individual variations between the treatment groups using a computer based software (SPSS 17.5), the significance was considered at the level of $p < 0.05$.

4.0 Objective I

To study the effect of quercetin on EGF – mediated signaling molecules involved in cell survival and proliferation of PC-3 cell line.

Parameters studied

Cell viability of Normal Prostate epithelial cells and PC-3 cell proliferation was assessed by MTT assay

Protein expression of EGFR, PI3k, PDK1, mTOR, Akt, Ras, Raf, ERK1/2, GSK-3β, Cyclin D1, FOXO1, NFkB, PCNA, Caspase-3, Bcl-2 and Bax by western blotting

Caspase-3 activity (Biovision, USA)

Cell death by acridine orange/ethidium bromide dual staining
Effect of quercetin on the viability of normal prostate epithelial cells by MTT assay

Normal prostate epithelial cells (PrEC) were purchased from Lonza, USA, seeded in 24 multiwell plates at a density of 5x 10^4 cells per well and treated with different doses of quercetin (25 µM, 50 µM, 75 µM, 100 µM & 125 µM). Quercetin didn’t show any cytotoxic effect on PrEC (Fig. 1).

EGF and quercetin on the proliferation of PC3 cells by MTT assay

Epidermal growth factor is a potent mitogenic factor that plays an important role in the growth, proliferation and differentiation of numerous cell types. PC-3 cells were cultured in 96 well plates, treated with different concentrations of EGF (12.5-100ng/ml) for 24 and 48 h. EGF stimulated the proliferation of PC-3 cells from 25ng/ml and at 50ng/ml maximum proliferation was observed (Fig. 2A). Quercetin (100µM) significantly decreased the EGF-induced PC-3 cell proliferation (Fig. 2B).

Effect of EGF on EGFR/p-EGFR protein levels in PC-3 cell line at different time durations

EGF acts by binding with high affinity to epidermal growth factor receptor (EGFR) on the cell surface and stimulating the intrinsic protein-tyrosine kinase activity of the receptor. The effects of EGF at different time points (0 h, 10 m, 30 m, 1 h, 3 h, 6 h, 12 h & 24 h) on the protein levels of EGFR, p-EGFR, were analyzed by western blotting. EGF treatment induced p-EGFR, EGFR protein levels as shown in (Fig. 3) at 10 m and at 30 m- 1 h. Maximum increase was observed at 1 h which sustained upto 6 h. p-EGFR protein levels were decreased after 6 h (Fig. 3).
Effect of EGF on Akt/p-Akt (Thr 308)/p-Akt (Ser 473) protein levels in PC-3 cell line at different time durations

Akt is a serine/threonine-specific protein kinase that plays a key role in cell survival and proliferation by regulating many downstream signaling pathways. The effects of EGF at different time points (0 h, 10 m, 30 m, 1 h, 3 h, 6 h, 12 h & 24 h) on the protein levels of Akt/p-Akt were analyzed by western blotting. EGF treatment induced p-Akt protein levels as shown in at 10 m and at 30 m. 1 h maximum increase was seen which sustained upto 6h, however after 6 h p-Akt protein levels were decreased (Fig. 4). Total Akt levels remained unchanged.

Effect of quercetin on EGF-mediated changes in EGFR/p-EGFR protein levels in PC-3 cell line

EGF signaling is important for cancer cell proliferation, migration and metastasis. The inhibitory effects of quercetin on EGF induced signaling in PCa cells were examined. Quercetin inhibited the EGF induced EGFR protein expression and its phosphorylated form p-EGFR (Fig. 5).

Effect of quercetin on EGF-mediated changes in PI3k and PDK1 protein levels

We next examined EGF-stimulated PI3K (p85), PDK1 the downstream signaling molecule of EGFR pathway and it was found that quercetin significantly decreased PI3K, PDK1 protein levels in PC-3 cell line (Fig. 6).

Effect of quercetin on EGF-mediated changes in mTOR/p-mTOR protein levels

The mammalian target of rapamycin (mTOR), a serine/threonine kinase is a downstream target of EGFR/PI3K/AKT pathway and it plays a critical role in cell
survival and proliferation. EGF induced the expression of mTOR, p-mTOR, whereas quercetin treatment significantly decreased mTOR/p-mTOR protein levels (Fig. 7).

**Quercetin on EGF-mediated changes in Akt and p-Akt protein levels**

Akt is a critical protein and is frequently activated in prostate carcinogenesis. Quercetin decreased the EGF induced Akt phosphorylations at threonine 308 and serine 473 in PC-3 cells (Fig. 8), however total Akt protein levels is unaltered.

**Quercetin on EGF- mediated changes in Ras, Raf and ERK1/2 protein levels**

ERK is a downstream component of Raf serine/threonine kinases. Raf activates the MAPK/ERK kinase (MEK)1/2 dual-specificity protein kinases, which then activate ERK1/2. EGF induced the protein levels of Ras, Raf, ERK1/2, MAPK. Quercetin significantly decreased the protein levels of Ras & Raf (Fig. 9), and ERK1/2 (Fig. 10) in PC-3 cells.

**Quercetin on EGF- mediated changes in p-GSK3β-GSK3β and Cyclin D1 protein expressions**

Glycogen synthase kinase-3- β (Gsk-3-β) is a downstream target of Akt and is negatively regulated by Akt. Akt phosphorylates Gsk-3-β which in turn regulates cyclins. Gsk-3-β phosphorylates cyclins which regulate cell cycle progression. Quercetin decreased Akt protein levels may have resulted in an increased expression of GSK in the present study which might have degraded cyclin D1 (Fig. 11).

**Quercetin on EGF- mediated changes in FOXO1 protein expression**

Akt phosphorylates FOXO and regulates cell survival by inhibiting apoptosis. EGF increased p-FOXO protein level and quercetin treatment significantly decreased
p-FOXO protein level in PC-3 cells, thereby quercetin may induce apoptosis in PC-3 cells by regulating FasL expression (Fig. 12).

**Effect of quercetin on EGF- mediated changes in PCNA, NFκB protein levels**

NFκB activation is regulated by Akt and ERK signaling pathway. The present study shows that quercetin significantly decreased the NFκB and PCNA expression thereby quercetin inhibited the NFκB mediated cell survival (Fig. 13).

**Quercetin on EGF- mediated changes in pro-apoptotic and anti-apoptotic protein levels**

Bcl-2 family is an important regulator of cell death with both anti-apoptotic (e.g. Bcl-2) and pro-apoptotic (e.g. Bax) members. Bcl-2 family regulates susceptibility to apoptosis, whereby over expression of Bcl-2 or Bcl-xL promotes survival, but over expression of Bax accelerates cell death. Bax over expression in PCa cells leads to induction of apoptosis. EGF induced the expression of Bcl-2 (Fig. 14) protein thereby favoring cell survival and proliferation however quercetin decreased the level of Bcl-2 protein thereby reversing EGF induced survival. Bax protein expression was decreased by EGF and increased by quercetin (Fig. 14).

**Quercetin on EGF-mediated changes in caspase-3 protein level and activity**

Caspases are cysteine proteases which are upregulated during apoptosis and cleave a broad range of cellular targets that ultimately result in apoptosis in diverse cell types, including PCa cells. This processing leads to cleavage of various death substrates, which in turn lead to morphological changes typical of apoptosis. Caspase-3 protein expression was increased by quercetin treatment (Fig. 14). Caspase-3 activity
was measured by caspase-3 activity kit from Biovision, USA and it was found that quercetin increased the activity of caspase-3 (Fig. 15).

**Visualization of apoptotic changes in PC-3 cells by Acridine orange/ethidium bromide (AO/Etbr) dual staining**

Acridine orange is taken up by both viable and dead cells. It fluoresces green when bound to double stranded DNA in living cells and fluoresce red when bound to single stranded DNA which dominates in dead cells. EtBr is excluded from living cells. However late apoptotic or necrotic cells have ruptured membranes that allow for the entrance of Etbr to intercalate into DNA and fluoresce red. Quercetin induced apoptosis in PC-3 cells was evidenced by the presence of red color, in AO/EtBr staining (Fig. 16).
The present study focused on evaluating the proficiency of quercetin on EGF-mediated signaling pathways involved in the progression of PCa in vitro. World health organization (WHO) estimated that approximately 80% of world population depends on traditional medicine to meet their primary health care needs. Quercetin is an active component of dietary flavonoids and possesses several biological activities, especially useful in cancer treatment. Quercetin has been reported to suppress cytokine and insulin like growth factor - induced invasiveness (Lee et al., 2004). Quercetin is a proved PI3K kinase inhibitor, and has got anticancer activity against various cancers including PCa. Various studies have shown that naturally occurring compounds, such as quercetin, are potential therapeutics for the prevention and treatment of human PCa (Cohen et al., 2000; Kolonel et al., 2000). Previous studies from our lab proved the anticancer activity of quercetin against PCa cell lines (Vijaybabu et al., 2005, 2006a,b; Senthilkumar et al., 2010, 2011). Further, quercetin showed chemopreventive effect against PCa in Sprague Dawley rats (Sharmila et al., 2013; 2014). Epidemiological evidences showed that dietary vegetables and fruits prevent cancer (Block et al., 1992). Therefore, quercetin was selected to check whether quercetin will interfere with EGF-induced PCa cell growth.

Prostate cancer is the second leading cause of cancer deaths in men, with an estimated 29,720 deaths in 2013 (Siegel et al., 2013). The mortality rate for metastatic PCa is extremely high (Han et al., 2001; Stephenson et al., 2006). We found that quercetin didn’t show any cytotoxicity against normal prostate epithelial cells. Quercetin treatment has been associated with antiproliferative effects (Nair et al., 2004) and induction of apoptotic mechanism, in cancer cell lines but not in normal cells (Chowdhury et al., 2005).
EGF plays a critical role during cancer progression. Prostate cancer becomes more dependent on growth promoting actions of EGF during androgen withdrawal. In this objective we found that EGF stimulated the proliferation of PC-3 cells and 50 ng/ml EGF was found to induce a maximum proliferation in PC-3 cells. However, quercetin was able to inhibit EGF-induced cell proliferation at 100 µM dosage. Wenfang et al., (2012) reported that EBP50 inhibits EGF-induced breast cancer cell proliferation by blocking EGFR phosphorylation. Melitin a flavonoid, suppresses EGF induces bladder cancer cell proliferation (Koji et al., 2012).

EGFR transmits a growth-inducing signal to cells that have been stimulated by an EGFR ligand (e.g., EGF and TGFα) (Carpenter and Cohen, 1990; Alroy and Yarden, 1997). EGF stimulated the expression of EGFR, p-EGFR and p-Akt protein levels in a time dependent manner, starting from 10 min onwards. EGF activated EGFR in PCa cell lines, primary tumors and androgen-independent metastatic tumors (Limonta et al., 1995). GFR transactivation may be ligand-dependent or ligand-independent, due to the activity of intracellular kinases, such as c-Src, which cause EGFR tyrosine phosphorylation. EGFR is a key factor in epithelial malignancies, and its activity enhances tumor growth, invasion, and metastasis (Normanno et al., 2006).

In normal tissues, EGF ligand activation is tightly regulated. EGFR is often perpetually stimulated in cancer because of the sustained production of EGFR ligands in the tumor microenvironment. Aberrant expression of EGFR by tumors typically confers a more aggressive phenotype (Grandis and Sok, 2004).

It is well known that growth factor receptors such as EGFR are frequently overexpressed in PCa. The progression and metastatic growth of PCa has been associated with a significant increase in the expression of EGFR and its ligands (Huang
et al., 2002). Quercetin decreased EGF-induced protein expression of EGFR, p-EGFR, therefore it may prevent PCa progression by regulating EGFR.

EGFR mediates its action by regulating two major downstream signaling pathways; PI3K/Akt and MAP kinase. EGF-induced protein expression of PI3K, PDK1 which may have increased Akt activation. However, quercetin significantly decreased PI3K, PDK1 and p-Akt protein levels. Akt is an important cell survival factor that stimulates progression of the cell cycle (Weng et al., 2001). Akt regulates survival, growth, metabolism and progression through the cell cycle (Engelman, 2009). Phosphorylated Akt is an attractive molecular target because it contributes to the development of cancer and confers resistance to conventional therapies. Akt prevents cells from undergoing apoptosis by inhibiting the pro-apoptotic factors Bad and caspase 9, as well as nuclear translocation of the Forkhead transcription factors (Cardone et al., 1998). Overexpression of Akt has been reported in many cancers including PCa since it favours cancer cell proliferation, invasion and angiogenesis (Engelman, 2009). Quercetin inhibited EGF-induced PDK1 and PI3K phosphorylation’s; thereby down-regulating Akt protein levels. Quercetin has been shown to inhibit EGFR and Akt protein levels (Senthilkumar et al., 2011) from our laboratory previously.

The full activation of Akt requires phosphorylation at Serine 473 position by mTOR (Mammalian target of rapamycin), which is a major downstream effector of Akt signaling and is activated via Akt-mediated inhibition of TSC (Tuberous sclerosis proteins) (Pene et al., 2002). AKT/mTOR and MAP kinase pathways are among the major signaling networks that have been implicated in advanced PCa. Deregulated expression of the PTEN occur with high frequency in PCa, leading to aberrant
activation of Akt kinase activity as well as its downstream effectors, including the mTOR signaling pathway (Malik et al., 2002; Kremer et al., 2006; Sparks and Guertin, 2010). In the present study quercetin prevented EGF- induced mTOR protein expression and its phosphorylation.

Evidences that ERK-MAPK signaling promotes cell proliferation, survival and metastasis, support current efforts to identify novel approaches to inhibit this pathway. Ras/Raf/MEK/ERK signaling cascades transmit signals from receptors to regulate gene expression and prevent apoptosis. This pathway has been reported to be activated in over 50% of acute myelogenous leukemia and acute lymphocytic leukemia and is also frequently activated in other cancer types (e.g., breast and prostate cancers) (Gioeli et al., 1999; Gioeli, 2005; Davis et al., 2014). Importantly, this increased expression is associated with a poor prognosis. EGF increased the protein levels of Ras, Raf and ERK1/2 in PC-3 cells in the present study.

The Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt pathways interact with each other to regulate tumorigenesis (Davis et al., 2014). Raf/MEK/ERK is usually associated with proliferation and drug resistance of hematopoietic cells, while activation of the Raf/MEK/ERK cascade is suppressed in some PCa cell lines which have mutations at PTEN and express high levels of activated Akt. Furthermore the Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt pathways also interact with the p53 pathway. Raf/MEK/ERK may promote cell cycle arrest in prostate cells and this may be regulated by p53 as restoration of wild-type p53 in p53 deficient PCa cells results in their enhanced sensitivity to chemotherapeutic drugs and increased expression of Raf/MEK/ERK pathway. In our study quercetin decreases the expression of Ras and Raf protein kiases. Notably, previous studies have demonstrated that the Akt/mTOR,
MAPK signaling pathways are alternatively or coordinately expressed in advanced PCa and function cooperatively to promote tumor growth and the emergence of hormone-refractory disease (Gioeli et al., 1999; Kinkade et al., 2008). Further, many prostate tumors exhibit deregulated growth factor signaling resulting in activation of the ERK-MAP kinase signaling (Gioeli et al., 1999). Quercetin decreased EGF-induced activation of ERK-MAP kinase pathway by inhibiting phosphorylation of ERK1/2. Flavonoids like fisetin have been shown to inhibit activation of extracellular signal-regulated kinase (ERK) (Gioeli et al., 2005).

GSK-3β plays a critical role in cell survival. This is most prominent at the G1–S transition of the cell cycle via phosphorylation and inhibition of glycogen synthase kinase 3-beta (GSK-3β). GSK-3β activity is controlled by phosphorylation and subcellular localization (Kremer et al., 2006). When phosphorylated by active Akt on serine 9, its kinase activity is inhibited and it loses the ability to prime cyclin D1 for degradation; thus, Akt-induced inactivation of GSK-3β stabilizes cyclin D1, whereas inhibition of Akt by Wortmannin results in the accelerated degradation of cyclin D1 (Jope and Jhonson, 2004; Dibble et al., 2009). Quercetin was able to increase GSK-3β expression which was decreased by EGF. Cyclin D1 is known as a proto-oncogene whose gene amplification and protein over expression are frequently observed in tumor cells. Cyclin D1 overexpression has been reported between 40 and 90% of cases of invasive breast cancer, while gene amplification is seen in about 5–20% of tumours (Michalides et al., 1996). Over expression of cyclin D1 has been reported in most of the cancers and also been linked to the development of endocrine resistance in breast cancer cells (Hodges et al., 2003; Hui et al., 2013). Akt phosphorylation inhibits the GSK3-β activation which phosphorylates and establishes cyclin D1 protein. In the
present study, EGF-induced cyclin D1 protein levels were significantly decreased by quercetin. Senthilkumar et al., (2011) has shown that quercetin inhibits PCa cell cycle progression by decreasing cyclin D protein levels.

Proliferating cell nuclear antigen (PCNA), a cofactor for DNA polymerase δ plays a central role in the cell cycle progression (Moldovan et al., 2007). PCNA is involved in a wide range of cellular functions including DNA replication, repair and epigenetic maintenance, and is often used as a diagnostic and prognostic marker. The activation of NF-κB typically occurs through site-specific phosphorylation and subsequent degradation of IκB. This allows the translocation of NF-κB into the nucleus to bind to NF-κB-specific DNA-binding sites and regulate gene transcription. NF-κB activation is regulated by Akt signaling pathway (Ozes et al. 1999). NF-κB plays an important role in the apoptotic process (Wang et al. 1998, 2013). EGF induced NF-κB and PCNA expression in PC-3 cells which suggests an increased cell proliferation and survival. Quercetin treatment significantly decreased NF-κB and PCNA protein levels, thereby decreasing PC-3 cell proliferation and survival. Previous studies from our lab reported that quercetin inhibits PCa cell survival by inhibiting NF-κB protein expression (Senthilkumar et al., 2011).

MAPKs regulates transcription factor NF-κB which acts independently or coordinately to regulate numerous genes involved in the regulation of MMPs expression (Rayet and Gelinhas 1999; Brandi et al., 2008). Rayet and Gelinhas (1999) studied that NF-κB has critical role in transcription of genes involved in apoptosis, inflammation and tumor progression. Senthilkumar et al., (2011) recently studied that quercetin inhibited the activation of NFκB and the suppression of NF-κB activation by quercetin was caused by a mechanism involving inhibition of I-κB kinase. Elumalai et
al., (2014) from our laboratory reported that nimbolide inhibited the activation of NF-kB through IκB kinase in breast cancer cells.

Forkhead box O (FOXO) transcription factors are involved in multiple signaling pathways and play critical roles in a number of physiological and pathological processes including cancer. FOXO target genes include Fas ligand (FasL), insulin-like growth factor-binding protein 1, the apoptotic regulator Bcl-2 interacting mediator of cell death (Bim) and others (Greer and Brunet, 2005).

FOXOs are phosphorylated by Akt at three consensus Akt sites, corresponding to Thr24, Ser256 and Ser319 of FOXO1 (Greer and Brunet, 2005; Cameron et al., 2008). This leads to the interaction of FOXOs with 14-3-3 proteins and the nuclear export of the FOXO-14-3-3 complex mediated by chromosomal region maintenance 1 (CRM1) and Ran GTPase. Translocation of FOXOs to the cytoplasm results in inhibition of target gene transcription (Burgering and Kops, 2002). Growth factor withdrawal leads to the PI3K–Akt pathway inactivation, FOXO dephosphorylation at its Akt sites, nuclear translocation and target gene activation (Webb and Brunet, 2014). Within the nucleus, FOXO triggers apoptosis by inducing the expression of death genes such as the FasL gene, and thereby participates actively in the process of apoptosis (Webb and Brunet, 2014). Quercetin decreased EGF- induced phosphorylation of FOXO1 protein in the present study, thereby decreasing the translocation of FOXO from nucleus to cytosol. This may lead to increased apoptosis in PCa cells.

An impaired apoptotic pathway is a major contributing factor in the development of hormone refractory prostate cancer (HRPC). Proapoptotic (Bax) and antiapoptotic (Bcl-2) molecules relative ratios regulate the sensitivity of cells toward
either survival or apoptosis. Bcl-2 inhibits apoptosis, and its overexpression is associated HRPC. Bax are in the Bcl-2 family and counteract the antiapoptotic function of Bcl-2. Overexpression of Bcl-2 seems to enable the PCa cells to survive in an androgen-deprived environment, and to confer resistance to anti-androgen therapy (Diehl et al., 1998). The functional role of Bak and Bax, which are in the same protein family as Bcl-2 and were cloned as Bcl-2–related genes, is to inhibit the protection from apoptosis that is provided by Bcl-2 (Alt et al., 2000). Quercetin decreased Bcl2 protein expression in PC-3 cells.

Caspases are a family of proteases involved as the central component of a proteolytic system in the apoptotic process. Caspases are classified into two groups according to their function and structure: the initiator caspases (caspase-2, 8, 9, 10) and the executioner caspases (caspase-3, 6, 7) (Kuribayashi et al., 2006). Activation of caspase is a hallmark of promoting apoptosis in response to death inducing signals originated from cell surface receptors and mitochondria (Budihardjo et al., 1999).

Caspase-3 is the ultimate executioner caspase that is essential for the nuclear changes associated with apoptosis, including chromatin condensation. An expanding body of evidence suggests that the caspase cascade is involved in the execution of apoptosis in PCa cells in response to diverse stimuli, including lovastatin and Fas-mediated signaling (McDonnell et al., 1992; Chittenden et al., 1995). In addition, blockade of caspases activity by the inhibitor CrmA has been shown to suppress androgen-ablation-induced apoptosis in LNCaP PCa cells in vitro and in vivo (Diaz et al., 1997). Furthermore, caspase-3 activation plays a role in apoptotic induction of other human cancers, such as osteocarcinoma, ovarian, gastric, and breast cancer (Bowen et al., 1999). Caspase-3 protein expression as well as its activity was increased
by quercetin. Quercetin has been shown to increase capase-3 activity (Henkels and Turchi, 1999). In particular, activation of caspase-3 plays the central role in the initiation of apoptosis (Salvesen and Dixit, 1999). This enzyme has substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves poly (ADP-ribose) polymerase (PARP), which is an important protein that appears to be involved in DNA repair, maintenance of chromosomal stability, and programmed cell death (Bursztajn et al., 2000). Quercetin may induce apoptosis by regulating caspase-3 activity in PC-3 cells. Our previous studies also demonstrated that quercetin treated PC-3 cells showed increased caspase-3 levels suggesting the mechanism of apoptosis induction (Vijayababu et al., 2006a). In the present study EGF alone treated cells showed decreased caspase-3 activity, however quercetin treatment increased caspase-3 activity.

Cell death was visualized by using acridine orange (Ao)/ ethidium bromide (Etbr) dual staining. Acridine orange is a vital dye that will stain both live and dead cells, whereas, ethidium bromide will stain only those cells that have lost their membrane integrity. Live cells appeared uniformly green. Early apoptotic cells stained yellow and late apoptotic cells showed condensed and often fragmented nuclei and the incorporated ethidium bromide stained red. It was found that number of dead cells increased significantly in quercetin treated group compared to that of EGF treated group. Vijayababu et al, (2006b) demonstrated that the increased level of IGFBP3 was associated with increased pro-apoptotic proteins and apoptosis in response to quercetin in PC-3 cells. Acridine orange and ethidium bromide staining of the cells showed apoptosis in quercetin treated cells.

It is concluded from this objective that quercetin prevented EGF- induced cell proliferation and survival. The present study demonstrated that quercetin inhibits EGF-
induced EGFR-PI3K-Akt and MAP kinase signaling pathways which further leads to the inhibition of GSK-3β, cyclin D1 and NFκB, thereby inhibiting PCa cell survival and proliferation. Further, quercetin induced cell death by regulating the levels of Bcl-xl/Bcl-2 ratio and increasing the activity of executioner caspases. These mechanisms may be exploited for the prevention and treatment of PCa.
Objective II

To evaluate the role of quercetin on EGF – induced epithelial to mesenchymal transition, migration and invasion via EGFR/PI3K/Akt pathway in PCa cell line

Parameters studied

Protein expression of E-cadherin and N-cadherin by immunocytochemistry

Protein expression of E-cadherin, N-cadherin, vimentin, ICAM-1, VCAM-1, E-selectin, p-selectin, MMP-2, MMP-9, Snail, Slug and Twist by western blotting

mRNA expression of E-cadherin, N-cadherin, vimentin, Snail, Slug and Twist by Real-Time PCR

Migration assay – Scratch/wound healing method

Invasion assay - Matrigel insert chamber (BD Bioscience, USA)
Effect of quercetin on EGF-mediated changes in the protein, mRNA expression of E-cadherin and N-cadherin

To determine the effectiveness of quercetin on EGF induced EMT, the characteristic markers of EMT E-cadherin and N-cadherin, were studied by Real Time PCR, western blotting and immunocytochemistry. Figures 17A & 18A represent the immunocytochemical localization of E-cadherin and N-cadherin, as indicated by red fluorescence and blue color (DAPI) represents nucleus. The mRNA expression of E-cadherin showed significant decrease by EGF treatment, whereas quercetin restored its expression (Fig. 17C). The protein expression of E-cadherin showed decreased expression in EGF treatment, while quercetin restored the protein levels (Fig. 17A & B). The obtained protein expression data were congruent with RT-PCR data. Contrary to E-cadherin, the mesenchymal phenotype marker N-cadherin mRNA (Fig. 18C) and protein expression showed significant increase by EGF treatment, whereas quercetin treatment decreased N-cadherin expression (Fig. 18A & B).

Quercetin on EGF-mediated changes in the protein and mRNA expression of Vimentin

Vimentin; an EMT marker undergoes upregulation during EMT process. The mRNA and protein expression of vimentin was significantly increased by EGF treatment, whereas quercetin treatment decreased the protein level (Fig. 19A & B).

Quercetin on the EGF-mediated changes in protein expressions of ICAM-1 and VCAM-1

ICAM-1 is a cell adhesion molecule and plays a crucial role during cell invasion and migration. The protein expression of ICAM-1 showed significant increase by EGF
treatment whereas quercetin brought down the protein expression of ICAM-1 (Fig. 20). VCAM-1 helps in tumor metastasis by mediating tumor-stromal interactions in various cancer types. The protein level of VCAM-1 was significantly increased by EGF, whereas quercetin decreased EGF induced V-CAM-1 protein expression in PC-3 cells (Fig. 20).

**Quercetin on the EGF- mediated changes in protein expressions of P-Selectin and E-Selectin**

Selectins (P-selectin & E-selectin) mediate cell adhesion and are involved in cancer cell metastasis. The protein levels of selectins were significantly increased by EGF, whereas quercetin decreased EGF induced selectins protein expression in PC-3 cells (Fig. 21).

**Quercetin on EGF- mediated changes in protein expressions of MMP-2 and -9**

Matrix metalloproteinases (MMPs) are a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for the tissue remodeling and degradation of the extracellular matrix (ECM), including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan. They are regulated by hormones, growth factors, and cytokines, and help in migration and invasion of cancer cells. Quercetin significantly decreased the protein expression of MMP-2, MMP-9 in PC-3 cells, thereby may have decreased cancer cell invasion and migration (Fig. 22).

**Quercetin on EGF- mediated mRNA & protein expression of Snail, Slug and Twist**

To determine the molecular mechanism by which quercetin regulates EMT, the effect of quercetin and EGF on transcriptional repressors Snail, Slug and Twist were
studied by Real Time PCR and western blotting. Treatment with EGF significantly increased Snail, Slug and Twist mRNA (Fig. 23) and protein levels (Fig. 24) in PC-3 cells whereas quercetin significantly decreased the expression, indicating that quercetin may up regulate E-cadherin by modifying its transcriptional repression. These results indicate that quercetin may regulate EMT at transcriptional level.

**Quercetin on EGF- induced cell migration and invasion in PC-3 cell line**

PC-3 cell migration was studied by using wound healing (scratch assay), which revealed EGF induced cancer cell migration (Fig. 25) was significantly decreased upon quercetin treatment. Further, to determine the effect of quercetin on EGF induced PC-3 cell invasion, we used BD Bio-Coat Matrigel Invasion chamber. The PC-3 Cell invasion showed significant increase by EGF treatment, while quercetin treatment prevented the invasiveness of cells (Fig. 26). The number of cells was counted manually and the percentage migration, invasion was calculated. Quercetin significantly inhibited EGF induced PC-3 cell invasion and migration.
Metastasis is the process by which a tumor cell leaves the primary tumor, travels to a distant site via the circulatory system, and establishes a secondary tumor. In order to metastasize, cancer cells must invade through the basement membrane and the extracellular matrix (ECM). Proteolysis of the ECM is an important step in metastasis and the process is associated with the upregulated production and activity of several ECM degrading proteases like MMPs. Nevertheless, the molecular mechanism of PCa metastasis still remains unclear.

It is known that tumor progression to malignancy requires a change from an epithelial phenotype to a fibroblast or mesenchymal phenotype, a re-programming known as an epithelial-mesenchymal transition (EMT). EMT is recognized as an important event in carcinoma progression. During EMT process epithelial cells lose their characteristic polarity, disassemble their cell-cell junctions and become highly motile (Thiery, 2002). Acquisition of migratory properties is a prerequisite for cancer progression so that the tumor cells can migrate and invade into surrounding tissue (Thiery and Sleeman, 2006).

E-cadherin is a prototypic type I cadherin that forms homophilic interactions through its extracellular immunoglobulin (Ig) domain, which connects to actin filaments indirectly via catenin through their cytoplasmic domains (Takeichi, 1995; Tepass et al., 2000). The appropriate expression of E-cadherin on the plasma membrane is essential for cells to retain an epithelial morphology. Evidence from previous studies has shown a correlation between loss of cell–cell adhesion during EMT and decreased E-cadherin function, resulting in the aggressiveness, de-differentiation and metastasis of many carcinomas (Hunt et al., 1997; Beavon, 2000). In transgenic mouse model loss of E-cadherin-mediated intercellular adhesion is a rate
limiting step in the progression from adenoma to invasive carcinoma in vivo (Perl et al., 1998).

PC-3 cells are useful and a reliable model for the evaluation of tumor progression events and for studying EMT. In the present study, quercetin prevented EGF induced EMT process by suppressing the expression of N-cadherin, and increasing E-cadherin expression in PC-3 cells. Reduced E-cadherin expression has been found in high-grade PCas and is associated with poor prognosis reflecting its critical role in tumor progression (Klymkowsky and Savagner, 2009). Therefore, quercetin appears to be a potential agent to target and prevent EMT in PCa.

“Cadherin switching” usually refers to a switch from the expression of E-cadherin to that of N-cadherin. N-cadherin promotes motility when expressed by epithelial cells and cells that express more E-cadherin remain less motile (Thiery, 2002). It has been shown that, when shRNA is used to block N-cadherin expression, the cells show less motility (Hazan et al., 2004), which suggest that N-cadherin specifically promotes cell motility and as a result it is implicated in cadherin switching when cell behavior is regulated.

Vimentin over-expression in different cancer cell lines and tissues, and its association with increased cancer cell growth, invasion and migration, suggests a possibility that vimentin is in fact participating in the promotion of these tumorigenic events and serves as an excellent target for cancer therapy. Vimentin functions as a positive regulator of EMT. The upregulation of vimentin appears to be a prerequisite for EMT induction. It has been shown that vimentin is an important regulator of cell motility (Umbas et al., 2005). Vimentin is a common marker of highly metastatic
cancer cells and as well possibly related to PCa stem- or progenitor cells. Proteome analysis indicates vimentin expression was correlated with invasion and metastases of androgen-independent PCas (Kaufhold and Bonavida, 2014). In the present study, quercetin upregulated E-cadherin and downregulated N-cadherin & vimentin in PC-3 cells and as a consequence of which it may have prevented EGF induced migration and invasion in PCa cells. Withaferin-A (WFA), a bioactive compound isolated from *Withania somnifera*, was shown to bind tetrameric vimentin at a unique binding pocket site between the pair of head-to-tail α-helical dimers and decrease migration, invasion and induce apoptosis in cancer cells (Bargagna *et al*., 2007).

E-cadherin transcriptional repressors such as Snail (SNAI1), Slug (SNAI2), ZEB-1, SIP-1, E12/E47 (Peinado *et al*., 2004), and Twist (Yang *et al*., 2004) have traditionally been implicated in promoting EMT in various systems of embryonic development and tumor progression. Further, to understand the molecular mechanism behind quercetin induced E-cadherin expression, we studied the effect of quercetin on transcriptional repressors Snail, Slug and Twist, which are EMT markers also. Snail regulates and is responsible for altered gene expression with EMT, and also indirectly regulates critical steps of EMT. In our study, we found that quercetin decreased the EGF induced mRNA and protein expression of Snail, Slug and Twist. Snail induces the expression of mesenchymal markers such as vimentin, fibronectin and MMPs (Olmeda *et al*., 2006). Snail is one of the several transcriptional factors that can suppress E-cadherin gene expression *via* binding to E-box sequences in the proximal E-cadherin promoter (Hemavathy and Ashraf, 2000). Therefore, quercetin might have induced the EGF suppressed expression of E-cadherin *via* Snail repression. Moreover, Twist has been shown to directly activate N-cadherin expression. It has been reported that, Twist
binds to an E-box in the first intron of the human N-cadherin gene and this upregulates expression of N-cadherin in PCa cells (Alexander et al., 2006). Thus, Twist seems to be an inducer of N-cadherin. In the present study, quercetin may have down-regulated N-cadherin by decreasing Twist expression. The inhibition of Slug, Snail or Twist action through interfering RNA (siRNA)or antisense transfer resulted in tumor metastasis or growth inhibition and increased sensitivity to the cytotoxic agents used in chemotherapy for solid cancers (Hu et al., 2008; Jin et al., 2010; Zhang et al., 2010).

PC-3 cells predominantly express EGFR and various evidences indicate that the EGFR family and PI3K/Akt signaling pathway can regulate Snail expression (Hipp et al., 2009), suggesting that inhibition of the EGFR signaling pathways may prevent the loss of E-cadherin function and thereby acquisition of invasive motility (metastasis). It has been shown that the ERK1/2 and PI3K/Akt pathways are involved in EGF-induced EMT (Ahmed et al., 2006; Chen et al., 2010). PI3K/Akt pathway plays an important role in human cancers including prostate carcinoma (Chin and Toker, 2010). Gan et al., (2010) reported that Akt pathway plays a central role in EGFR-driven PCa cell migration by activating EMT. In the present study, we found that EGF- induced protein expression of p-EGFR, p-Akt, p- PI3K activity was significantly decreased by quercetin, suggesting that quercetin may have prevented EGF- induced EMT by inhibiting PI3K/Akt pathway. PI3K-Akt and its associated regulatory signaling pathways are potential targets for therapeutic intervention and molecular based approaches for management of PCa. Quercetin is a potent inhibitor of PI3K/Akt pathway. Our earlier studies also revealed the role of quercetin in inhibition of invasion, migration of PCa cell line (Senthilkumar et al., 2011).
Intercellular adhesion molecule-1 (ICAM-1, also called CD54) is an inducible surface glycoprotein that mediates cell–cell adhesion (Zimmerman and Blanco, 2008). ICAM-1 is crucial for trans-endothelial migration of leukocytes from the capillary bed into the surrounding tissue (Duperray et al., 1997), but it may also facilitate migration of other cell types (Roche et al., 2003; Yang et al., 2010). ICAM-1 plays a key role in breast and lung cancer cell invasion (Grothey et al., 1998). In the present study, quercetin prevented EGF induced ICAM-1 expression. The knockdown of ICAM-1 expression reduces the invasiveness of breast cancer cells (Chen et al., 2011). Since, ICAM-1 plays a critical role in tumorigenesis; disruption of ICAM-1 may prove useful for preventing cancer cell metastasis.

Vascular cell adhesion molecule-1 (VCAM-1) is a cell surface molecule that mediates cell adhesion. When aberrantly expressed in breast cancer cells, VCAM-1 mediates distinct tumor-stromal interactions that are unique to lung and bone microenvironments and facilitate metastasis to these sites (Ruco et al., 1996; Lu et al., 2011). Little is known about the function of VCAM-1 in other cancers. However, VCAM-1 expression has been reported in gastric, renal carcinomas and in PCa (Lu et al., 2011), where it might play roles similar to those recently reported in breast cancer (Shin et al., 2006; Ivaska, 2011). Quercetin significantly decreased EGF- induced expression of VCAM-1, therefore it may help in preventing cancer metastasis in PCa.

Flurries of recent evidence suggest that E-Selectin is involved in the attachment and transmigration of cancer cells including PCa (Wu et al., 2007). Selectins (P-selectin & E-selectin) mediate cell adhesion and are involved in cancer cell metastasis (Myers et al., 1998). Heidemann et al., (2014) reported that selectins mediate lung cancer metastasis. The inhibition of selectin expression correlates with inhibition of
metastasis. Similarly, it has been shown that anti-E-selectin-agents, impairs lung metastasis (Khatib et al., 2002). The up-regulation of selectins in PCa, together with its limited expression in normal body tissues, makes it a potential therapeutic target. Josep et al., (2007) reported that cancer metastasis is impaired in P-selectin deficient mice. Quercetin decreased EGF- induced protein levels of P-selectin and E-selectins in PC-3 cells, thereby may prevent EGF- induced cancer cell metastasis. Previous studies from our lab have proved that DADS inhibits selectins protein expression in PC-3 cells (Arunkumar et al., 2012).

Matrix metalloproteinases (MMP) are 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). MMP-2 and 9 are expressed in human epithelial cancer (Ovary, Prostate, Colorectal, Colon) and there levels seem to be related to the metastatic potential and malignancy (Naylor et al., 1994; Liabakk et al., 1996; Lamson and Brignall 2000). During tumor metastasis, the balance between the active protease and their inhibitors is disrupted, generally leading to an elevated MMP expression (Stamenkovic, 2000). Epidermal growth factor mediated induction of MMPs expression was PI3K-dependent and activation of this signaling pathway also led to increased expression of MMPs (Zhang et al., 2010). Growth factors increase the enzymatic activity of MMP-2 and MMP-9 in DU145 cells (Saikali et al., 2008). In the present study also EGF- induced the expression of MMP2 & 9 in PC-3 cells, thereby inducing invasion and migration in these cells. EGF-induced MMP-9 secretion facilitates tumor invasion and metastasis in various tumor cells, such as ovarian cancer,
head and neck squamous carcinoma, and lung cancer (O-charoenrat et al., 2000). Aberrant expressions of MMPs and TIMPs in PCa and their correlations with invasion and metastasis have been documented in various studies (Lichtinghagen et al., 2003; Zeng et al., 2004, 2006).

MMPs have been implicated in process leading to cancer invasion and metastasis (Liotta et al., 1980; Liotta, 1986) and also play a major role in tumor angiogenesis (Stetler-Stevenson, 1999). MMP -2 and MMP -9 are important contributors to the process of invasion, metastasis and angiogenesis in various tumors including PCa (Stetler-Stevenson, 1999). Quercetin has been shown to be chemopreventive in cancer prevention in several animal models and cancer cell lines (Lamson and Brignall 2000).

Flavonoids are competitive inhibitors for the ATP binding site on a Variety of enzymes such as PKC, a region of considerable homology among kinase. Among flavonoids tested, quercetin exerted the strongest inhibitory effects on cell growth, kinase activity and MMP secretion in many tumour cells (Lamson and Brignall 2000; Zhang et al., 2004). Our previous studies demonstrated a marked decrease in the secretion of MMP2 and MMP 9 in PC-3 cells on quercetin treatment (Vijayababu et al., 2006). Quercetin may cause the inhibition of MMP9 at activation or secretion.

MMP2 and MMP 9 expression were regulated by MAP kinase signaling pathways including ERK and MAPK signaling cascades in human vascular endothelial cells. Quercetin decreased the expression of MMP9 via protein kinase C pathway in murein melanoma cells (Lamson and Brignall 2000; Zhang et al., 2004). It is well
known that quercetin is an inhibitor of sever kinase including MAPKinase and tyrosin kinases (Liu and Liang, 2002).

Quercetin mediated inhibition of invasion occurred via the downregulation of MMP-2 and -9 may be through the inhibition of PI3K/Akt along with parallel inhibition of ERK signaling. Regulation of MMP through PI3K/Akt has been extensively studied, apart from this NFkB also plays a key role in the regulation of MMP. In the present study, quercetin inhibited the levels of NF-kB, which may also affect the MMP-2 and -9 in PC-3 cells. The expression of MMPs are primarily regulated at the transcriptional level through nuclear factor-kappa B (NF-kB) (O-charoenrat et al., 2000). Phytochemicals like epigallocatechin-3-gallate, indole-3-carbinol, sanguinarine, decreased the activity of MMP-2 and -9, as well as increased TIMP-1 and -2 levels (Takada et al., 2005; Hazgui et al., 2008; Choi et al., 2009). Present study also shows marked inhibition of MMP-2 and -9 protein levels following quercetin treatment. Taken together, these data suggest that the anti-invasive activity of quercetin in PC-3 cells was associated with inhibition of MMP-2 and -9 activities.

Further, quercetin inhibited EGF- induced PCa cell migration (scratch assay) and invasion (BD Matrigel invasion assay). Since, quercetin inhibited EGF- induced EMT and MMPs which are the primary requirements for cancer cell migration and invasion. Further, EGF is a critical factor that induces cancer cell migration and invasion. Quercetin inhibited EGFR, p-EGFR and ERK1/2 protein levels which are involved in cancer cell invasion and migration. Our results are supported by the findings of Elumalai et al., (2014), who found that nimbolide inhibits breast cancer cell invasion and migration. Diallyl disulfide, a garlic component, has been shown to inhibit PCa cell invasion and migration (Arunkumar et al., 2012).
To conclude, the present work reveals the potency of dietary flavonoid quercetin to reverse EGF induced cadherin switching, by downregulating EGF-induced EMT markers. Further, quercetin decreased cell adhesion proteins and MMP-2 & MMP-9 protein levels, thereby prevents EGF-induced migration and invasion of PC-3 cells. Thus, quercetin can target EMT and may be used as chemotherapeutic drug in cancer metastasis.
Objective III

To evaluate the effect of quercetin on EGF – induced capillary tube formation (angiogenesis) in HUVEC cells

Parameters Studied

Cell viability of HUVEC cells by MTT assay

Scratch assay/wound healing assay

Tube formation assay
**Effect of quercetin on cell viability of HUVEC cells**

Human umbilical vein endothelial cells (HUVEC) were purchased from Himedia, Mumbai, India and treated with different doses of quercetin (25 µM, 50 µM, 75 µM, 100 µM & 125 µM). Quercetin didn’t show any cytotoxic effect on HUVEC cells upto 100 µM, however, quercetin at 125 µM decreased the viability of HUVEC cells (Fig. 27).

**Effects of the quercetin on EGF- Induced HUVEC Migration**

Endothelial cell migration is the key process in angiogenesis and EGF plays crucial role in cell migration. To determine whether quercetin could inhibit EGF-induced endothelial cell migration, scratch assay/wound-healing assay was done to explore the changes in migration following treatment with EGF that had been preincubated with or without quercetin. EGF treatment for 24 h significantly increased HUVEC cell migration (Fig. 28), however, when HUVECs were treated with EGF + quercetin (100 µM) the cell migration was significantly decreased. This shows that quercetin was effective in preventing EGF- induced endothelial cell migration.

**Tube formation assay/ Capillary-like Tube Formation Assay**

HUVEC cells were plated in 6 well matrigel coated plates at a density of 6x10^4 cells per well and allowed to attach for 48 h in endothelial cell expansion medium. After that cells were treated with quercetin (100 µM) and EGF (50 ng/ml) for 24 h (Fig. 29). Tube formation was examined and photographed using an inverted microscope (10X). EGF (50 ng/ml) induced tube formation in HUVEC cells, resulting in elongated and tube like structures, however quercetin (100 µM) effectively reduced the width and length of endothelial tubes (Fig. 29).
Cancer has the ability to spread to adjacent or distant organs, which makes it life threatening. Tumor cells can penetrate blood or lymphatic vessels, circulate through the intravascular stream, and then proliferate at another site: metastasis (Folkman, 1971; Shields, 2014). For the metastatic spread of cancer tissue, growth of the vascular network is important. The processes whereby new blood and lymphatic vessels form are called angiogenesis and lymphangiogenesis, respectively. Both have an essential role in the formation of a new vascular network to supply nutrients, oxygen and immune cells, and also to remove waste products (Folkman, 1971; Shields, 2014). Angiogenic and lymphangiogenic factors are increasingly receiving attention, especially in the field of neoplastic vascularization.

Angiogenesis, the growth of new blood vessels as sprouts or offshoots of the pre-existing microvasculature, is a physiological event occurring in the development of organisms, wound healing and the reproductive cycle, but it is also involved in pathologic processes such as inflammation, tumour growth and metastasis (Carmeliet, 2000). Aberrant angiogenesis has been implicated in a variety of diseases such as cancer, atherosclerosis, arthritis, obesity, pulmonary hypertension, diabetic retinopathy, and age-related macular degeneration. These conditions collectively affect nearly 10% of the global population (De Falco, 2014). Much effort has focused on identifying new therapeutic agents that inhibit pathological angiogenesis. The early stages of angiogenesis can be divided into three steps: endothelial cell proliferation, migration, and tube formation. Angiogenesis can be stimulated by a large number of pro-angiogenic cytokines, such as VEGF, EGF, TNF-α, bFGF and IL-8 (D’Andrea et al., 2006; Li et al., 2003). All these proangiogenic agents act through distinct membrane receptors (Waugh and Wilson, 2008; Brooks et al., 2012) which result in the activation
of extensively overlapping intracellular cascades finally activating common effector molecules, such as NF-κB or HIF-1 (Waugh and Wilson, 2008). In addition, recent evidences indicate that direct interactions may occur between integrin activated pathways and signalling from VEGF receptors (Paesler et al., 2012) and EGF receptors (Hu et al., 2005). During neovascularization, vascular endothelial cell proliferation and migration occur initially, followed by organization into a network of tube-like structures and the formation of new capillaries (Wang et al., 2012).

HUVEC cells represent a valid in vitro model which provides seminal insights into the cellular and molecular events leading to neovascularization in response to inflammation and hypoxia in cancer, ischemic events, and in embryogenesis (Xu et al., 2013). Growth factors such as VEGF, EGF, IGF-I and FGF2 are known to promote angiogenesis. Angiogenesis, the growth of new capillaries from pre-existing vessels, occurs as a result of dynamic endothelial cell functions such as migration and tube formation that are essential to the organized formation of vessel sprouts (Folkman and Shing, 1992; Cezar-De-Mello et al., 2006). We, therefore, evaluated these two critical angiogenic processes in this study. The results of cell migration (scratch assay) showed that quercetin inhibited EGF- induced HUVEC cell migration.

According to our results, quercetin is able to strongly inhibit EGF- induced angiogenesis, as indicated by the reduction of tube formation/network formation, and this occurs without affecting cell viability. In our experimental conditions, quercetin did not affect cell viability, suggesting that its antiangiogenic activity is likely independent from cytotoxicity. This latter observation deserves further consideration because angiogenesis represents a key step in cancer metastasis. Pratheeshkumar et al., (2012) reported that quercetin inhibits VEGF- induced angiogenesis in endothelial cells
and nude mice. Quercetin-49-O-b-D-glucopyranoside (QODG) inhibits angiogenesis by Suppressing VEGFR2-mediated signaling in Zebrafish and endothelial cells (Lin et al., 2012). Fanelli et al. (2014) reported that a novel compound cyclic RGD peptidomimetic inhibited cell proliferation, migration and angiogenic activity in human endothelial cells. Further, it was reported that Gold nanoparticles inhibit VEGF165-induced migration and tube formation of endothelial cells via the Akt pathway (Yunlong, 2014).

In conclusion, the data of the present objective shows that the quercetin inhibits EGF- induced HUVEC cell migration and tube formation/angiogenic processes without affecting their viability. We propose therefore this compound as a candidate modulator of angiogenesis, feasibly devoid of the adverse effects of cytotoxic analogues.
Prostate cancer is the most frequently diagnosed malignancy. The mortality rate due to metastatic PCa is very high. The metastatic PCa patients are estimated to have a median survival of 12–15 months even with chemotherapies. Epidermal growth factor plays a key role in the transformation of PCa to a metastatic stage and the PCa becomes more reliant on the growth promoting actions of EGF during androgen withdrawal. Quercetin is a key bioflavonoid, which is known to have anticancer effects against various cancer cell lines including PCa cell lines. Previous studies from our laboratory also reported anticancer effects of quercetin against PCa. Nevertheless the regulatory mechanism of quercetin on EGF- induced PCa progression is not well recognized. So the present study was designed to study the effects of quercetin on EGF- mediated signaling involved in PCa cell survival, proliferation, migration and invasion.

Quercetin (25-125µM) didn’t show any cytotoxicity towards normal prostate epithelial cells, which suggest that quercetin explicitly, affects cancer cells.

EGF- induced protein levels of EGFR and Akt in time dependent mode. EGF-induced PCa cell proliferation was inhibited by quercetin (100µM).

Quercetin inhibits EGF- induced EGFR, PI3K, mTOR, Akt, NFkB protein levels. Further, quercetin repressed EGF- induced changes in GSK-3β, Cyclin D1.

Quercetin also inhibited Ras-Raf-ERK1/2 pathway. Therefore, quercetin inhibits proliferation and progression of PCa cells.

EGF- mediated changes in Bcl-2-Bax protein levels were altered by quercetin.

Quercetin increased caspase-3 activity. Acridine orange/ethidium bromide staining revealed that quercetin induced apoptosis in PC-3 cells.

Quercetin inhibited EGF- induced EMT in PC-3 cells by increasing E-cadherin expression and decreasing N-cadherin, vimentin protein levels in PC-3 cells. Further,
Quercetin inhibited EGF- induced EMT by regulating transcriptional repressors Snail, Slug and Twist.

Quercetin significantly decreased the EGF- induced protein expression of MMP-2 & MMP-9.

Quercetin decreased the EGF- induced invasive and migratory potential of PC-3 cells as assayed by matrigel coated invasion assay and scratch/wound healing assay.

HUVEC cells serve as an ideal and reliable model for studying angiogenesis in \textit{in vitro} conditions. Angiogenesis is a multifaceted process, which is vital for metastatic spread, of cancer cells. Quercetin didn’t affect the viability of HUVEC cell up to 100\,\mu M dosage, as studied by MTT assay. EGF- induced HUVEC cell migration and tube formation was inhibited by quercetin, which suggests that quercetin may inhibit angiogenesis.

To conclude from the present study, quercetin effectively inhibited EGF-induced cell survival, proliferation, migration and invasion of PCa cells. On the basis of our findings, we propose that quercetin exhibits anti-proliferative, anti-invasive, anti-migrative, and apoptotic effects through the suppression of the EGFR-PI3K-Akt and MAP kinase pathways. Further, quercetin inhibited EGF- induced tube formation in HUVEC cells, which serves as an \textit{in vitro} model for angiogenesis.

The present study adds to the ample literature that quercetin may provide some faith for treating and preventing PCa progression. However, it is obligatory to conduct further studies in animal models and then clinical studies to fully determine the anticancer efficiency of quercetin. However, further studies in animals and humans are mandatory to determine the full potential of this enthralling molecule for cancer prevention or treatment.