et al., 1998). Thus, Akt dependent phosphorylation and inactivation of its substrate GSK-3\text{β} prevents the degradation of cyclin D1 which in turn facilitates the G1/S progression.

Akt also inactivates the cdk inhibitor proteins p21 and p27, thereby promoting cdk activity and cell cycle progression (Liang and Slingerland, 2003). The mechanism by which p21 induces cell cycle arrest is not clearly known till date. Recently it is stated that depletion of PLK-1 (polo-like kinases) which is a potent regulator of multiple cell cycle functions, including activation of cell division control protein 2 (cdc2), mitotic entry, bipolar spindle formation, centrosome maturation, and cytokinesis (Pesin and Orr-Weaver, 2008) reduces the Murine double minute 2 (Mdm2) expression, a negative regulator of p21 protein stability (Kreis et al., 2009). Thus depletion of PLK-1 will lead to up-regulation of p21 and will further inhibit cell cycle progression. On the other hand it is also mentioned that p21 controls PLK-1 transcription at the promoter region as a negative regulator in cancer cells (Salvatore et al., 2007). Seo et al. (2011) reported that natural isoflavones and flavones induced PLK-1 down-regulation causing G2/M cell cycle arrest and mediating apoptosis and in LNCaP and PC-3 cells.
In last 10 decades, scientists are interested in working with the extracts of neem and various constituents isolated from different parts of the neem tree (*A Indica*) to have a thorough knowledge on its anticancer effects. In that aspect nimbolide, a natural triterpenoid present in the edible parts of the neem tree (*A Indica*) has been found to possess anticancer effect. Nimbolide treatment to human colon carcinoma (HT-29) cells at 2.5-10 µM resulted in moderate to very strong growth inhibition. Flow cytometric analysis showed that nimbolide treatment of HT-29 cells at 2.5 µM at 48 h decreased the total cell population by 18 % in the G2/M phase while the cells in G0/G1 phase increased to 52.3 % indicating the inhibition of cell cycle progression. Further, western blot analysis revealed that nimbolide-mediated G2/M arrest was accompanied by the up-regulation of p21, cyclin D2, Chk2; and down-regulation of cyclin A, cyclin E and cdk2 (Roy *et al.*, 2006). Recently, in another study, ENLE has been shown to inhibit cell proliferation by up-regulating the level of p21 and p27. In addition, ENLE treatment also showed cell differentiation and induction of apoptosis in DMBA-induced mammary carcinoma in swiss albino female rats, further supported by the inhibition of the histopathological lesions in the mammary tissues (Alakilli, 2010). All the above inferences, correlates with the present data, where a significant decrease in the p-Akt by ENLE treatment results with the significant decrease in the protein expression of cyclin D1 in both the cell lines with a marked increase in the level of p21. Thus, the inhibitory effect of ENLE treatment on
the cell cycle progression of both PC-3 and LNCaP cell lines has been well demonstrated.

Activated Akt not only regulates cell proliferation but it also regulates apoptosis. Akt is thought to prevent apoptosis by activating the transcription factor nuclear factor-κB (NFκB), which in turn induces the expression of antiapoptotic genes (Ozes et al., 1999), such as the expression of the anti-apoptotic protein, FADD-like ICE (FLICE)-inhibitory protein (FLIP), which inhibits activation of caspase-8 (Panka et al., 2001). Another mechanism by which Akt inhibits cell death pathways is by direct phosphorylation and inactivation of proteins involved in apoptosis, including Bad, procaspase-9, and members of the Forkhead transcription factor family (Datta et al., 1997; Cardone et al., 1998). Phosphorylation of Bad by Akt at serine (Ser) residues 112 and 136 enables the 14-3-3 protein to interact with and sequester the inactivated Bad protein in the cytoplasm (Datta et al., 1997). Akt phosphorylates and inactivates the procaspase-9 protease by phosphorylating at Ser-196. Once the caspase-9 is inactivated it can no longer induce the activation of its downstream caspases such as caspase-3, 6 or 7 thus inhibiting apoptosis (Cardone et al., 1998). In the absence of active Akt, non-phosphorylated forkhead localizes in to nucleus where it can either induce transcription of FasL and thereby promote apoptosis, or stimulate transcription of p27Kip1 and induce withdrawal from the cell cycle (Medema et al., 2000).
In one of the studies it was observed that quercetin a constituent identified to be present in neem (Manikandan et al., 2008; Vinothini et al., 2009) has been found to possess apoptotic effect against prostate cancer (LNCaP) cells. Quercetin at 100 µM concentration induced apoptosis in LNCaP cells by a rapid decrease in the inhibitory Ser (473) phosphorylation of Akt leading to inhibition of its kinase activity. At the same time it also caused a decrease in Ser (136) phosphorylation of Bad, which is a downstream target of Akt. The un-phosphorylated form is the active form of Bad which further induced apoptosis to LNCaP cell by activating its downstream caspases (Lee et al., 2008). The current study also demonstrates that ENLE inhibits phosphorylation of Akt which further leads to inhibition of Bad phosphorylation as a result the active non-phosphorylated form of the Bad is capable of inducing apoptosis to both PC-3 and LNCaP prostate cancer cells. Therefore it confirms that ENLE by inhibiting PI3K/Akt signaling pathway inhibits cell proliferation and induces apoptosis in prostate cancer cells.

**ENLE regulates EGFR and downstream signaling molecules as well as the key metastatic proteins**

Signal transduction through the epidermal growth factor (EGF) receptor (EGFR) axis has been implicated in mediating multiple processes involved in tumor progression and metastasis, including invasion,
angiogenesis, proliferation, and inhibition of apoptosis (see Normanno et al., 2001). Prostate cancer commonly overexpresses EGF and its receptor EGFR (Sherwood et al., 1998; Davies et al., 1988). EGFR is known to be essential for growth and maintenance of epithelial cancer tissues including prostate cancer. Phosphorylation of EGFR permits mitogenesis and angiogenesis of prostate cancer cells. The EGFR mediates its function through tyrosine kinase signaling pathways such as Ras/ Raf/ Erk pathway. Therefore, inhibiting the activation of growth factor receptors, especially EGFR, may be a promising strategy for the treatment of prostate cancer (Festuccia et al., 2005). In the present study, ENLE was used to investigate various critical cellular signaling pathways, including EGFR signaling, in human prostate cancer cell lines PC-3 and LNCaP cells. It has been reported that the EGFR-blocking agents like erlotinib and gefitinib which are tyrosine kinase inhibitors inhibits proliferation, induce apoptosis, and suppress metastasis in human NSCLC cells in vitro and in xenograft models (Raben et al., 2005). Similarly in another study, it has been documented that phenylethyl isothiocyanate (PEITC) in combination with curcumin blocked EGFR activation in PC-3 cells and further inhibited the activation of Akt and NF-κB thus inducing programmed cell death (Kim et al., 2006). Recently it has been cited that, quercetin one of the substance to be present in the fractions of ethanolic extract to suppresses the phosphorylation of EGFR thereby block the activation of EGFR pathway in PC-3 cells (Senthilkumar et al., 2011). In the
current study, a significant decrease in the expression of EGFR and EGF at 100 µg/ml of ENLE treatment in both PC-3 and LNCaP cell lines correlates with previous reports suggesting that ENLE acts as a potent inhibitor of EGFR and its ligand EGF, thus blocking the activation of EGFR pathway. Thus, ENLE decreases the EGFR system thereby inhibits cell proliferation of PC-3 and LNCaP cells.

Ras signaling is activated in a large fraction of human tumors (Harris et al., 1992). Activation of the growth-factor receptors leads to increased Ras activation in advanced prostate cancer. Ras activation as a result of increased EGF receptor (ErbB-1) signaling was reported in 40% of prostate tumors and occurred with greater frequency in advanced stages (Di Lorenzo et al., 2002). Constitutively active mutants of Ras are commonly encountered in some types of cancer but they appear to be relatively rare in prostate cancer. In spite of this, prostate cancer cells exhibit elevated levels of activated MAP kinases, which are targets of Ras, in correlation with tumor stage and grade (Gioeli et al., 1999). It has also been demonstrated that Ras activation plays a vital role in altering prostate cancer cells toward decreased hormone-dependence and increased malignant phenotype (Bakin et al., 2003). Expression of constitutively active form of H-Ras in LNCaP cells was sufficient for progression toward androgen-independence in terms of tumorigenicity and it is correlated with the activation of MAP kinase signaling. Ras activates Raf which is a serine/threonine (S/T) kinase which in
turn phosphorylates MAPK including extracellular signal regulated kinases (ERK). ERKs can directly phosphorylate many transcription factors including ets-1, c-jun and c-myc. ERK can also phosphorylate and activate the 90 kDa ribosomal S6 kinase (p90Rsk), which then leads to the activation of the transcription factor CREB (Steelman et al., 2004).

As mentioned earlier that nimbolide constituent of neem leaves effectively inhibited proliferation of colon cancer cells through inhibition of cyclin A leading to S phase arrest. It also caused the activation of caspase-mediated apoptosis through the inhibition of ERK1/2 and activation of p38 and JNK1/2. Further nimbolide effectively retarded tumor cell migration and invasion through inhibition of metalloproteinase-2/9 (MMP-2/9) expression, both at the mRNA and protein level (Babbykutty et al., 2011). Similarly, in the current study, ENLE significantly decreased the protein expression of p-ERK 1/2 as well as EGFR, N-Ras and Raf-1 upstream regulators of ERK 1/2 at 100 µg/ml concentration compared with the control. ENLE inhibiting cell proliferation and metastasis of both PC-3 and LNCaP cell lines could have probably be achieved due to the presence of nimbolide. Therefore present study confirms that, ENLE significantly decreased the proliferation and metastasis of prostate cancer by inhibiting the expression of down-stream signaling molecules of EGFR pathway or EGFR induced MAPK pathway.
IL-8 expression enhances tumorigenicity and metastasis (Inoue et al., 2000). The level of circulating IL-8 are increases in advanced prostate cancer at a stage when the tumors no longer respond to antiandrogens (Aalinkeel et al., 2004; Lehrer et al., 2004). It is reported previously that prostate cancer cells are subjected to an autocrine/paracrine IL-8-signaling stimulus as a consequence of the increased expression of IL-8 and its receptors, CXCR1 and CXCR2, in tumor cells of human prostate biopsy tissue (Murphy et al., 2005). A weak to moderate IL-8 expression was detected only on the apical membrane of the majority of normal prostate epithelial cells but a moderate to strong IL-8 expression was detected in prostate cancer cells on the nonapical part of the membrane and within the cytoplasm of prostate cancer cells. This stimulus detected in low Gleason grade cancer of the prostate reaches a maximal level in androgen-independent disease (Murphy et al., 2005). Exogenous administration of IL-8 to two AR-expressing prostate cancer cell lines (LNCaP and 22Rv1) resulted in the proliferation of the cells under steroid-depleted conditions. Furthermore, the IL-8-promoted proliferation of either cell line was abrogated following blockade of the AR with the AR antagonist, bicalutamide, indicating the capacity of IL-8 signaling to induce androgen-independent proliferation of the LNCaP cell line (Lee et al., 2004; Araki et al., 2007). Recently in another study it is confirmed that depletion of endogenous expression of IL-8 over 90% by siRNA decreased PC-3 and DU145 cell proliferation, cell cycle
progression, angiogenic potential and up-regulated spontaneous apoptosis to cancer cells (Singh and Lokeshwar, 2009).

IL-8 signaling has been shown to induce the activation of the classic MAPK signaling cascade, with downstream phosphorylation of Erk1/2 detected in both neutrophils (Knall et al., 1996) and cancer cells (Venkatakrishnan et al., 2000; Mac Manus et al., 2007; Luppi et al., 2007). IL-8 signaling regulates the activity of the MAPK signaling cascade that constitutes a number of serine/threonine kinases that are colocalized via their interaction with scaffolding proteins in close proximity to cell-surface receptors. The substrate specificity of these kinases results in the activation of distinct signaling cascades, the best characterized of which is the Raf-1/MAP/ERK kinase cascade. Stimulation of PC-3 cells with recombinant human IL-8 induced a potent phosphorylation of Erk1/2 (p42/p44) MAPK and p38 MAPK. Elevated phosphorylation of Erk1/2 has been observed in proliferating prostatic epithelial cells and has been associated with the initiation of prostate cancer (Uzgare et al., 2003). Moreover it has been confirmed that IL-8 signaling induces a sustained activation of both the p42/44 MAPK and PI3K signal transduction cascades in androgen independent prostate cancer cells (AIPC) (Chen et al., 2006).

Venkatakrishnan et al. (2000) have showed that stimulation of ovarian epithelial cells with recombinant human IL-8 resulted in the rapid
activation of MAPK p42/44 and that EGFR participated in the transduction of GPCR-mediated IL-8 signals. In human colon carcinoma cell line, Itoh et al. (2005) observed that IL-8-induced EGFR transactivation mediated by an ADAM-dependent pathway, in which HB-EGF plays an important role as the major ligand for the activation of the pathway. IL-8 stimulated proliferation of two NSCLC (A549 and NCI-H292), involving transactivation of the EGFR (Luppi et al., 2007). The findings of the current study are in line with those observations, and extend these by showing that similar mechanisms are operative in PC-3 and LNCaP cells, where ENLE treatment has significantly decreased the mRNA expression of IL-8 at both 50 and 100 µg/ml concentration. This might have probably resulted in the decreased transactivation of EGFR and its down-stream signaling molecules, as there is a significant decrease in the expression Ras, Raf and p-Erk1/2. Consequently, this may further inhibit the metastasis of PC-3 and LNCaP prostate cancer cells.

Matrix metalloproteinase (MMP) is a zinc dependent protease that plays a major role in proteolytic degradation of ECM components and aid in tumor invasion and metastasis. MMP, secreted as latent zymogens, are activated by plasmin and their activity is regulated by a family of tissue inhibitors of metalloproteinase (TIMP) (Khasigov et al., 2003). During tumor metastasis, the balance between the active protease and their inhibitors is disrupted, generally leading to an elevated MMP expression (Stamenkovic,
Tissue inhibitors of metalloproteinases (TIMPs) have been shown to perform several biological functions in blocking tumor metastasis, principally by their action of inhibiting matrix metalloproteinases (MMPs) at different steps of the metastatic process (Bratland et al., 2003). Gene expression profiles of prostate neoplasms have recently been reported, which indicate that TIMP-2 expression is down-regulated in prostatic intraepithelial neoplasia compared to adjacent normal prostate epithelium (Dhanasekaran et al., 2001). Moreover, TIMP-2 expression in stroma cells has been shown to be lost with progression from low grade to high-grade prostate cancer (Wood et al., 1997). And the synthetic androgen analogue R1881 has been shown to inhibit the expression of the metalloproteinase inhibitor TIMP-2 in a time-dependent manner in the prostate carcinoma cell line LNCaP (Bratland et al., 2003).

Inoue et al. (2000) have observed that the PC-3P prostate cancer cells transfected with the full-length sense IL-8 cDNA overexpressed IL-8-specific mRNA and protein, which resulted in up-regulation of matrix metalloproteinase 9 (MMP-9) mRNA, and collagenase activity, resulting in increased invasion through Matrigel, in vitro. Similarly, orthotopic implantation of such IL-8 overexpressing cells in athymic nude mice showed increased metastasis and tumorigenicity in vivo. Whereas antisense transfection of the PC-3M-LN4 a highly metastatic prostate cancer cell line, with antisense IL-8 greatly reduced the MMP-9 expression, collagenase
activity, and invasion relative to its control. This suggests that the activity of MMP-9 in human prostate cancer cells is directly correlated with the expression of IL-8 (Inoue et al., 2000). One of the earlier studies have reported that in ovarian cancer cells EGFR activation has lead to the stimulation of matrix metalloproteinase (MMP-9) production and promoted the migration and invasion in ovarian cancer cells (Karen et al., 2008). A recent study showed that siRNA mediated knockdown of MMP-9 and uPAR inhibited the invasiveness of PC-3 and DU145 cells as assayed by matrigel coated invasion assay and migration of prostate cancer cells. Further, it induced apoptosis in both prostate cancer cells in vitro and in vivo (Nalla et al., 2010). The findings of the current study are in agreement with other reports where a similar trend was observed. Both PC-3 and LNCaP cell lines upon ENLE treatment resulted in decreased expression and activity of MMP-9 with a significant increase in the TIMP-2 expression. Accordingly, invasive potential of PC-3 and LNCaP cell lines considerably decreased upon ENLE treatment at 50 and 100 µg/ml concentrations as evaluated by matrigel-coated invasion assay. The decrease in the expression and activity of MMP-9 would have been probably due to a decrease in the IL-8, EGFR expression and its down-stream signaling molecules by ENLE treatment.

Tumor cell invasion and metastasis are complex processes which involve three stages as adhesion to the extracellular matrix, digestion of the matrix to release cells from the primary tumor mass, and migration of the
tumor cells to secondary targets. A high level of expression of one or more proteases often correlates with the migration of cancer cells through the digested extracellular matrix and contributes to tumor cell invasion and metastasis of virtually all malignancies, including prostate cancer (Boyd et al., 2003; Rao, 2003). One such key protease, urokinase plasminogen activator (uPA), and its receptor uPAR play important roles in cancer cell invasion and metastasis (Nishimura et al., 2003; Mamoune et al., 2004). Evidences support that plasma levels of uPA and uPAR were markedly elevated in men with prostate cancer metastatic to bone. They were significantly higher in patients with clinically localized and metastatic prostate cancer than in healthy men. Moreover the higher circulating levels of uPA and uPAR were decreased significantly after the prostate was removed suggesting that there is a direct local production of uPA and uPAR by these malignant cells (Shariat et al., 2007). Earlier report indicates that in human prostate epithelial cells (PrEC) and hormone-responsive LNCaP cells the uPA promoter is hypermethylated, whereas in hormone-insensitive PC-3 cells the promoter is unmethylated and hence there is maximal expression of uPA in PC-3 cells than in LNCaP cells. Treatment of LNCaP cells with 5’-azacytidine, a potent demethylating agent results in demethylation of uPA promoter and in the induction of uPA mRNA expression (Pakneshan et al., 2003).
Urokinase plasminogen activator (uPA) receptor is one of the major regulators of extracellular matrix degradation and tissue remodeling, and it has been implicated in ligand-independent activation of EGFR. This uPA receptor is a glycosylphosphatidyl-inositol-anchored receptor on the cell surface that converts pro-uPA to the active protease. uPAR laterally associates with several transmembrane receptors, including integrins and EGFR. In many cell types, when uPA binds to uPAR, it activates signal transduction and protein tyrosine kinases, including focal adhesion kinase (FAK) (Kjoller, 2002) and ERK (Jo et al., 2005). Knockdown of uPA-uPAR expression either with uPA-shRNA or uPAR-shRNA significantly inhibited the growth of PC3 cells in vitro as well as in vivo and ultimately resulted in apoptotic cell death. Suppression of the uPA-uPAR system leads to a decrease in the downstream signaling molecules of Erk and Stat 3 in uPA-shRNA or uPAR-shRNA transfected PC-3 cells (Pulukuri et al., 2005). There are reports stating that uPA-uPAR-mediated signaling up-regulates the production of matrix metalloproteases, which induce extracellular matrix degradation and, in turn, tumor invasion and metastasis (Legrand et al., 2001). The results of the present study are in consistent with the previous reports as there was a very minimal level of expression observed in LNCaP when compared with the PC-3 cells. Further our data reveal that ENLE treatment at 50 and 100 µg/ml to PC-3 and LNCaP prostate cancer cell lines has significantly inhibited the mRNA expression of uPA and its receptor uPAR. Altogether the
Datas of the present study highlights that the ENLE has significantly decreased the invasion and metastatic potential of prostate cancer cells by decreasing IL-8, uPA and uPAR mRNA expression thus inhibiting the transactivation of EGFR and its downstream molecules such as N-Ras, Raf-1 and p-ERK 1/2 which finally leads to suppression of MMP-9 expression and its activity.

**ENLE induces apoptosis in prostate cancer cells**

Apoptosis is a physiological cell death process in which individual cells are eliminated or removed from the body in a temporal manner or in response to a specific signal without affecting neighboring cells or eliciting an inflammatory response. Caspase activation occurs through the release of apoptogenic factors from the mitochondria, including cytochrome C, Smac/DIABLO and Omi/HtrA2. Released cytochrome C allows the formation of a high-molecular weight complex, the apoptosome, which consists of the adapter protein Apaf-1 and caspase-9, which is activated following recruitment into the apoptosome (Wolf and Green, 1999). Active caspase-9 then cleaves and activates the effector caspases, such as caspase-3 and 7, which execute the apoptotic program (Sudheer et al., 2006). The release of cytochrome C is regulated by the pro and anti-apoptotic Bcl-2 family proteins, which either induce or prevent the permeabilization of the outer mitochondrial membrane. In the current study, ENLE treatment at 50 and 100 µg/ml
concentrations induced the mRNA expression of pro-apoptotic proteins (Bax and Bad) in PC-3 and LNCaP cells lines with a significant increase in the protein expression of Bad. ENLE significantly enhanced the release of cytochrome C leading to caspase-3 activation in a dose-dependent manner. The increased activity of caspase-3 further cleaved PARP protein into 115 and 85 kDa peptides finally inducing apoptosis to prostate cancer cells. Apoptosis was further confirmed by TUNEL assay. The characteristic red fluorescence emission which indicates the apoptotic nature was prominently observed in cells treated with 100 µg/ml of ENLE in both PC-3 and LNCaP cell lines.

Although a number of therapeutically useful compounds have been identified from neem leaf, most of the pharmacological properties have been reported only with crude extracts (Chattopadhyay, 1998; Subapriya and Nagini, 2003, 2005). *A indica* leaf extract has been reported effectively to suppress oral squamous cell carcinoma induced by DMBA (Balasenthil *et al.*, 1999) and MNNG-induced stomach and gastric carcinogenesis in rats (Arivazhagan *et al.*, 2000; Dasgupta *et al.*, 2004).

Manikandan *et al.* (2008) have shown that neem leaf fractions exert greater inhibitory effect on hamster buccal pouch carcinogenesis. Both ethyl acetate and methanolic fractions of neem leaf extract showed *in vitro* antioxidant activity as well as *in vivo* antiproliferative and antiangiogenic
effects and possessed significant chemopreventive potential. Similarly, neem leaf fraction inhibited DMBA-induced mammary carcinogenesis by modulating phase I and phase II xenobiotic metabolizing enzymes, oxidative stress as well as by inhibition of cell proliferation and induction of apoptosis (Vinothini et al., 2009). Alakilli (2010) have shown that ENLE inhibited cell proliferation, induced differentiation and apoptosis in DMBA-induced mammary carcinogenesis. ENLE decreased the level of PCNA (proliferating cell nuclear antigen) which is said to be highly expressed in the nuclei of proliferating cells during S-phase and at the same time it also decreased the level of anti-apoptotic protein Bcl-2 thus favoring apoptosis. Further, it is reported from our laboratory, that ENLE induced apoptosis in breast cancer cell lines such as MCF-7 and MDA MB-231 by up-regulating the expression of pro-apoptotic proteins and leading to cleavage of PARP protein (Elumalai et al., 2011).

These studies strongly supports the current work where ENLE treatment at 50 and 100 µg/ml concentration to both PC-3 and LNCaP cells increased the mRNA expression of pro-apoptotic gene such as Bax and Bad and increased the protein level of Bad, which further leads to release of cytochrome C from mitochondria and activates the caspase-3. ENLE thus activating caspase-3 leads to cleavage of PARP protein into two peptides of 115 kDa and 85 kDa thus inducing apoptosis in both androgen-dependent and androgen-independent prostate cancer cells.
Thus, it is demonstrated that ENLE exerts its anticarcinogenic properties through the inhibition of cell proliferation, induction of differentiation and apoptosis to the cancer cells. The anticancer effects of ENLE observed in the present study may be related to its constituent phytochemicals in it. ENLE is said to contain a number of potent antioxidants and anticarcinogens including β-carotene, flavonoids, terpenoids and various limonoids (Subapriya and Nagini, 2005). Chattopadhyay (1998) has reported that ENLE contain six major compounds: (i) quercetin-3-O-β-D-glucoside; (ii) myricetin-3-O-rutinoside; (iii) quercetin-3-O-rutinoside; (iv) kaempferol-3-O-rutinoside; (v) kaempferol-3-O-β-D-glucoside; (vi) quercetin-3-O-L-rhamnioside. Yet another study revealed quercetin and nimbolide to be major constituents of crude ethanolic extract analysed by HPLC analysis (Manikandan et al., 2008; Vinothini et al., 2009). Azadirachtin, a limonoid present in neem leaf (Gogate, 1996) is reported to have the cytotoxicity and inhibitory effects on cell proliferation in human glioblastoma cell lines (Akudugu et al., 2001). Quercetin and kaempferol, the flavonoids present in neem leaf have been documented to block carcinogenesis (Le Marchand, 2002). The antiproliferative effects of quercetin have been documented in experimental animal models and humans. Quercetin has been reported to inhibit the growth of tumor cells in malignant cell lines and downregulate the expression of Bcl-2 and mutant p53 protein (Lamson and Brignall, 2000). Studies from our laboratory have documented that the quercetin to possesses
anticancer effects against prostate cancer in both in vivo and in vitro (Vijaybabu et al., 2005; 2006). Quercetin increased the level of IGFBP-3 associated with increased level of pro-apoptotic proteins thus inducing apoptosis to prostate caner cells (Vijaybabu et al., 2006). It induced apoptosis via modulating the Bax: Bcl-2 protein ratio. Quercetin inhibits IGF signaling molecules and protein levels IGF-IRb, PI3K, p-Akt, cyclin D1 and inducing extrinsic and intrinsic pathway mediated apoptosis. (Senthil kumar et al., 2010)

Further, protein interaction assay revealed that during treatment with quercetin, Bcl-xL dissociated from Bax and then associated with Bad. Moreover quercetin decreases the Bcl-xL:Bax ratio and increases translocation and multimerization of Bax to the mitochondrial membrane. This translocation is accompanied by cytochrome C release, and procaspases-3, -8 and -9 cleavage and increased poly (ADP-ribose) polymerase (PARP) cleavage thus inducing apoptosis to prostate cancer cells (Lee et al., 2008). Hence, ENLE mediating its anticancer and apoptotic effects in prostate cancer (PC-3 and LNCaP) probably might be due to the presence of several flavonoids, β-carotene, terpenoids and various limonoids.