Quercetin Synergistically Potentiates the Anti-Angiogenic Effect of 5- Fluorouracil on HUVEC Cell Line

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Research Article

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Abstract

background: Since tumors need oxygen and nutrients to grow and spread, angiogenesis has an essential role in cancer growth and metastasis. So, inhibition of angiogenesis at the initial stage of cancer is a critical strategy in metastasis inhibition. 5-Fluorouracil (5-FU) is a chemotherapy drug that has also been shown to have anti-angiogenic effects. Quercetin, a natural polyphenolic compound, has anti-angiogenic effects. The present study was conducted to investigate the enhancement of the anti-angiogenic effect of 5-FU in combination with Quercetin (Que) and compare it with the application of 5-FU alone.

Method and Results: Following the treatment of human umbilical vein endothelial cells (HUVECs) with Que, 5-FU and their combination, the cell viability, migration, and gene expression of VEGFR2 and VEGFR1 were assessed through MTT assay, wound healing assay, and real-time RT-PCR, respectively. In vivo angiogenesis was evaluated using chicken chorioallantoic membrane (CAM) assay. Our study showed that cell viability, migration, gene expression of VEGFR2 and VEGFR1, and angiogenesis significantly decreased following Que and 5-FU alone treatment, and the decrease in combination state was significant compare to 5-FU alone.

Conclusions: In summary, the present study showed that the combination of Que with 5-FU improves its anti-angiogenic effects. Therefore, this combination can be suggested for future in vivo studies.

Introduction

Cancer is a major public health problem and a leading cause of death worldwide[1]. The ability of cancer to spread to adjacent or distant organs has made it life-threatening. Tumor cells can penetrate blood or lymphatic vessels, circulate through the intravascular stream, and proliferate at another site: metastasis. So vascular network growth is critical for tumor growth and metastatic spread[2, 3].

Angiogenesis is the process in which new blood vessels sprout from an existing vascular system. It is a complex and physiological process that is influenced by various biomolecules include angiogenic factors such as vascular endothelial growth factor (VEGF), angiogenin1, and basic fibroblast growth factor (b-FGF) and anti-angiogenic factors such as angiostatin, endostatin, and thrombospondin-1 [4–8]. Angiogenesis is physiologically involved in several processes, such as embryogenesis and wound healing[3, 5]. Studies have shown that tumors can lead to angiogenesis progression and angiogenesis also causes tumor growth and metastasis to other organs[8, 9]. When tumors grow to 1–2 mm in diameter, the inner cells of the tumor become hypoxic, which inhibits the degradation of the Hypoxia-inducible factor 1-alpha (HIF-1α). Hypoxia- Thus, HIF-1α is increased and binds to the Response Element (HRE) region of genes involved in angiogenesis, such as the VEGF-expressing gene, so by this way, the expression of VEGF is increased [10–13].

At the next step, VEGF binds to its receptors on vascular endothelial cells and increases the proliferation and migration of endothelial cells and finally angiogenesis[14, 15]. VEGF has three main tyrosine kinase receptors, Vascular endothelial growth factor receptor 1 (VEGFR1) and Vascular endothelial growth factor

receptor 2 (VEGFR2), which are involved in angiogenesis, and VEGFR3, which is involved in lymphangiogenesis. VEGFR2 is expressed mainly in endothelial cells, while VEGFR1 is expressed in hematopoietic stem cells and inflammatory cells in addition to endothelial cells. VEGFR2 plays a key role in angiogenesis; however, the role of VEGFR-1 in angiogenesis is unclear [16–18]. The binding of VEGF to its receptor activates signaling pathways which resulted in increased expression of Matrix metalloproteinase (MMPs), change in cell membrane integrin profile, increased expression of cyclin D1, reduced Vascular endothelial (VE)-cadherin connections, and as a result, increase in invasion, migration, and cell proliferation and ultimately increased angiogenesis [19–21].

There are several therapeutic techniques for treating cancers, which the most common of them include: chemotherapy, radiation therapy, and surgery. Chemotherapy also can be used as tumor progression-preventing therapy through angiogenesis suppression. Mentioned drugs have adverse effects on normal tissues which accompany the pain and as a result, enduring them is difficult for patients [22, 23]. On the other hand, natural compounds have been considered because of extensive biological activity and low toxicity. So studies with the aim of decrease in usage dose and subsequently side effects of chemotherapy drugs have focused on combining these drugs with natural compounds [24, 25].

5-Fluorouracil (5-FU) is a chemotherapy drug used to treat various cancers such as breast, cervical, and gastrointestinal cancer [26]. It is revealed that 5-FU inhibits the thymidylate synthase (TS), which is the critical enzyme in DNA synthesis and repair. In addition, it has been shown that 5-FU inhibits angiogenesis and migration by suppressing the expression of angiogenic factors, including VEGF and MCP-1.[27, 28].

Quercetin (Que) is a typical flavonoid present in fruits and vegetables, including apple, garlic, radish, red onion, and green tea [29, 30]. Previous studies have shown that Que could inhibit the apoptosis, angiogenesis, cell cycle, and migration process of cancer cells [31]. Que suppresses angiogenesis by targeting the expression of VEGFR-2 and its signaling pathway. Studies have shown that Que can inhibit VEGF-induced migration, invasion, proliferation, and tube formation of human umbilical vein endothelial cells (HUVECs) by suppressing the activation of the VEGFR-2-induced AKT / mTOR / P70S6K axis. [32–34]. In addition, it has been reported that Que could inhibit angiogenesis through the inhibition of the ERK signaling pathway and the expression of VEGFR-2[35].

Therefore, Que can be used as an enhancer of the anti-angiogenic effect combined with drugs such as 5-FU, which have anti-angiogenic properties [this way, in addition to enhancing the anti-angiogenic effect of these drugs, usage dose, and subsequently, side effects of these drugs can be decreased. In this study, we evaluated the effects of 5-FU and Que alone and in combination on the viability, migration, and VEGFR1 and VEGFR2 gene expression of human umbilical vein endothelial cells (HUVECs) and in vivo neovascularization.

2. Materials and methods

2. Materials And Methods

2 – 1. Cell Culture

Human umbilical vein endothelial cells (HUVECs) were provided from Pasteur Institute (Tehran, Iran). HUVECs were cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all reagents were purchased from Bio-Idea Tehran, Iran). Cells were incubated at 37 $^{\circ}$C with 95% humidity and 5% CO_2.

2.2. Preparation of treatments

5-FU at 50 mg/ml was obtained from Ebewe Pharmacy (Unterach, Austria). By adding the right amount of drug as a supplement to the medium, we obtained the desired concentration. Que was purchased from Sigma–Aldrich (St. Louis, Missouri, United States) and dissolved in dimethylsulfoxide (DMSO, Bio-Idea), and the stock solution was prepared at 200 μM, which diluted with medium to prepare different concentrations. The concentration of DMSO in the cell culture medium was < 0.1%. Thus, it has not affected any cell functions.

2–3. Cell viability assay (MTT)

The MTT assay was used to measure the cell viability in culture; for this purpose, 6× 10^3 (HUVECs) cells per well were seeded in 96-well plates and incubated for 24 h. Then the cells were treated with different concentrations of 5-FU (2.5, 5, 10, 20, 40, 80, and 160μM), Que (10; 40; 70; 100; 130 and 160μM), and various combination state with 2.5 and 5μM of 5-FU plus 70, 100, and 130μM of Que for 24h and 48h. After that, the medium was replaced by MTT solution at a final concentration of 0.5mg/ml and the cells were incubated for 4h in a dark place. Then MTT solution was removed and 150μl DMSO (Merck, Darmstadt, Germany) was added and shacked for 15 minutes. The plates were read using a microplate reader (BioTek ELx800 Winooski, Vermont, United States) at a wavelength of 570 nm. IC50 values were calculated using computer software Graphpad Prism 8 (La Jolla, CA). The assay was performed in triplicate with four replicates per sample.

2–4. Wound-healing Migration Assay

Cell migration was evaluated by wound healing assay. To this end, 2 × 10^5 Cells / well were seeded in 6-well plates and incubated at 37 $^{\circ}$C to reach 90% confluency. Then cells were scratched using the sterile yellow tip and washed with PBS to remove debris and subsequently treated with 5μM 5-FU, 130μM Que, and their combination. The wells were photographed at 0 h, 24 h, and 48 h, in random microscopic zones, and the width of the scratch was measured by NIH Image J software (National Institutes of Health, Bethesda, USA). Subsequently, the percentage of wound closure was calculated according to the following formula: %wound closure = $[(T_0 - T_{48})/T0] \times 100$.

2–5. Real-time RT-PCR

Total RNA was extracted with Hybrid-R RNA isolation kit from harvested cells according to manufacturer's instructions (GeneAll, Songpa-gu, Seoul, South Korea). RNA purity and integrity were confirmed with A260/A280 ratio (~ 1.8-2.0) and agarose gel electrophoresis, respectively. cDNA was synthesized from the extracted RNAs using the cDNA synthesis kit based on the manufacturer's instructions (Yehta Tajhiz Azma, Iran). In brief, 2µl of cDNA was amplified in each 20µl PCR reaction mix containing 10µl of 2x SYBR Green Master Mix (Yehta Tajhiz Azma, Iran), 1µl of each 10µM forward and reverse primers and 6µl DEPC water. HPRT was preferred as the internal reference. Results were analyzed using an Applied Biosystem with software version 2.3 (StepOne™, USA). The reaction conditions were as follows: 94˚C for 3 min; 94˚C for 40 sec, 59˚C for 30 sec, 72˚C for 30 sec for 40 cycles; 72˚C for 5 min. The primer sequences were shown in Table 1.

2–6. CAM assay

To evaluate in vivo angiogenesis CAM assay was performed. Fertilized chicken eggs (purchased from the Department of Poultry, School of Veterinary Medicine, Shiraz University) were incubated at 37 ˚C and 60% humidity and randomly divided into 4 groups: control, 5µM 5-FU, 130µM Que, and 5-FU plus Que (n = 4 per group). Briefly, on the second day of incubation 1 cm^2 square window was made at the top of the live eggs and 2ml of albumin was aspirated at the opposite side. On the 5th day, the chorioallantoic membrane (CAM) is developed, so sterile methylcellulose discs, which were loaded with the drug, were applied to the CAMs. The drug-treated eggs were incubated at 37 ˚C and 60% humidity for 48 hours. Then the CAM tissues under the filter discs were removed, washed in phosphate-buffered saline (PBS), and fixed by Formaldehyde 4%. Lastly, images (150X) was taken using a stereomicroscope (Leica Zoom 2000). The images were analyzed using online Wimasis Image Analysis Software. The number of total branch points and total vessel network lengths were used as indicators of angiogenesis.

2–7. Statistical analysis

Values are presented as the mean ± S.E.M and results were analyzed using SPSS software version 22. Data were compared by one-way analysis of variance (ANOVA) and LSD analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1- Effects of 5-FU, Que, and their combination on cell viability.

MTT assay was used to determine IC50 values, choose the appropriate concentration of drugs for combination, and eventually evaluate the combined effect. As shown in Fig. 1a, different concentrations of Que reduced cell viability compared to the control untreated sample. This reduction was dose-dependent and at 160 µM, it was revealing a maximum inhibition of 55% after 48h of incubation. Different concentrations of 5-FU also decreased cell viability in a dose-dependent manner and maximum inhibition of 53% was observed at 160 µM after 48h treatment (Fig. 1b). IC50 values for Que and 5-FU at 48h were 130µM and 160 µM, respectively. At the next step, according to the results of alone treatments,

six combination states of 5-FU (2.5 and 5μM), which have a few effects on growth inhibition of HUVECs, and Que (130, 70, and 100μM) were used for treatment. As shown in Fig. 2, all combination states showed a significant reduction in cell viability at 24 and 48h compared to 5-FU alone treatment. These results showed that Que was significantly enhanced the effect of 5-FU on cell viability. The highest growth inhibition was observed in the combination of 130μM Que and 5μM 5-FU, so this combination state was used in the rest of the experiments. The comparison of the combination treatment of 24h with 48h showed that reduction of cell viability was also time-dependent, in addition to being dose-dependent.

3.2- Effects of 5-FU, Que, and their combination on cell migration.

The wound-healing assay was performed to evaluate the migration rate of HUVECs. The anti-migration effect of 130μM Que, 5μM 5-FU, and their combination at 24h and 48h was shown in Fig. 3. The results revealed that Que significantly inhibited cancer cells' migration, and the percentage of wound closure ,as an indicator of cell migration, after 24h and 48h was 40.1% and 42.6%, respectively. Also, in 5-FU-treated cells, the percentage of wound closure after 24h and 48h was 62.9% and 77.3%, respectively. The combination of Que and 5-FU compared to 5μM 5-FU alone significantly inhibited migration of HUVECs, and the width of the scratch was almost similar to that of at 0h. The percentage of wound closure in combined treatment after 24h and 48h was 5.5% and 16.3%, respectively. These results indicate that Que significantly increases the anti-migration effect of 5-FU.

3.3- Effects of 5-FU, Que, and their combination on the gene expression of VEGFR-1 and VEGFR-2

VEGF regulates endothelial cell proliferation, migration, differentiation, tube formation, and subsequently angiogenesis. To exert these effects, VEGF should bind to its receptor and activate signaling pathways, so increased expression of related receptors (VEGFR-1 and VEGFR-2) can increase VEGF effect on angiogenesis; therefore, the effects of Que and 5-FU on VEGFR-1 and VEGFR-2 genes expression were assessed alone and in combination. As shown in Fig. 4 a and b, 5μM 5-FU significantly reduced the gene expression of VEGFR1 and VEGFR2 to 0.93 and 0.95 fold, respectively. Also, Que significantly decreased the gene expression VEGFR1 and VEGFR2 to 0.44 and 0.51 fold, respectively. However, the combination of Que with 5-FU could significantly reduce the gene expression of VEGFR1 and VEGFR2 compared to 5-FU alone, by 0.14 and 0.16 fold, respectively.

3.4- Effects of 5-FU, Que, and their combination on CAM Angiogenesis

CAM assay was used to investigate the anti-angiogenic effect of the treatments. In Fig. 5a, the angiogenesis of CAM with and without treatment was depicted in the first row, and their analysis by the Wimasis software was shown in the second row. As shown in Fig. 5b, the number of total branch points and total vessel network lengths as two indexes of angiogenesis showed a similar pattern following treatments. It means that these indexes significantly decreased by Que and 5-FU compared to control, and the decrease was significant in their combination compared to 5-FU alone.

4. Discussion

The metastatic property of tumors is one of the leading causes of poor prognosis and cancer death [3]. To grow up more than 2 mm and migrate to other tissues, tumors need a blood supply, so the formation of the new vascular network from existing vessels (angiogenesis) is needed; accordingly, tumor vascularization is a key prognostic index of tumor grading. So inhibition of angiogenesis can be a promising therapeutic strategy to prevent cancer progression [38]. Invasion, migration, proliferation, and tube formation of endothelial cells are essential steps in the angiogenic cascade, which should be target by anti-angiogenic strategies to prevent new vessel formation. [39]. 5-FU is one of the key chemo drugs to treat various cancers, including breast and colorectal cancer, which has an anti-angiogenic effect in addition to cytotoxic effect. However, the long-term application of 5FU causes drug resistance in the cancer cells and the development of destructive effects on the normal tissues [23, 40]. Therefore, finding a way to reduce the dose of chemotherapy agents while maintaining or enhancing their therapeutic effects has become an interesting research topic[40]. In the present study Que as a natural compound with anti-angiogenic effects, was used to enhance the anti-angiogenic effect of 5-FU. Our results indicate that Que potentiates the effect of 5-FU on growth inhibition, migration, and new vessel formation of the endothelial cells.

To evaluate the effect of Que, 5-FU, and their combination on endothelial cell viability MTT assay was performed. The results showed that alone application of Que and 5-FU significantly reduce the viability of endothelial cells. However, the combination state significantly reduced endothelial cell viability compared to 5-FU alone. So Que could enhance the effect of 5-FU on endothelial cell viability. These results are in line with other studies that revealing the combination of 5-FU with interferon-alpha and methylglyoxal (MG) enhances its effect on growth inhibition of endothelial cells and MCF7, respectively[41].

The Wound healing assay was used to determine of Migration rate. The results showed that the percentage of wound closure is significantly decreased in Que and 5-FU alone treatment. In the combined application, the decrease significantly was more than 5-FU alone treatment. The results showed that Que enhances the anti-migration effect of 5-FU on HUVEC .these observations were time-dependent, so the effects were more potentiated at 48h than 24h. The results of this part of the study are in agreement with previous reports, which shown enhancing the anti-migration effect of 5-FU in combination whit RU-A1, calcium supplementation, and resveratrol on hepatocellular carcinoma (HCC), and colorectal cancer (CRC), respectively [42–44].

Previous studies have shown that VEGF as a key pro-angiogenic factor, regulates angiogenesis steps, including proliferation, migration, and tube formation of endothelial cells through two important receptors so call as, VEGFR1 and VEGFR2 [2, 45]. In the present study, we showed that alone treatment of Que and 5-FU significantly decreased the gene expression of both receptors, especially VEGFR1, compared to the control group. However, gene expression of both receptors showed a significant decrease following combination treatment compared to treatment of each drug alone. These results are in agreement with other studies, which have shown decreased expression of VEGFRs by Que and 5-FU [35, 46], but to the best of our knowledge, combination treatment of them and evaluation of enhancing effect of Que on the downregulation of mentioned receptors has not performed previously.

To evaluate the effects of Que, 5-FU, and their combinations on angiogenesis, CAM assay as a reliable in vivo method was performed, and the number of total branch points and total vessel network lengths were used as indicators of angiogenesis. Our results showed that both mentioned angiogenic indexes significantly decreased by applying Que and 5-FU alone compared to the untreated control group. The combination state caused a significant reduction in angiogenesis than 5-FU alone, which indicates enhancement of 5fu anti-angiogenic effect by Que. The results of this part are consistent with previous studies that have reported a significant decrease in microvessel density following co-treatment of 5-FU and resveratrol in a tumor xenograft model [36]. Also, in agreement with our results, it has been reported that co-treatment of deoxypodophyllotoxin and 5-FU causes a significant reduction in neovascularization[47].

4. Conclusions

In conclusion, the present study showed that the combination of Que with 5-FU improves 5-FU anti-angiogenic effects compared to the application of 5-FU alone. Therefore, this combination can be suggested for future in vivo studies.

Declarations

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Conflicts of interest/Competing interests : We have no conflict of interest to declare.

Authors' contributions:

Dr . Mojtaba Rashidi: made substantial contributions to the conception and design of the work

Dr. Ghorban Mohammadzadeh: drafted the work and revised it

Arash Sanaei: the acquisition, analysis, interpretation of data

Research involving human and/or animal rights: This article does not contain any studies with human participants or animals performed by any of the authors.

Ethics approval code: IR.AJUMS.MEDICINE.REC.1399.006

Consent to participate : all of the Authors declare Consent to participate

Consent for publication : all of the Authors declare Consent for the publication

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References

1. Siegel RL, Miller KD, Jemal A (2019) Cancer statistics, 2019. Cancer J Clin 69:7–34
2. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M (2006) Angiogenesis in cancer. Vasc Health Risk Manag 2:213
3. Rajabi M, Mousa SA (2017) The role of angiogenesis in cancer treatment. Biomedicines 5:34
4. Mousa SA, Davis PJ (2017) Angiogenesis and anti-angiogenesis strategies in cancer; in Anti-Angiogenesis Strategies in Cancer Therapeutics 1–19. Elsevier
5. Teleanu RI, Chircov C, Grumezescu AM, Teleanu DM (2020) Tumor angiogenesis and anti-angiogenic strategies for cancer treatment. Journal of clinical medicine 9:84
6. Chertok V, Chertok A, Zenkina V (2017) ENDOTELIAL-DEPENDENT OF THE REGULATION OF ANGIOGENESIS. Tsitologiia 59:243–258
7. Karamysheva A (2008) Mechanisms of angiogenesis. Biochemistry 73:751–762
8. Moriya J, Minamino T (2017) Angiogenesis, cancer, and vascular aging. Frontiers in cardiovascular medicine 4:65
9. Appelmann I, Liersch R, Kessler T, Mesters RM, Berdel WE (2010) Angiogenesis inhibition in cancer therapy. Angiogenesis Inhibition, 51–81
10. Akil A, Gutiérrez-García AK, Guenter R et al (2021) Notch Signaling in Vascular Endothelial Cells, Angiogenesis, and Tumor Progression: An Update and Prospective. Frontiers in Cell Developmental Biology 9:177
11. Zhang E-Y, Gao B, Shi H-L et al (2017) 20 (S)-Protopanaxadiol enhances angiogenesis via HIF-1α-mediated VEGF secretion by activating p70S6 kinase and benefits wound healing in genetically diabetic mice. Exp Mol Med 49:e387–e387

12. Zhou Y, Li S, Li J, Wang D, Li Q (2017) Effect of microRNA-135a on cell proliferation, migration, invasion, apoptosis and tumor angiogenesis through the IGF-1/PI3K/Akt signaling pathway in non-small cell lung cancer. Cellular physiology biochemistry 42:1431–1446
13. Phillips CM, Lima EA, Woodall RT, Brock A, Yankeelov TE (2020) A hybrid model of tumor growth and angiogenesis: In silico experiments. Plos one 15:e0231137
14. Melincovici CS, Bosca AB, Susman S et al (2018) Vascular endothelial growth factor (VEGF)-key factor in normal and pathological angiogenesis. Rom J Morphol Embryol 59:455–467
15. Apte RS, Chen DS, Ferrara N (2019) VEGF in signaling and disease: beyond discovery and development. Cell 176:1248–1264
16. Weddell JC, Chen S, Imoukhuede P (2017) VEGFR1 promotes cell migration and proliferation through PLCγ and PI3K pathways. NPJ systems biology applications 4:1–11
17. Kiselyov A, Balakin KV, Tkachenko SE (2007) VEGF/VEGFR signalling as a target for inhibiting angiogenesis. Expert opinion on investigational drugs 16:83–107
18. Liu G, Chen T, Ding Z, Wang Y, Wei Y, Wei X (2021) Inhibition of FGF-FGFR and VEGF-VEGFR signalling in cancer treatment. Cell Proliferation, e13009
19. Wu J-b, Tang Y-l, Liang X-h (2018) Targeting VEGF pathway to normalize the vasculature: an emerging insight in cancer therapy. OncoTargets therapy 11:6901
20. Carmeliet P (2005) VEGF as a key mediator of angiogenesis in cancer. Oncology 69:4–10
21. Viallard C, Larrivée B (2017) Tumor angiogenesis and vascular normalization: alternative therapeutic targets. Angiogenesis 20:409–426
22. Shewach DS, Kuchta RD (2009) Introduction to cancer chemotherapeutics. Chemical reviews 109:2859–2861
23. Altun I, Sonkaya A (2018) The most common side effects experienced by patients were receiving first cycle of chemotherapy. Iranian journal of public health 47:1218–1219
24. Mokhtari RB, Homayouni TS, Baluch N et al (2017) Combination therapy in combating cancer. Oncotarget 8:38022
25. Rejhová A, Opattová A, Čumová A, Slíva D, Vodička P (2018) Natural compounds and combination therapy in colorectal cancer treatment. Eur J Med Chem 144:582–594
26. Khallaf RA, Salem HF, Abdelbary A (2016) 5-Fluorouracil shell-enriched solid lipid nanoparticles (SLN) for effective skin carcinoma treatment. Drug Deliv 23:3452–3460
27. López EP-F, García FG, Jornet PL (2019) Combination of 5-Florouracil and polyphenol EGCG exerts suppressive effects on oral cancer cells exposed to radiation. Archives of oral biology 101:8–12
28. Karaayvaz M, Zhai H, Ju J (2013) miR-129 promotes apoptosis and enhances chemosensitivity to 5-fluorouracil in colorectal cancer. Cell death disease 4:e659–e659
29. Larson AJ, Symons JD, Jalili T (2012) Therapeutic potential of quercetin to decrease blood pressure: review of efficacy and mechanisms. Advances in nutrition 3:39–46

30. Bischoff SC (2008) Quercetin: potentials in the prevention and therapy of disease. Current Opinion in Clinical Nutrition Metabolic Care 11:733–740
31. Kashyap D, Mittal S, Sak K, Singhal P, Tuli HS (2016) Molecular mechanisms of action of quercetin in cancer: recent advances. Tumor Biology 37:12927–12939
32. Pratheeshkumar P, Budhraja A, Son Y-O et al (2012) Quercetin inhibits angiogenesis mediated human prostate tumor growth by targeting VEGFR-2 regulated AKT/mTOR/P70S6K signaling pathways. PloS one 7:e47516
33. Song W, Zhao X, Xu J, Zhang H (2017) Quercetin inhibits angiogenesis–mediated human retinoblastoma growth by targeting vascular endothelial growth factor receptor. Oncology Letters 14:3343–3348
34. Zhu Y, Liu X, Zhao P, Zhao H, Gao W, Wang L (2020) Celastrol suppresses glioma vasculogenic mimicry formation and angiogenesis by blocking the PI3K/Akt/mTOR signaling pathway. Front Pharmacol 11:25
35. Zhao D, Qin C, Fan X, Li Y, Gu B (2014) Inhibitory effects of quercetin on angiogenesis in larval zebrafish and human umbilical vein endothelial cells. Eur J Pharmacol 723:360–367
36. Lee SH, Koo BS, Park SY, Kim YM (2015) Anti–angiogenic effects of resveratrol in combination with 5–fluorouracil on B16 murine melanoma cells. Mol Med Rep 12:2777–2783
37. Song D, Meng T, Xu W et al (2015) 5-Fluoruracil blocked giant cell tumor progression by suppressing osteoclastogenesis through NF-kappaB signals and blocking angiogenesis. Bone 78:46–54
38. Hansen S, Grabau D, Sørensen FB, Bak M, Vach W, Rose C (2000) Vascular grading of angiogenesis: prognostic significance in breast cancer. British journal of cancer 82:339–347
39. Zhao Y, Adjei AA (2015) Targeting angiogenesis in cancer therapy: moving beyond vascular endothelial growth factor. Oncologist 20:660
40. Kuczynski EA, Sargent DJ, Grothey A, Kerbel RS (2013) Drug rechallenge and treatment beyond progression—implications for drug resistance. Nature reviews Clinical oncology 10:571
41. Ghosh S, Pal A, Ray M (2017) 5-FU synergistically inhibits MCF-7 in combination with methylglyoxal. Clin Oncol 2:1353
42. Park M, Sundaramoorthy P, Sim JJ, Jeong K-Y, Kim HM (2017) Synergistically anti-metastatic effect of 5-flourouracil on colorectal cancer cells via calcium-mediated focal adhesion kinase proteolysis. Anticancer research 37:103–114
43. Buhrmann C, Yazdi M, Popper B et al (2018) Resveratrol chemosensitizes TNF-β-induced survival of 5-FU-treated colorectal cancer cells. Nutrients 10:888
44. Bartucci M, Hussein MS, Huselid E et al (2017) Synthesis and characterization of novel BMI1 inhibitors targeting cellular self-renewal in hepatocellular carcinoma. Target Oncol 12:449–462
45. Park K, Amano H, Ito Y et al (2016) Vascular endothelial growth factor receptor-1 (VEGFR-1) signaling enhances angiogenesis in a surgical sponge model. Biomed Pharmacother 78:140–149

46. Zhang Y, Zhu S, Xu X, Zuo L (2019) In Vitro Study of Combined Application of Bevacizumab and 5-Fluorouracil or Bevacizumab and Mitomycin C to Inhibit Scar Formation in Glaucoma Filtration Surgery. Journal of ophthalmology 2019
47. Xiang R, Guan X-W, Hui L, Jin Y-X, Chen S-W (2017) Investigation of the anti-angiogenesis effects induced by deoxypodophyllotoxin-5-FU conjugate C069 against HUVE cells. Bioorg Med Chem Lett 27:713–717

Tables

Table 1
Sequences of used primers

Gene		SEq. (5 – 3)
VEGFR1	F	CTGCTACCACTCCCTTGA
	R	TCCACTCCTTACACGACAA
VEGFR2	F	TGGAGGAGGAGGAAGTAT
	R	CGTCTGGTTGTCATCTGG
HPRT	F	GACCAGTCAACAGGGGACAT
	R	CCTGACCAAGGAAAGCAAAG

Figures

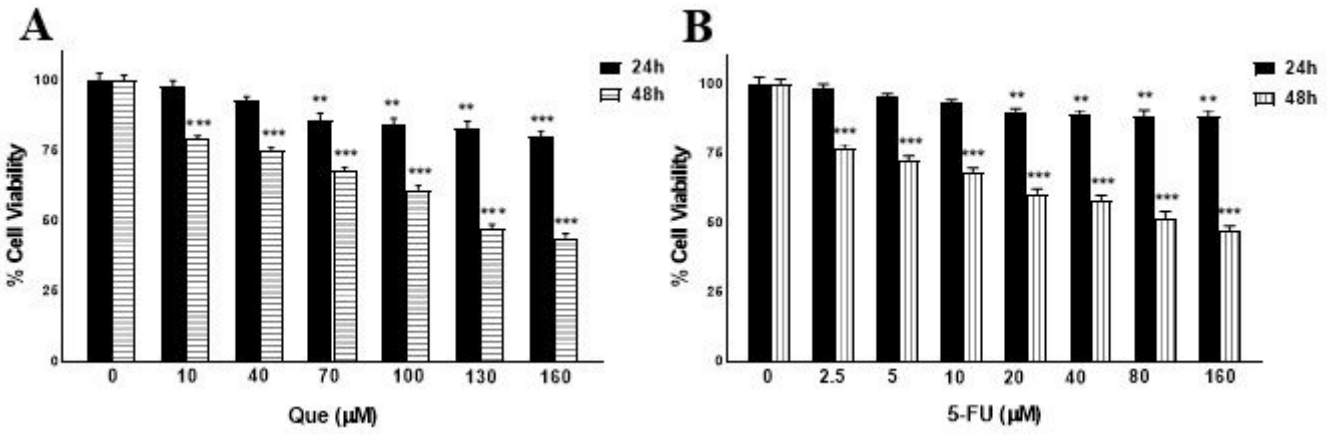

Figure 1

Effects of Que and 5-FU on cell viability of HUVECs. (a) HUVECs were treated with different concentrations of Que for 24 and 48h. Cell viability was assessed using MTT assay. (b) HUVECs were treated with different concentrations of 5-FU for 24 and 48h. Cell viability was assessed using MTT

assay. Results are shown as the mean ± S.E.M of three independent experiments. **P < 0.01; ***P < 0.001 compared with control

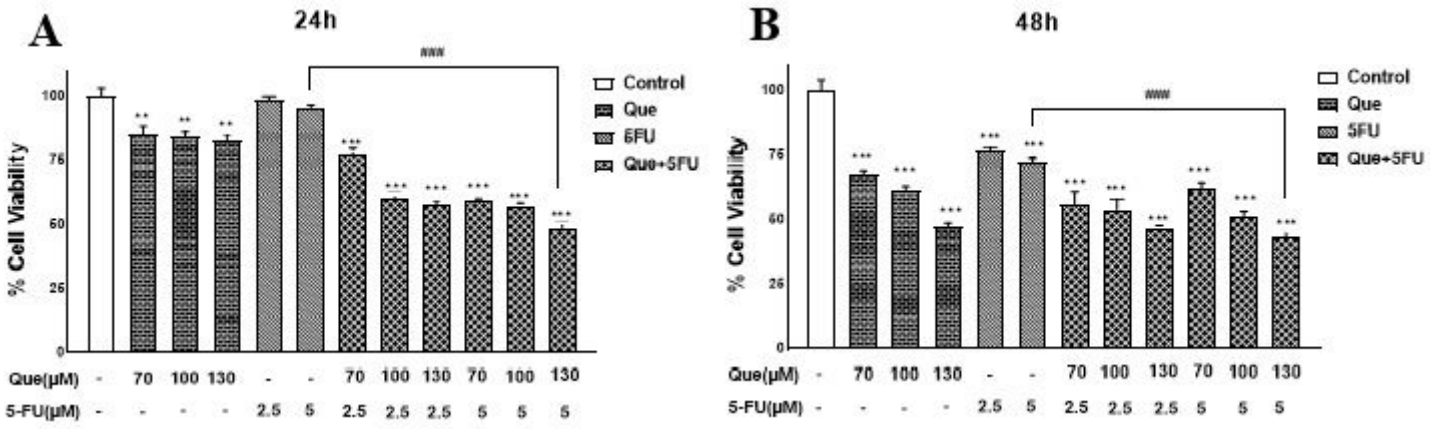

Figure 2

Effect of Que and 5-FU combinations on cell viability of HUVECs. (a) Viability of HUVECs after treatment with Que (70, 100, and 130μM) combined with 5-FU (2.5 and 5μM) for 24h was measured using MTT assay. (b) Viability of HUVECs after treatment with Que (70, 100, and 130μM) combined with 5-FU (2.5 and 5μM) for 48h was measured using MTT assay. Results are shown as the mean ± S.E.M of three independent experiments. **P < 0.01 and ***P < 0.001 compared with control, ###p<0.001 compared with 5-FU-alone group

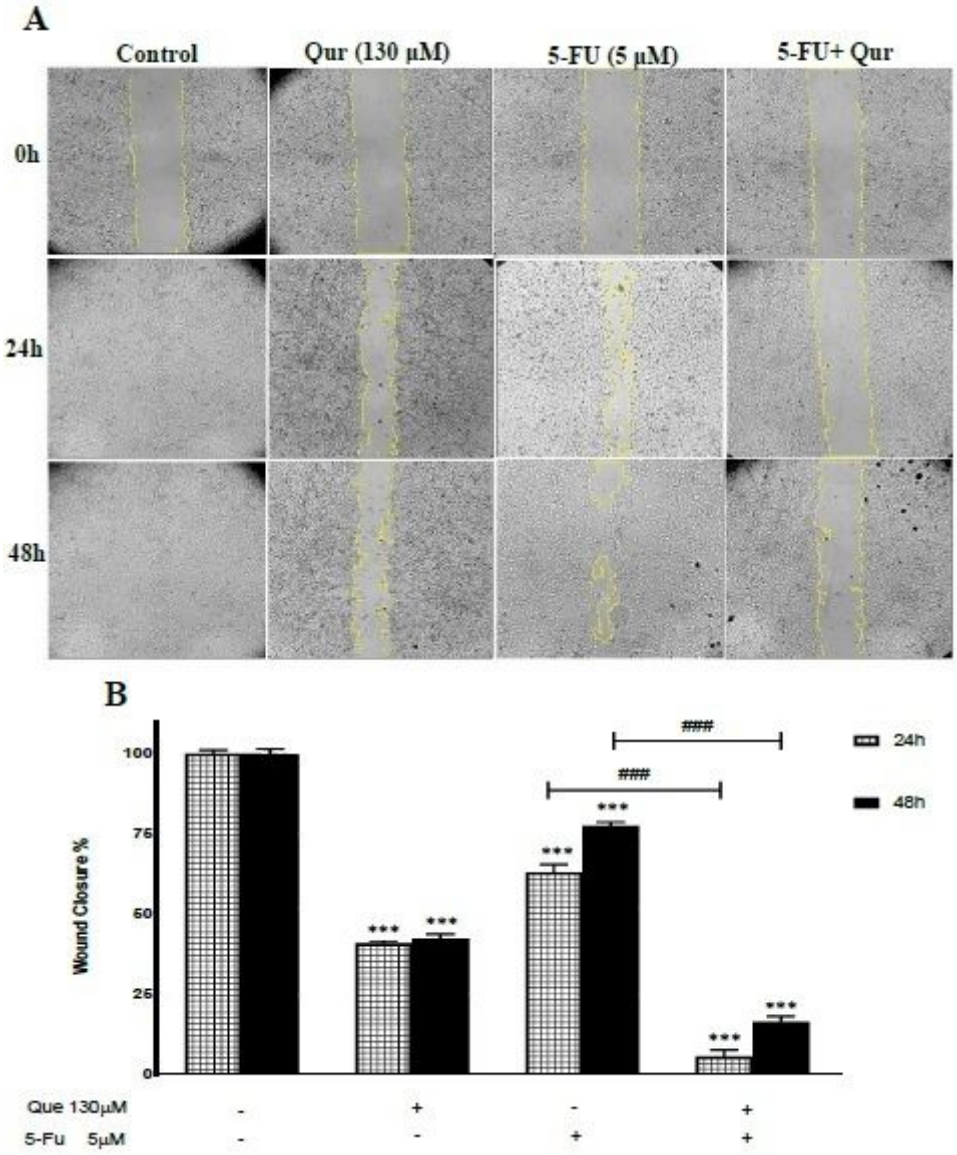

Figure 3

wound healing assay of HUVECs treated with 5-FU and Que. (a) image of HUVECs migration following treatment with Que (130µM), 5FU(5µM), and their combination for 0h, 24h, and 48h. (b) Quantitative analysis of the anti-migration effect of Que (130µM), 5-FU (5µM), and their combination for 24h and 48h. Results are shown as the mean ± S.E.M of three independent experiments. ***P < 0.001 compared with control; ###p<0.001 compared with 5-FU-alone group

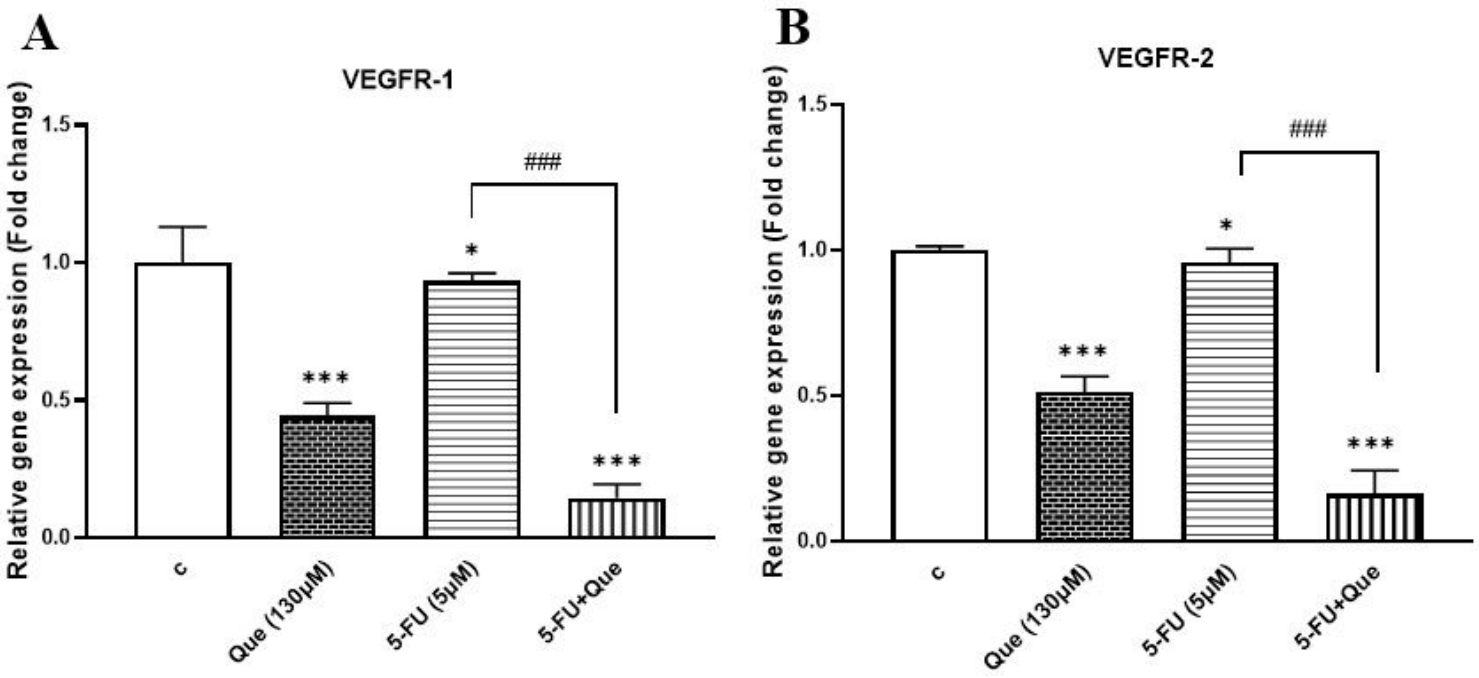

Figure 4

Effect of Que, 5-FU and their combination on the VEGFR-1 and VEGFR-2 gene expression in HUVECs. Que and 5-FU and their combination reduced gene expression of VEGFR-1 and VEGFR-2. (a) Expression of VEGFR-1 gene was evaluated in HUVECs untreated control cells, treated cells with Que(130μM), 5-FU(5μM), and combination of Que+5-FU using quantitative Real-time PCR. (b) Expression of VEGFR-2 gene was evaluated in HUVECs untreated control cells, treated cells with Que(130μM), 5-FU(5μM), and combination of Que+5-FU using quantitative Real-time PCR. Results are shown as the mean ± S.E.M of three independent experiments. *P < 0.05 and ***P < 0.001 compared with control; ###p<0.001 compared with 5-FU-alone group

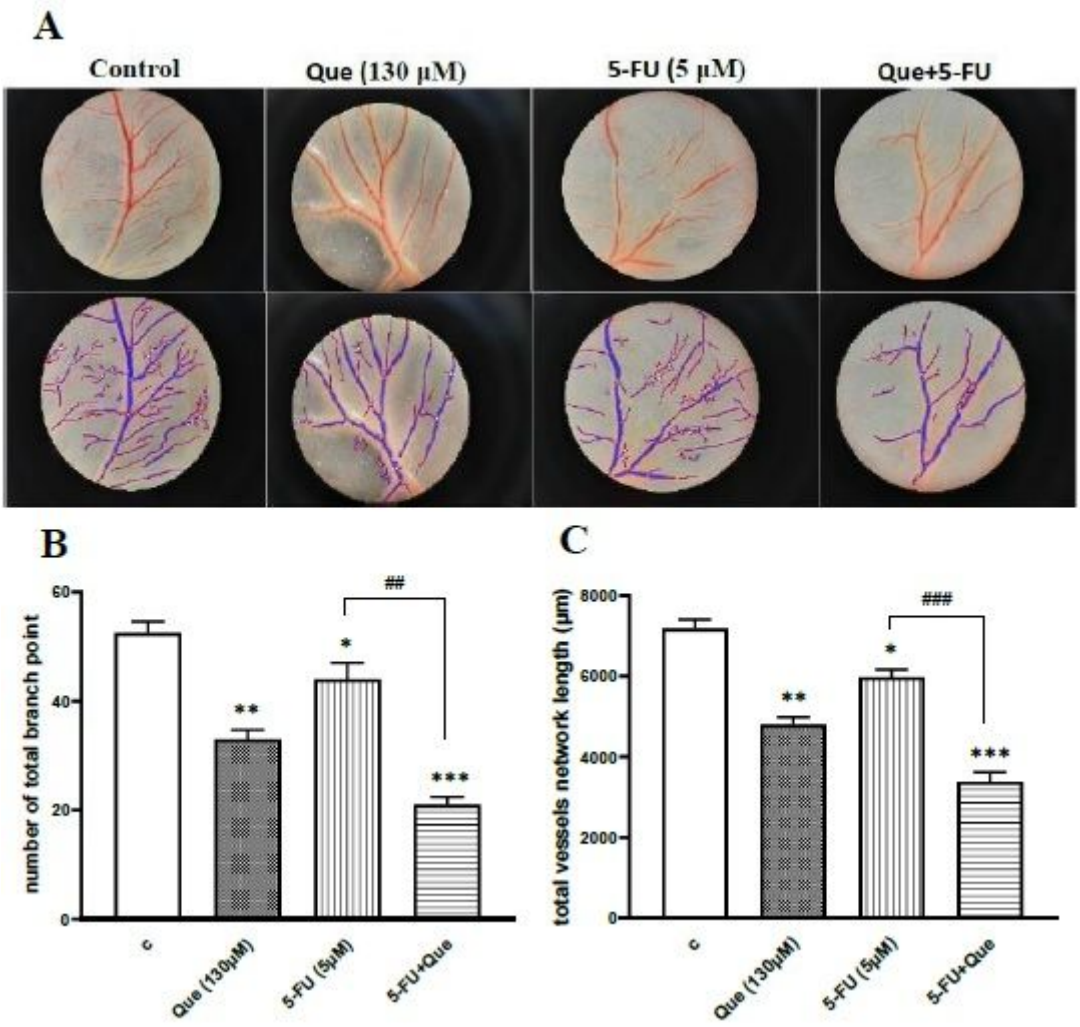

Figure 5

effect of Que, 5-FU and their combination on in vivo angiogenesis. (a) The first row is the images of angiogenesis of CAM following treatment with Que (130µM), 5FU (5µM), and their combination (n = 4 eggs per group). The second row is the images of software analysis of each mentioned condition (Bb) quantitative analysis of the anti-angiogenic effect of Que (130µM), 5-FU (5µM), and their combination based on the number of the total branch point. (c) Quantitative analysis of the anti-angiogenic effect of Que (130µM), 5-FU (5µM), and their combination based on total vessels network length. Results are presented as the mean ± S.E.M of at least three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with control; ###p<0.001 compared with 5-FU-alone group