Quercetin, a natural dietary flavonoid acts as a chemopreventive agent against prostate cancer in *in vivo* model by inhibiting EGFR signaling pathway

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Abstract

Prostate cancer incidence and mortality rates have increased over the past years. The purpose of the present study was to examine the molecular mechanism underlying the chemopreventive effects of quercetin on prostate cancer in an *in vivo* model. Sprague-Dawley male rats were divided into four groups; Group I: vehicle control (propylene glycol), Group II: chemical- induced cancer model (MNU+T); Group III: chemical- induced cancer model + quercetin (200 mg/kg.b.wt); Group IV: quercetin (200mg/kg.b.wt). Serum levels of quercetin were assessed by high performance liquid chromatography (HPLC). EGFR, PI3K/Akt protein levels were significantly increased in chemical- induced cancer rats, which were brought back to normalcy in both DLP & VP (dorsolateral prostate & ventral prostate) by quercetin supplementation. Also, the protein expression levels of proliferating cell nuclear antigen (PCNA), N-Cadherin, Vimentin, Cyclin D1 exhibited significant increase in both DLP & VP of chemical- induced cancer rats. However, simultaneous quercetin supplementation significantly decreased PCNA, N-cadherin, vimentin, cyclin D1 protein levels compared to chemical-induced cancer rats. E-cadherin expression was decreased in chemical induced cancer animals. Simultaneous quercetin supplementation prevented it. Real Time PCR was used to study the mRNA expression of Snail, Slug and Twist. Quercetin significantly decreased Snail, Slug, Twist mRNA levels in chemical- induced cancer rats. To conclude from the present study, quercetin was effective in preventing prostate cancer progression by inhibiting EGFR signaling pathway and by regulating cell adhesion molecules in Sprague Dawley rats.

1. Introduction

Prostate cancer is the most commonly diagnosed cancer and the second most frequent cause of death in males in USA. African Americans have the highest rates of prostate cancer incidence.[1] In Europe, the incidence has tripled in the last 40 years, with about 328,000 men diagnosed with prostate cancer in 2008.[2]

Epidermal growth factor receptor (EGFR) is a key factor in epithelial malignancies, and its activity enhances tumor growth, invasion, and metastasis.[3] EGFR is a member of the ErbB family of tyrosine kinase receptors that transmit growth-inducing signal to cells that have been stimulated by an EGFR ligand (e.g., TGFα and EGF).[4,5] The activation of the EGFR signaling pathway stimulates downstream signaling cascades involved in cell proliferation (Ras/mitogen-activated protein kinase [MAPK]) and anti-apoptosis (phosphatidylinositol 3-kinase [PI3K]/Akt).[6] Protein kinase B/Akt is activated by the phosphoinositide 3-kinase (PI3-K) pathway. Generation of PIP3 and PI(3, 4)P2 is necessary for the localization of PKB to the membrane surface. Protein dependent kinase (PDK1) phosphorylates Akt at Threonine 308. The fully active multiphosphorylated Akt then dissociates from the plasma membrane and targets substrates located in the cytoplasm and nucleus. It causes the activation of genes involved in diverse cellular processes.[7]

Overexpression of TGFα and EGFR by many carcinomas correlates with the development of cancer metastasis, resistance to chemotherapy and poor prognosis.[8,9] Epidermal growth factor (EGF) regulates cancer metastasis by regulating epithelial to mesenchymal transition (EMT). [10,11] As a feature of aggressive tumors, EMT is characterized by reduced E-cadherin and increased N-cadherin expression, contributing to a stroma-oriented cellular adhesion profile with increased tumor cell motility and invasive properties.[12,13] Vimentin intermediate filaments, in addition to their potential interactions with microfilaments and microtubules, participate in many other specialized cell functions.[14] It is required to maintain the architecture of the cytoplasm, and aberrant vimentin expression during EMT is suggested to be an essential element for epithelial plasticity and tumor cell metastasis.[15] EMT trans-differentiation processes involve the conversion of adherent epithelial cells into individual migratory cells, leading to changes in cell phenotype into more loose mesenchymal-like cells, and promoting local invasion and metastatic dissemination of tumor cells.[16,17]

PCNA is a ubiquitously expressed protein that plays crucial roles in many vital cellular processes.[18,19] It is synthesized in all stages of the cell cycle with a half-life of

approximately 20 h and is elevated in early S phase to support cell cycle progression.[19] PCNA gene deregulation and post-translational modulation are hallmarks of malignant cells. Tumor cells, regardless of their origins, express higher levels of PCNA.[20,21] Expression levels of PCNA correlate positively with other pathological indices in prostate cancer and can serve as an independent prognosis marker.[22] Overexpression of PCNA is also a reliable biomarker for other tumor types.[20]

Epidemiological and dietary intervention studies in animals and humans have suggested that bioflavonoids such as quercetin may play a beneficial role in inhibiting, reversing or retarding tumorigenesis in many types of cancers, including prostate cancer.[23,24] Quercetin (3,3',4',5,7-pentahydroxyflavone), a naturally occurring flavonoid, is a component of most edible fruits and vegetables, with the highest concentrations being found in onions, apples, and red wine.[23,25] Several studies have shown that quercetin has a broad range of pharmacological properties that include antioxidant, anticancer and anti-inflammatory activities.[26] Quercetin has been shown to exert anticancer effects on prostate cancer and breast cancer cell lines.[27,28,29] However, studies on the effects of quercetin on *in vivo* models are lacking. Previous studies from our lab have shown that quercetin prevents ROS production and exerts chemopreventive effect on prostate cancer *in vivo* in Sprague Dawley rats.[30,31] In the present study, we focused on epidermal growth factor signaling involved in tumor progression and EGFR regulation of cell adhesion molecules in prostate cancer induced Sprague Dawley male rats. EGFR plays a critical role in prostate cancer progression and *in vivo* studies are lacking on the effects of quercetin on EGFR signaling.

2. Materials and Methods

2.1. Chemicals

Quercetin, testosterone propionate, and MNU were purchased from Sigma–Aldrich Chemicals Private Ltd. (MO, USA). Real time PCR kit (SYBR-Green) was purchased from Invitrogen, USA. Primary antibodies such as mouse monoclonal proliferating cell nuclear antigen (PCNA), E-Cadherin, N-Cadherin, Cyclin D1 antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and EGFR, p-EGFR, p-PI3k, p-PDK1, Akt, p-Akt, mTOR, GSK-3β were purchased from Cell Signaling Technologies (USA). Secondary antibody was purchased from Santa Cruz Biotechnology Inc. Other chemicals were obtained from SISCO Pvt. Ltd. Research Laboratories, India. All the chemicals were extra pure and of analytical grade.

2.2. Animals

Healthy adult male Sprague-Dawley rats weighing 160—200 g (60 d old) were used in the present study. The animals were housed in clean polypropylene cages and maintained in an air-conditioned animal house with constant 12 h light and 12 h dark schedule. At all times during the studies, rats were permitted free access to drinking water. The animals were fed with standard rat pellet diet (Lipton India Ltd., Mumbai, India). Experiment was approved by the Institute animal ethical committee (IAEC-No 01/01/12).

2.3. Induction of Prostatic Carcinogenesis Using Carcinogen and Hormone

Prostate cancer was induced in rats using MNU and Testosterone undecanoate by a modified protocol.[32,33] First, each rat received daily intraperitonealy (i.p.) injections of testosterone undecanoate (50 mg/kg bodyweight) for 21 consecutive days. At day 23, rats received i.p. injections of 100 mg testosterone undecanoate/kg body weight in 0.3 ml propylene glycol for 3 days. At day 27, all the rats received a single i.v. dose (50 mg/kg body weight) of MNU (dissolved in saline at 10 mg/ml), via the tail vein. One week after MNU administration, rats received i.p. injection of 4 mg testosterone undecanoate/kg body weight alternatively for 16 weeks.

2.4. Experimental Design

A total of 40 rats were divided into four groups. Each group consists of 10 animals.

Group I: Rats received vehicle (propylene glycol) alone by intra peritoneal (i.p) injection.

Group II: Rats prostate cancer was induced by using carcinogen and hormone.

Group III: Rats were induced prostate cancer with simultaneous supplementation of quercetin 200 mg/kg body weight thrice a week for 16 weeks through oral gavage. Quercetin supplementation was begun a week before the administration of initial dose of testosterone undecanoate administration and throughout the studies. The dose was selected based on the earlier studies.[30,31]

Group IV: Rats received quercetin (200 mg/kg body weight) alone (oral gavage).

After the treatment period, rats were sacrificed by cervical decapitation. Prostatic fluid was removed. Both ventral and dorsolateral prostate lobes were dissected from the adhering connective tissue and washed several times with physiological saline, weighed accurately, and separated. Blood was collected for the estimation of quercetin in serum.

2.5. Sample preparation

100 mg ventral and dorsolateral prostate tissue were weighed separately and accurately. 1ml of RIPA (Radio immuno precipitation assay buffer) containing 50mM Tris, 150mM NaCl, 1mM EDTA, 0.5% Sodium deoxycholate, 0.1% NP-40 with 1×protease, phosphatase

inhibitor cocktails was added homogenized, followed by centrifugation at 5000g for 10 min. The pellet was discarded and the supernatant was used for immunoblot/western blot analysis. Protein concentrations were determined by Lowry's method.[34] The samples were stored at -80°C for further study.

2.6. Western Blot Analysis

The tissue homogenate (30-50μg) samples were mixed with 5X sample buffer, boiled for 5 min and were electrophoresed in 10% SDS polyacrylamide gel and then transferred into PVDF membranes. Next the membrane was blocked in PBS containing 5% BSA for 1 h at room temperature. The membranes were incubated with primary antibodies against EGFR, N-Cadherin, E- Cadherin, Vimentin, Akt (1: 2000) and p-EGFR, p-PI3K, p-PDK1, p-Akt, p-Erk, (1: 1000) in Tris-buffered saline at 4°C overnight. After washing, the membranes were incubated with HRP conjugated antimouse IgG (1: 5000) or HRP conjugated goat-antirabbit IgG (1: 5000) secondary antibodies at room temperature. Protein bands were detected using chemiluminescence system (ECL kit) and quantified in Chemi Doc XRS Imaging System, Bio-Rad (USA).

2.7. RNA Isolation and Real Time PCR

The total RNA was isolated by using TRI Reagent (Sigma).[35] 1.5 μg of total RNA was used to synthesize Complementary DNA using a cDNA synthesis kit (IScript, Bio-Rad, USA). Real time-PCR was carried out in MX3000p PCR system (Stratagene, Europe). Reaction was performed using Kapasybr green fast pcr master mix PCR kit (It contains all the PCR components along with SyBR green dye). The primers used for SYBR Green reverse transcription-qPCR (RT-qPCR) were as follows: Snail sense sequence, 5-AGAAGCCTTTCTCCTGCTCC -3 and antisense sequence,5-CACTGGTATCTCTTCACATCCGA -3 (100bp); Slug sense sequence, 5-AGCAGTTCCATTCCACTCCC -3 and antisense sequence, 5-CTTCAATGGCATGGGGGTCT -3 (191bp); Twist sense sequence, 5-GCCGGAGACCTAGATGTCATT-3 and antisense sequence, 5-GGCCTGTCTCGCTTTCTCTT -3 (185bp); Beta actin sense sequence 5-CGCGAGTACAACCTTCTTGC-3 and antisense sequence 5-ATACCCACCATCACACCCTG -3 (200bp). The data were normalized by comparing threshold cycle ratios between the genes of interest and housekeeping gene β-actin. The data were analyzed by comparative CT method.[36]

2.8. HPLC analysis of serum samples

A LC-2010, Shimadzu, Japan (C18) waters (250 x 4.6 mm i.d.) columns was used for HPLC analysis. The chromatograms were monitored at 370 nm using a UV-Vis detector. The elution system consisted of two solvents, A: 2.5% acetic acid in water, B: 80% acetonitrile in A as 7:3 ratio. The analysis was carried out at room temperature 1 mL/min–1.

Chromatographic Conditions

The HPLC system used consisted of an autosampler, an ultraviolet detector, and a software system that controlled all the equipment and carried out data processing. The system was fitted with a 5-μm C18 Hypersil based desactivated silicat analytic column (150 x 34.6 mm ID) (Life Sciences International, Cergy, France). The ultraviolet detector was set at 370 nm, and the mobile phase consisted of 73% solvent A and 27% solvent B, where solvent A = H_2O/H_3PO_4 (99.5:0.5) and solvent B = acetonitrile. For deconjugated samples, elution was isocratic (flow rate 1.5 ml/min). To visualize and separate the conjugated metabolites of flavonols, the chromatographic conditions of elution were as follows (flow rate 1 ml/min): 0–2 min, 85% solvent A/15% solvent B; 2–22 min, 85% solvent A/15% solvent B = 60% solvent A/40% solvent B; 22–24 min, isocratic for 3 min, then returned to initial conditions and equilibration for 8 min.

2.9. Statistical Analysis

Data were expressed as mean ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Student–Newman–Keul's (SNK) tests for comparison between treatment and control groups using Statistical Package for Student version 17.5 (SPSS) software. Statistical significance was considered at *P < 0.05*.

3. Results

3.1. Effect of quercetin on body weight and organ weight (DLP &VP) of chemical-induced cancer model male rats

The body weight of chemical- induced cancer (MNU+T treated) rats showed significant decrease as compared with control animals, whereas simultaneous treatment with quercetin showed increased body weight. Although no significant change was observed the body weight in quercetin alone treated rats (Table 1). Ventral prostate and dorsolateral prostate weights were significantly increased in chemical- induced cancer (MNU + T-treated) male rats as compared to control rats, whereas, simultaneous quercetin treatment showed decreased ventral and dorsolateral prostate weight compared to MNU + T-induced rats. However quercetin alone treated group did not show any significant change in organ weight (Table 2).

3.2. Effect of quercetin on the protein expression of Proliferating cell nuclear antigen (PCNA) protein expression in DLP &VP of chemical- induced cancer model male rats

PCNA serves as a marker for various cancers. The protein expression level of PCNA was significantly increased in both DLP & VP of chemical- induced cancer rats as compared to control rats, whereas, simultaneous quercetin supplementation significantly decreased PCNA expression in both DLP & VP of chemical- induced cancer rats (Fig. 1a & b). There was no significant change in PCNA expression in both DLP & VP of quercetin alone treated rats.

3.3. Effect of quercetin on the protein expression of EGFR and p-EGFR protein expression in DLP & VP of chemical- induced cancer model male rats

EGFR, p-EGFR protein expression levels showed significant increase in both DLP & VP of chemical- induced cancer rats, whereas simultaneous quercetin supplementation decreased both EGFR and p-EGFR expression levels in DLP &VP (Fig. 2a & 2b). Although no significant change was observed in quercetin alone treated group in EGFR, p-EGFR protein levels in both DLP & VP.

3.4. Effect of quercetin on the protein expression of p-PI3K, p-PDK1, p-AKT, AKT protein expression levels in DLP & VP of chemical- induced cancer model male rats

The downstream signaling of EGFR includes PI3K/Akt, plays a crucial role in cancer progression. Akt is a major cell survival protein, and is activated by both PI3K and PDK1. The protein expression of p-PI3K, p-PDK1, Akt, p-Akt were significantly increased in chemical- induced cancer group, whereas, simultaneous quercetin supplementation decreased p-PDK1, p-PI3K and Akt protein levels in both DLP & VP (Fig. 3, 4). However, quercetin alone treated group in both DLP & VP didn't show significant change in the expression level of these proteins.

3.5. Effect of quercetin on the protein expression of p-ERK ½ in DLP & VP of chemical- induced cancer male rats

The protein expression of p-ERK1/2, which is activated by EGFR via Ras/Raf cascade; revealed increased expression level in both DLP & VP of chemical- induced cancer rats (Fig. 5a, 4b). Simultaneous quercetin supplementation significantly decreased the expression level of p-ERK ½ protein in both DLP & VP. There was no significant change in p-ERK1/2 protein levels in both DLP & VP of quercetin alone treated rats.

3.6. Effect of quercetin on the protein expression of mTOR, GSK3β, Cyclin D1 protein expression in DLP & VP of chemical- induced cancer male rats

Mammalian target of rapamycin (mTOR) belongs to the phosphatidylinositol 3-kinase-related kinase protein family, and is a downstream target of Akt. GSK-3 β is integrally tied to the pathways of cell proliferation via cyclins. The protein expressions of mTOR, Cyclin D1 were significantly increased in both DLP & VP of chemical- induced cancer rats, whereas simultaneous quercetin supplementation decreased mTOR, cyclin D1 in both DLP & VP. There was no significant change in mTOR, cyclin D1 in quercetin alone treated rats. GSK3β protein expression significantly decreased in both DLP & VP of chemical- induced cancer rats, whereas, simultaneous quercetin supplementation decreased the protein levels of GSK3β in both DLP&VP. Quercetin alone treated rats didn't show any significant change in GSK3β protein in both VP & DLP (Fig. 6a, b).

3.7. Effect of quercetin on the mRNA & protein expression of N-cadherin, E-cadherin, and vimentin in DLP & VP of chemical- induced cancer male rats

During cancer progression cadherins are deregulated, thereby promoting cancer cell invasion and migration. The mRNA, protein expression level of N-cadherin & vimentin were significantly increased in both DLP & VP of chemical- induced cancer rats (Fig. 7&8), whereas, simultaneous quercetin supplementation decreased N-cadherin, vimentin mRNA and protein expressions in both DLP & VP. E-cadherin mRNA, protein expression was significantly decreased in both DLP &VP of chemical- induced cancer rats, whereas, simultaneous quercetin supplementation significantly increased E-cadherin expression level in both DLP & VP (Fig. 7&8).

3.8. Effect of quercetin on the mRNA expression of Snail, Slug and Twist in DLP & VP of chemical- induced cancer male rats

Snail, Slug and Twist are transcriptional repressors that regulate cancer metastasis by regulating cadherin proteins. The mRNA expression of Snail, Slug & Twist were significantly increased in both DLP & VP of chemical- induced cancer rats, whereas, simultaneous quercetin supplementation decreased the mRNA expression levels in both DLP & VP (Fig. 9 & 10). Although, no significant change in the mRNA expressions of Snail, Slug and Twist of quercetin alone treated rats.

3.9. HPLC profile of quercetin

Figure 11, A, B, C shows representative chromatograms of the plasma from control rats, chemical- induced cancer rats with simultaneous quercetin supplementation and from rats adapted to 200 mg/kg b.wt quercetin diet thrice a week. The HPLC profile from control rats showed two small peaks 1&2, which are unlikely to be flavonol metabolites, these peaks may be oxyhemoglobin since there intensity increases parallel to the degree of hemolysis, with

maximum absorption at 415-420nm. Chemical- induced cancer rats with simultaneous quercetin supplementation presented four peaks 1,2,3,4. The first peak is unlikely to be a flavonol, peak 2 & 3 correspond to conjugated metabolites of quercetin and peak 4 corresponds to quercetin. The HPLC profile from rats adapted to the quercetin diet is characterized by three peaks noted 1, 2, and 3 (retention times: 11.859, 12.419, 14.271 min), corresponding to the quercetin and conjugated metabolites of quercetin. These metabolites had spectra (from 250 to 450 nm) that fitted with flavonoid structure.

4. Discussion

Prostate cancer causes enormous losses each year considering its high morbidity and mortality. Quercetin, a major bioflavonoid in the human diet has been shown to induce apoptosis and have antiproliferative effects *in vitro* in various prostate cancer cell lines such as PC3, DU145, LNCap, but very few studies are available on the anticancer effects of quercetin in *in vivo* models. ROS stimulates EGFR signaling through inactivation of the phosphatases that dephosphorylate EGFR tyrosine residues. It has been suggested that various cellular stresses that contribute to the production of intracellular ROS cause increased EGFR signaling via this mechanism.[37] Sharmila *et al*., reported that quercetin acts as a chemopreventive agent by scavenging free radicals (H_2O_2), inhibiting lipid peroxidation and increasing GSH level in Sprague Dawley rats.[30] However, the molecular mechanism underlying the chemoprevention of quercetin are not known. In the present study, we focused on the chemopreventive effects of quercetin on EGFR signaling pathway involved in prostate cancer progression *in vivo* in Sprague Dawley rats. Since, EGFR signaling plays a major role in prostate cancer progression and is an ideal target to prevent various cancers including prostate cancer. Quercetin and its metabolites were detected in the serum by HPLC. Quercetin can be absorbed in the intestine after luminal hydrolysis by phlorizin hydrolase (LPH), an enzyme at the brush border membrane of intestinal cells, involved in the *in vivo* intestinal uptake of quercetin-sugars. HPLC analysis demonstrated detectable levels of quercetin and its conjugated metabolites in the serum indicating sufficient intestinal absorption of quercetin.

Proliferating cell nuclear antigen (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.[38,39] PCNA has been used as an independent marker for various cancers.[40,41,42] PCNA expression was significantly increased in both VP & DLP of chemical- induced cancer rats, as compared to control rats. Simultaneous quercetin

supplementation significantly decreased PCNA expression in both VP & DLP of chemical-induced cancer rats.

Aberrant overexpression of epidermal growth factor (EGF) receptors has been associated with hormone-refractory and metastatic prostate cancer (PCa).[43,44] Activation of EGFR signaling cascade promotes the proliferation, survival and invasion of PCa cells.[45] EGFR and p-EGFR protein expression were significantly increased in chemical- induced cancer rats as compared to control rats. However simultaneous quercetin supplementation significantly decreased EGFR, p-EGFR protein expression in both DLP&VP of chemical-induced cancer SD rats.

EGFR regulates two major signaling pathways PI3K/Akt and MAPK/ERK pathway. The phosphatidylinositol 3-kinase (PI3K) signaling pathway is aberrantly active in many human cancers. The p-PI3k protein expression was significantly increased in chemical-induced cancer rats compared to control rats. Whereas simultaneous quercetin supplementation decreased p-PI3K protein expression in both DLP & VP of chemical-induced cancer male rats. Quercetin has been shown to decrease PI3k protein expression in prostate cancer *in vitro*.[28] Previous studies have shown that quercetin acts as a potential PI3K inhibitor, an enzyme involved in the pivotal cell survival pathway.[29]

The best characterized downstream target of PI3K activation is the Akt kinase, which influences cancer cell metabolism, survival, growth, proliferation, angiogenesis, and migration.[46] Protein dependent kinase1 (PDK1) phosphorylates Akt at Thr 308. Akt activation contributes to tumorigenesis and tumor metastasis as well as chemotherapeutic resistance.[7] p-PDK1, p-Akt protein levels were significantly increased in DLP & VP of chemical- induced cancer rats and simultaneous quercetin supplementation prevented p-PDK1, p-Akt significantly in both DLP & VP of chemical- induced cancer rats. Senthilkumar *et al.,* has shown that quercetin downregulates p-Akt and PDK1 levels in prostate cancer *in vitro*.[27]

Receptor tyrosine kinase RTK/ERK pathway is said to be one of the important link in cancer development, regulating proliferation, differentiation, cell migration and survival.[47] Activation of MAPK cascade, leads to signal transduction from cell surface to the DNA in the nucleus, transferred by proteins influencing regulation, transcription, and synthesis of the proteins.[48,49] The phosphorylation of ERK1/2 was significantly increased in both DLP &VP of chemical- induced cancer male rats, whereas simultaneous quercetin supplementation significantly decreased p-ERK1/2 protein levels in both DLP & VP. Senthilkumar *et al.,* has shown that quercetin inhibits ERK1/2 phosphorylation in prostate cancer PC-3 cell line.[28] The

specificity of MAPK interactions and the effector molecules stimulated depends largely on the MAPK subtypes involved. In particular, extracellular signal-regulated kinases (ERK1/2)/MAPKs are primarily stimulated by growth factors and modulate cell growth and differentiation.[50]

Alterations of components of the PI3K/Akt/mTOR pathway, including mutation, altered expression, and copy number alterations, have been reported in 42% of primary prostate tumors and 100% of metastatic tumors.[6,7,50] In the present study, mTOR, protein expression was significantly increased in both DLP & VP of chemical- induced cancer male rats, whereas, simultaneous quercetin supplementation decreased mTOR, protein expression in both DLP & VP of rats. Epigallocatechin-3-gallate- a flavonoids has been shown to induce apoptosis in human pancreatic carcinoma cells via PI3K/Akt/mTOR pathway.[52]

Akt is involved in the cell survival pathway by regulating the activities of many key signaling molecules including GSK-3β. GSK-3β phosphorylates and inactivates crucial cell cycle regulators and transcription factors including cyclin D1.[53] A correlation between increased levels of cytoplasmic GSK-3β and clinical stage, Gleason score and high expression of the androgen receptor (AR) has been found in prostate cancer.[53,54] p-Akt is increased in chemical- induced cancer DLP & VP, which was brought back to normalcy by quercetin. Decreased GSK-3β was observed in both DLP & VP of chemical- induced cancer rats. Quercetin treatment brought back to normalcy. Akt phosphorylation inhibits the GSK-3β activation which phosphorylates and degrades cyclin D1 protein. In the present study quercetin inhibits Akt phosphorylation thereby there is an increase in GSK-3β which causes the inhibition of cyclin D1 protein expression which leads to inhibition of cell cycle progression. Quercetin was shown to arrest cell cycle progression in PC-3 cells.[28]

It has been shown that EGF induces epithelial-mesenchymal transition (EMT), a crucial mechanism for the acquisition of metastatic capabilities. Several mechanisms, such as mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)-dependent upregulation of Twist, Snail or Slug may mediate the effect of EGF on EMT and acquired invasiveness.[55] E-cadherin protein expression was significantly decreased in both DLP & VP of chemical- induced cancer male rats, however simultaneous quercetin supplementation prevented E-cadherin decrease in chemical- induced cancer rats. There was a significant increase in mRNA and protein expression of N-cadherin & vimentin in chemical- induced cancer rats, whereas quercetin prevented N-cadherin, vimentin increase in cancer induced animals. Further, simultaneous quercetin supplementation significantly decreased the mRNA

expression of Snail, Slug & Twist in both DLP & VP of chemical- induced cancer male rats. Overexpression of vimentin has been associated with enhanced metastatic potential in breast amongst other malignancies.[54,57] Snail, a transcriptional repressor is a key EMT regulator.[56] Snail genes are regulators of cell survival, adhesion, migration and the triggering of the EMT is just one of the mechanisms used to promote cell movement.[59] Luteolin a flavonoid has been shown to regulate snail, slug and twist mRNA in vitro.[60]

To conclude, quercetin prevents prostate cancer growth via EGFR signaling and prostate cancer progression by regulating cell adhesion molecules like E-cadherin, N-cadherin and vimentin via snail, slug and twist gene. Interestingly quercetin was effective in preventing carcinogenesis in both DLP & VP. Therefore, quercetin may prove to be an effective chemopreventive agent against prostate cancer.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgement

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Legends:

Fig. 1 A & B represents effect of quercetin on Proliferating cell nuclear antigen (PCNA) protein expression in DLP & VP of chemical- induced cancer male rats. PCNA expression was significantly increased in cancer induced rats. However, simultaneous quercetin supplementation decreased PCNA levels in both DLP & VP of chemical- induced cancer rats. Each bar represents the mean ± SEM of three independent observations. "ᵃ" Represents statistical significance between control versus others, "ᵇ" represents Cancer Induced vs others and "ᶜ" represents Cancer Induced + Quercetin vs others. Significance at $p<0.05$ level using Student's–Newman–Keuls test.

Fig. 2 A & B represents effect of quercetin on EGFR and p-EGFR protein expression in DLP & VP of cancer-induced male rats. EGFR and p-EGFR protein levels were significantly increased in both DLP & VP of chemical- induced cancer male rats, which was brought back to normalcy by simultaneous quercetin supplementation. Each bar represents the mean ± SEM of three independent observations. "ᵃ" Represents statistical significance between control versus others, "ᵇ" represents Cancer Induced vs others and "ᶜ" represents Cancer Induced + Quercetin vs others. Significance at $p<0.05$ level using Student's–Newman–Keuls test.

Fig. 3 A & B represents effect of quercetin on p-PDK1, p-PI3K protein expression in DLP & VP of cancer-induced male rats. p-PDK1 and p-PI3K protein levels were significantly increased in both DLP & VP of chemical- induced cancer rats, which was brought back to normalcy by simultaneous quercetin supplementation. Each bar represents the mean ± SEM of three independent observations. "ᵃ" Represents statistical significance between control versus others, "ᵇ" represents Cancer Induced vs others and "ᶜ" represents Cancer Induced + Quercetin vs others. Significance at $p<0.05$ level using Student's–Newman–Keuls test.

Fig.4 A & B shows the effect of quercetin on Akt protein expression in DLP & VP of cancer-induced male rats. Akt expression was significantly increased in chemical- induced cancer rats. However, simultaneous quercetin supplementation significantly decreased Akt levels in both DLP & VP of cancer induced rats. Each bar represents the mean ± SEM of three independent observations. "ᵃ" Represents statistical significance between control versus others, "ᵇ" represents Cancer Induced vs others and "ᶜ" represents Cancer Induced + Quercetin vs others. Significance at $p<0.05$ level using Student's–Newman–Keuls test.

Fig.5 A & B shows the effect of quercetin on p-ERK1/2 protein expression in DLP & VP of cancer-induced male rats. p-ERK1/2 expression was significantly increased in cancer induced rats. However, simultaneous quercetin supplementation significantly decreased p-ERK1/2

levels in both DLP & VP of chemical- induced cancer rats. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus others, "b" represents Cancer Induced vs others and "c" represents Cancer Induced + Quercetin vs others. Significance at *p<0.05* level using Student's–Newman–Keuls test.

Fig.6 A & B shows the effect of quercetin on mTOR, GSK3β and Cyclin D1 protein expression in DLP & VP of cancer-induced male rats. mTOR & cyclin D1 protein levels were significantly increased in both DLP & VP of cancer induced rats, which was brought back to normalcy by simultaneous quercetin supplementation. Gsk-3β levels was decreased in cancer induced rats, simultaneous quercetin supplementation brought it back to normalcy in both DLP & VP of chemical- induced cancer rats. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus others, "b" represents Cancer Induced vs others and "c" represents Cancer Induced + Quercetin vs others. Significance at *p<0.05* level using Student's–Newman–Keuls test.

Fig.7 A & B shows the effect of quercetin on E-Cadherin, N-Cadherin and vimentin mRNA expression in dorsolateral prostate of cancer induced male Sprague Dawley rats. N-Cadherin and Vimentin mRNA levels were significantly increased in cancer induced rats, however quercetin supplementation brought it back to normalcy in DLP of chemical- induced cancer rats. E-cadherin expression was decreased in chemical- induced cancer rats, and quercetin restored E-cadherin expression in chemical- induced cancer rats. The data was analyzed by the comparative CT method. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus others, "b" represents Cancer Induced vs others and "c" represents Cancer Induced + Q vs others. Significance at *p<0.05* level using Student's–Newman–Keuls test.

Fig.8 A & B shows the effect of quercetin on E-Cadherin, N-Cadherin and vimentin mRNA expression in ventral prostate of cancer induced male Sprague Dawley rats. N-Cadherin and Vimentin mRNA levels were significantly increased in chemical- induced cancer rats, however quercetin supplementation brought it back to normalcy in VP of chemical- induced cancer rats. E-cadherin expression was decreased in chemical- induced cancer rats, and quercetin restored E-cadherin expression in chemical- induced cancer male rats. The data was analyzed by the comparative CT method. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus others, "b" represents Cancer Induced vs others and "c" represents Cancer Induced + Quercetin vs others. Significance at *p<0.05* level using Student's–Newman–Keuls test.

Fig.9 A & B shows the effect of quercetin on vimentin, N-cadherin and E-cadherin protein expression in DLP & VP of chemical- induced cancer male rats. N-cadherin & vimentin protein levels were significantly increased in both DLP & VP of chemical- induced cancer male rats, which was brought back to normalcy by simultaneous quercetin supplementation. E-cadherin protein level was decreased in chemical- induced cancer male rats, simultaneous quercetin supplementation brought it back to normalcy in both DLP & VP of chemical-induced cancer male rats. Each bar represents the mean ± SEM of three independent observations. Statistical significance at *p<0.05*. "a" control vs others; "b" CI vs CI+Q–treated rats. "a" Represents statistical significance between control versus others, "b" represents Cancer Induced vs other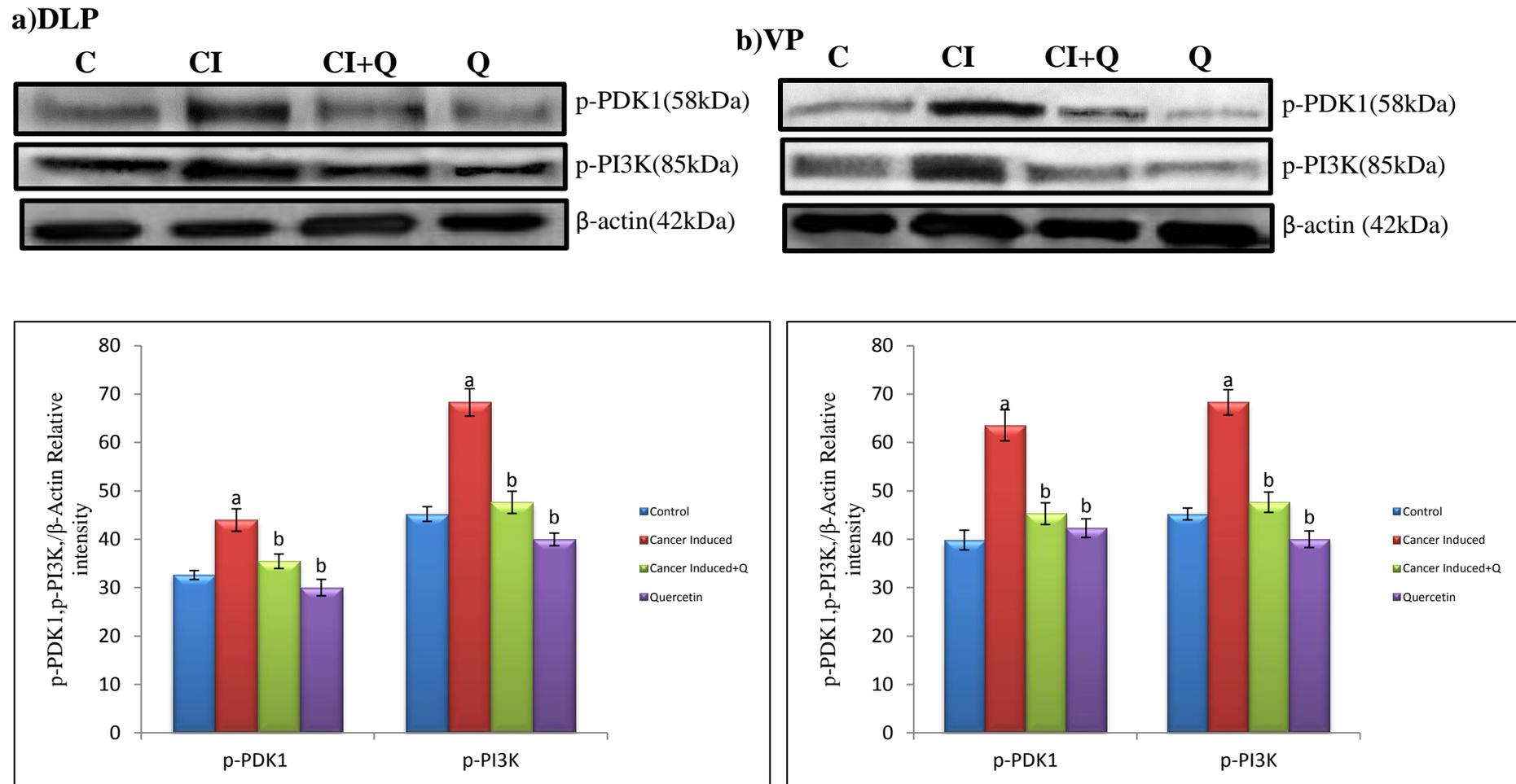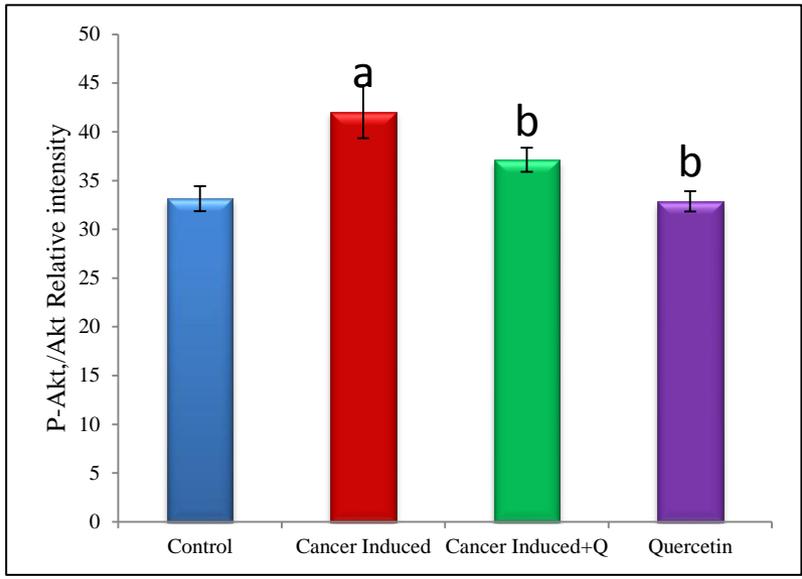s and "c" represents Cancer Induced + Quercetin vs others. Significance at *p<0.05* level using Student's–Newman–Keuls test.

Fig.10 A & B shows the effect of quercetin on Slug, Twist and Snail mRNA expression in dorsolateral prostate of cancer induced male Sprague Dawley rats. Slug, Twist and Snail mRNA levels were significantly increased in chemical- induced cancer male rats, however quercetin supplementation brought it back to normalcy in DLP of chemical- induced cancer male rats. The data was analyzed by the comparative CT method. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus others, "b" represents Cancer Induced vs others and "c" represents Cancer Induced + Quercetin vs others. Significance at *p<0.05* level using Student's–Newman–Keuls test.

Fig.11 A & B shows the effect of quercetin on Slug, Twist and Snail mRNA expression in ventral prostate of cancer induced male Sprague Dawley rats. Slug, Twist and Snail mRNA levels were significantly increased in chemical- induced cancer male rats, however quercetin supplementation brought it back to normalcy in VP of chemical- induced cancer male rats. The data was analyzed by the comparative CT method. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus others, "b" represents Cancer Induced vs others and "c" represents Cancer Induced + Quercetin vs others. Significance at *p<0.05* level using Student's–Newman–Keuls test.

Fig.12 A, B, C shows the HPLC profile of quercetin in serum of control, CI+Q and Quercetin alone treated rats. A) Two small peaks were observed in control rats, which are unlikely to be flavonol metabolites. B) The HPLC profile from cancer induced rats with simultaneous quercetin supplementation is characterized by four peaks noted (retention times: 4.152, 12.219, 12.928, 15.459 min), the first peak is unlikely a flavonol, peaks 2 & 3 correspond to the conjugated metabolites of quercetin and peak 4 corresponds to quercetin. C) Rats fed with

quercetin presented three peaks 1,2,3 (retention times 11.859, 12.419, 14.271) , peaks 1 & 2 correspond to conjugated metabolites of quercetin and peak 3 corresponds to quercetin.

TABLE 1

Body weight (g) of Sprague Dawley rats treated with N-methyl-N-nitrosourea (MNU) + testosterone (T), quercetin (Q), and vehicle

Group	1st wk	5th wk	10th wk	15th wk	20th wk
Control	193	207	229	252	282
Cancer induced (MNU & T)	218[a]	234[a]	209[a]	198[a]	183[a]
Cancer induced + quercetin (200 mg/kg b.wt)	205[a,b]	224[a]	228[a,b]	236[a,b]	260[a,b]
Quercetin (200 mg/kg b.wt)	214[a,b,c]	231[b,c]	257[a,b,c]	290[a,b,c]	308[a,b,c]

Each value represents mean $\pm$ SEM of 10 animals. The statistical significance was considered at the level of $P < 0.05$, following Student's–Newman–Keul's test. b.wt = body weight.

[a]Control vs. others. [b]Cancer Induced vs. Cancer Induced + Quercetin-treated rats. [c]Cancer Induced + Quercetin vs. quercetin alone.

TABLE 2

Effect of Quercetin (Q) on organ weight of ventral prostate (VP) and dorsolateral prostate (DLP) of Sprague Dawley rats treated with N-methyl-N-nitrosourea (MNU) +testosterone (T), Q, and vehicle

	Absolute organ weight (mg)	
Group	VP	DLP
Control	342 ± 5.78	241 ± 4.59
Cancer Induced (MNU+T)	814 ± 8.74[a]	392 ± 7.29[a]
Cancer Induced + quercetin(200mg/kg b.wt.)	597 ± 10.52[a,b]	317.82 ± 9.28[a,b]
Quercetin (200mg/kg b. wt.)	$374+6.59$[b,c]	$278+8.05$[a,b,c]

Each value represents mean ± SEM of 10 animals. The statistical significance was considered at the level of $P < 0.05,$ following Student's–Newman–Keul's test.

 VP = ventral prostate; DLP = dorsolateral prostate;

[a]Control vs. others. [b]Cancer induced vs. Cancer Induced+Quercetin-treated rats. [c]Cancer Induced + Quercetin vs. quercetin alone.

Fig 1 : Effect of quercetin on Proliferating cell nuclear antigen (PCNA) protein expression in DLP & VP of chemical- induced cancer model male Sprague Dawley rats.
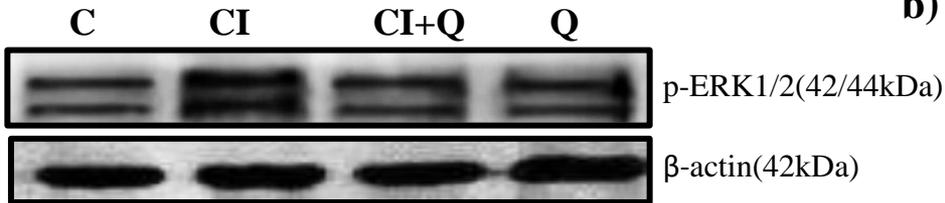
a) DLP

b)VP
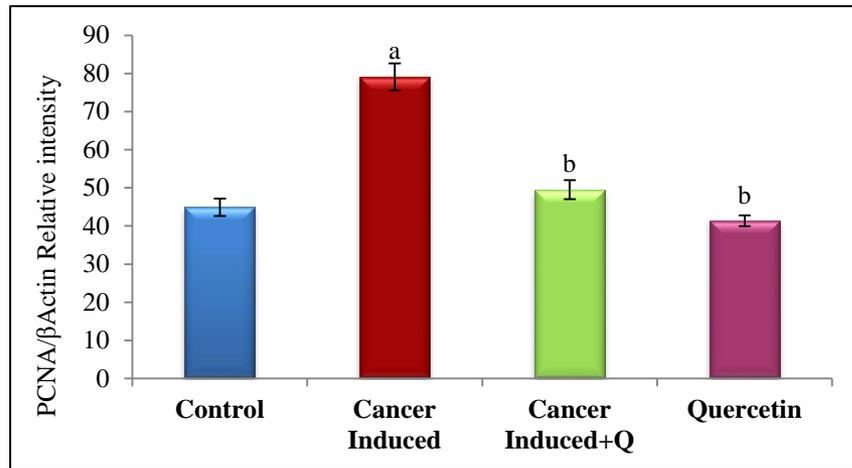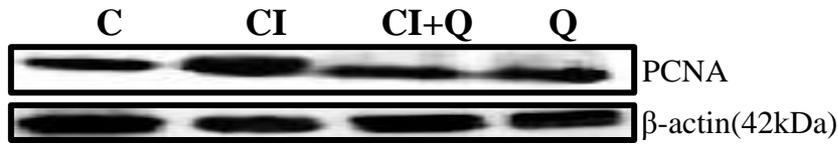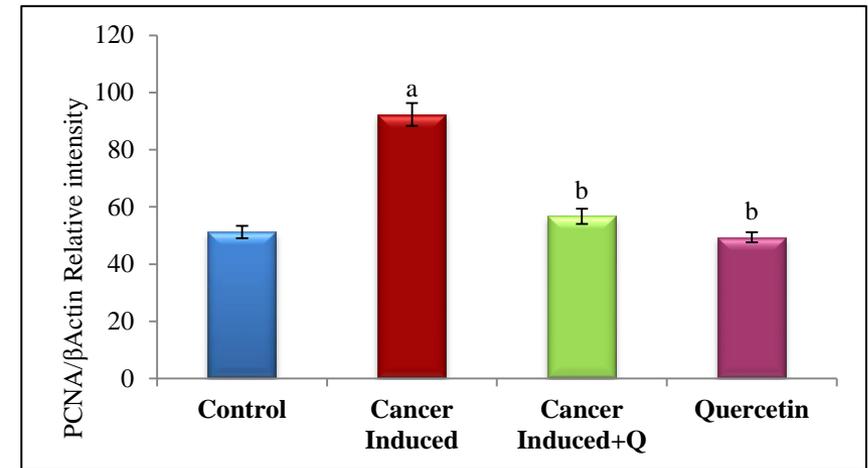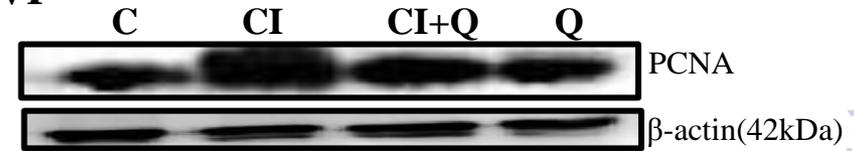
C- control; CI- Cancer-Induced (Testosterone and MNU); CI+Q- Cancer-Induced + Quercetin (200mg/kg body.wt); Q- Quercetin (200mg/kg body.wt)
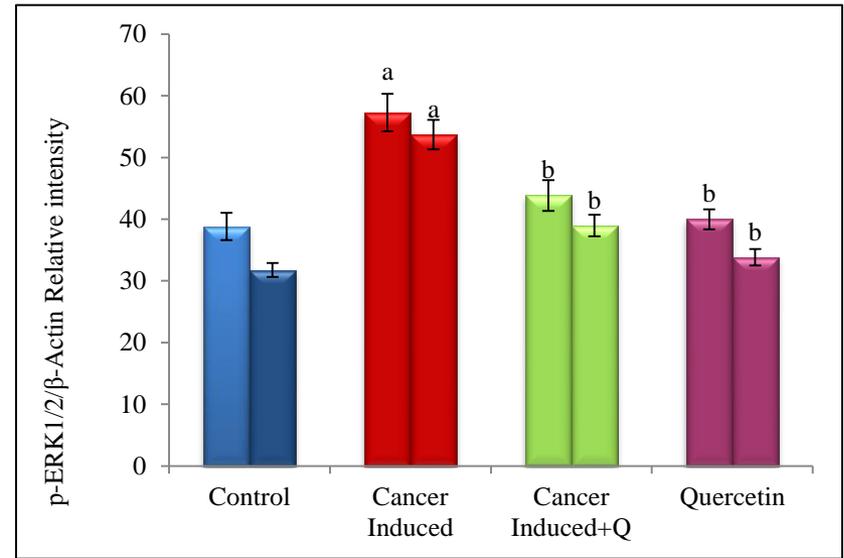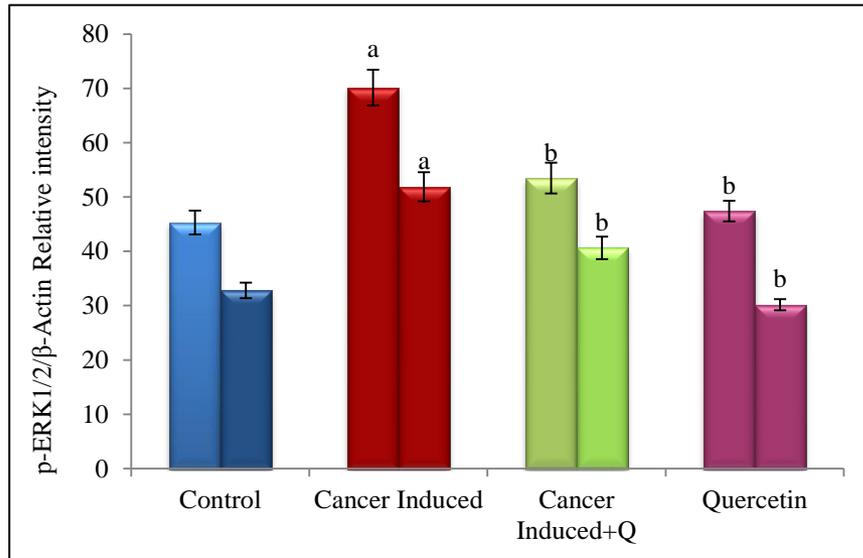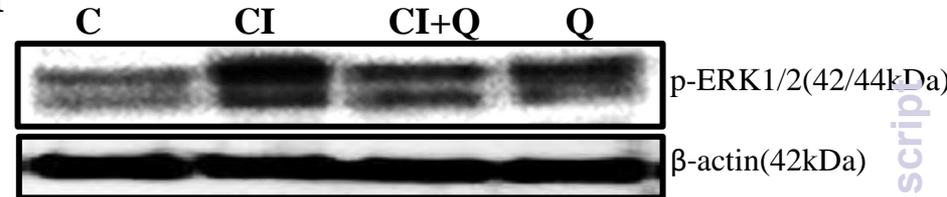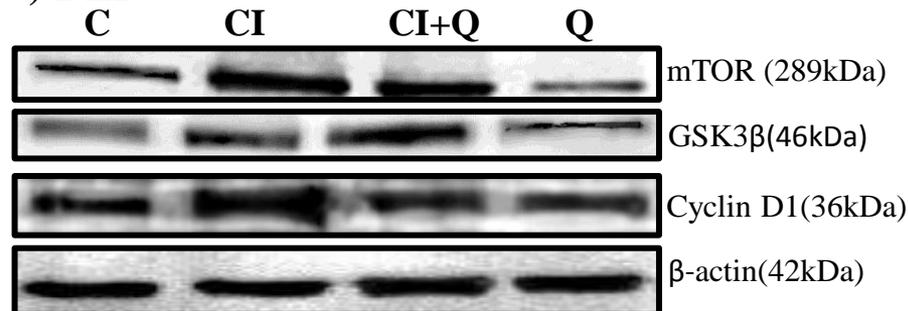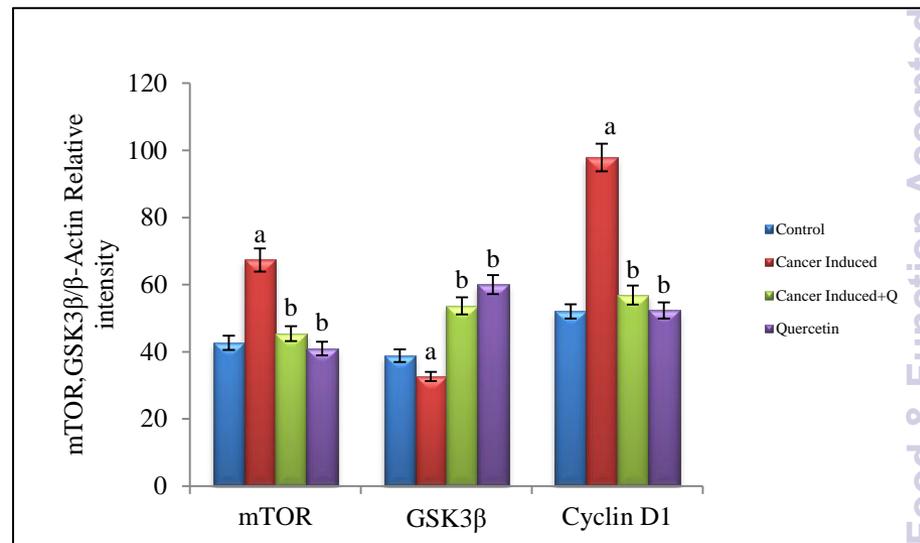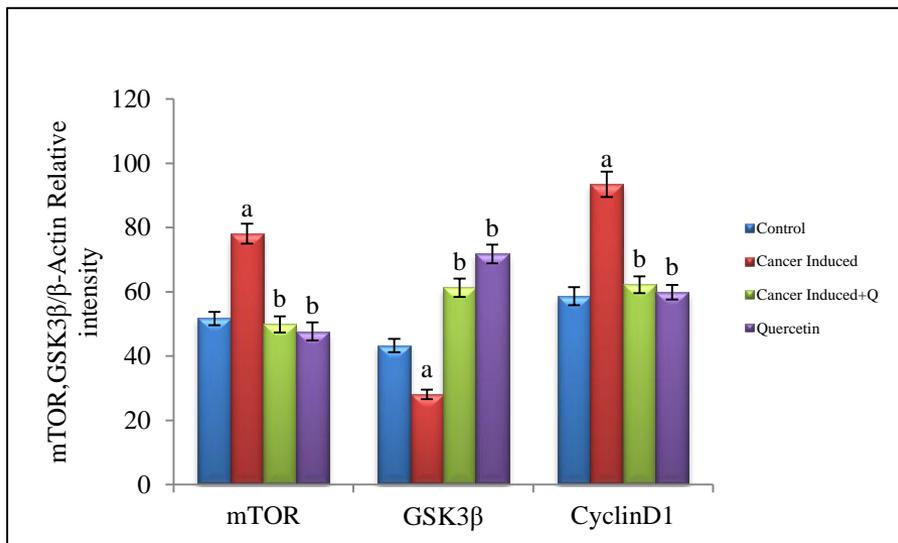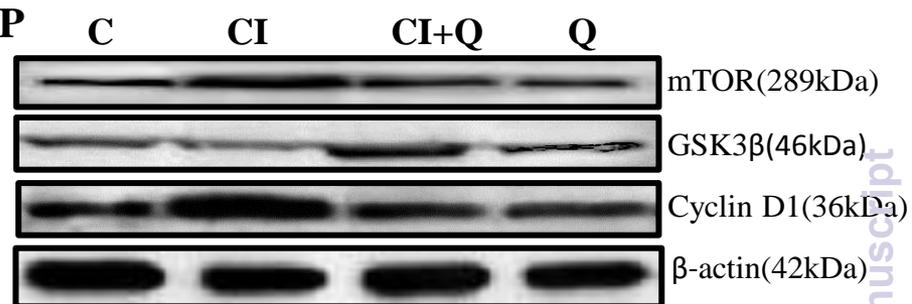
Each bar represents the mean ± SEM of three independent observations. Statistical significance at *p<0.05*. [a] control vs others; [b] Cancer Induced vs others.

Fig 25: Effect of quercetin on EGFR and p-EGFR protein expression in DLP & VP of chemical- induced cancer model male Sprague Dawley rats.
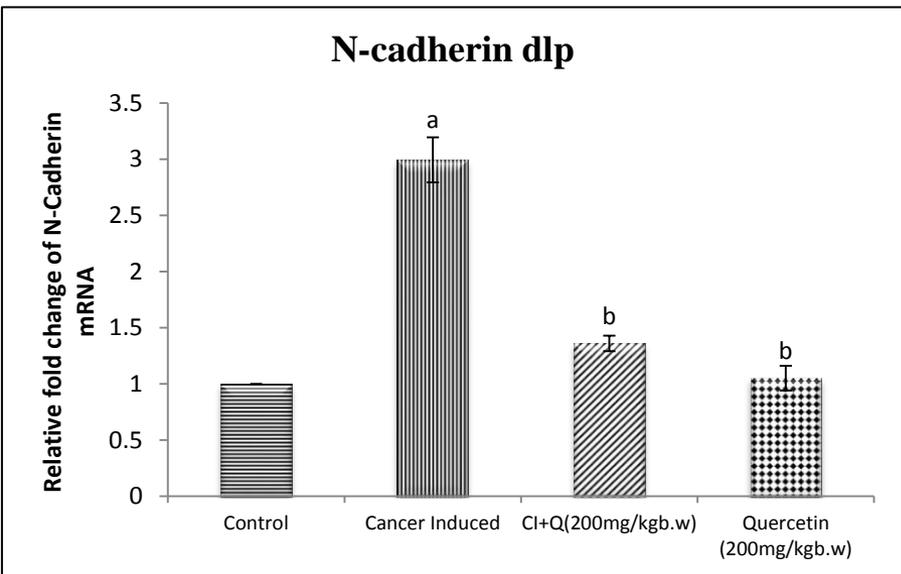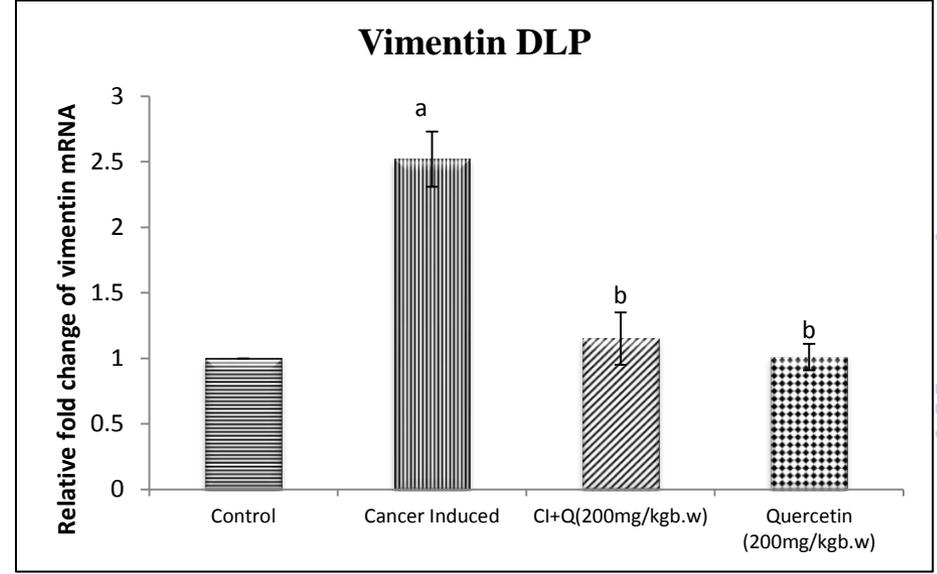
a) DLP

b) VP
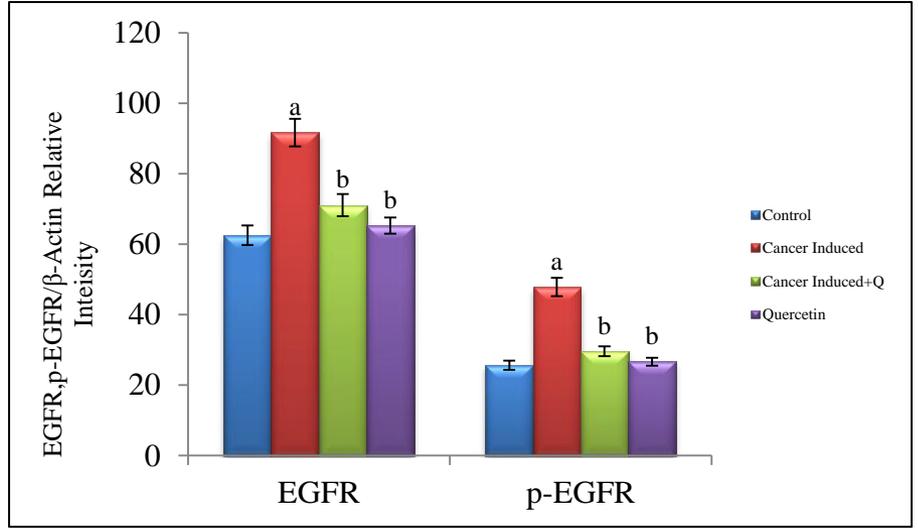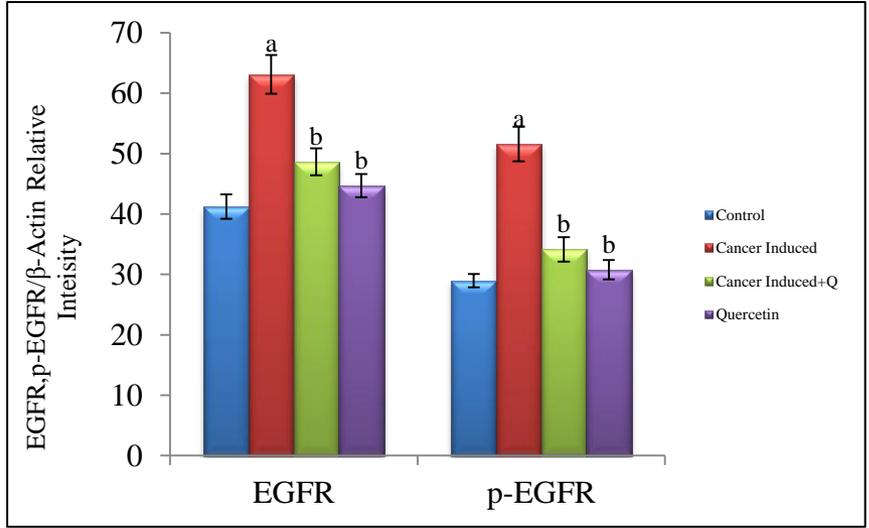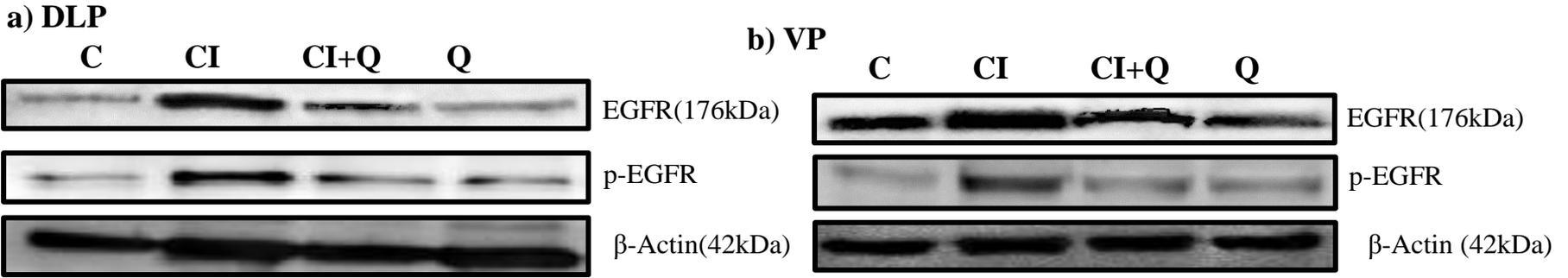
C- control; CI- Cancer-Induced (Testosterone and MNU); CI+Q- Cancer-Induced + Quercetin (200mg/kg body.wt); Q- Quercetin (200mg/kg body.wt)

Each bar represents the mean ± SEM of three independent observations. Statistical significance at *p<0.05* [a] control vs others; [b] Cancer Induced vs others.

Fig 3: Effect of quercetin on p-PDK1, p-PI3K, protein expression in DLP & VP of chemical-induced cancer model male Sprague Dawley rats.
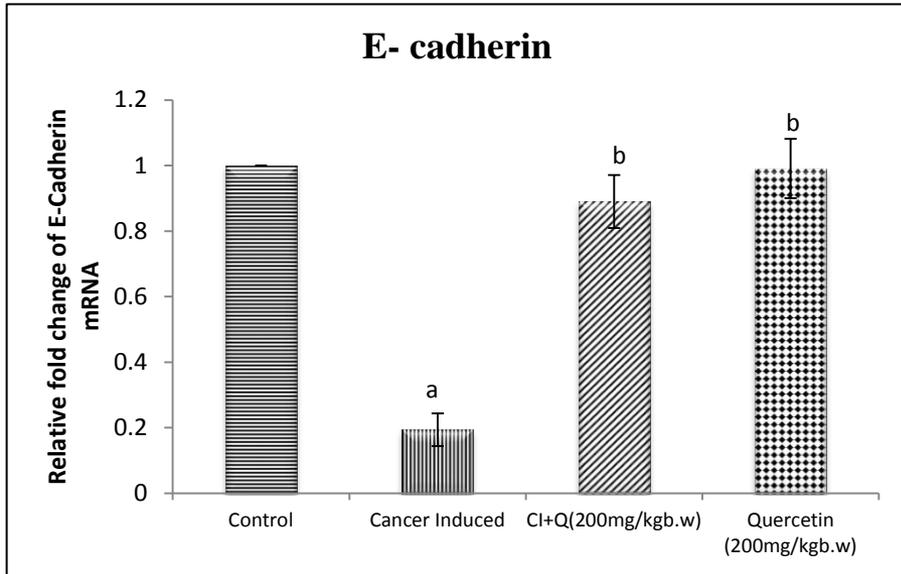
a)DLP

b)VP

C- control; CI- Cancer-Induced (Testosterone and MNU); CI+Q- Cancer-Induced + Quercetin (200mg/kg body.wt); Q-Quercetin (200mg/kg body.wt)
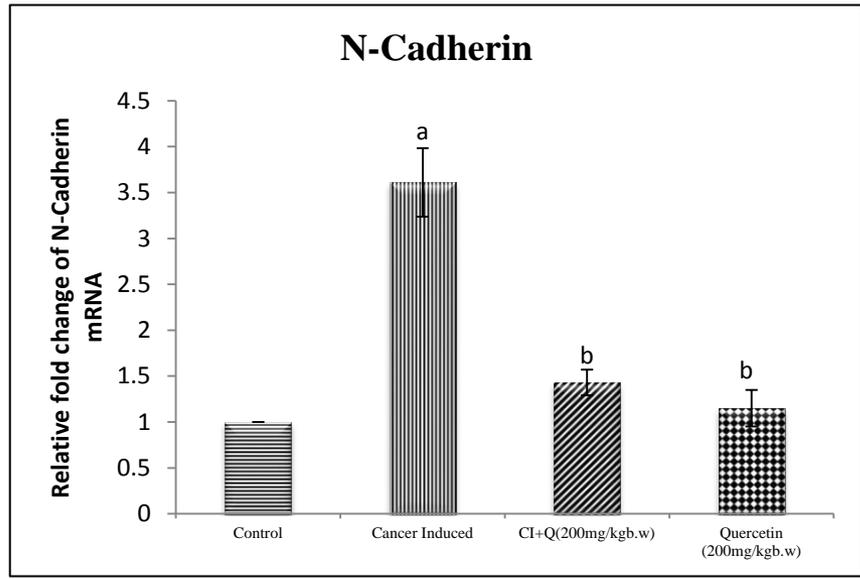
Each bar represents the mean ± SEM of three independent observations. Statistical significance at $p<0.05$ [a] control vs others; [b] Cancer Induced vs others.

Fig 4: Effect of quercetin on Akt protein expression in DLP & VP of chemical- induced cancer model male Sprague Dawley rats.

a)DLP

b)VP

C- control; CI- Cancer-Induced (Testosterone and MNU); CI+Q- Cancer-Induced + Quercetin (200mg/kg body.wt); Q-Quercetin (200mg/kg body.wt)
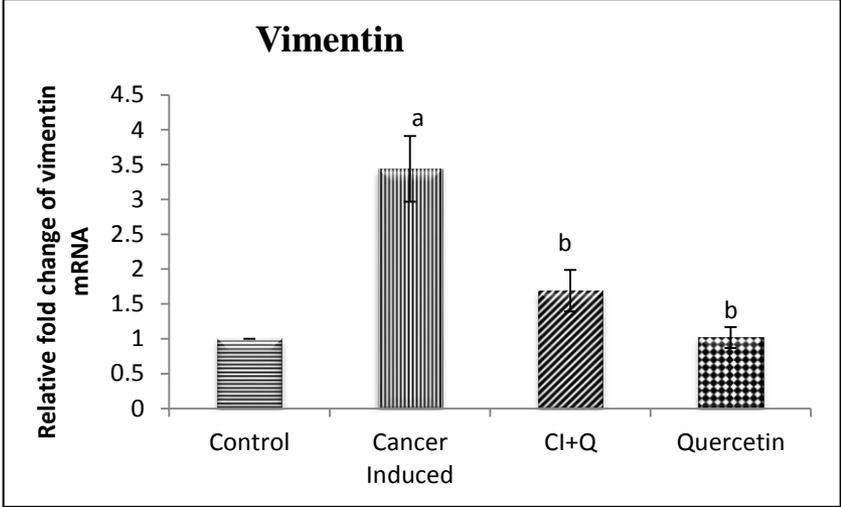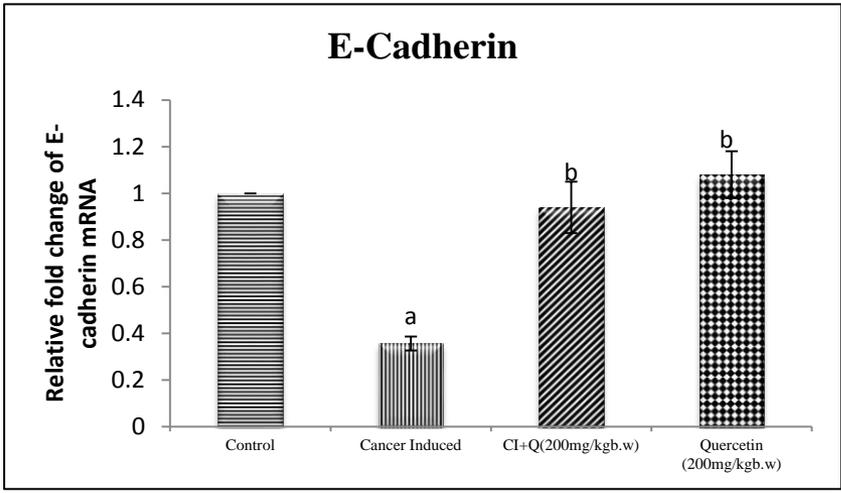
Each bar represents the mean ± SEM of three independent observations. Statistical significance at $p<0.05$ [a] control vs others; [b] Cancer Induced vs others.

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Fig 5: Effect of quercetin on p-ERK1/2 protein expression in DLP & VP of chemical- induced cancer model male Sprague Dawley rats.

a) DLP

| C | CI | CI+Q | Q |

p-ERK1/2(42/44kDa)

β-actin(42kDa)

b)VP

| C | CI | CI+Q | Q |

p-ERK1/2(42/44kDa)

β-actin(42kDa)

C- control; CI- Cancer-Induced (Testosterone and MNU); CI+Q- Cancer-Induced + Quercetin (200mg/kg body.wt); Q- Quercetin (200mg/kg body.wt)

Each bar represents the mean ± SEM of three independent observations. Statistical significance at $p<0.05$ [a] control vs others; [b] Cancer Induced vs others.

Fig 6: Effect of quercetin on mTOR, GSK3β and Cyclin D1 protein expression in DLP & VP of chemical- induced cancer model male Sprague Dawley rats.

a) DLP

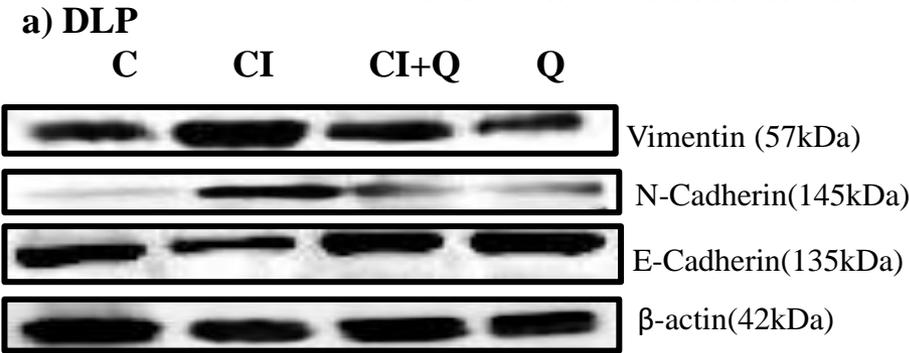**b) VP**

C- control; CI- Cancer-Induced (Testosterone and MNU); CI+Q- Cancer-Induced + Quercetin (200mg/kg body.wt); Q- Quercetin (200mg/kg body.wt)

Each bar represents the mean ± SEM of three independent observations. Statistical significance at *p<0.05* [a] control vs others; [b] Cancer Induced vs others.

Fig.7. Effects of quercetin on E-Cadherin, N-Cadherin and vimentin mRNA expression in dorsolateral prostate of chemical- induced cancer model male Sprague Dawley rats

E- cadherin

Vimentin DLP

N-cadherin dlp

The data was analyzed by the comparative C_T method. Each bar represents the mean ± SEM of three independent observations. "a"Represents statistical significance between control versus others, and "b" represents Cancer Induced vs others. Significance at $P<0.05$ level using Student's–Newman–Keuls test.
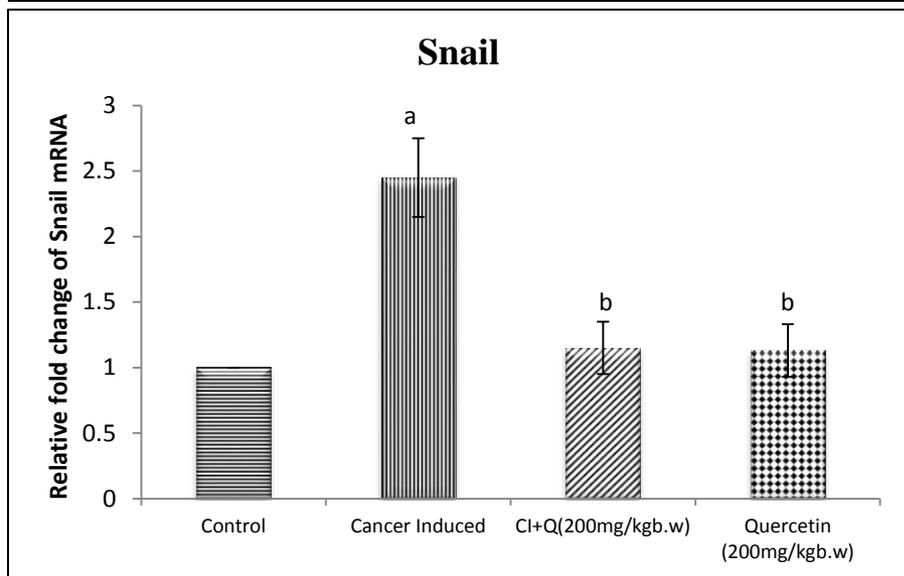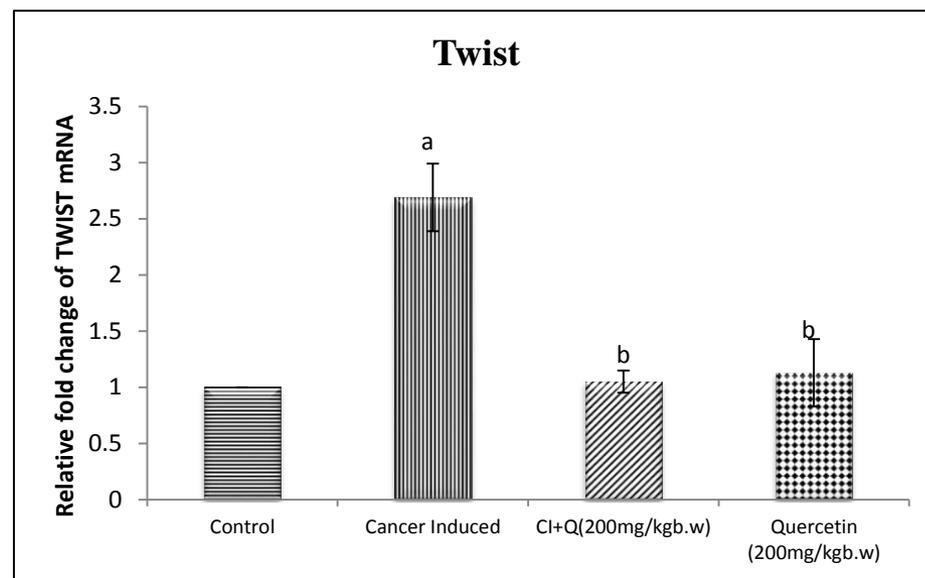
Fig.8:Effects of quercetin on E-Cadherin, N-Cadherin and vimentin mRNA expression in ventral prostate of chemical- induced cancer model male Sprague Dawley rats

The data was analyzed by the comparative C_T method. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus others, and "b" represents Cancer Induced vs others. Significance at *P<0.05* level using Student's–Newman–Keuls test.

Fig.9. Effect of quercetin on vimentin, N-cadherin and E-cadherin protein expression in DLP & VP of chemical- induced cancer model male Sprague Dawley rats.

a) DLP

b) VP
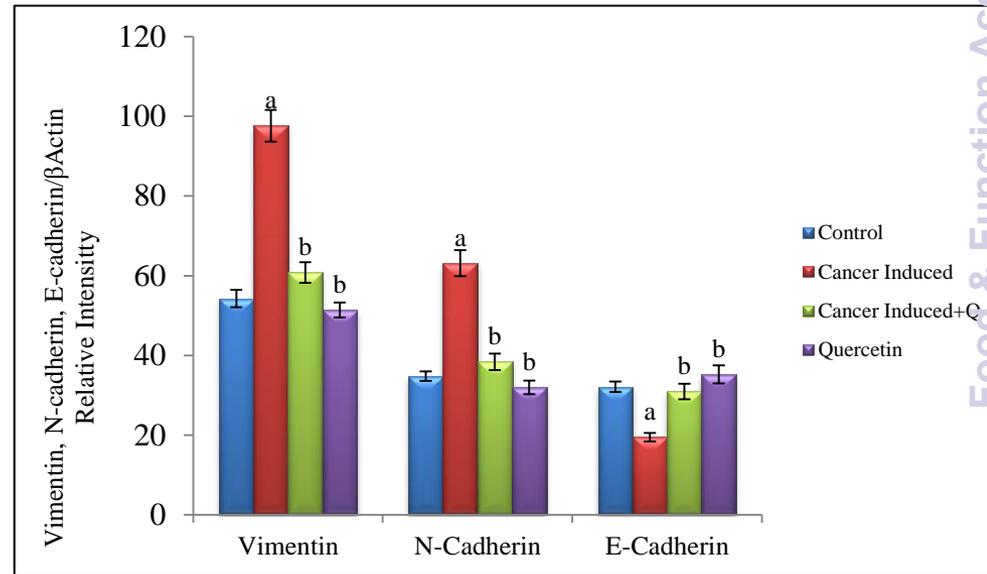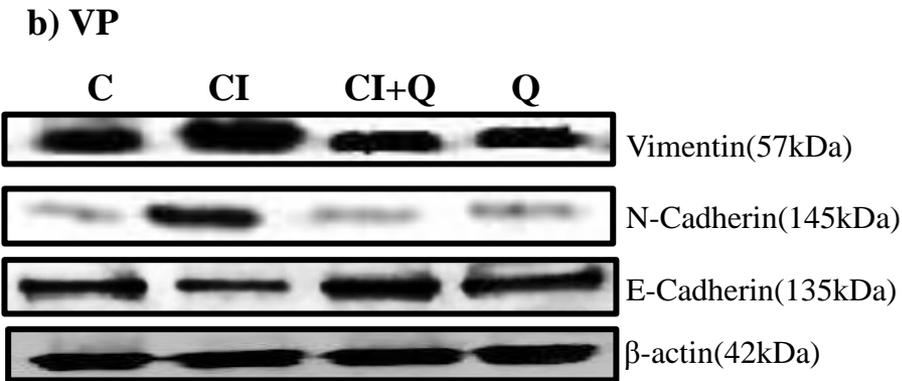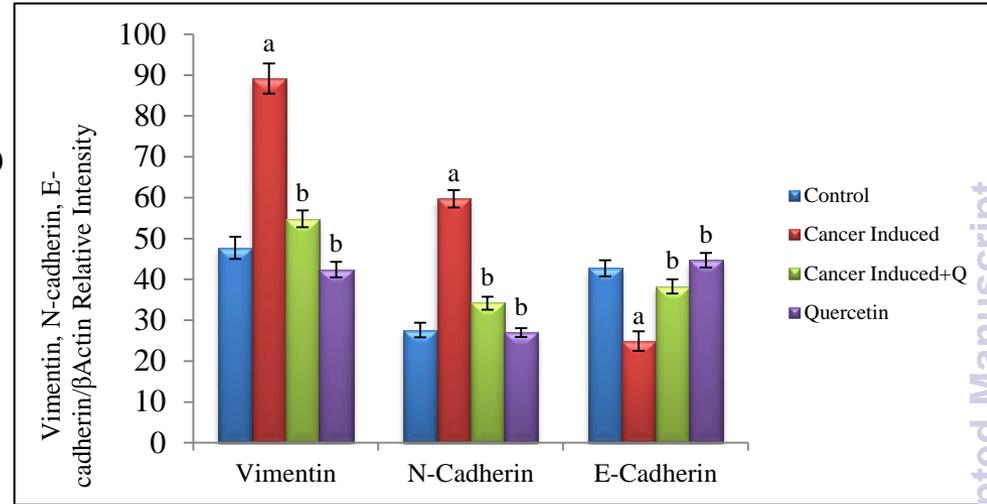
C- control; CI- Cancer-Induced (Testosterone and MNU);
CI+Q- Cancer-Induced + Quercetin (200mg/kg body.wt);
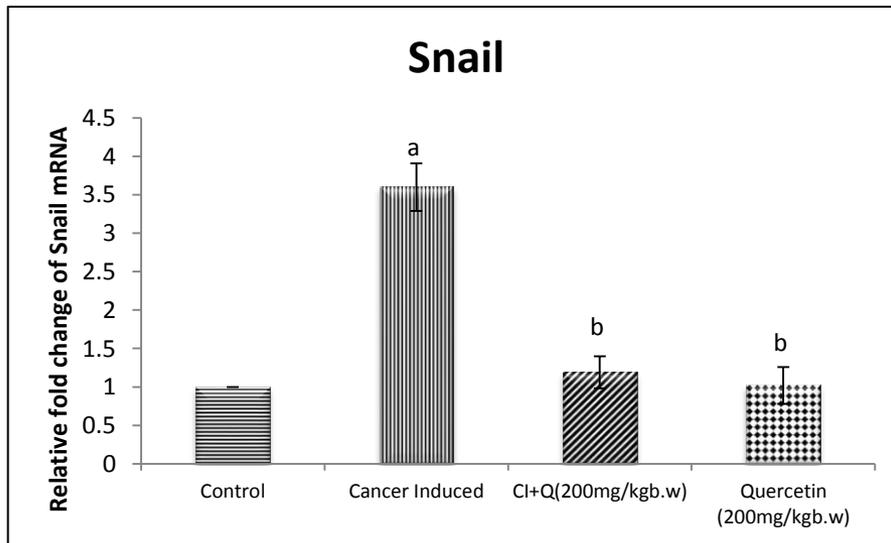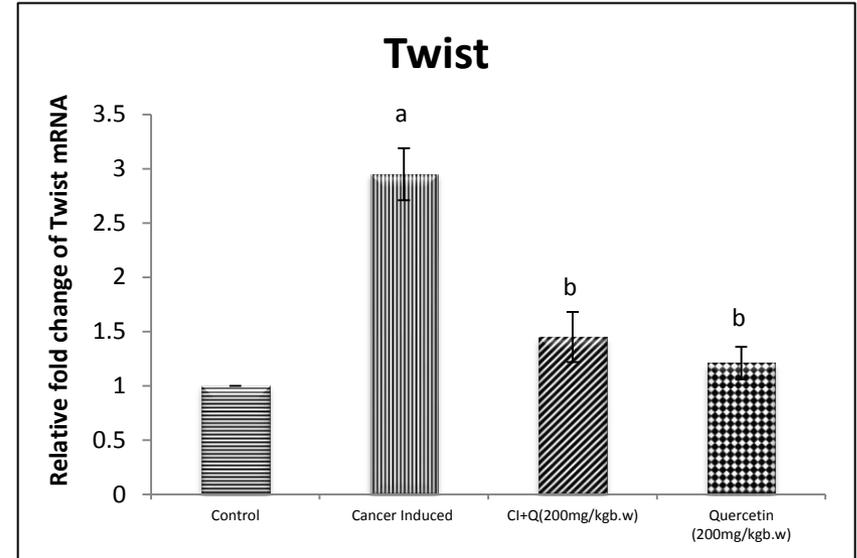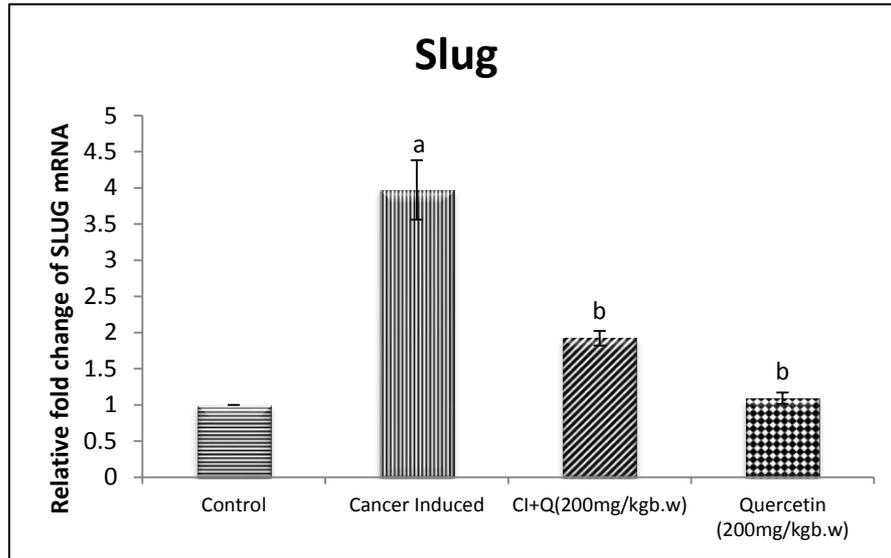**Q-Quercetin (200mg/kg body.wt)**

Each bar represents the mean ± SEM of three independent observations. Statistical significance at *p<0.05.* [a] control vs others; [b] Cancer Induced vs others.

Fig.10. Effects of quercetin on Slug, TWIST and Snail mRNA expression in dorsolateral prostate of chemical- induced cancer model male Sprague Dawley rats
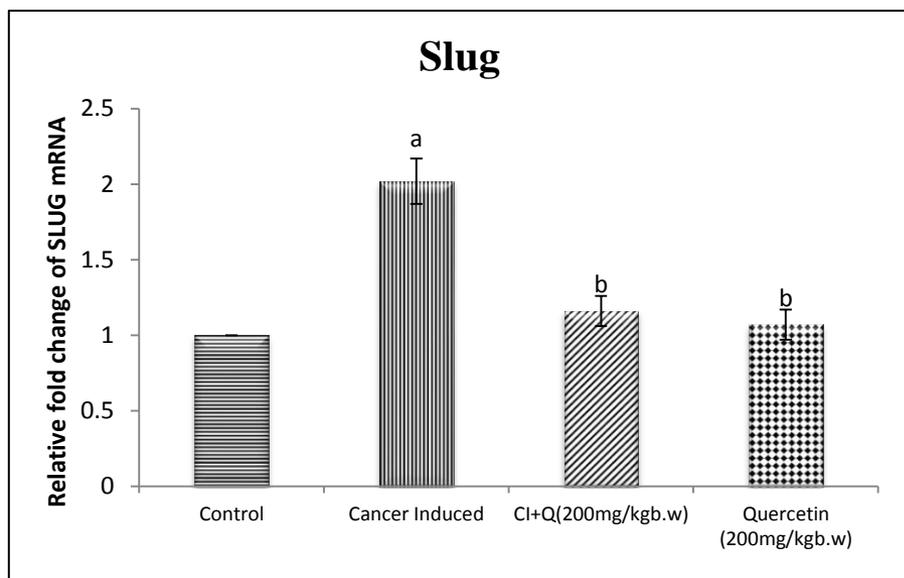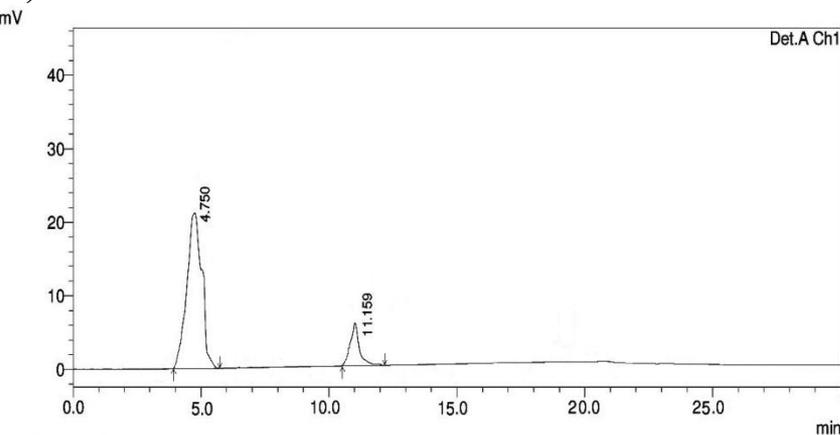

The data was analyzed by the comparative C_T method. Each bar represents the mean $\pm$ SEM of three independent observations. "a" Represents statistical significance between control versus others, and "b" represents CI vs others. Significance at $P<0.05$ level using Student's–Newman–Keuls test.

Fig.11. Effects of quercetin on Slug, Twist and Snail mRNA expression in ventral prostate of chemical-induced cancer model male Sprague Dawley rats

The data was analyzed by the comparative C_T method. Each bar represents the mean ± SEM of three independent observations. "a" Represents statistical significance between control versus others, and "b" represents CI vs others. Significance at $P<0.05$ level using Student's–Newman–Keuls test.
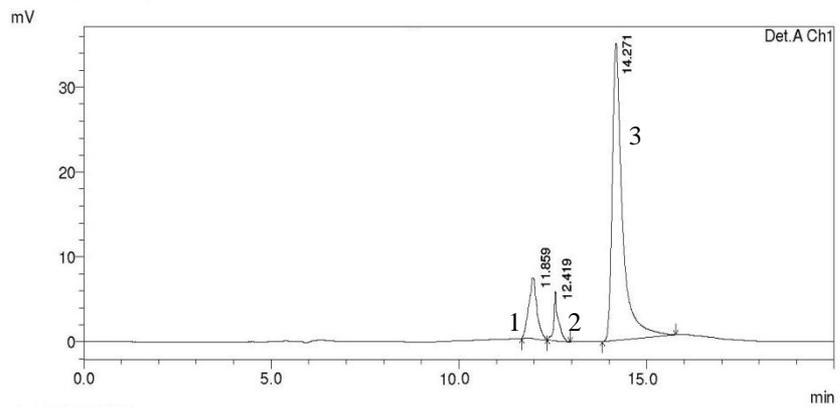
Fig. 12. HPLC profile of quercetin in serum of control, CI+Q and Quercetin alone treated Sprague Dawley rats.

A) Control

PeakTable

Detector A Ch1 370nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.750	17545	3200	79.459	77.938
2	11.159	2168	706	20.541	22.062
Total		19713	3906	100.000	100.000

B) CI+Q

PeakTable

Detector A Ch1 370nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.152	162540	13951	15.151	15.003
2	12.219	428080	21404	22.459	27.682
3	12.928	224126	14491	16.533	18.253
4	15.459	1114874	43773	45.857	39.062
Total		1929620	93169	100.000	100.000

C) Quercetin

PeakTable

Detector A Ch1 370nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	11.859	17545	1404	11.751	8.243
2	12.419	11685	1027	8.764	6.190
3	14.271	1144762	43501	79.485	85.567
Total		1173992	45931	100.000	100.000

Fig. 12. A) Two small peaks were observed in control rats, which are unlikely to be flavonol metabolites.
B) The HPLC profile from cancer induced rats with simultaneous quercetin supplementation is characterized by four peaks noted (retention times: 4.152, 12.219, 12.928, 15.459 min), the first peak is unlikely a flavonol, peaks 2 & 3 correspond to the conjugated metabolites of quercetin and peak 4 corresponds to quercetin.
C) Rats fed with quercetin presented three peaks 1,2,3 (retention times 11.859, 12.419, 14.271) , peaks 1 & 2 correspond to conjugated metabolites of quercetin and peak 3 corresponds to quercetin.