Original Article

Effect of Quercetin and Doxorubicin on SW480 Colorectal Cancer Cells

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Abstract

Background: Quercetin is one of the important flavonoids that have antioxidant/pro-oxidant properties and apoptotic impact on cancer cells. Doxorubicin is a DNA intercalating agent that is utilized to manage a variety of cancers. Cardiotoxicity limits the usage of doxorubicin despite the fact that it is powerful in the treatment of cancer. **Objectives:** To evaluate the impact of quercetin on oxidative stress, apoptosis, and its effect on the therapeutic cytotoxic profile of doxorubicin in SW480 colorectal cancer cell lines. **Materials and Methods:** SW480 cancer cells and Vero (nontumoral) cells were provided by the Tissue Culture Laboratory in the College of Medicine, University of Babylon. Assessment of the safety of quercetin concentrations on Vero cells by MTT assay to be then selected in combination with IC50 of doxorubicin. Anticancer effects of each of quercetin and doxorubicin alone and in combination on SW480 cells were evaluated using MTT assay and enzyme-linked immunosorbent assay enzyme-linked immunosorbent assay analysis of glutathione (GSH), malondialdehyde (MDA) and caspase 3 levels. **Results:** Vero cells, in a 48-h period, had cell viability of 81.27% and 75% at high concentrations of quercetin (100 and 200 µM), respectively. However, cell viability was 98.12% and 96.86% when Vero cells were incubated with 25 and 50 µM, respectively. Each one of quercetin and doxorubicin significantly (*$P < 0.05$) and (**$P < 0.001$) inhibited cell proliferation and induced apoptosis by increasing the level of oxidative stress in SW480 cells in a dose-dependent manner. At low concentrations, quercetin significantly reduced the cytotoxicity, oxidative stress, and apoptosis induction of doxorubicin. **Conclusion:** Using quercetin at low concentrations decreases the therapeutic impacts of doxorubicin by lowering oxidative stress damage. Quercetin supplementation while receiving chemotherapy can result in resistance to chemotherapies.

Keywords: Apoptosis, doxorubicin and quercetin, oxidative stress

Introduction

Colorectal cancer (CRC) is a commonly seen gastrointestinal cancer in clinical practice and contributes to over 930,000 fatalities, according to the Global Cancer Statistics 2020 released by the International Agency. It ranks second among all malignancies in terms of overall mortality, followed by lung cancer, and fifth in China with 280,000 fatalities. The findings of disease screening indicate that the incidence of CRC ranks third globally and second in China.[1] Current standard treatments for CRC include surgery, radiation, chemotherapy, and targeted therapy, whereas the emergence of CRC invasion and metastasis at intermediate and advanced stages led to inadequate efficacy and became a major cause of death.[2] The average survival period for people with advanced CRC has doubled in the past 10 years due to advances in early diagnosis and new medications. Unfortunately, they generally die within 3 years, as their survival chances are still low.[3] Therefore, a challenge in the current study is to find safe and effective drugs that can prevent tumor cell invasion and metastasis. Flavonoids have recently attracted a lot of attention due to their potential chemotherapeutic and chemo-preventative effects. Quercetin (3,3′,4′,5,7-pentahydroxyflavone), a major flavonoid, is

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the most important component in the nightshade found in a variety of fruits and vegetables.[4] Today, quercetin is consumed as a dietary supplement and has the potential to fight cancer when consumed regularly. Studies in vivo and in vitro have suggested that quercetin ingestion could have biological benefits, such as antioxidant, anticancer, and anti-inflammatory properties, at an acceptable dosage.[5] One of the most important characteristics of quercetin is proapoptotic action, which is brought about by upregulating proapoptotic molecules, including P53, BAX, caspase-3, and caspase-9 or downregulating antiapoptotic proteins (Bcl-2 family proteins). Quercetin can prompt activation of intrinsic and extrinsic pathways of apoptosis. In the intrinsic pathway, quercetin causes depolarization of mitochondrial membrane potential by elevating the intracellular levels of ROS and Ca2, lead to release of cytochrome *c* and activation of caspase-3, -8, and -9.[6] Doxorubicin is a DNA intercalating medication that is utilized to manage a variety of neoplastic diseases and cancers, such as leukemia, breast cancer, cervical cancer, and other types of carcinoma. Its molecular mechanism of action involves intercalating with DNA and the destruction of topoisomerase II enzyme, ultimately resulting in cell death by stopping the biological process of DNA replication. Another established anticancer mechanism for doxorubicin involves its capacity to cause oxidative stress through the generation of reactive oxygen species (ROS), where intracellular oxidoreductase reacts with doxorubicin metabolites to create semiquinone radicals and ROS.[7] Cardiotoxicity, nephrotoxicity, alopecia, and hematological suppression limit the usage of doxorubicin despite the fact that it is powerful in the treatment of cancer. Activation of ROS-scavenging mechanisms reduces ROS levels and lessens DNA damage, resulting in the enhancement of chemoresistance. The failure of treatment with mono-administration of doxorubicin is mostly attributable to the severe adverse effects. Despite the fact that doxorubicin treatment has demonstrated major potential in delaying the progression of the disease over the years, clinically utilized doses exhibit insufficient antitumor activity, while larger doses frequently cause systemic toxicity in patients.[8] The ability to have the most impact on cancer cells while causing the least amount of harm to nearby normal cells is one of the main requirements for possible anticancer medications.

Materials and Methods

Materials

Fetal bovine serum (FBS), RPMI 1640, penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco England (UK). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and quercetin were purchased from Sigma (Germany). Dimethyl sulfoxide (DMSO) was purchased from Roth (Germany). Enzyme-linked immunosorbent assay (ELISA) kits, caspase 3 were procured from Elabscience Biotechnology (USA) used sandwich-ELISA principle, GSH ELISA kits were procured from Elabscience Biotechnology used competitive-ELASA principle and Lipid Peroxidation kits were purchased from Bilişim Destek Hizmetleri (Turkey) and measured by thiobarbituric acid reactive substances. Doxorubicin was purchased from Pfizer.

Cells and cell culture

The SW480 CRC cell and vero cell lines (purchased from the Tissue Culture Laboratory in the College of Medicine, The University of Babylon) were grown in RPMI-1640 media supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 95% air and 5% CO_2 in a humidified 37°C incubator.

Drug preparation

Quercetin was initially dissolved in DMSO at a concentration of 40 mM and kept at 4°C and shielded from the light. Noteworthy, the stock solution of quercetin should be prepared at low concentrations to avoid precipitation in serial dilution. Various concentrations of quercetin that had been freshly prepared in full culture media, added to the cells in various experiments. DMSO concentration never exceeded 0.5% in all experiments, which has no impact on SW480 cells. Doxorubicin (20 mg/10 mL) was diluted in new complete culture media before usage and given to the cancer cells at various concentrations.

Cytotoxicity assay

The viability of Vero cells under different concentrations of quercetin (12.5, 25, 50, 100, 200 µM) was estimated by utilizing the colorimetric MTT assay to determine the effective concentrations of quercetin that kill cancer cells without harming normal cells in order to be used in combination with chemotherapy. Likewise, the viability of SW480 cells under different treatment conditions was estimated by utilizing the colorimetric MTT assay. Briefly, Cells were seeded in 96-well plates (1×10^4 cells/well) and maintained overnight at 37°C; old media was aspirated, and the cells were treated with 12.5, 25, 50, 100, and 200 µM of quercetin and 1, 2, 4, 8, 16 µM of doxorubicin alone and depending on the concentration that has no harmful effect on Vero cells, various combinations of quercetin (25, 50 µM) with IC_{50} of doxorubicin 8 µM were prepared at 48 h. To achieve a final concentration of 0.5 mg/mL, 1 mL of MTT solution (5 mg/mL) was added to 10 mL of medium. Then, pour 100 µL of the resultant solution into each well and incubate for 4 h at 37°C or until intracellular purple formazan crystals can be seen under a microscope. Then MTT was removed, and add solubilizing solution (100 µL DMSO) was added and triturate. Incubation continues for 30 min at 37°C or at room temperature or until the purple crystals dissolve

and account for the absorbance at 570 nm. The results were shown as a ratio to the control (untreated cells). The following equation was used to compute the viability rate: viability rate (%) = (OD_{sample}/$OD_{control}$) × 100, where OD_{sample} and $OD_{control}$ represent the optical densities of cells that treated and control (cell treated with RPMI), respectively. The concentration that inhibits the proliferation of cells to 50% (IC_{50}) in comparison to the control was obtained from a usage curve fitting way from three independent experiments in a triplicate design for each concentration.

ELISA assay

SW480 cells were seeded in 96-well plates (1 × 104 cells/well) and maintained overnight at 37°C; old media was aspirated, the cells were treated by 25, 50, 100, 200 µM and 2, 4, 8 µM of quercetin and doxorubicin respectively and the combination of quercetin (25, 50 µM) with IC_{50} 8 µM of doxorubicin at 48 h. Supernatants were harvested and kept at -20°C until further analysis. GSH, MDA, and Caspase 3 level in culture supernatants were measured using an ELISA kit and following the instructions of the manufacturer.

Ethical approval

Not applicable, as no patients were included in the study.

Results

Cytotoxic effect of quercetin on Vero cells

The impacts of different concentrations of quercetin on the proliferation vero cell line were determined by MTT cytotoxicity assay. Vero cells, in 48 h incubation period, had cell viability 81.27% and 75% at high concentrations of quercetin 100 and 200 µM, respectively [Figure 1].

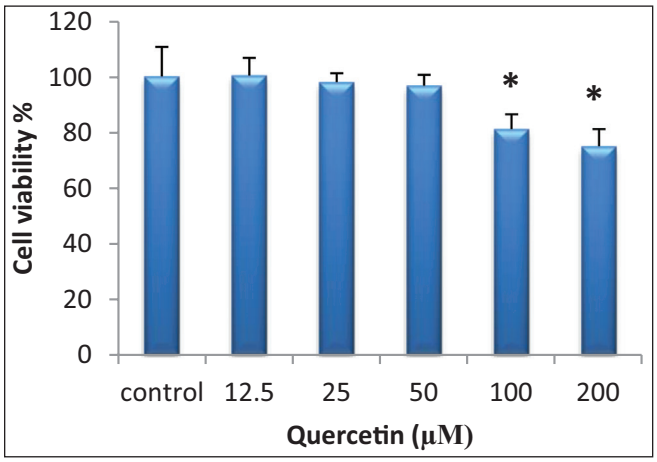

Figure 1: Cytotoxicity evaluation in Vero cells were treated with various concentrations of quercetin. The data were expressed as mean ± SD of three independent experiments in triplicate design for each concentration. *Means $P < 0.05$ and **, $P < 0.001$ for significant difference between concentrations in comparison to control. Control: cells treated with RPMI

However, cell viability was 98.12% and 96.86% when Vero cells were incubated with low concentrations of quercetin (25 and 50 µM, respectively), which were then used in subsequent experiments in combination with IC50 of doxorubicin on SW480 cancer cells.

Cytotoxicity of different treatments on SW480 cancer cells

The impacts of different treatments on cell proliferation in the SW480 cell line were determined by MTT cytotoxicity assay. Cell proliferation was significantly reduced following treatment with doxorubicin or quercetin in a concentration-dependent manner in the SW480 cell line [Figure 2A and B]. The IC_{50} for quercetin and doxorubicin were determined to be about 185 µM and 8 µM after 48 h treatment of SW480 cells, respectively. Cotreatment of SW480 cells with quercetin and doxorubicin resulted in a remarkable increase in the viability of these cells to doxorubicin chemotherapy [Figure 2C]. Quercetin at a low concentration significantly reduced the efficacy of doxorubicin on SW480 cancer cells in comparison to doxorubicin alone.

ELISA assay

Impact of different concentrations of quercetin and doxorubicin and their combinations on GSH level in SW480 cells

GSH was analyzed in SW480 cells treated with quercetin and doxorubicin alone and in combination. Results in Figure 3A and B show a significant decrease in GSH level in concentration dependent manner in comparison with the control group in SW480 cells treated with quercetin and doxorubicin alone after incubation for 48 h at 37°C. Cotreatment of SW480 cells with quercetin and doxorubicin resulted in a significant increase in GSH levels [Figure 3C]. Quercetin at low concentration significantly induces the level of GSH on SW480 cancer cells in comparison to doxorubicin alone (*$P < 0.05$) and (** $P < 0.001$).

Impact of different concentrations of quercetin and doxorubicin and their combinations on MDA level in SW480 cells

Analysis of MDA was achieved in SW480 cells treated with quercetin and doxorubicin alone and in combination. Results in Figure 4A and B show a significant increase in MDA level in a concentration-dependent manner in comparison with the control group in SW480 cells treated with quercetin and doxorubicin alone after incubation for 48 h at 37°C. Cotreatment of SW480 cells with low concentrations of quercetin and IC50 of doxorubicin resulted in a significant decrease in MDA level [Figure 4C]. Quercetin at low concentration significantly induces the level of MDA on SW480 cancer

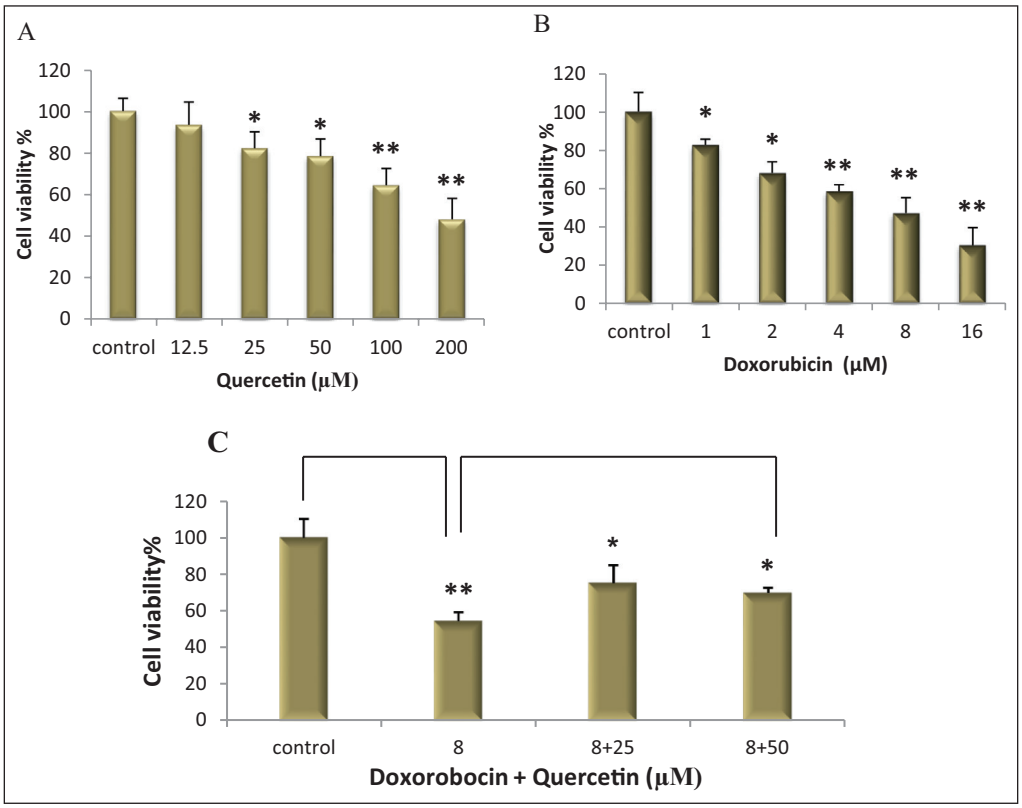

Figure 2: Cytotoxicity evaluation in SW480 cancer cells were treated with (A) various concentrations of quercetin, (B) various concentrations of doxorubicin, (C) a combination of 25, 50 μM of quercetin with IC50 8 μM of doxorubicin. The data were expressed as mean ± SD of three independent experiments in triplicate design for each concentration. *Means $P < 0.05$ and **$P < 0.001$ for significant difference between concentrations in comparison to control. Control: cells treated with RPMI

cells in comparison to doxorubicin alone (*$P < 0.05$) and (** $P < 0.001$).

Impact of different concentrations of quercetin and doxorubicin and their combinations on caspase 3 level in SW480 cells

Caspase-3 was studied in the SW480 cells line. Figure 5A and B show that caspase-3 was upregulated in a dose-dependent manner in these cells after incubation with different concentrations of quercetin and doxorubicin alone in comparison with the control group. Figure 5C shows that the combination of a low concentration of quercetin with IC_{50} of doxorubicin reduces the level of caspase 3 in comparison with doxorubicin alone (*$P < 0.05$).

Discussion

The antiproliferative effects of quercetin were initially assessed on vero and SW480 cells following incubation with different concentrations of quercetin for 48 h. Results show that quercetin show a toxic effect on normal cells, only at high concentrations, in addition to cancer cells [Figure 1]. Quercetin significantly decreases the proliferation of SW480 cancer cells in a concentration-dependent manner [Figure 2A]. These findings were in agreement with Lin *et al.*,[9] who demonstrate that quercetin significantly inhibits colon cancer cell viability in a concentration-dependent manner. Doxorubicin is an effective and mostly utilized anthracycline chemotherapeutic agent in many human cancers. The major anticancer activities of doxorubicin are DNA intercalation, topoisomerase II inhibition, and free radical formation, leading to cell death or growth inhibition.[10] Present results show that doxorubicin reduces the viability of cells in a dose-dependent manner [Figure 2B]. This study augments previous studies indicated that doxorubicin can decrease the proliferation of HT29 cancer cells in a concentration-dependent manner.[11] Importantly, a combination of low concentrations of quercetin with IC50 of doxorubicin shows a decrease in the cytotoxic efficacy of doxorubicin [Figure 2C]. So, quercetin at low concentrations displays a significant decrease in the antiproliferative effect of doxorubicin. Controversy, Mingxiang *et al.*[12] demonstrate that quercetin has been used with a variety of chemotherapeutic drugs, including doxorubicin and cisplatin. Due to its lipophilic nature, quercetin can cross cell membranes and produce a synergistic effect with doxorubicin and other chemotherapeutic drugs. Findings of the present study show that quercetin induces a significant decrease in GSH levels in high concentrations when compared with the

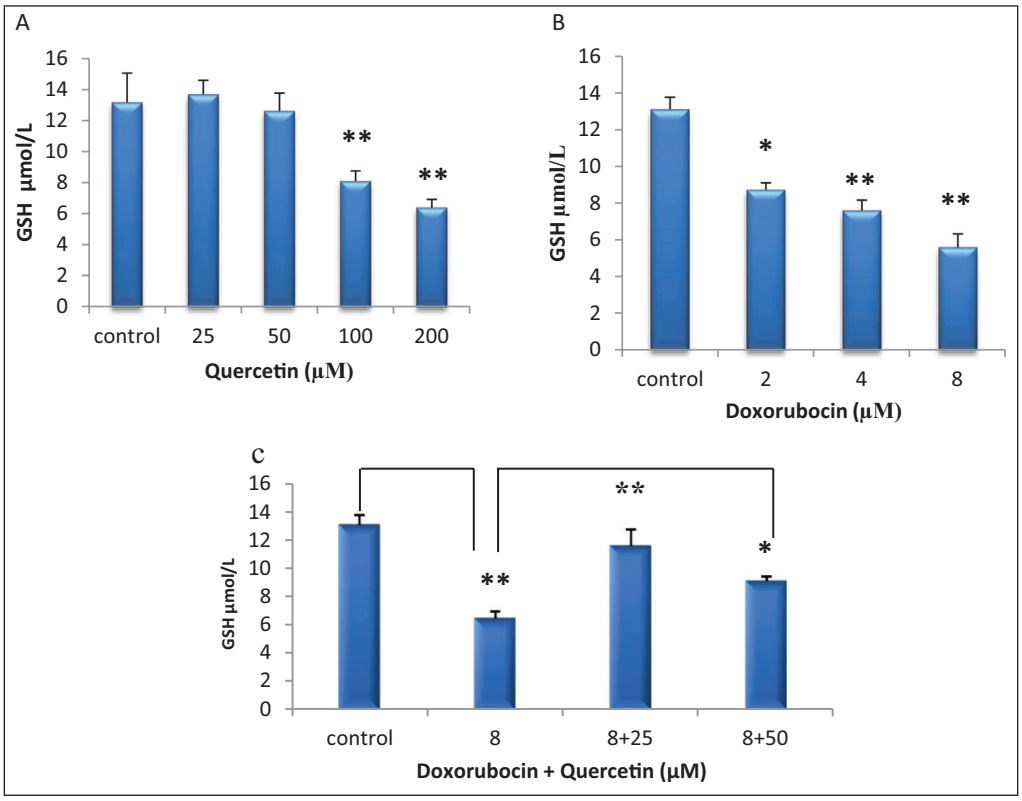

Figure 3: Evaluation of GSH level in SW480 cancer cells were treated with (A) various concentrations of quercetin, (B) various concentrations of doxorubicin, (C) a combination of 25, 50 µM of quercetin with IC50 8 µM of doxorubicin. Results are presented as mean ± SD. GSH: glutathione, control: cells treated with RPMI

control group [Figure 3A] and a significant increase in MDA levels in high concentrations [Figure 4A]. This data explain that quercetin at high concentration induces oxidative stress and subsequently produce a cytotoxic effect. Additionally, present findings confirmed the idea that programmed cell death comes from ROS overproduction and antioxidant defense suppression, which is thought to be one of the key mechanisms underlying quercetin antitumor action.[13] According to previous studies, ROS exert dual function in the cellular process in a concentration-dependent manner since in moderate levels, they play a role in cancer onset and development through inflammation, DNA mutation, and cellular damage, while in higher levels, they act as an anticancer agent by induction of apoptosis.[14] Free radicals can attack unsaturated lipids in a cell, resulting in a chain of reactions. These reactions are terminated by the production of lipid breakdown products, lipid alcohols, aldehydes, and malondialdehyde (MDA). Therefore, measurement of MDA concentration is a common method for the determination of primary toxic effects caused by free radicals in experiments in vitro.[15] Glutathione (GSH) is the most prevalent non-protein thiol found in mill molar concentrations in mammalian tissues. It functions as a regulator of cellular redox status and an essential intracellular antioxidant, protecting cells from harm from xenobiotics, reactive oxygen and nitrogen species, and lipid peroxides. Elevated GSH levels in tumor cells are linked to tumor progression and increased resistance to chemotherapeutic medicines, despite the fact that in healthy cells, they are essential for the elimination and detoxification of carcinogens.[16] Based on the results of previous studies, anticancer properties of doxorubicin, at least in part, mediated through the induction of oxidative DNA damage, which is in line with those of the present study. The findings of the present study indicated that doxorubicin significantly decreases GSH levels in a dose-dependent manner [Figure 3B]. Significantly, the MDA level increased in the SW480 cell line after exposure for 48 h to doxorubicin in a dose-dependent manner [Figure 4B]. Doxorubicin causes oxidative damage in cancer cells, and it relies on redox cycling associated with the release of iron from cells. The drug–iron combination catalyzes the formation of stronger radicals from O_2 and H_2O_2. In cancer cells, the oxidative damage pathway has been regarded as a key anticancer strategy.[17] Importantly, a combination of quercetin with doxorubicin leads to a remarkable increase in antioxidant levels, including GSH [Figure 3C] and a decrease in MDA level [Figure 4C]. Similarly, Henidi *et al.*[18] demonstrated that quercetin elucidates vascular protective effects (due to its antioxidant activity) but ameliorates doxorubicin-induced antibreast cancer properties against MCF-7 and MDA-MB-231 cell lines with profound and moderate antagonistic interaction,

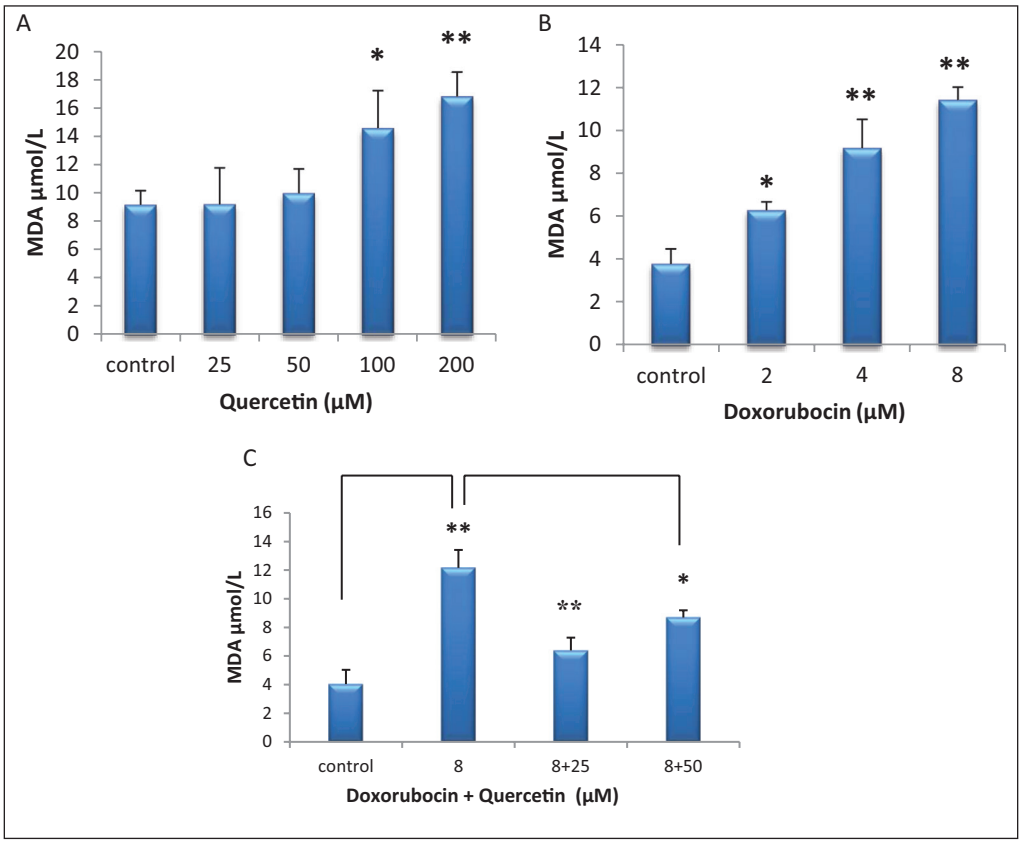

Figure 4: Evaluation of MDA level in SW480 cancer cells were treated with (A) various concentrations of quercetin, (B) various concentrations of doxorubicin, (C) a combination of 25, 50 µM of quercetin with IC_{50} 8 µM of doxorubicin. Results are presented as mean ± SD. MDA: malondialdehyde, control: cells treated with RPMI

respectively. Doxorubicin and anthraquinones, in general, have a unique chemical structure, which is thought to be responsible for the intracellular ROS release phenomena. The capacity of quercetin to quickly (immediately) scavenge ROS is a potential explanation for the antagonistic interaction between doxorubicin and quercetin in the current work. Quercetin may have an intracellular ROS-scavenging activity that reduces the intracellular active form of doxorubicin by scavenging intracellular ROS caused by doxorubicin. Controversy, Staedler *et al.*[19] demonstrated that doxorubicin and quercetin synergistically suppressed cell proliferation and promoted apoptosis by lowering GSH in breast cancer cell lines. The results show a significant increase in apoptotic protein (caspase 3) when SW480 cells have been treated with a high concentration of quercetin [Figure 5A]. According to Na *et al.*,[20] quercetin therapy may accelerate the apoptosis of SW480 CRC cancer cells because it increases the expression of the proapoptotic proteins (Bax and caspase-3). Previous study demonstrated that quercetin induces proapoptotic signaling pathways, which lead to cell death.[21] Caspase-3 is an essential factor in the execution of apoptosis, and cleaved caspase-3 is an activated form of caspase-3. Apoptosis is known to involve a number of different potential pathways. The death receptor-triggered extrinsic apoptotic pathway, which is controlled by Fas/FasL, is one of the crucial routes that is activated by TNF-α and FasL through caspase-8. Another pathway is the mitochondria-initiated intrinsic apoptotic pathway, in which cytochrome c is released as a result of mitochondrial dysfunction that results in the collapse of the mitochondrial membrane potential, which triggers caspase-9, which in turn activates caspase-3. The two pathways, intrinsic and extrinsic, result in caspase-3 activation, then endonucleases finally cause intranucleosomal DNA fragmentation, and the last stages of apoptosis take place.[22] In the intrinsic pathway, quercetin, at high concentration, causes depolarization of mitochondrial membrane potential by elevating the intracellular levels of ROS and Ca2, lead to release of cytochrome c and activation of caspase-3.[23] Doxorubicin shows a significant increase in caspase 3 levels in high concentrations [Figure 5B]. Pilco-Ferreto and Gloria[17] demonstrated that doxorubicin enhances apoptosis by downregulation of Bcl-2 protein expression (antiapoptotic protein) and upregulating of Bax, caspase-8 and caspase-3 (proapoptotic protein) in breast cancer cell line. Ueno *et al.*[24] demonstrate that doxorubicin induces apoptosis through activation of caspase-3, suggesting that apoptosis has an important role in the progression of cardiomyopathy

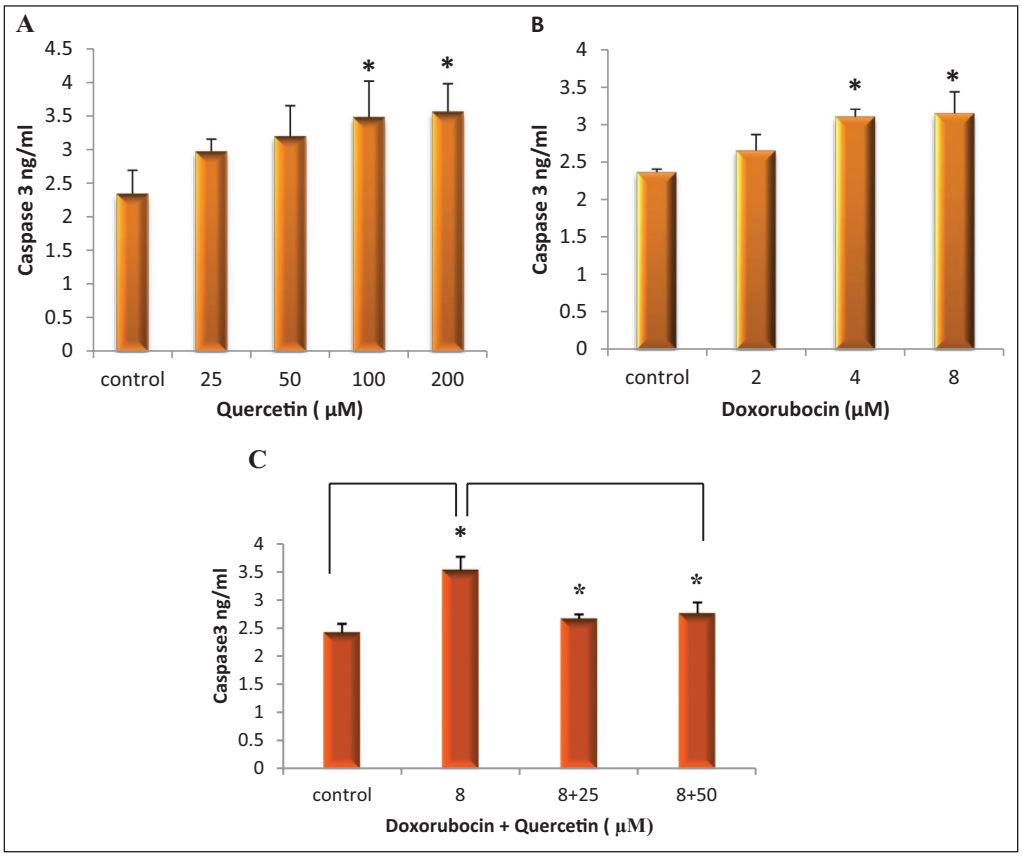

Figure 5: Evaluation of caspase 3 level in SW480 cancer cells were treated with (A) various concentrations of quercetin, (B) various concentrations of doxorubicin, (C) a combination of 25, 50 µM of quercetin with IC50 8 µM of doxorubicin. Results are presented as mean ± SD. Control: DMSO-treated cells

due to doxorubicin. In the previous study, Childs *et al.*[25] demonstrated that doxorubicin finally enhances oxidative stress. It results in the opening of mitochondrial membrane permeability transition pore of the mitochondrial membrane and exerts of proapoptotic proteins involving cytochrome c from the mitochondrial matrix. Since the membrane potential could not be adequately managed in such a situation, this release of cytochrome c is understood to be brought on by mitochondrial impairment. Cytochrome *c* triggers caspase-3 by interacting with Apaf-1 and caspase-9, which causes apoptosis. However, Combination treatment of quercetin with IC_{50} of doxorubicin exhibits a decrease in caspase-3 level in comparison with positive control because quercetin combination with doxorubicin decreases the level of oxidative stress, decreases cytochrome *c* release, and inactivate caspase-3 which responsible of apoptosis [Figure 5C].

Conclusion

Based on the findings, quercetin alone exhibits a powerful anticancer effect in SW480 cancer cells. Cotreatment of quercetin at low concentrations was noticed to reduce intracellular ROS levels and excess the expression of endogenous antioxidants, proposing a mechanism through which quercetin attenuates the effects of antineoplastic medications. Further, in vivo studies to ascertain the short- and long-term effects of quercetin, alone and in combination, on the effectiveness of cancer chemotherapy and the development of side effects.

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Conflicts of interest

There are no conflicts of interest.

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