RESULTS
Effect of ENLE on cell viability in PC-3 and LNCaP cell lines

To determine the effect of ENLE on cell viability in prostate cancer cells, the PC-3 and LNCaP cell lines were treated with different concentration of ENLE for 24 and 48 h and assayed by MTT method. ENLE decreased the cell viability gradually with increased concentration in both the cell lines. 50% of viable cells was observed at 100 µg/ml in both PC-3 and LNCaP cell lines at 24 h (Fig. 1). From these results the IC$_{50}$ value was calculated as 100 µg/ml for both the cells. Hence, for further studies ENLE of 50 and 100 µg/ml concentrations for 24 h were considered.

Effect of ENLE on the expression of PI3K and PTEN

Effect of ENLE on PI3K levels was studied in both PC-3 and LNCaP prostate cancer cell lines by western blot analysis. A significant decrease in the protein level of PI3K was observed at 100 µg/ml concentration of ENLE in both PC-3 and LNCaP cell lines (Fig. 2). As the PTEN is a negative regulator of PI3K, the mRNA level of PTEN was assessed upon ENLE treatment. There was a significant increase in the mRNA of PTEN in LNCaP cell line at both 50 and 100 µg/ml concentrations (Fig. 3).

Effect of ENLE on the expression of Akt and phospho-Akt

Protein levels of Akt and p-Akt (phospho-specific Ser 473 Akt antibody) were measured by immunobblott. Data revealed that the
phosphorylation of Akt was inhibited by ENLE treatment at 50 and 100 µg/ml concentrations in both PC-3 and LNCaP cell lines (Fig. 4).

**ENLE regulates expression of cell cycle proteins cyclin D1 and p21**

As Akt is known to regulate the cell cycle, the expression of cyclin D1 and cell cycle inhibitory protein p21 were examined. ENLE significantly decreased the level of cyclin D1 at 100 µg/ml concentration. Conversely there was a significant increase in the p21 expression at 50 and 100 µg/ml ENLE treatments in both PC-3 and LNCaP cell lines (Fig. 5).

**Effect of ENLE on the mRNA expression of EGF**

RT-PCR analysis showed that ENLE significantly decreased the mRNA expression of EGF at 100 µg/ml concentration when compared with the control in both cell lines, and a significant decrease was also observed at 50 µg/ml concentrations of ENLE treatment in PC-3 cells but not in LNCaP cell line (Fig. 6).

**Effect of ENLE on the mRNA expression of uPA and uPAR**

The mRNA expression of uPA and uPAR was assessed by RT-PCR in both PC-3 and LNCaP cell lines. ENLE significantly decreased the mRNA expression of uPA and uPAR at 100 µg/ml concentrations (Fig. 7 and 8).
ENLE downregulated the protein expression of EGFR

ENLE significantly reduced the protein level of EGFR at 50 and 100 µg/ml concentrations in both the cell lines (Fig. 9).

ENLE downregulated signaling molecules of EGFR pathway

The downstream signaling molecules of EGFR pathway was studied by immunoblotting. ENLE significantly reduced the protein levels of N-Ras, Raf-1 and p-ERK 1/2 at 50 and 100 µg/ml concentrations in both the cell lines (Fig. 10 and 11).

Effect of ENLE on the mRNA expression of IL-8 and MMP-9

ENLE significantly reduced the mRNA expression of IL-8 and MMP-9 at 50 and 100 µg/ml concentrations in both the cell lines (Fig. 12 and 13), thus inhibiting metastasis of prostate cancer cells.

ENLE down regulates key proteins that control metastasis

The protein levels of MMP-9 and TIMP-2 were examined by immunoblot. ENLE treatment significantly decreased the level of MMP-9 in both the cells at 100 µg/ml and increased the protein expression of TIMP-2 at 50 and 100 µg/ml concentrations (Fig. 14).

Zymography assay showed that MMP-9 was active only in the untreated, control cells but not in the ENLE treated cells of both PC-3 and LNCaP cell lines as evident by the white band on blue background (Fig. 15).
**ENLE inhibited invasion of PC-3 and LNCaP cells**

Invasion was determined by transwell invasion assay. ENLE at both 50 and 100 µg/ml concentrations significantly inhibited invasion of PC-3 and LNCaP cells (Fig. 16). Quantitative data derived from three independent experiments supported that ENLE effectively prevented the invasion of PC-3 and LNCaP cell lines.

**ENLE regulates expression of Bcl-2 family members**

The Bcl-2 protein family consists of both pro- apoptotic (Bax and Bad) and anti-apoptotic (Bcl-2) proteins that regulate mitochondrial outer membrane integrity, cytochrome C release and caspase activation leading to apoptosis. The mRNA expression of pro-apoptotic protein Bax was significantly up-regulated by ENLE treatment in both PC-3 and LNCaP cell lines (Fig. 17). ENLE at 100 µg/ml concentrations significantly increased both the mRNA and protein level of Bad in PC-3 and LNCaP cell lines (Fig. 18).

**ENLE induces the release of cytochrome C and activates caspase-3**

Apoptosis involves the permeabilization of the outer mitochondrial membrane and release of cytochrome C which further leads to activation of caspase-3. In the current study, cytochrome C was analysed by immunoblotting. ENLE significantly increased the protein level of cytochrome C at 100 µg/ml concentrations in both the cell lines (Fig. 19).
Caspase-3 is the final executioner caspase which promotes the cleavage of cellular substrates like PARP thus leading to apoptosis. The activity of caspase-3 was examined by CPP32 protease assay kit in PC-3 and LNCaP cells. ENLE increased caspase-3 activity in both PC-3 and LNCaP cells in a dose dependent manner (Fig. 20).

Activation of caspase-3 leads to cleavage of several substrates including PARP. Therefore, cleavage of PARP was determined by western blot analysis. ENLE induced the cleavage of PARP into 115 kDa and 85 kDa fragments through activating caspase-3 (Fig. 21). These data suggest that ENLE induced apoptosis to both prostate cancer cell lines.

**In situ apoptosis assay in PC-3 and LNCaP cells**

*In situ* apoptosis assay i.e., Terminal deoxynucleotidyl transferase mediated biotinylated UTP nick end-labeling (TUNEL) was performed in both the cell lines to access the DNA strand breaks in apoptotic cells. There was an increased red fluorescence in 100 µg/ml of ENLE treated PC-3 and LNCaP cells. The red fluorescence represents the apoptotic cells (Fig. 22).