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Quercetin attenuates metastatic ability of human metastatic ovarian cancer cells *via* modulating multiple signaling molecules involved in cell survival, proliferation, migration and adhesion

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ABSTRACT

Ovarian cancer is the most deadly gynaecology related cancer due to its high metastasizing ability. Quercetin is the most abundant flavonoids received increased interest due to its anti-cancer properties. Although the anti-cancer property of quercetin is very well known, its anti-metastatic effect on metastatic ovarian cancer cells and their underlying molecular mechanism remains to be elucidated. Quercetin treatment at 50 μM and 75 μM concentration inhibit human metastatic ovarian cancer PA-1 cell survival and proliferation *via* inactivating PI3k/Akt, Ras/Raf pathways and EGFR expression. It also alters the expression of N-cadherin in PA-1 cells. Quercetin also decreases the secretion of gelatinase enzyme, proteolytic activity of MMP-2/-9, and both MMPs gene expression in metastatic ovarian cancer PA-1 cells. In addition to this quercetin inhibits the migration of PA-1 cells. Treatment of quercetin with PA-1 cells also downregulates the tight junctional molecules such as Claudin-4 and Claudin-11 while upregulates the expression of occludin. It is further validated by cell adhesion assay in which quercetin reduces the adhesion of PA-1 ovarian cancer cells. Results suggest that quercetin inhibits cell survival, proliferation, migration, and adhesion which plays crucial role in ovarian cancer metastasis. Hence, it could be a valuable therapeutic drug for the treatment and prevention of metastatic ovarian cancer.

1. Introduction

Ovarian cancer is one of the major causes of gynaecologic associated malignancies with an estimated death of 184,799 worldwide [1]. In India, incidence of ovarian cancer increases due to its longetivity and ranks 2nd globally [2]. Although ovarian cancer is less prevalent than breast cancer epidemiological studies have predicted three times higher mortality rate by the year 2040 [3]. Due to its rapid proliferation and metastasis, treating ovarian cancer is very difficult which leads to higher morbidity and mortality [4]. Thus, there is desperate need to find therapy for metastatic ovarian cancer. Increased expression of epidermal growth factor receptor (EGFR) was associated with invasive ovarian cancer [5]. Aberrant EGFR mediated signaling promotes ovarian cancer development, progression and metastasis [6]. It has been reported that PI3k/Akt and Ras/Raf pathways are activated in ovarian cancer contributing to cell survival, proliferation and metastatic progression [7,8].

During metastasis, ovarian cancer cells undergo detachment from the primary sites then migrate, invade, adhere to the peritoneal cavity and other organs [9]. Epithelial-to-mesenchymal transition (EMT) plays a crucial role in the invasion and metastasis of ovarian cancer cells [10]. In ovarian cancer, the major cause of EMT is due to dysfunction of cell adhesion molecules, tight junctional proteins and matrix metalloproteinases [11,12]. Although, several chemotherapeutic drugs like

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PI3k, phosphoinositide 3-kinase; AKT/PKB, Protein kinase B; mTOR, mammalian target of rapamycin; PCNA, Proliferating cell nuclear antigen; Ras, rat sarcoma; Raf, rapidly accelerated fibrosarcoma; MEK, MAPK extracellular kinase; ERK, extracellular signal-regulated kinase; c-Fos, FBJ murine osteosarcoma; AP-1, Activator protein 1; MMPs, matrix metalloproteinases; uPA, urokinase-type plasminogen activator; PAI-1, Plasminogen activator inhibitor-1; Bcl-2, B-cell lymphoma 2; JNK, c-Jun N-terminal kinases; STAT3, Signal transducer and activator of transcription 3; SIRT1, Sirtuin 1; PRL-3, Protein-tyrosine phosphatase of regenerating liver 3; CLDN-4, Claudin-4; EMT, Epithelial-to-mesenchymal transition; LPA, Lysophosphatidic acid; FUT1, fucosyltransferase 1; TGF-β1, transforming growth factor β1; MAPK, mitogen-activated protein kinase.

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cisplatin, carboplatin and paclitaxel are available still treatment and management of ovarian cancer is challenging due to its metastatic ability.

Nowadays plant-derived flavonoids have received increased interest due to its anti-cancer properties and also dietary polyphenols have become not only important potential chemopreventive, but also therapeutic natural agents. Since, ovarian cancer is often diagnosed at metastatic condition flavanoid that kills the metastatic ovarian cancer cells may be useful against this type of tumors. Quercetin is a bio-flavonoid present in various edible fruits and vegetables. It is also well known for its various biological properties that include antioxidant, anti-inflammatory, and anticancer activities [13]. Several studies have proved that intake of vegetables, fruits, and black tea with a rich source of quercetin lowers the incidence of ovarian cancer [14–20]. However, the effect of quercetin and its underlying molecular mechanisms are still remains poorly understood in metastatic ovarian cancer.

Our previous study demonstrated that quercetin induces intrinsic pathway mediated apoptosis in human metastatic ovarian cancer cells [21]. The present study was aimed to unravel the anti-metastatic activity of quercetin on human metastatic ovarian cancer PA-1 cells. Alongside, we also elucidated the effect of quercetin on signaling molecules involved in cell survival, proliferation, adhesion, and migration of ovarian cancer in PA-1 cells.

2. Materials and methods

2.1. Chemicals

Quercetin, Dulbecco's Modified Eagle's Medium (DMEM), Bovine serum albumin (BSA), Acrylamide, Bis-acrylamide, Ammonium persulfate (APS), N, N, N′, N′-Tetramethylethylenediamine (TEMED), Dimethyl sulfoxide (DMSO) and TRI reagent were purchased from Sigma Aldrich Chemicals Pvt. Ltd., (St. Louis, MO, USA). Antibiotics from HiMedia (Mumbai, India), Trypsin-EDTA, Fetal Bovine Serum (FBS) were obtained from Gibco (Invitrogen, Carlsbad, CA, USA), and Polyvinylidene Difluoride (PVDF) membrane was purchased from Millipore (Billerica, MA, USA). Primary antibodies EGFR, pEGFR, PI3k, pPI3k, Akt, pAkt^{Ser473}, pAkt^{Thr308}, mTOR, pmTOR, survivin were purchased from Cell Signaling Technology (Danvers, MA, USA) whereas N-Ras, Raf-1, MEK, ERK½, PCNA, c-Fos, c-Jun, N-cadherin, claudin-11, occludin, MMP-2, MMP-9 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies horseradish peroxidase conjugated rabbit-anti mouse IgG, goat-anti rabbit IgG and FITC conjugated goat-anti rabbit IgG were obtained from GENEi (Bangalore, India). Remaining chemicals were purchased from Sisco Research Laboratories Pvt. Ltd., (Andheri East, India). Chemicals used in this work were extra pure and culture grade.

2.2. Human metastatic ovarian cancer cell line (PA-1)

Human metastatic ovarian cancer cell line (PA-1) was procured from National Centre for Cell Science (NCCS), Pune. PA-1 cells were cultured in DMEM supplemented with 10% FBS and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2 incubator. Cells were passaged using trypsin and exponentially growing cells were used for all the experiments.

2.3. Immunoblotting

PA-1 cells were cultured on 100 mm petridish and treated with vehicle control (0.01% DMSO), 50 μM and 75 μM of quercetin for 24 h. After treatment period the cells were washed with ice cold PBS and lysed in RIPA buffer containing protease inhibitor cocktails. The total protein concentration was estimated using Bradford's method [22]. Briefly, 50 μg of total protein was separated with 10%–15% SDS-PAGE gel, transferred to PVDF membrane and blocked with blocking buffer. The membranes were incubated with primary antibodies against EGFR, pEGFR, PI3k, pPI3k, Akt, pAkt^{Ser473}, pAkt^{Thr308}, mTOR, pmTOR, survivin, PCNA, N-Ras, Raf-1, MEK, ERK½, c-Fos, c-Jun, claudin-11, occludin, MMP-2 and MMP-9 (1:250 to 1:4000) for overnight in Tris-buffered saline. β-actin (1:5000) was used as a loading control. After washing, the membranes were incubated with HRP-conjugated rabbit-antimouse IgG or goat–antirabbit IgG secondary antibody at a dilution of 1:10000. Protein band were detected by enhanced chemiluminescence kit in ChemiDoc XRS Imaging System (Bio-Rad, USA).

2.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the samples using TRI reagent as

Table 1
Primers list.

Target Gene	Gene bank accession number	Primer sequence (5'→3')	Annealing temperature (°C)
EGFR	NM_005228.5	F-AGGCACGAGTAACAAGCTCAC R-ATGAGGACATAACCAGCCACC	60
PI3k	NM_006218.4	F-TGGATGCTCTACAGGGCTTT R-GTCTGGGTTCTCCCAATTCA	58
Akt	NM_005163.2	F-TCTATGGCGCTGAGATTGTG R-CTTAATGTGCCCGTCCTTGT	58
mTOR	NM_004958.4	F-GCACTGTGGGCCGACTCAGT R-GCATTGGAGACCAGGTGCCC	64
N-Ras	NM_002524.5	F-GAGCTTGAGGTTCTTGC R-AGTATGTCCAACAAACAGG	53
Raf-1	NM_001354689.3	F-CAGCCCTGTCCAGTAGC R-GCCTGACTTTACTGTTGC	56
MMP-2	NM_004530.6	F-CTCTCCTGACATTGACCTTGG R-CCGGTCCTTGAAGAAGAAGAT	58
MMP-9	NM_004994.3	F-GAACCAATCTCACCGACAGG R-GCCACCCGAGTGTAACCATA	59
Claudin-11	NM_005602.6	F-GGCTGGTGTTTTGCTCATTCTGC R-AGCACCAATCCAGCCTGCATAC	63
Claudin-4	NM_001305.5	F-TCATCGGCAGCAACATTGTC R-GCAGTGCCAGCAGCGAGT	60
Occludin	NM_002538.4	F-TTTGTGGGACAAGGAACACA R-TCATTCACTTTGCCATTGGA	57
GAPDH	NM_002046.7	F-GTCAAGGCTGAGAACGGGAA R-AAATGAGCCCCAGCCTTCTC	58

(F- Forward primer; R- Reverse primer).

described earlier [23]. 2 μg of total RNA was used to synthesize first-strand complementary DNA (cDNA) using a cDNA synthesis kit (iScript, Bio-Rad, USA) according to the manufacturer's protocol.

Real-time PCR was performed in a CFX96 real-time detection system (Bio-Rad, USA) using Takara SYBR Green kit (Takara Bio Inc., Japan) as per the manufacturer's instruction. Gene-specific primers were used to determine EGFR, PI3k, Akt, mTOR, N-Ras, Raf-1, claudin-11, claudin-4, occludin, MMP-2 and MMP-9 mRNA expression. Primer sequences of the genes are listed in Table 1. The mRNA expression was normalized with GAPDH as a reference gene. The Ct values were obtained from the amplification, and relative fold changes was calculated by the $2-\Delta\Delta Ct$ method [24].

2.5. Immunocytochemistry

PA-1 cells were cultured on glass coverslips and treated with vehicle control and quercetin for 24 h. Cells were washed with PBS, fixed with 100% methanol at −20 °C for 20 min and then permeabilized with 0.5% Triton X-100 for 5 min at room temperature. After permeabilization, cells were washed with PBS and blocked with 5% BSA in PBS for 2 h. For labelling, cells were washed with PBS and incubated with rabbit polyclonal antibody against N-cadherin (1:250) for overnight at 4 °C. Subsequently, the cells were washed with PBS and incubated with FITC-conjugated goat anti-rabbit IgG (1:500) for 1 h at room temperature. Finally, after three washes with PBS, cells were incubated with DAPI for 10 min at room temperature and mounted by coverslips facing down on glass slides using PBS-Glycerol solution (9:1). Images were acquired using EVOS FLoid Cell Imaging Station.

2.6. Gelatinase microplate assay

The MMP-2/9 activity in the conditional medium was assessed by gelatinase microplate assay [25]. In brief, PA-1 cells were treated with vehicle control (0.01% DMSO), 50 μM and 75 μM quercetin. After treatment, serum free conditioned medium was collected and protein concentration was estimated. In a 96 well plate, 5 μg of protein sample from each treatment group was added in 6 wells and the volume was made up to 60 μl with calibration buffer. For each treatment group, 100 μl of solution A was added to the first three wells and 100 μl of solution I was added to the next three wells. Plates were incubated for 2 h at 37 °C. Further, 120 μl of protein assay dye reagent was added to all wells and incubated for 5 min at room temperature. The absorbance was measured at 595 nm. MMP-2/9 activity was calculated for each group by subtracting the absorbance between solution I and solution A. Gelatinase activity was represented as absorbance difference at 595 nm (AD at 595 nm).

2.7. Gelatin zymography

Zymography was used to determine the gelatinolytic activity of both MMP-2/9 in quercetin treated PA-1 cells [26]. PA-1 cells were cultured in 100 mm culture dish and incubated until it reaches the confluency. Then, cells were treated with quercetin (50 and 75 μM) and vehicle (0.01% DMSO) for 24 h in serum free medium. Serum free conditioned medium was collected and centrifuged to remove cell debris after the treatment period. Collected conditioned medium was filtered and concentrated by 15 ml Amicon Ultra-15 centrifugal filter (Merck Millipore Ltd., Ireland). Protein concentration in the supernatant was estimated by Bradford's protein assay. 50 μg of protein from each treatment group was equally mixed with 2X non-reducing loading buffer and incubated at room temperature for 30 min. Samples were loaded on 7.5% SDS-polyacrlyamide gel co-polymerized with 0.1% gelatin and electrophoresed. After electrophoresis, the gels were washed with 2.5% Triton X-100 twice for 30 min at room temperature and incubated in incubation buffer for overnight at 37 °C. Following incubation, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 for 30 min and destained with destain solution until required resolution is obtained. Areas of proteolytic activity were visible as clear bands on blue background of the stained gel. Bands were detected by Gel Doc XRS Imaging System (Bio-Rad, USA).

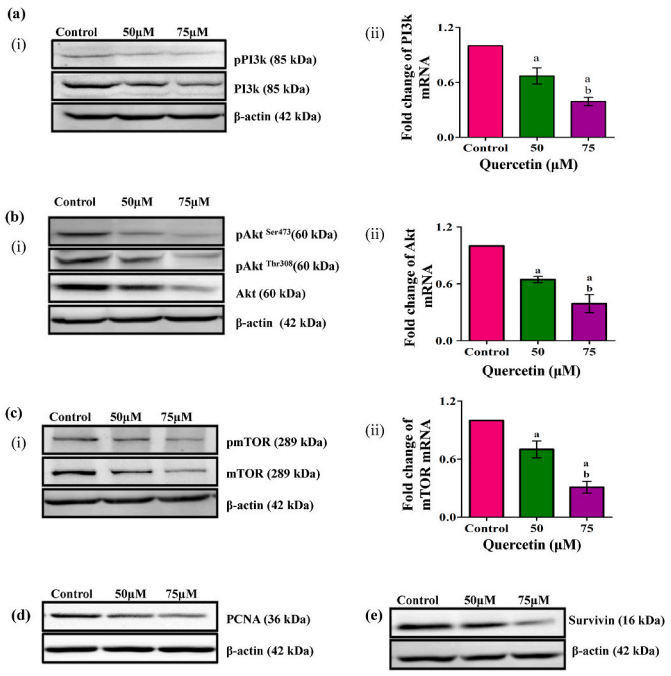

Fig. 1. Effects of quercetin on PI3k/Akt/mTOR pathway in human metastatic ovarian cancer PA-1 cell line.
PA-1 cells were treated with vehicle control, 50 μM, and 75 μM of quercetin for 24 h (a–e). Protein sample from control and quercetin-treated cell lysates was separated in SDS-PAGE and protein levels were detected by Western blot analysis (a–i) PI3k, (b–i) Akt, (c–i) mTOR, (d) PCNA and (e) Survivin. The membranes were stripped and reprobed for β-Actin as a loading control. Real-time PCR was used to analyze mRNA expressions of (a-ii) PI3k, (b-ii) Akt, and (c-ii) mTOR. Target gene expression was normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents the mean ± SEM of three independent observations. "a" represents the statistical significance between control vs quercetin treated groups and "b" represents the statistical significance between the quercetin treated group at $p < 0.05$.

2.8. Scratch wound migration assay

PA-1 cells were seeded in a 35 mm plate and grown until it reaches the confluency. Monolayer of the cells were wounded by scratching with 200 μL micropipette tip and washed with PBS to remove the floating non-adherent cells. Cells were incubated with vehicle control (0.01% DMSO), 50 μM and 75 μM quercetin for 24 h. After 24 h treatment, cells were washed by PBS and fixed with 4% paraformaldehyde and then stained with 0.5% crystal violet solution. Thereafter, the cells were washed with PBS. Images were taken at 0 h and 24 h independently on Nikon Eclipse-80i, Microscope (Japan) at 10x magnification.

2.9. Cell adhesion assay

Cell adhesion assay was used to assess the metastatic ability of human metastatic ovarian cancer PA-1 cells [27]. PA-1 cells were seeded on 6-well microplate at a density of 3×10^5 and treated with or without quercetin for 24 h. Cells were trypsinized and transferred to a collagen I coated 6-well plate (Gibco, USA). Further, the cells were incubated at 37 °C for 30 min, medium was discarded and gently washed with PBS to remove debris. Then the PA-1 cells were fixed with 4% PFA, stained with 0.5% crystal violet and photographed using Nikon Eclipse-80i,

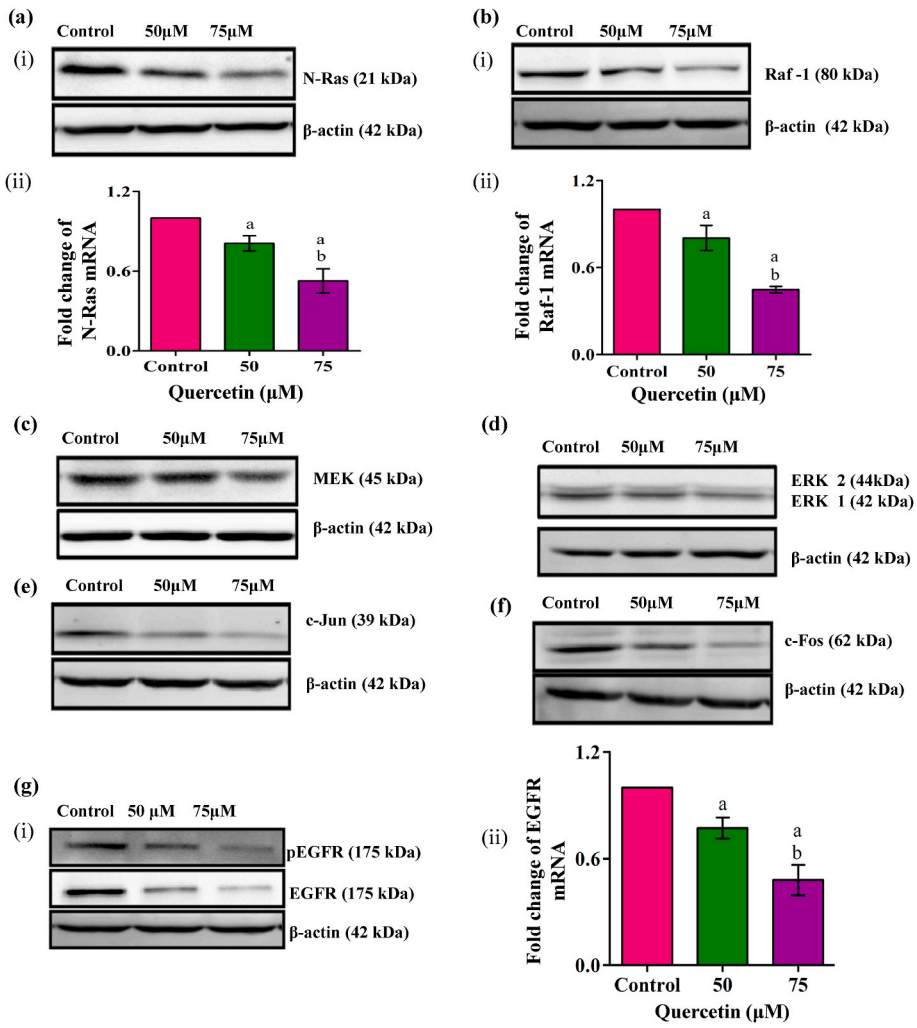

Fig. 2. Effects of quercetin on Ras/Raf pathway in human metastatic ovarian cancer PA-1 cell line.
PA-1 cells were treated with vehicle control, 50 μM, and 75 μM of quercetin for 24 h (a–g). Protein sample from control and quercetin-treated cell lysates was separated in SDS-PAGE, and protein levels were detected by Western blot analysis (a–i) N-Ras, (b–i) Raf-1, (c) MEK, (d) ERK½, (e) c-Jun, (f) c-Fos and (g) EGFR. The membranes were stripped and reprobed for β-Actin as a loading control. Real-time PCR was used to analyze mRNA expressions of (a-ii) N-Ras, (b-ii) Raf-1, and (g-ii) EGFR. Target gene expression was normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents the mean ± SEM of three independent observations. "a" represents the statistical significance between control vs quercetin treated groups and "b" represents the statistical significance between the quercetin treated group at $p < 0.05$.

Microscope.

2.10. Statistical analysis

The data were expressed as mean ± SEM of three independent observations. For all the measurements, one-way ANOVA followed by Student's–Newman–Keuls (SNK) test was used to assess the statistical significance between control and treated groups. A statistical significance was considered at $p < 0.05$. Statistical analysis was performed using the GraphPad Prism 5 software (La Jolla, CA, USA).

3. Results

3.1. Quercetin suppresses survival and proliferation in human metastatic ovarian cancer PA-1 cell line

To investigate whether quercetin inhibits the cell survival and proliferation of human metastatic ovarian cancer we assessed the molecules involved in PI3k/Akt pathway.

Therefore, PA-1 cells were treated with vehicle control, 50 and 75 μM concentration of quercetin for 24 h to examine the expression of proteins in PI3k/Akt pathway. Real-time PCR analysis of PI3k and Akt showed that quercetin treatment (50 and 75 μM) decreased the mRNA expression of these genes in PA-1 cells. Further, we investigated the total and phosphorylated form of PI3k and Akt expressions in PA-1 cells using western blot. Similar to mRNA expression, quercetin decreased both total and phosphorylated forms of PI3k/Akt protein levels when compared to control (Fig. 1a and b).

We next evaluated the effect of quercetin on mTOR protein expression a downstream effector of Akt. As shown in Fig. 1c, real-time PCR results revealed decrease in the mRNA expression of mTOR in the quercetin treated PA-1 cells when compared to control. It was also further supported by immunoblot analysis which evidenced that quercetin at 50 and 75 μM concentration decreased total and phosphorylated form of mTOR protein level in human metastatic ovarian cancer PA-1 cells.

In order to identify the role of PI3k/Akt/mTOR signaling in quercetin mediated inhibition of PA-1 cell survival and proliferation, we further investigated survivin and proliferating cell nuclear antigen (PCNA). For this, PA-1 cells were treated with vehicle control, 50 and 75 μM concentration of quercetin for 24 h. Exposure of quercetin decreased the protein levels of PCNA and survivin in PA-1 cells assessed by Western blot (Fig. 1d and e). Collectively, these results confirmed that quercetin inhibits PA-1 cell survival and proliferation *via* downregulation of PI3k/Akt/mTOR pathway.

To validate our findings, we next evaluated the effect of quercetin on Ras/Raf signaling molecules in PA-1 cell survival and proliferation. So, we treated the PA-1 cells with quercetin for 24 h and assessed the expression of proteins involved in Ras/Raf signaling. The gene expression of N-Ras and Raf-1 were analysed by western blotting and real-time PCR. We found that compared to vehicle control group quercetin treated PA-1 cells shows decreased N-Ras and Raf-1 gene expression (Fig. 2a and b). Further effect of quercetin on MEK and ERK½ protein expression were analysed by Western blot. As shown in Fig. 2c and d, quercetin

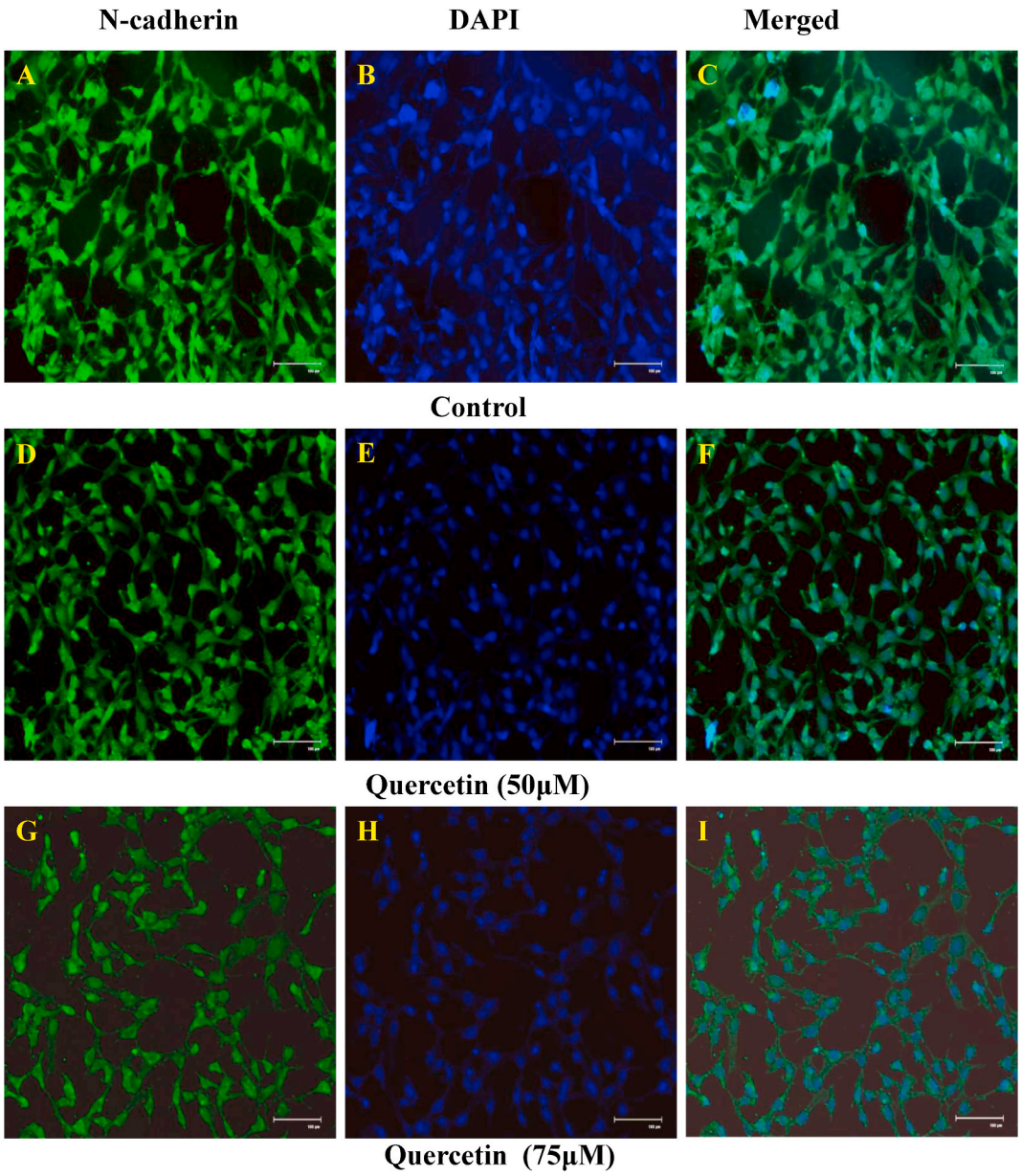

Fig. 3. Effects of quercetin on N-cadherin immunolocalization in human metastatic ovarian cancer PA-1 cell line.
For immunolocalization, untreated and quercetin (50 and 75 μM) treated PA-1 cells were analysed for N-cadherin protein (mesenchymal cell marker). Cells were seeded on the coverslip and processed for immunofluorescence with N-cadherin antibody (Green) and nuclei were counterstained with DAPI (blue) in control and quercetin treated PA-1 cells. Images were acquired using EVOS FLoid Cell Imaging Station at 100 μM scale bar. N-cadherin of Control (A), 50 (D), and 75 μM (G) quercetin treated PA-1 cells. DAPI nuclear stain of Control (B), 50 (E), and 75 μM (H) quercetin treated PA-1 cells. Merged images of Control (C), 50 (F), and 75 μM (I) quercetin treated PA-1 cells. Typical results were obtained from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

decreased MEK and ERK½ protein level at 50 and 75 μM concentration in PA-1 cells. Therefore it shows that quercetin reduces both MEK and ERK½ protein levels in PA-1 cell line.

In addition, we analysed the expression of Activator protein 1 (AP-1) transcription factor in human metastatic ovarian cancer cell. PA-1 cells were treated with 50 and 75 μM concentration of quercetin for 24 h. Western blot analysis revealed that quercetin reduced the c-Jun and c-Fos protein level in PA-1 cells (Fig. 2e and f). From the above observation, it was clear that quercetin inhibits metastatic ovarian cancer cell proliferation and survival by decreasing the signaling molecules involved in the Ras/Raf pathway in ovarian cancer cells.

3.2. Quercetin reduces EGFR gene expression in human metastatic ovarian cancer PA-1 cell line

In order to elucidate the effect of quercetin on EGFR that transduces several signaling pathway, we treated PA-1 cells with quercetin (50 and 75 μM) for 24 h and performed real-time PCR and immunoblot analysis. The result shows that quercetin treatment decreased the mRNA expression of EGFR in PA-1 cells when compared to control. Quercetin also decreased both total and phosphorylated form of EGFR protein levels in PA-1 cells (Fig. 2g). These results indicate that quercetin downregulates EGFR gene expression in PA-1 cell line.

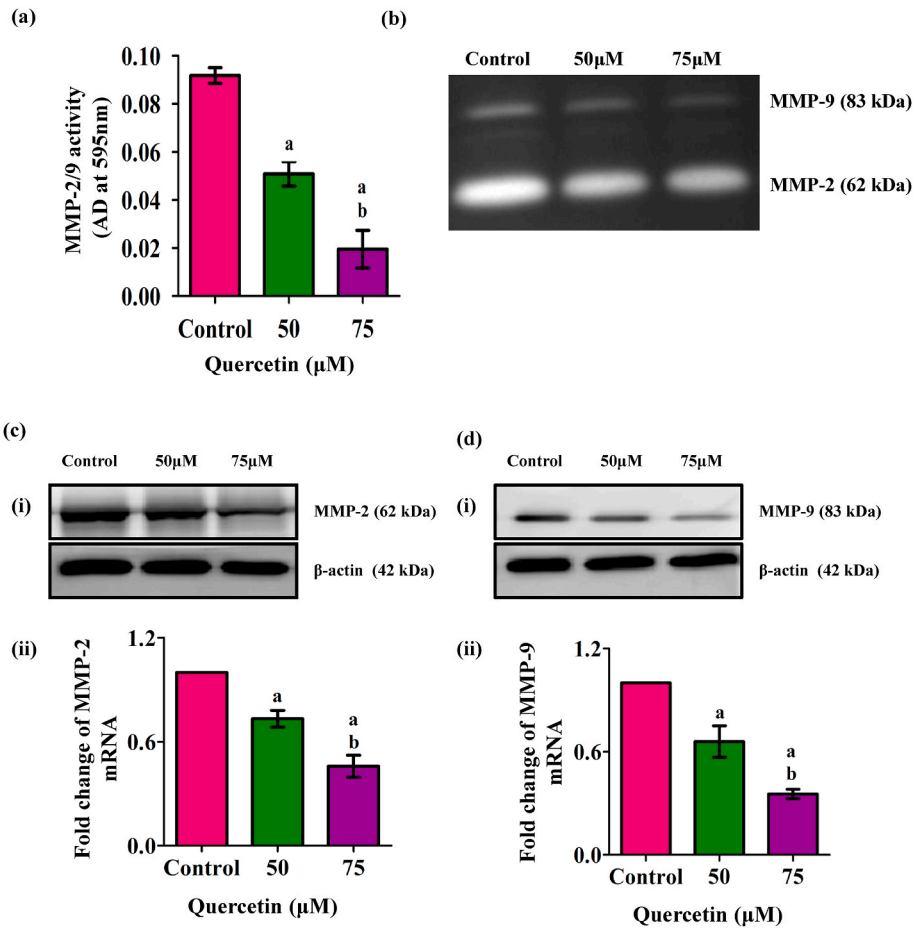

Fig. 4. Effects of quercetin on MMP-2/9 in human metastatic ovarian cancer PA-1 cell line.
PA-1 cells were cultured in the presence and absence of quercetin and serum-free conditional medium was collected to determine MMP-2/9. Protein samples from control and quercetin-treated cells were used to determine the MMP-2/-9 enzymatic activity by gelatinase microplate assay (a). Protein sample from control and quercetin-treated cells was used to examine the gelatinolytic activity of MMP-2/9 by gelatin zymography (b). Protein sample from control and the quercetin-treated cell was separated in SDS-PAGE and protein levels were detected by Western blot analysis (c–i) MMP-2 and (d–i) MMP-9. The membranes were stripped and reprobed for β-Actin as a loading control. Real-time PCR was used to analyze mRNA expressions of (c-ii) MMP-2 and (d-ii) MMP-9. Target gene expression was normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents the mean ± SEM of three independent observations. "a" represents the statistical significance between control vs quercetin treated groups and "b" represents the statistical significance between the quercetin treated group at $p < 0.05$.

3.3. Quercetin inhibits the migration and adhesion in human metastatic ovarian cancer PA-1 cell line

To determine whether quercetin alters the mesenchymal marker N-cadherin involved in EMT process, we performed immunofluorescence staining to evaluate the expression of N-cadherin protein in PA-1 cells. Our data suggested that N-cadherin expression was reduced in quercetin treated PA-1 cells when compared to vehicle control group (Fig. 3). This shows that quercetin treatment attenuates mesenchymal marker N-cadherin and inhibits the EMT process in human metastatic ovarian cancer PA-1 cells.

We then explored action of quercetin on gelatinase (MMP-2/9), an enzyme that disrupts the extracellular matrix and makes way for migratory cancer cells. So, gelatinase microplate assay was done to detect the enzymatic activity of MMP-2/9. As shown in Fig. 4a, treatment of PA-1 cells with quercetin at 50 and 75 μM decreased the MMP-2/9 activity but the gelatinase activity was stronger in control group. Gelatin zymography is used to detect the enzymatic ability of MMP-2/9 to hydrolyze gelatin. Similar to microplate assay, zymography data showed that quercetin decreased the enzymatic ability of MMP-2/9 in PA-1 cells whereas it shows more gelatinolytic activity in vehicle treated control group (Fig. 4b). In order to understand the molecular mechanism of quercetin on MMP-2/9 we checked the gene expression of both MMPs by real-time PCR and western blotting. We observed that mRNA levels of both MMP-2/9 were reduced in quercetin treated PA-1 cells but not in vehicle control group. The Western blot results was also concordance with the mRNA levels of MMP-2/9 which shows that quercetin decreased both MMP-2/9 protein levels when compared to control group (Fig. 4c and d).

Effect of quercetin on PA-1 cell migration was determined by scratch wound healing assay. The PA-1 cells treated with vehicle control showed more number of migrated cells which exhibit a complete wound closure activity after 24 h. On the other hand, cells treated with quercetin (50 and 75 μM) decreased the migration of PA-1 cells which inhibits the wound closure activity after 24 h of treatment (Fig. 5). This result suggests that quercetin inhibited PA-1 cell migration in a dose-dependent manner, indicating that quercetin curtail the migration of metastatic ovarian cancer cells.

Finally, we explored the role of quercetin on tight junction proteins that contribute to invasion and adhesion of cancer cells. Therefore, we investigated the tight junctional molecules such as claudin-11, claudin-4 and occludin in quercetin treated ovarian cancer. We found that quercetin modulated the tight junctional proteins in PA-1 ovarian cancer cells. Both claudin-11 and claudin-4 in quercetin treated PA-1 cells showed reduced gene expression in a dose dependent manner (Fig. 6a and b). In contrast to these results, treatment of PA-1 cells with quercetin shows increased occludin gene expression compared to untreated vehicle control (Fig. 6c). Next we analysed the adhesion behaviour of PA-1 cells by cell adhesion assay. Untreated and treated PA-1 cells were allowed to adhere on collagen type I coated surface for 30min. In vehicle treated control, the adherent cells were more whereas quercetin treatment inhibits the PA-1 cell adhesion and shows reduced adherent cells (Fig. 7). Collectively, these results suggest that quercetin reduced the cell migration, adhesion and attenuates the metastatic ability of human metastatic ovarian cancer PA-1 cell line *via* alteration of multiple signaling molecules.

4. Discussion

Ovarian cancer is often detected at advanced or metastatic stage and

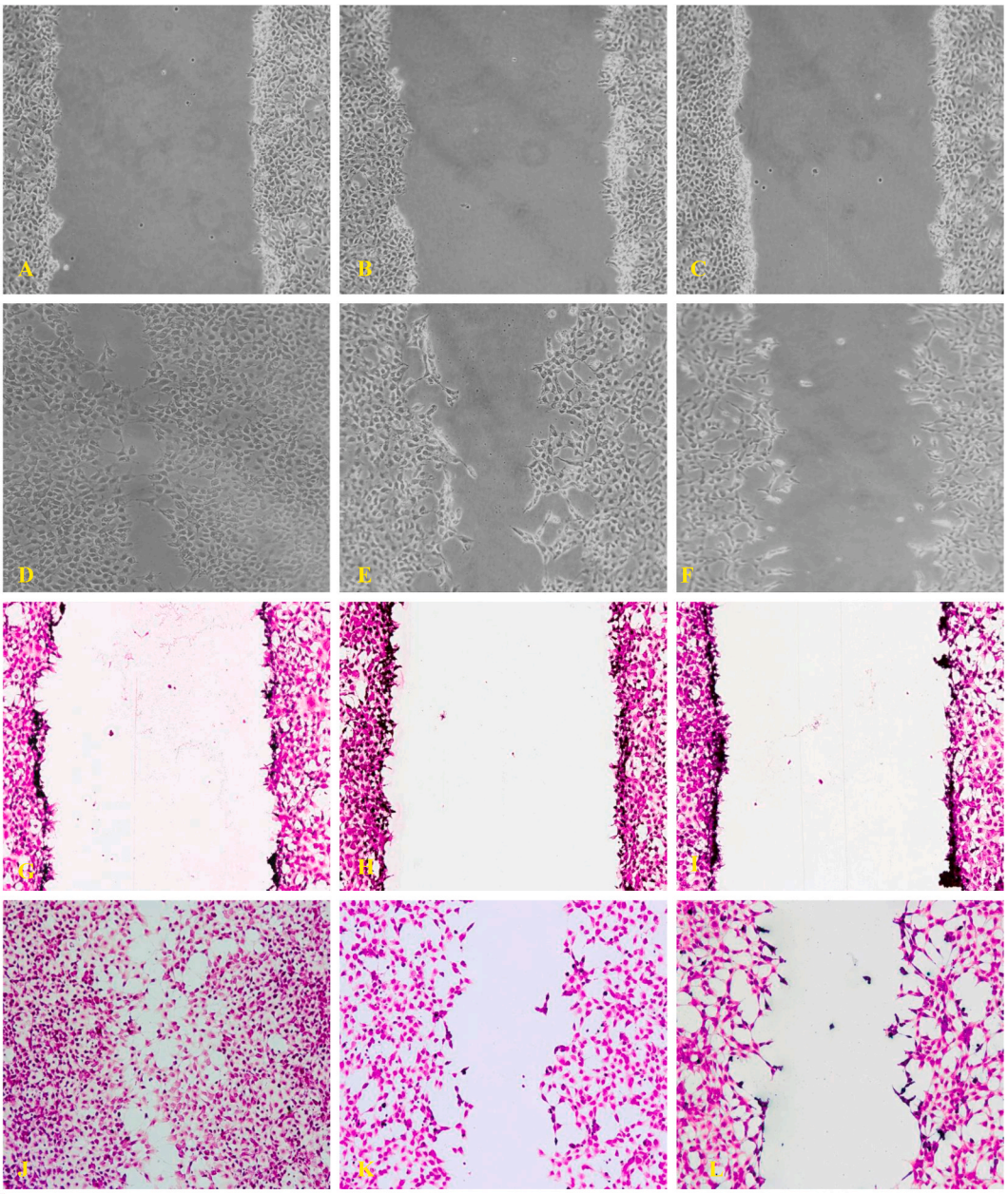

Fig. 5. Effect of quercetin on cell migration of human metastatic ovarian cancer PA-1 cell line.
For cell migration assay, confluent PA-1 cells were scratched with 200 μl tip and treated with vehicle control, 50 μM, and 75 μM of quercetin for 24 h. Images were captured at 0 and 24 h respectively. PA-1 cells images were acquired using phase-contrast microscope at 20X magnification (100 μM scale bar): Control (A), 50 (B), and 75 μM (C) quercetin treated PA-1 cells for 0 h whereas Control (D), 50 (E), and 75 μM (F) quercetin treated PA-1 cells for 24 h. PA-1 cells were stained with 0.5% crystal violet and images were acquired using Nikon Eclipse-80i, Japan microscope at 10X magnification (100 μM scale bar): Control (G), 50 (H), and 75 μM (I) quercetin treated PA-1 cells for 0 h whereas Control (J), 50 (K), and 75 μM (L) quercetin treated PA-1 cells for 24 h. Typical results were obtained from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

clinical management of metastatic ovarian cancer remains a major challenge [28]. Quercetin aglycone induces apoptosis through caspase-3 activation and PARP deactivation in ovarian cancer cells SKOV-3 and OVCAR-8 [29]. It also induces protective autophagy and apoptosis through ER stress *via* the p-STAT3/Bcl-2 axis in ovarian cancer [30]. Quercetin inhibits DNA double-strand break repair and enhances radiosensitivity *via* p53-dependent endoplasmic reticulum stress pathway in human ovarian cancer cells [31]. However, the anti-metastatic effect of quercetin on metastatic ovarian cancer cells and their underlying molecular mechanisms are still remains to be elucidated. Therefore, in this study we evaluated the anti-metastatic property of quercetin on human metastatic ovarian cancer cells.

It has been reported that quercetin is present in variety of vegetables such as onions (284–486 mg/kg), kale (110 mg/kg), French beans (32–45 mg/kg), broccoli (30 mg/kg), lettuce (14 mg/kg) and tomatoes (8 mg/kg). It is also present in fruits namely red grapes (15 mg/kg) and apples, with the highest concentration (21–72 mg/kg) [17]. It has been reported that 200 μM concentration of quercetin for 24 h did not altered the cell viability in normal ovarian cells (HOSE cells) which shows the non-toxic effect of quercetin on normal ovarian cells [32]. Our earlier study reported that ovarian cancer cell viability decreased gradually with increased concentration of quercetin with IC_{50} value of 75 μM for 24 h in human metastatic ovarian cancer cells and also induces apoptosis [21]. Hence, for further studies we considered 75 μM as high dose and

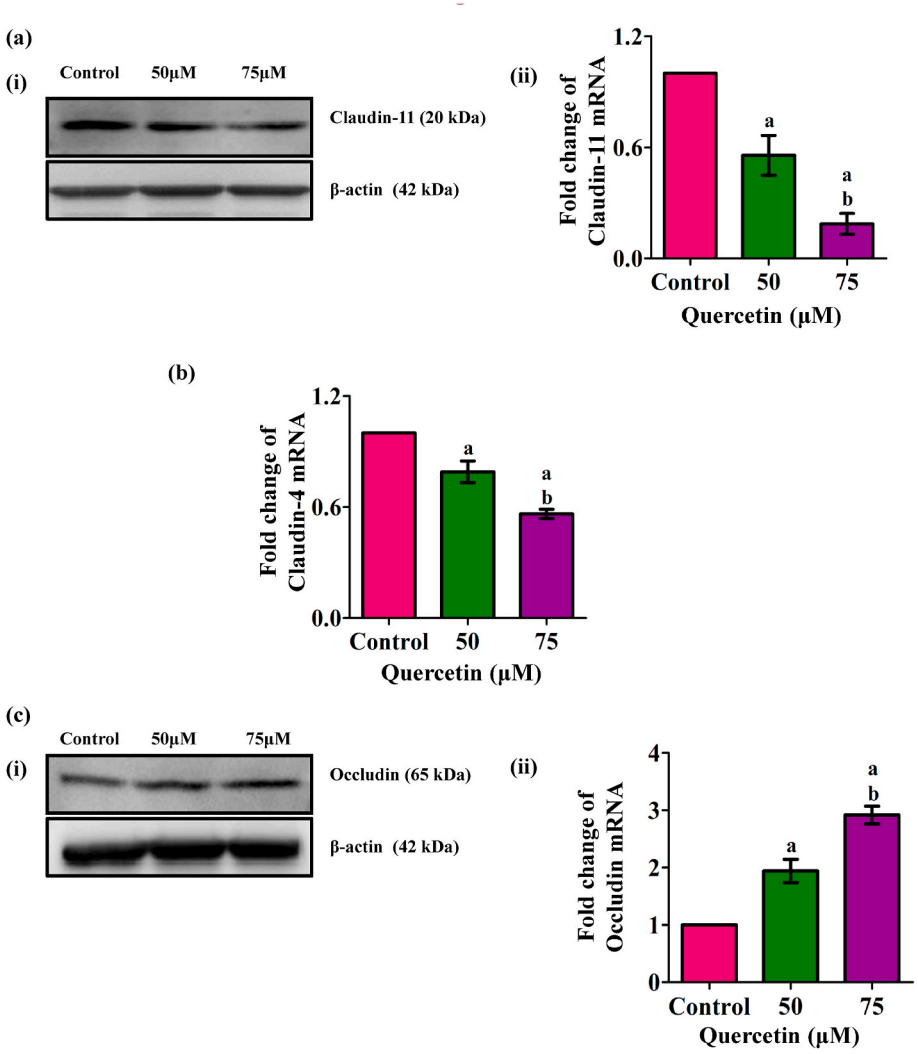

Fig. 6. Effects of quercetin on tight junctional proteins in human metastatic ovarian cancer PA-1 cell line.
PA-1 cells were treated with vehicle control, 50 μM, and 75 μM of quercetin for 24 h (a–c). Protein sample from control and quercetin-treated cell lysates was separated in SDS-PAGE, and protein levels were detected by Western blot analysis (a–i) Claudin-11 and (c–i) occludin. The membranes were stripped and reprobed for β-Actin as a loading control. Real-time PCR was used to analyze mRNA expressions of (a-ii) claudin-11, (b) claudin-4, and (c-ii) occludin. Target gene expression was normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents the mean ± SEM of three independent observations. "a" represents the statistical significance between control vs quercetin treated groups and "b" represents the statistical significance between the quercetin treated group at $p < 0.05$.

50 μM as low dose which is in the range of clinically relevant/non toxic dose and bioavailability to identify the effect of quercetin on signaling molecules involved in cell survival, proliferation, migration and adhesion.

Among several pathways PI3k/Akt/mTOR plays the most critical role in the regulation of cell survival and proliferation in ovarian cancer [33]. Thus, we investigated the effect of quercetin on gene expression of molecules involved in the PI3K/Akt/mTOR signaling pathway that favours cell survival and proliferation in ovarian cancer.

Quercetin acts as a competitive inhibitor for the ATP binding site of PI3K kinase active domain [34]. Luteolin inhibited the phosphorylation of PI3k and Akt *via* suppression of MMP-2/-9 and induces apoptosis in human melanoma [35]. Quercetin also inhibits the EGF induced phosphorylation of PDK1 and PI3k thereby it suppresses Akt phosphorylation in prostate cancer cells [36]. In accordance with previous reports our study showed that quercetin reduced gene expression of PI3K and Akt in PA-1 ovarian cancer cells may be due to the binding of quercetin with PI3k which suppresses its kinase activity and downstream molecules Akt. It is also clear from our results that quercetin inhibits phosphorylation of PI3k and Akt *via* downregulation of MMP-2/-9 in human metastatic ovarian cancer PA-1 cells. Quercetin acts as a dual-inhibitor for both PI3K and mTOR [37]. Quercetin inhibits growth of prostate cancer cells by decreasing the phosphorylation of mTOR through AKT inhibition [38]. It also inhibits glycolysis *via* downregulation of Akt/mTOR and suppresses the motility of breast cancer [39]. It is evidenced from our data that quercetin might also binds with mTOR ATP binding site and downregulates mTOR gene expression in human metastatic ovarian cancer PA-1 cells due its dual inhibitory capacity of both PI3K and mTOR. Our results also suggest that inhibition of Akt by quercetin decreased mTOR gene expression and its phosphorylation in PA-1 cell line.

Quercetin inhibited Akt/mTOR and its activation in PA-1 cells. It also decreases the levels of phosphorylated form of both Akt/mTOR more when compared to total form, which shows that phospho forms are strongly inhibited by quercetin. Study showed that perifosine decreases the levels of p-Akt/Akt and p-mTOR/mTOR. Perifosine inhibits mTOR complex assembly and induces autophagy in human lung cancer cells by reduced levels of mTOR, raptor, rictor, 70-kDa ribosomal S6 kinase, and 4E-binding protein 1 that can function as both downstream and upstream of Akt [40]. It is clear from this study the decreased phosphorylated form of Akt/mTOR might be due to the inhibition of Akt/mTOR axis.

Quercetin suppressed the migration and invasion of ovarian cancer cells but not cell proliferation *via* decreasing the expression of uPA and MMP-2 without alteration in PAI-1 and PCNA protein levels [41]. In contrast to the above report our results indicates that quercetin down-regulated PCNA protein level and suppresses proliferation of human metastatic ovarian cancer PA-1 cells. Gene knockout or inhibition of survivin suppresses the growth of primary ovarian tumor and its metastasis [42]. Combination of quercetin and sodium butyrate induces apoptosis by downregulation of survivin and antiapoptotic protein in rat C6 and human T98G glioblastoma cells [43]. But in our study quercetin

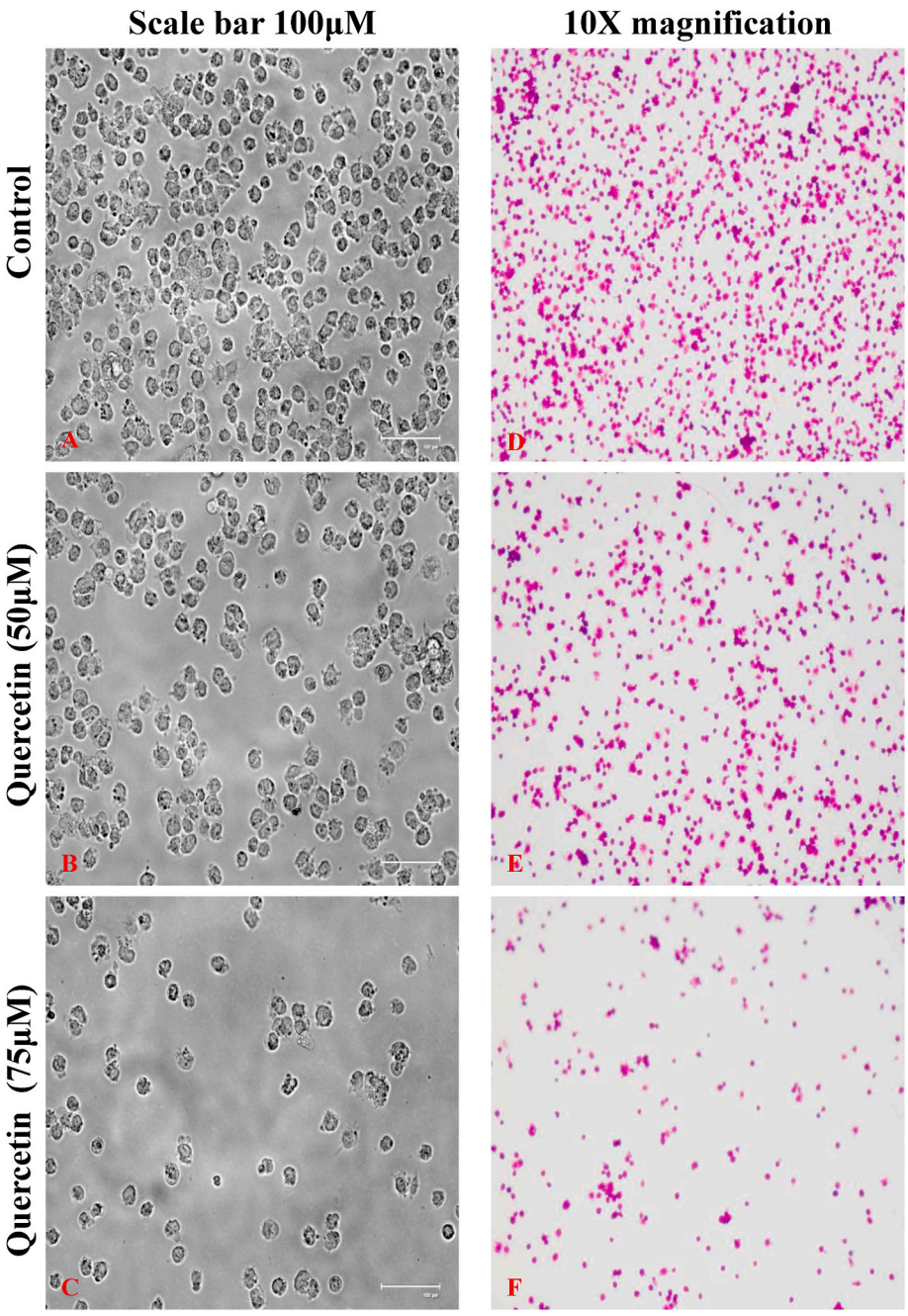

Fig. 7. Effect of quercetin on cell adhesion of human metastatic ovarian cancer PA-1 cell line.
PA-1 cells were treated with vehicle control, 50 µM, and 75 µM of quercetin for 24 h. The cells were trypsinized, seeded on a collagen-coated plate and incubated at 37 °C for 30min. Images of cells without stain were captured using EVOS FLoid Cell Imaging Station at 100 µM scale bar: Control (A), 50 (B), and 75 µM (C) quercetin treated PA-1 cells. Images of cells stained with crystal violet were captured using Nikon Eclipse-80i, Japan microscope at 10X magnification at (100 µM scale bar): Control (D), 50 (E), and 75 µM (F) quercetin treated PA-1 cells. Typical results were obtained from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

alone decreased the survivin protein expression and inhibits the survival of human metastatic ovarian cancer PA-1 cells. From our results it was confirmed that quercetin inhibited the survival and proliferation of human metastatic ovarian cancer PA-1 cells *via* reduced expression of both survivin and PCNA proteins. We found that quercetin inhibits human metastatic ovarian cancer cell survival and proliferation *via* downregulation of PI3k/Akt/mTOR pathway.

Crosstalk between PI3k/Akt and Ras/Raf signaling pathways occurs in human ovarian cancer cells [44]. So, targeting both pathways could be better for metastatic ovarian cancer treatment because inhibiting one pathway may be compensated by other pathway. Consequently in addition to PI3K/Akt pathway we assessed the effect of quercetin on Ras/Raf signaling molecules that might regulate cell survival and proliferation in metastatic ovarian cancer.

Quercetin induces degradation of Ras thereby it blocks the cell proliferation and survival of colon cancer [45]. Combination of sulforaphane, quercetin and catechins inhibits pancreatic cancer cells growth by downregulation of Ras [46]. Results from our study show that quercetin alone decreases Ras expression in PA-1 ovarian metastatic cancer cell line. Consistent with these previous observations, we identified that quercetin downregulates Ras protein in human metastatic PA-1 ovarian cancer cells which may be due to the degradation of Ras protein. Quercetin nanoparticles suppress the liver cancer cell proliferation by inactivation of Raf protein [47]. Structural findings suggest that quercetin can directly bind with Raf and suppressed the kinase activity of Raf [48]. It was clear from our study that quercetin inhibits the gene expression of Raf because of the downregulation of its upstream molecule Ras in ovarian cancer PA-1 cells. Our observations are consistent with the notion that downregulation of Raf may be due to direct binding of quercetin with Raf protein and suppression of its kinase activity in human ovarian cancer PA-1 cell line.

Quercetin induced apoptosis requires alteration in expression of Bcl-

2 family in addition to activation of MEK-ERK pathway and inactivation of Akt in human lung cancer cells [49]. It also induces apoptosis in human melanoma cancer cells through activation of JNK, p38, ERK½ and modulation of apoptotic molecules [50]. In contrast to these reports our study demonstrated quercetin decreased the protein levels of both MEK and ERK½ due to the inactivation of Akt in human metastatic ovarian cancer PA-1 cell line. AP-1 is the downstream transcription factors of MEK/ERK that favours cell survival and proliferation of cancer cells [51]. c-Jun promotes the carcinogenesis and tumor progression of ovarian cancer cells in ovarian cancer patients [52]. It has been showed that quercetin inhibits proliferation of prostate cancer cells by down-regulation of c-Jun [53]. In accordance with previous finding, results from our study showed that the metastatic ovarian cancer PA-1 cells treated with quercetin decreased the expression of c-Jun.

Report suggests that Lysophosphatidic acid (LPA) treatment activates AP-1 transcription factor *via* VEGF and stimulates the growth of human ovarian cancer cells [54]. Upregulation of c-Fos activates fucosyltransferase 1 (FUT1) and Lewis y antigen *via* transforming growth factor β1 (TGF-β1) mediated mitogen-activated protein kinase (MAPK) pathway associated with ovarian cancer cells proliferation [55]. Consistent with these reports, our results suggest that quercetin decreased the c-Jun and c-Fos protein level in ovarian cancer cells *via* inhibition of Ras/Raf signaling. These results provide evidence that quercetin inhibits cell survival and proliferation of human metastatic ovarian cancer PA-1 cells mediated through reduced expression of c-Jun/c-Fos which might inhibits AP-1 dimer formation and suppresses AP-1 binding at DNA binding site. Further in the present study quercetin inhibits cell survival and proliferation of human metastatic ovarian cancer PA-1 cell line *via* inactivation of molecules in Ras/Raf pathway.

Regarding mechanism that inhibits the human metastatic ovarian cancer PA-1 cell survival and proliferation, we found that quercetin modulated PI3k/Akt and Ras/Raf signaling pathway.

EGFR activates downstream molecules through tyrosine phosphorylation leads to cancer cell growth [56]. Quercetin suppressed the EGFR tyrosine kinase activity in A431 cell line [57]. 2′-Hydroxyflavanone sensitizes chemoresistance pancreatic cancer cells *via* inhibition of EGFR phosphorylation and suppression of signal transducer and activator of transcription 3 (STAT3) pathway [58]. In the present study, quercetin decreased the EGFR and its phosphorylation. It is possible that, the inhibitory effect of quercetin might be due to inhibition of tyrosine kinase activity and phosphorylation of EGFR in human metastatic PA-1 ovarian cancer cell line. Further, constitutive EGFR signaling activates PI3k/Akt and Ras/Raf pathways in cancer [59]. Therefore, quercetin inhibition of these signaling molecules could also be mediated through downregulation of EGFR. However, further study targeting EGFR is needed for complete understanding of this mechanism.

Available therapies are ineffective mainly due to the metastatic properties of malignant ovarian cancer cells. Hence, we further investigated the role of quercetin on metastatic property of ovarian cancer. One of the major events that occur during metastasis is cell motility or migration that invades other tissue [60]. LPA induces cadherin switching *via* increased mesenchymal marker N-cadherin and repressed Sirtuin 1 (SIRT1) in ovarian cancer cells. Further they reported that mesenchymal marker N-cadherin localization was diminished at cell-cell contact and cell membrane in SIRT1 activated as well as resveratrol treated ovarian cancer cells [61]. The present study revealed that N-cadherin expression was reduced in quercetin treated PA-1 ovarian cancer cells. In consistent with previous report our findings confirmed that quercetin inhibits the cadherin switching which might suppress the EMT process in human metastatic ovarian cancer by altering the expression of N-cadherin.

Quercetin downregulates MMP-2/9 *via* inhibition of MAPK and PI3k signaling pathways, resulting in the suppression of migration and invasion in human oral cancer cells [62]. It also suppresses the expression of p-Akt1, MMP-2/9 as a result it inhibits the migration and invasion of human hepatocellular carcinoma cell [63]. Available data from our

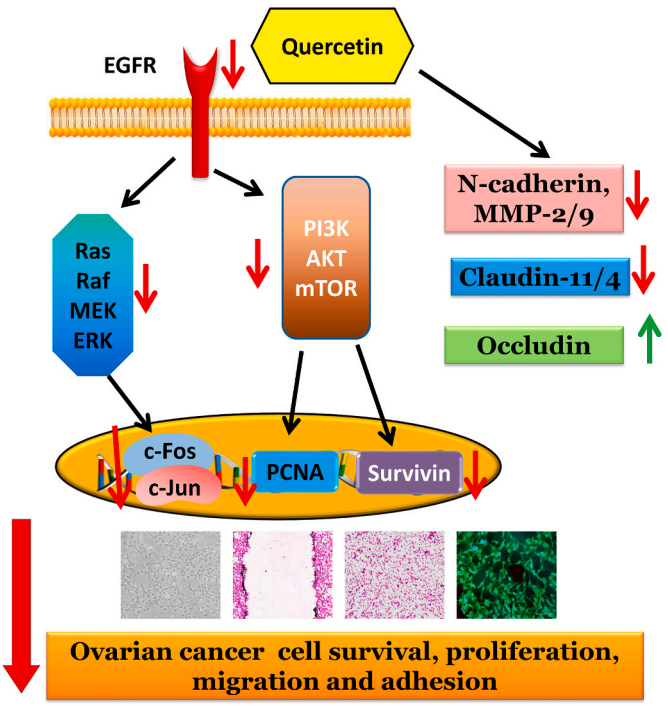

Fig. 8. Schematic representation of the study that explains antimetastatic effect of quercetin *via* modulation of intracellular signaling molecules.

study indicates that quercetin inhibits MMP-2/-9 which is due to the suppression of ERK and Akt pathways in PA-1 cells which correlates with our previous result. In accordance with this finding it was evidenced from our study that quercetin might be helpful in the prevention of extracellular matrix degradation in metastatic ovarian cancer cells. Silibinin inhibits migration and invasion of breast cancer cells *via* down-regulation of MMP-2/-9 [64]. In our present work, we showed that quercetin has the ability to stop migration of human metastatic ovarian cancer PA-1 cells. Results obtained from cell migration assay indicated that quercetin blocks the migration of PA-1 cells due to the inhibition of PI3k and Ras *via* downregulation of MMP-2/-9.

Epigenetic modification in the promoter region of CLDN-4 gene induces its overexpression in ovarian cancer [65]. Quercetin targets several epigenetic regulators of certain genes in cancer cells and inhibits the growth of cancer cells [66]. From our result it is clear that quercetin reduced the mRNA expression of CLDN-4 gene in PA-1 cells. It suggests that quercetin might target any epigenetic regulator that involves in epigenetic modification of CLDN-4 gene in PA-1 cells and inhibits its overexpression. Upregulation of Claudin-11 promotes tumor progression in mouse ovarian serous cystadenoma [67]. EMT mediates squamous cell carcinoma migration *via* snail induced expression of claudin-11 [68]. Quercetin decreased the transcription factors snail, slug and twist responsible for EMT process in prostate cancer [13]. Quercetin, in our study actually decreased gene expression of claudin-11 in PA-1 ovarian cancer cells which may be due to inhibition of snail mediated activation of claudin-11. Occludin is a component of cellular tight junction which was repressed by transcription factor like snail and slug, concertedly mediates EMT in ovarian cancer [69]. Quercetin significantly decreased expressions of EMT related transcription factors such as snail and slug in A549 and HCC827 cells [70]. From the above previous reports it was evidenced that quercetin may repress the activity of EMT related proteins snail and slug which could mediate the upregulation of occludin gene expression in PA-1 ovarian cancer cells. In accordance with our results, quercetin inhibits EMT process in PA-1 ovarian cancer cells *via* upregulation of occludin due to its tumor suppressor activity in PA-1 ovarian cancer cells. Collectively, available data suggests that quercetin might inhibit the intercellular adhesion *via*

modulating tight junctional proteins such as claudin-4, -11 and occludin in human metastatic ovarian cancer PA-1 cells.

Collagen has been associated with the resistance of paclitaxel therapy and also induces metastatic phenotype in ovarian cancer [71,72]. Chinese herbal medicine *Wenxia Changfu* formula reverses cell adhesion-mediated drug resistance by downregulating Integrin β1/PI3k/Akt in lung cancer cells [73]. It is clearly evidenced from our result that quercetin treated human metastatic ovarian cancer PA-1 cells showed reduced adhesion ability to extracellular matrix (collagen I) when compared to vehicle control. The fact that quercetin reduced the cell adhesion of human metastatic ovarian cancer PA-1 cells was due to the inactivation of PI3k/Akt pathway. These results revealed that quercetin disrupts the adhesion between the cell-extracellular matrix and cell–cell junctions which might be responsible for the prevention of metastatic property of PA-1 ovarian cancer cells. Altogether, we demonstrated that quercetin inhibits cell migration and adhesion *via* alteration of cell adhesion molecules and MMPs owing to suppression of metastatic capacity in human ovarian metastatic cancer PA-1 cells.

Overall, our present study confirmed that quercetin exerts anti-metastatic effects *via* inhibition of cell survival, proliferation, migration and adhesion of human ovarian metastatic cancer PA-1 cells through modulating multiple signaling molecules (Fig. 8).

5. Conclusion

The present study revealed the anti-metastatic activity of quercetin in human metastatic ovarian cancer PA-1 cells by suppression of cell survival, proliferation, migration and adhesion. Therefore, we suggest that quercetin might be a potential flavanoid to control metastasis of ovarian cancer. Though quercetin exerts its anti-metastatic activity in highly metastatic ovarian cancer PA-1 cells, a better understanding in ovarian cancer animal model would be essential and further investigations are needed.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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