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## Article

# An in-Depth Study on the Anti-Oxidant and Anti-Apoptotic Potentials of Quercetin in Human Colon Cancer Cell Lines

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**Abstract:** (1) Background: Colon cancer is one of the leading causes of cancer morbidity and mortality globally. It is a multi-step process that involves genetic and epigenetic modifications leading to histological and morphological changes. Several complementary therapeutic options have been analyzed, shedding light on plant-based medication as a potential treatment for colon cancer. Flavonoids such as quercetin are known to have anti-cancer and anti-inflammatory properties. This *in vitro* study examines quercetin's anti-inflammatory, anti-apoptotic, anti-angiogenesis effects and antioxidant properties in colon cancer cells. (2) Methods: The antioxidant capacity of quercetin-treated cells was investigated using biochemical assays, and angiogenesis and cytokine levels were assessed using enzyme-linked immunosorbent assay (ELISA). The epigenetic modulation and differential expression of aging, apoptotic, and proliferation genes, and Histone deacetylases (HDACs) were also investigated. (3) Results: In this study, the quercetin-treated group significantly reduced the antioxidant enzymes, cytokines, and VEGF levels, altering the expression of epigenetic factors. Quercetin also induced significant senescence in colon cancer cells. Moreover, a considerable increase was observed in the apoptotic and hTERT genes. In contrast, a decrease in *p53*, proliferation genes, and HDACs was observed, providing a basis for the clinical use of quercetin in cancer treatment. (4) Conclusion: *In vitro* studies showed that quercetin treatment efficiently induces senescence and apoptosis in colon cancer cells. We also found that quercetin effectively modulated the expression of *p53*, Wnt1, CTNNB1, and HDACs, indicating that it could be used to treat colon cancer.

**Keywords:** quercetin; antioxidant; apoptotic; anti-inflammatory activity; colon cancer

## 1. Introduction

Colon cancer is known to be one of the most common malignancies, leading up to 1.15 million deaths in 2020 globally [1]. Age, race, personal history, intestinal inflammatory condition, western diet, alcohol consumption, smoking, and obesity are considerable threats to colon cancer [2]. However, oxidative stress, inflammation, and angiogenesis are typical hallmarks of colon cancer [3]. Several treatment approaches, such as surgery, chemotherapy, radiation therapy, targeted drug therapy, combinational therapy, and immunotherapies, are presently accessible for colon cancer [4]. Unfortunately, these therapies have low tumor sensitivity and higher toxicity, which reduces overall therapy effectiveness. The interplay of inflammation in colon cancer is well-established by many epigenetic and pharmacological analyses [5]. Inflammatory cytokines promote tumor cell growth, interrupt differentiation, and aid in cancer cell survival [6]. Inflammation is mediated by cytokines released by tumor cells, suggesting that targeting cytokines may be helpful for both preventive and

therapeutic strategies for colon cancer [7]. However, cancer treatment is often ineffective as tumors spread to distant organs. As a result, the discovery of novel therapeutic approaches remains a critical goal in the ongoing fight against colon cancer. Since ancient times, studies have attempted to examine plant-based drugs to treat various diseases as it has fewer side effects [8,9]. Recent research has discovered many plant-based polyphenol compounds with beneficial pharmacological properties and therapeutic value, including anti-tumor activity. Flavonoids are a type of dietary polyphenol widely studied to reduce the risk of cancer development, particularly colon cancer [10]. Evidence supports that polyphenol family members can influence cell proliferation, cellular differentiation, and apoptosis-related gene expression by modulating the associated signaling pathways [11]. One commonly used polyphenol is quercetin which is well known for its anti-proliferative, antioxidant, and anti-inflammatory effects, which make it act as an anti-cancerous agent through broad-spectrum mechanisms from anti-carcinogenesis to anti-metastasis [12,13]. Quercetin can also regulate signaling pathways associated with cellular proliferation and inflammation cascades, including the PI3K, NF $\kappa$ B, MAPK, Wnt pathway, and protein kinase B (Akt). Cytotoxic activity of colon cancer cells is mediated by inhibition of NF $\kappa$ B and induction of apoptosis via *p53* activation, suppression of inflammation via downregulation of Cox2, which is commonly upregulated in colon cancer, and cell cycle arrest due to downregulation of cell cycle genes. Another way quercetin may affect colon cancer cell proliferation is by increasing the expression of the G-protein coupled cannabinoid receptor, CB1-R, which can then bind to quercetin, inhibiting cell growth and migration via the Wnt, Akt, PI3K, and STAT3 pathways. In the existence of a CB1-R antagonist, this effect was abrogated [14]. The mechanisms by which flavonoids may decrease colon carcinogenesis are not entirely understood. However, they have been linked to antioxidant, pro-apoptotic, and anti-inflammatory properties. As a result, more research is needed to determine the association between quercetin and colon cancer. Our previous in vitro study demonstrated quercetin's anti-cancer activity and epigenetic regulations in normal, primary, and metastatic colon cancer cells [11]. In this in vitro study, we further focused on analyzing anti-oxidant, anti-inflammatory, apoptosis-induction, and anti-angiogenesis properties of quercetin in normal, primary, and metastatic colon cancer cell lines. Additionally, we have analyzed the effect of quercetin on epigenetic alterations by analyzing Histone deacetylase (HDAC) expression in colon cancer cells. Therefore, this study focused on further detailed parameters validating quercetin's benefits, particularly on the modulation of HDACs and related epigenetic modulators.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

Quercetin and DMSO was acquired from Sigma-Aldrich, USA. Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffer saline (PBS), Trypsin and antibiotic-antimycotic solution (penicillin, amphotericin B, streptomycin was acquired from Gibco, Thermo Fisher Scientific, USA.

### 2.2. Cell culture

The following colon cell lines were utilized in this study: L132 (Normal lung epithelial cell), HCT 116, Colo 320 (Primary colon cancer cell), and Colo 205 (Metastatic colon cancer cell), all of which were acquired from NCCS, Pune, India. The normoxic condition (37 °C with 5% CO<sub>2</sub>) was used to maintain the cell lines cultured in DMEM and supplemented with 10% FBS and 1% antibiotic.

### 2.3. Preparation of quercetin stock solution

20mM Quercetin stock was prepared based on our previous study and stored at 4 °C [11].

## 2.4. Cell viability analysis

### 2.4.1. Cell Counting Kit-8 (CCK-8) assay

Based on the method reported in our previous study [11], the cell viability assay was performed using a CCK-8 assay kit (Sigma-Aldrich, USA) for the normal, primary, and metastatic cell lines post-treatment with quercetin.  $2 \times 10^3$  cells were seeded into 96-well plates and maintained under normoxic conditions for 24 h. After 24 h, the medium was discarded, and fresh medium was added, along with various concentrations of quercetin ( $Q_{20\mu M}$ ,  $Q_{40\mu M}$ ,  $Q_{80\mu M}$ ,  $Q_{120\mu M}$ ,  $Q_{160\mu M}$ ,  $Q_{320\mu M}$ ) and kept for 72 h. Post 72 h of treatment, 10  $\mu$ l of the CCK-8 reagent was added to every well and kept for 4 h. At 450 nm, the absorbance was analyzed utilizing an ELISA plate reader [11]. Finally, the IC<sub>50</sub> value is determined, and the graph was plotted (data not shown).

### 2.4.2. Experimental groups:

After dose determination, the following experimental groups were taken forward in the present study, L132- (Control,  $LQ_{80\mu M}$ ,  $LQ_{120\mu M}$ ), HCT116- (Control,  $HQ_{80\mu M}$ ,  $HQ_{120\mu M}$ ), Colo320- (Control,  $CQ_{80\mu M}$ ,  $CQ_{120\mu M}$ ) and Colo 205- (Control,  $CQ_{80\mu M}$ ,  $CQ_{120\mu M}$ ). All the control groups were supplemented with DMEM+10%FBS +DMSO (solvent), and two further groups for each cell line were treated with 80 $\mu$ M and 120 $\mu$ M quercetin concentrations.

## 2.5. Assessment of the antioxidant activity

### 2.5.1. Sample preparation

$4 \times 10^4$  cells were treated with the preferred doses of quercetin (80 $\mu$ M and 120  $\mu$ M) for 72 h. After treatment, the cell lysate (the treated cells and control cells were centrifuged at 5000 rpm for 10 mins and the supernatant was collected) for all the cell lines and stored at -20 °C for the following analysis.

### 2.5.2. Lipid peroxidase activity

The lipid peroxidase (LPO) assay was analyzed using the spectrophotometric method with minor alterations [15]. The cell lysate (1 mL) was mixed thoroughly with TCA-TBA-HCL buffer (2 mL) in a centrifuge tube and heated for 15 min in a boiling water bath. After cooling, centrifugation was done at 1000 rpm for 10 minutes to remove the precipitate. The absorbance was measured at 535nm using Milli-Q water as a "blank."

### 2.5.3. Catalase activity

The catalase activity was evaluated utilizing the spectrophotometric procedure with minimal modification [16]. A reaction mixture comprising phosphate buffer (50mM), H<sub>2</sub>O<sub>2</sub> (15mM), was prepared to which 0.1mL of cell lysate was added. The optical density (O.D.) at 240nm was measured over three minutes at intervals of 30 seconds.

### 2.5.4. Superoxide dismutase activity

The Superoxide dismutase (SOD) activity was examined using the spectrophotometric method with a minor alteration [17]. 1mL assay mixture was prepared by adding sodium phosphate buffer (0.05 M, pH 8.0), Ethylenediaminetetraacetic acid (EDTA) (0.01M), pyrogallol (0.27 mM solution of pyrogallol was made in 100mM HCl) and cell lysate. The O.D. was analyzed at 420 nm against Tris-EDTA buffer at the start of the experiment and after 1 min of the addition of pyrogallol.

### 2.5.5. Total thiol content activity

The reaction mixture was prepared by adding 0.1mL Sulfosalicylic acid 4%, 0.5mL sodium phosphate buffer (0.1M). Then, 0.5mL of reaction mixture was incubated for 75 min with 0.65mL of

sample and 6.6  $\mu$ l DTNB at room temperature (RT). After incubation, the test solution vortexed for 15 sec, and the absorbance was measured at 412 nm.

## 2.6. Assessment of free radical scavenging activity

### 2.6.1. ABTS free radical scavenging activity

The method for ABTS scavenging activity was followed as per the protocol with slight alterations [18]. A mixture of potassium persulfate (2.45 mM) and the ABTS (7mM, Cat. No. 40157, SRL, India) was prepared as a stock and were in a (1:1, v/v) ratio and maintained in the dark for 16 h at RT. Then, the stock solution was diluted by adding 1 mL of ABTS stock solution in methanol (60 mL), with spectrophotometric absorbance of  $0.706 \pm 0.01$  units at 734 nm. The test samples from various treated groups (10 $\mu$ l) were incubated with diluted ABTS solution (200 $\mu$ l) for 7 min, and the absorbance was taken at 734 nm.

### 2.6.2. DPPH free radical scavenging activity

DPPH (Cat. No. D9132, Sigma-Aldrich, USA). DPPH stock solutions were prepared in a methanol buffer. Methanol buffer was made by combining 40 mL of 0.1M acetate buffer (0.1 M, pH 5.5) with 60 mL methanol. 100 $\mu$ l cell lysate was incubated with DPPH stock solution in the dark for 30 min at 30 °C. The O.D. was measured spectrophotometrically at 517 nm.

## 2.7. Senescence-associated $\beta$ -galactosidase (SA- $\beta$ -gal) activity

The SA- $\beta$ -gal analysis uses  $\beta$ -galactosidase to convert X-gal (5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside), a colorless substrate, into a blue product that accumulates in senescent cells due to upregulation of  $\beta$ -galactosidase activity. The EZdetect<sup>TM</sup> cell senescence detection kit (Cat. No. CCK063, HiMedia Ltd, India) was used to measure the activity of SA- $\beta$ -gal in control and treated cells. The procedure was followed exactly as directed by the manufacturer. The cell senescence was observed, and images were acquired utilizing an inverted microscope.

## 2.8. Gene expression analysis by qPCR

For extracting the total RNA (L132, HCT116, Colo305, Colo205), the cells were treated with TRIzol<sup>TM</sup> Reagent (Invitrogen, Thermo Fisher Scientific, Cat. No. 15596026). The purity and concentration of isolated RNA were quantified using a spectrophotometer at 260 nm. cDNA was generated utilizing a reverse transcription kit (Cat. No. RT-RTCK-03, Eurogentec, Belgium). The Syber Green qPCR master mix (Cat.NO. UF- RSMT-B0701, Takyon, Eurogentec, Belgium) was used to perform the qPCR for the following genes (*p53*, *Bax*, *Bcl-2*, *CTNNB1*( $\beta$ -catenin), *Wnt-1* and *GAPDH*).  $\Delta$ Ct values and fold change for each gene were obtained by normalizing the Ct value of the target gene to *GAPDH* expression, and the graphs were plotted.

## 2.9. Epigenetic modification assays

### 2.9.1. HDAC quantification assay

TRIzol<sup>TM</sup> Reagent (Cat. No. 15596026, Invitrogen, Thermo Fisher Scientific) was utilized to isolate total RNA for HDAC quantification. The purity and concentration of isolated RNA were quantified using a spectrophotometer at 260 nm. cDNA was prepared using a reverse transcription kit (Cat. No. RT-RTCK-03, Eurogentec, Belgium). The RT2 SYBR Green ROX qPCR Mastermix (Cat. No. 330520, Qiagen, USA) and RT2 qPCR primer assay (*HDAC1*, *HDAC2*, *HDAC4*, *HDAC5*, *HDAC8*, *HDAC10*) (Cat. No. 330001, Qiagen, USA) were used to perform qPCR for the epigenetic regulator genes.



### 2.9.2. hTERT quantification assay

The hTERT expression was quantified utilizing the RT2 first-strand synthesis kit (Cat. No. 330401, Qiagen, USA) and RT2 qPCR hTERT primer assay (Cat. No. 330001, Qiagen, USA) kit by the manufacturer's protocol. The fold expression was analyzed, and graphs were plotted with statistical significance using GraphPad Prism V9.

### 2.10. Inflammatory cytokines and angiogenic factor expression analysis by ELISA

Protein quantification for the following, Interleukin-6 (IL6), Interleukin-10 (IL10), and Tumor necrosis factor (TNF- $\alpha$ ) levels of control and quercetin-treated cells using an IL10 ELISA kit (Cat No. E0102Hu), IL6 ELISA Kit (Cat No. E0090Hu) and TNF- $\alpha$  ELISA Kit (Cat No. E0082Hu, Bioassay Technology Laboratory, China) was conducted by the manufacturer's instructions.

### 2.11. Vascular Endothelial Growth Factor (VEGF) expression

The level of VEGF was quantified from the cell lysate obtained from the quercetin-treated cells utilizing the VEGF ELISA Kit (Cat No. E0080Hu, Bioassay Technology Laboratory, China) in accordance with the manufacturer's instructions.

### 2.12. Statistical analysis

The GraphPad V9 software was utilized for statistical analysis. Employing the unpaired student's t-test, the significant difference between the treatment and control groups was determined. Derived from three independent experimental studies, the data are expressed as mean  $\pm$  standard error. P value =0.05 was considered statistically significant for the present study.

## 3. Results

### 3.1. Cell viability analysis

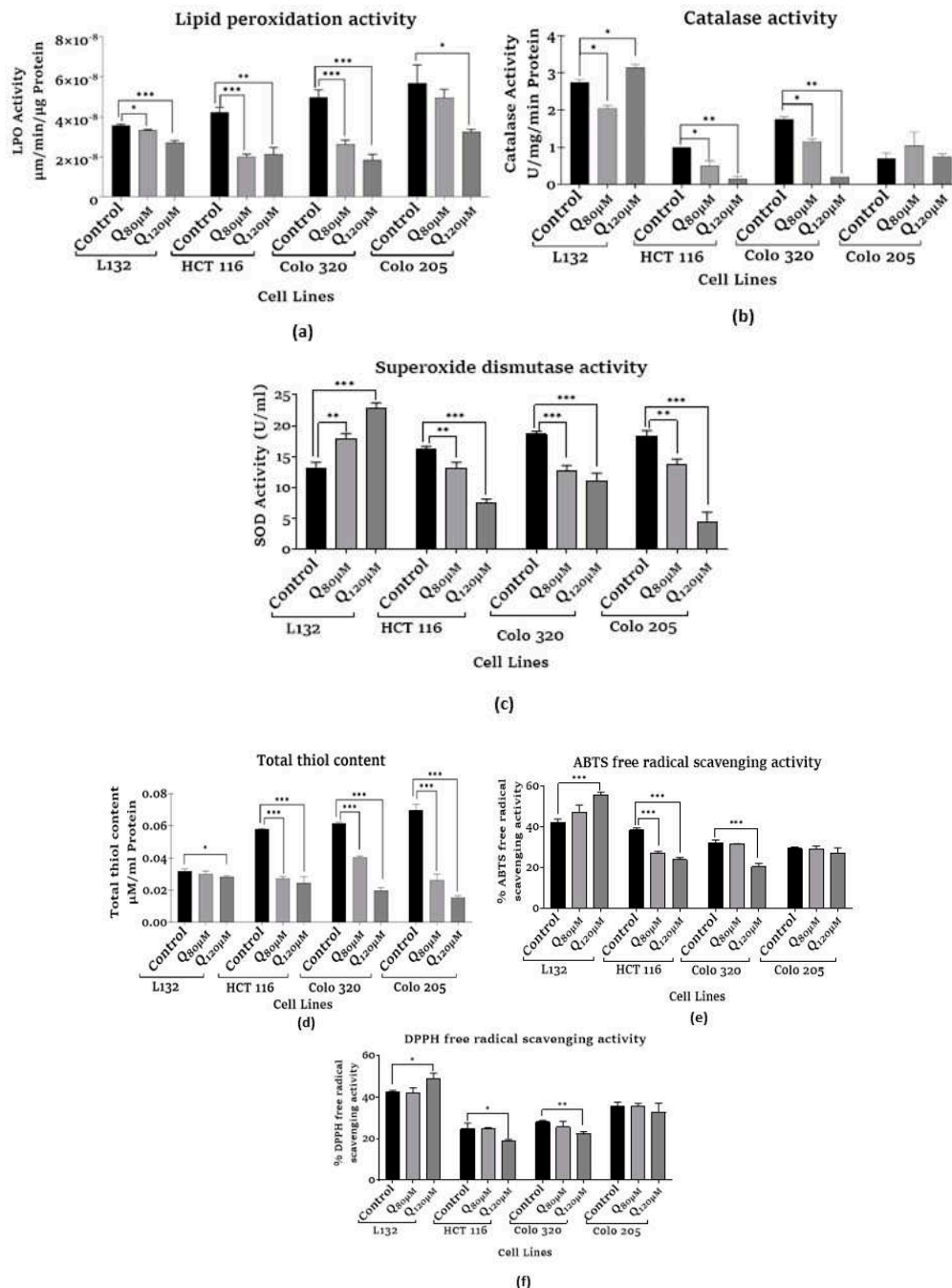
#### 3.1.1. CCK-8 assay

The CCK-8 cell viability assay was carried out in control as well as normal lung epithelial cell, primary and metastatic colon cancer cells to identify and to calculate the IC<sub>50</sub> value of quercetin, which has been concurred from our previous study, [11] the dose was determined and taken forward for the current study. Based on the results of the CCK8 assay, two doses of 80 $\mu$ M and 120  $\mu$ M quercetin were taken for further treatments, which is explained in our previously published in vitro work (data not shown here) [11].

### 3.2. Assessment of antioxidant activity

#### 3.2.1. Assessment of lipid peroxidation (LPO) assay

The lipid peroxidase activity is elucidated in Figure 1a. The results demonstrated that quercetin at a concentration of 80 $\mu$ M/mL showed decreased LPO activity in HCT116 (p=0.0101), Colo320 (p=0.0007), and Colo 205 respectively, while at a concentration of 120  $\mu$ M/mL was significantly decreased in Colo320 (p=0.0003) and Colo205 (p=0.0105) when compared to their respective controls. Whereas HCT116 Q<sub>120 $\mu$ M</sub> (p=0.0011) was slightly increased compared to HCT116 Q<sub>80 $\mu$ M</sub>. (p=0.0002). Although no significant decrease in LPO level was observed in L132 (LQ<sub>80 $\mu$ M</sub>, p=0.0101, LQ<sub>120 $\mu$ M</sub>, p=0.0004) treated groups compared to their respective control group.



**Figure 1.** (a-c): Biochemical assays were performed to measure the inhibitory effect of quercetin on antioxidant enzymes (a) Lipid peroxidase activity (b) Catalase activity (c) Superoxide dismutase activity was measured (spectrophotometric method) normal cells (L132), primary, and metastasis colon cancer cells. (d-f). Biochemical assays were performed to measure the inhibitory effect of quercetin on antioxidant enzymes (d) Total thiol content (e) ABTS free radical scavenging activity (f) DPPH free radical scavenging activity was measured (spectrophotometric method) in normal cells (L132), primary, and metastasis colon cancer cells.

### 3.2.2. Assessment of catalase activity

The catalase activity is represented in Figure 1b. The results shows that catalase activity is significantly decrease in Q<sub>120</sub>μM of HCT116 (p=0.0034) and Colo 320 cell line (p=0.001) when compared to their respective control groups. Whereas in Q<sub>120</sub>μM of L132 (p=0.0299) and Q<sub>80</sub>μM of Colo 205 was seen to be increased compared to its control group.

### 3.2.3. Assessment of superoxide dismutase activity

The superoxide dismutase activity is demonstrated in Figure 1c. The results shows that SOD levels have significantly decreased in  $Q_{120\mu M}$  of HCT116 ( $p=0.0001$ ) and Colo 205 ( $p=0.0002$ ) cells, respectively, compared to that of their control groups. In L132 cells, both the treatment group showed a significant increase ( $LQ_{80\mu M} p=0.0039$ ,  $LQ_{120\mu M} p=0.0003$ ) in SOD activity compared to their respective control. Although in Colo 320 cells ( $CQ_{80\mu M} p=0.0006$ ,  $CQ_{120\mu M} p=0.0006$ ), there were reduced SOD levels, which did not have a significant difference between the treatment groups compared to their control group.

### 3.2.4. Assessment of total thiol content

The free thiol groups efficiently protect cells from free radical damage. The total thiol content is shown in Figure 1d. The results indicated that total thiol content had been significantly decreased in Colo 320 ( $CQ_{80\mu M}$ ,  $p=0.0001$ ,  $CQ_{120\mu M}$ ,  $p=0.0001$ ) and Colo 205 ( $CQ_{80\mu M}$ ,  $p=0.0001$ ,  $CQ_{120\mu M}$ ,  $p=0.0001$ ) cells when compared to their control groups. Although in HCT116 ( $HQ_{80\mu M}$ ,  $p=0.0001$ ,  $HQ_{120\mu M}$ ,  $p=0.0001$ ) cells thiol content was found to be reduced but did not have a significant difference between the treatment groups when compared to their control group. However, in L132 treated groups, quercetin did not affect the overall thiol content.

## 3.3. Assessment of free radical scavenging activity

### 3.3.1. Assessment of ABTS free radical scavenging activity

The ABTS free radical scavenging activity is represented in Figure 1e. Here, a significant inhibition in ABTS free radical scavenging activity in HCT116  $Q_{80\mu M}$  and  $Q_{120\mu M}$  treatment groups ( $p=0.001$ ) was seen than in their control group. Likewise, there was a considerable reduction in free radical scavenging activity in Colo 320 ( $p=0.0008$ ), but no changes were observed in Colo 205 groups. In contrast, quercetin administration significantly increased ABTS free radical scavenging capacity in L132 cells ( $p=0.0005$ ).

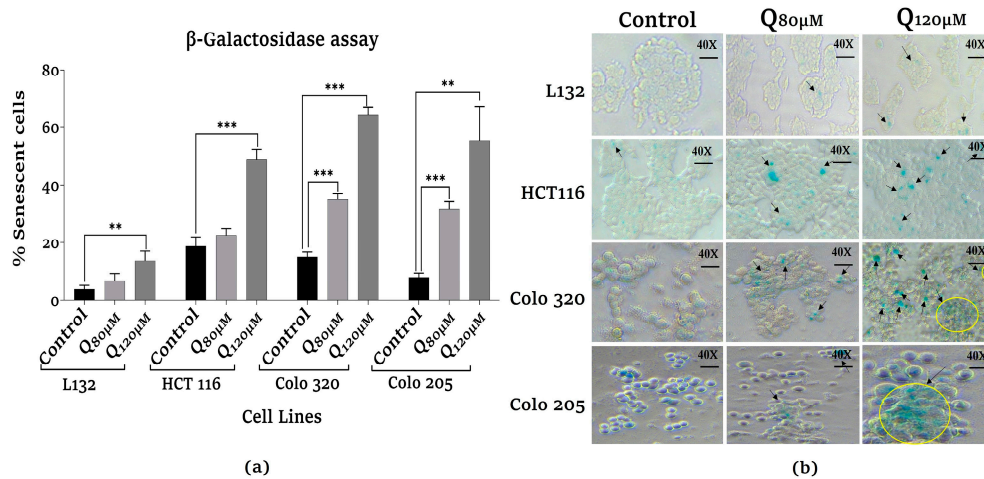
### 3.3.2. Assessment of DPPH free radical scavenging activity

The DPPH scavenging activity is represented in Figure 1f. The DPPH activity was reduced in HCT116 ( $HQ_{120\mu M}$ ,  $p=0.0229$ ) and Colo 320 ( $Q_{120\mu M}$ ,  $p=0.0017$ ) respectively. In contrast, L132 showed an increased level of activity when compared to their control group. However, there was no change in Colo 205 group.

## 3.4. Assessment of senescence-associated $\beta$ -galactosidase activity analysis

In SA-  $\beta$ -Gal cell senescence analysis, the number of senescent cells increased significantly in all cell lines upon quercetin treatment. In L132 ( $Q_{120\mu M}$ ,  $p=0.0091$ ) and HCT116 ( $Q_{120\mu M}$ ,  $p=0.0005$ )  $Q_{120\mu M}$  treatment group, the number of senescent cells was much higher than that of their control. Whereas in treated Colo 320 ( $Q_{80\mu M}$ ,  $p=0.0002$ ,  $Q_{120\mu M}$ ,  $p=0.0001$ ), a significantly large number of senescent cells were observed at both doses, Figure 2a. Similarly, a very high number of senescent cells was seen at both concentrations in Colo 205 ( $Q_{80\mu M}$ ,  $p=0.0002$ ,  $Q_{120\mu M}$ ,  $p=0.0024$ ). The senescent cells in each group have been indicated by arrows in Figure 2b. These findings suggest that  $Q_{120\mu M}$  shows a significant increase in cell senescence.



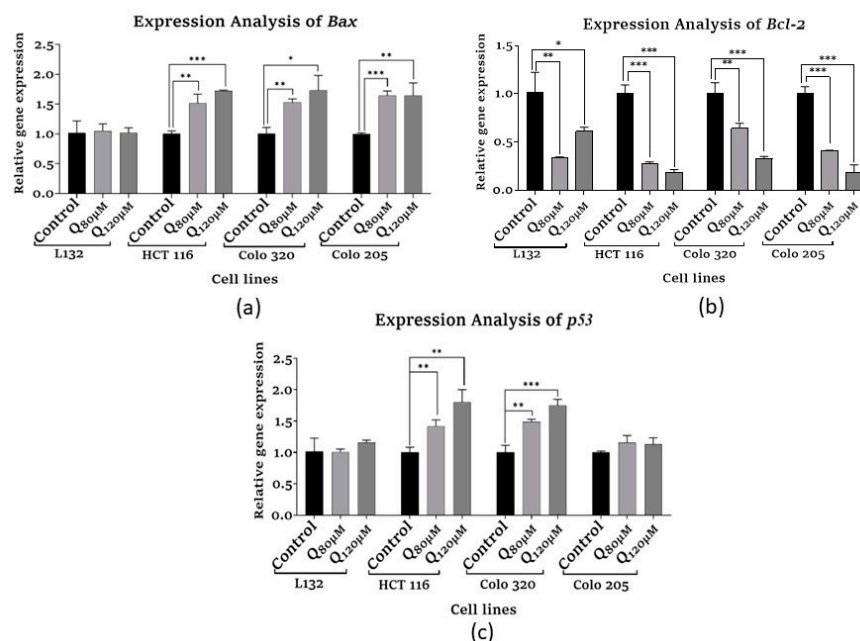


**Figure 2.** (a) Graphical presentation, (b) morphological presentation of SA- β-Gal cell senescence analysis in control and quercetin-treated normal cells (L132), primary, and metastasis colon cancer cells.

### 3.5. Gene expression analysis by qPCR

#### 3.5.1. Apoptosis-related and Tumor suppressor gene expression

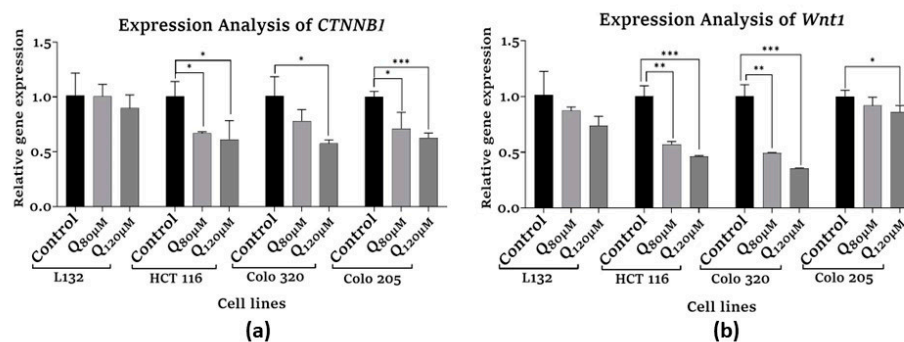
In this study, quercetin therapy significantly enhanced *Bax* expression in HCT116 (HQ80μM,  $p=0.0054$ , HQ120μM,  $p=0.0001$ ), Colo320 (CQ80μM,  $p=0.0016$ , CQ120μM,  $p=0.0103$ ) and Colo205 (CQ80μM,  $p=0.0002$ , CQ120μM,  $p=0.0072$ ), whereas there were no significant changes in L132 in comparison to their respective control groups, Figure 3a. In contrast, the *Bcl-2* expression was drastically downregulated in all the cell lines with respect to their control groups; L132 (LQ80μM,  $p=0.0048$ , LQ120μM,  $p=0.0305$ ), HCT116 (HQ80μM,  $p=0.0003$ , HQ120μM,  $p=0.0001$ ), Colo320 (CQ80μM,  $p=0.0068$ , CQ120μM,  $p=0.0005$ ) and Colo205 (CQ80μM,  $p=0.0002$ , CQ120μM,  $p=0.0002$ ), Figure 3b. Likewise, the *p53* gene was significantly upregulated in quercetin-treated HCT116 (HQ80μM,  $p=0.0050$ , HQ120μM,  $p=0.0031$ ) and Colo 320 (CQ80μM,  $p=0.0021$ , CQ120μM,  $p=0.0009$ ). Whereas no changes in the expression were observed in the Colo 205 and L132 cells, Figure 3c.



**Figure 3.** Analysis of apoptosis-related gene and Tumor suppressor expression (a) *Bax*, (b) *Bcl-2*, (c) *p53* in control and quercetin-treated normal cells (L132), primary, and metastasis colon cancer cells.

### 3.5.2. Proliferation-related gene expression

The impact of quercetin on primary cell proliferation was found to be more effective than the metastatic cell line. The proliferation-related gene *Wnt1* was downregulated in HCT116 (HQ<sub>80</sub>μM, p=0.0015, HQ<sub>120</sub>μM, p=0.0006) and Colo320 (CQ<sub>80</sub>μM, p=0.0010, CQ<sub>120</sub>μM, p=0.0004) in both treated concentrations than the control group. Likewise, a higher dose of quercetin treatment significantly downregulated *CTNNB1* expression in HCT116 (HQ<sub>80</sub>μM, p=0.0124, HQ<sub>120</sub>μM, p=0.0325), Colo320 (CQ<sub>120</sub>μM, p=0.0132) and Colo320 (CQ<sub>80</sub>μM, p=0.0323, CQ<sub>120</sub>μM, p=0.0006) cancer cell lines than the control group, respectively Figure 4 a, b. No statistically significant variation in gene expression was seen in either of the genes in L132 cells.

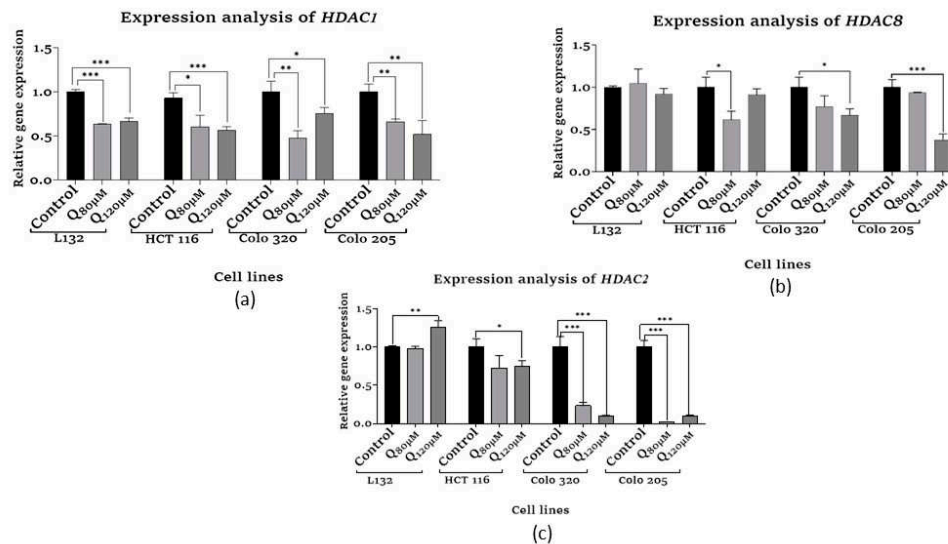


**Figure 4.** Analysis of proliferation-related gene expression (a)  $\beta$ -catenin (*CTNNB1*), (b). *Wnt1* in control and quercetin-treated normal cells (L132), primary, and metastasis colon cancer cells.

### 3.6. Epigenetic modification assays

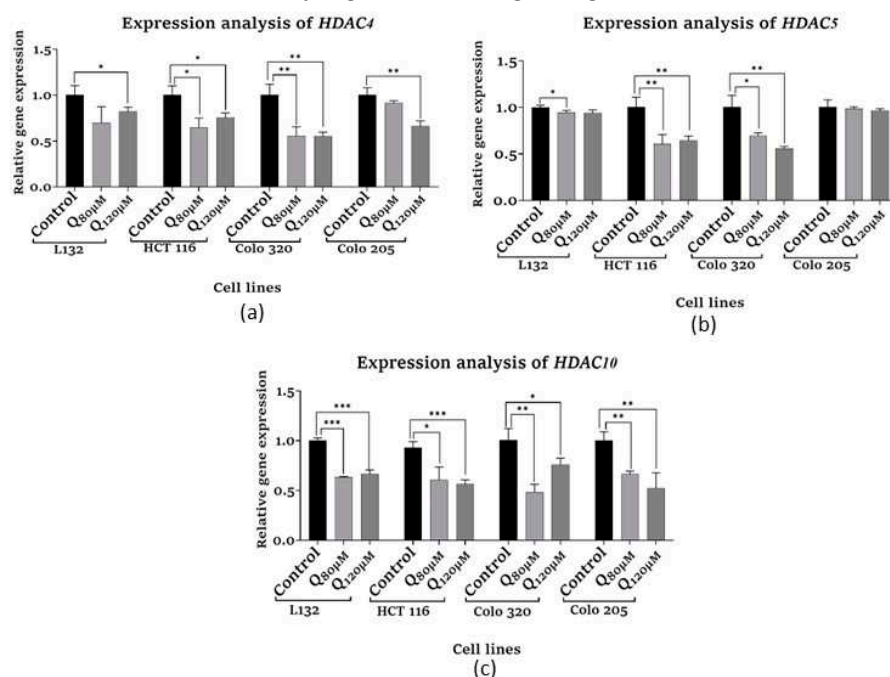
#### 3.6.1. HDAC Quantification assay

HDACs expression analysis was performed to investigate the impact of quercetin on class I HDACs (*HDAC1*, *HDAC2*, *HDAC8*) and class II HDACs (*HDAC4*, *HDAC5*, *HDAC10*), expression in colon cancer cells, where quercetin showed a significant decrease in HDACs expression. Interestingly, we observed that members of both HDACs family, class I and II were downregulated in quercetin-treated colon cancer cells. In brief, quercetin showed significant downregulation in the expression of *HDAC1* in L132 (LQ<sub>80</sub>μM, p=0.0001, LQ<sub>120</sub> μM, p = 0.0003), HCT116 (HQ<sub>80</sub>μM, p=0.0168, HQ<sub>120</sub>μM, p=0.0010), Colo320 (CQ<sub>80</sub>μM, p=0.0031, CQ<sub>120</sub>μM, p=0.034) treatment groups and Colo205 (CQ<sub>80</sub>μM, p=0.0031, CQ<sub>120</sub>μM, p=0.0096) cells at both the concentration, Figure 5a. Similarly, *HDAC2* expression was reduced in quercetin-treated (L132Q<sub>120</sub> μM, p=0.0051), (HCT116Q<sub>120</sub> μM, p=0.0269) treatment group, whereas in Colo320 (Q<sub>80</sub>μM, p=0.0007, Q<sub>120</sub> μM, p=0.0003), and Colo205 (Q<sub>80</sub>μM, p=0.0001, Q<sub>120</sub>μM, p=0.0001) cells at both the concentration, Figure 5b. In contrast, quercetin treatment reduced the *HDAC8* expression in (HCT116Q<sub>80</sub>μM, p=0.0118), (Colo320Q<sub>120</sub>μM, p=0.0131), and (Colo205Q<sub>120</sub>μM, p=0.0007) treatment groups whereas no considerable differences were seen in L132 cells, Figure 5c.



**Figure 5.** Epigenetic modification analysis HDAC I family expression (a). *HDAC1*, (b) *HDAC2* (c) *HDAC8* in control and quercetin-treated normal cells (L132), primary, and metastasis colon cancer cells.

Previous studies showed that *HDAC4* plays a significant role in colon cancer maintenance and malignant transformation [19]. Here, quercetin-treatment groups showed a decrease in *HDAC4* L132 (LQ<sub>120</sub>μM,  $p=0.0482$ ), HCT116 (HQ<sub>80</sub>μM,  $p=0.0126$ , HQ<sub>120</sub>μM,  $p=0.0184$ ), Colo320 (CQ<sub>80</sub>μM,  $p=0.0067$ , CQ<sub>120</sub>μM,  $p=0.0031$ ) and Colo205 (CQ<sub>120</sub>μM,  $p=0.0040$ ) expression in all the cell lines, Figure 6a. A similar pattern of expression inhibition was observed for *HDAC10* L132 (LQ<sub>80</sub>μM,  $p=0.0001$ , LQ<sub>120</sub>μM,  $p=0.0003$ ), HCT116 (HQ<sub>80</sub>μM,  $p=0.0168$ , HQ<sub>120</sub>μM,  $p=0.0010$ ), Colo320 (CQ<sub>80</sub>μM,  $p=0.0031$ , CQ<sub>120</sub>μM,  $p=0.0341$ ) and Colo205 (CQ<sub>80</sub>μM,  $p=0.0031$ , CQ<sub>120</sub>μM,  $p=0.0096$ ), Figure 6c. In contrast, *HDAC5* expression was observed to be downregulated in HCT116 (HQ<sub>80</sub>μM,  $p=0.0094$ , HQ<sub>120</sub>μM,  $p=0.0058$ ) and Colo 320 (CQ<sub>80</sub>μM,  $p=0.0136$ , CQ<sub>120</sub>μM,  $p=0.0035$ ) at both the doses whereas in L132 and Colo 205 cells whereas quercetin treatment did not show any significant changes, Figure 6b.



**Figure 6.** Epigenetic modification analysis HDAC II family expression (a) *HDAC4*, (b) *HDAC5* (c) *HDAC10* in control and quercetin-treated normal cells (L132), primary, and metastasis colon cancer cells.

### 3.6.2. hTERT quantification assay

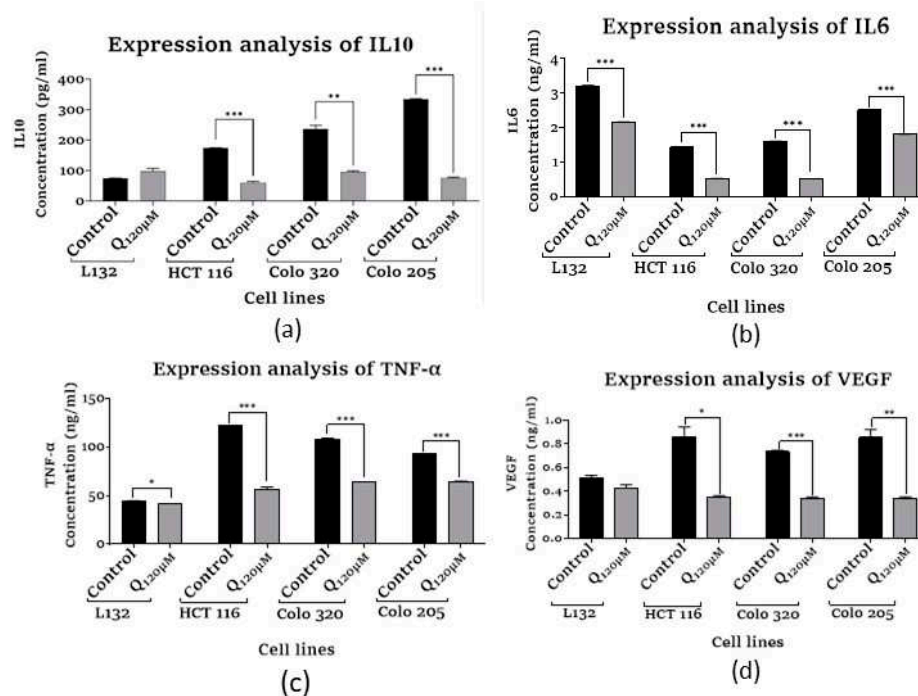
In this analysis, quercetin-treated groups showed significant downregulation in the expression of hTERT in (Colo320Q<sub>80</sub>μM,  $p=0.0065$ , Q<sub>120</sub>μM,  $p=0.458$ ) and (Colo205Q<sub>80</sub>μM,  $p=0.0016$ ) treatment group at a lower dose when compared to higher dose. In HCT116 (HQ<sub>80</sub>μM  $p=0.0077$ , HQ<sub>120</sub>μM,  $p=0.0089$ ) quercetin significantly reduced the hTERT expression at both doses. Although a reduction in the hTERT expression was observed only in the L132Q<sub>120</sub>μM ( $p=0.0052$ ) group when compared to L132Q<sub>80</sub>μM, Figure 7.

**Figure 7.** Epigenetic modification analysis hTERT expression in control and quercetin-treated normal cells (L132), primary, and metastasis colon cancer cells.

### 3.7. Inflammatory cytokines and angiogenic factor expression analysis

#### 3.7.1. Assessment of Human Interleukin 10

The results showed that there was significant decrease in IL10 levels of HCT116, Colo320 and Colo 205 with their respective control groups. Whereas, we observed mild increase in IL10 level in L132 with respect to its control depicted in Figure 8a.



**Figure 8.** Effect of Quercetin on inflammatory cytokines and angiogenesis factor expression in normal cells (L132), primary, and metastasis colon cancer cells. (a) Human IL10 expression (b) Human IL6 expression (c) Human TNF-α. (d) Human VEGF. .

#### 3.7.2. Assessment of Human Interleukin 6

Interleukin-6 is involved in immune response as well as inflammation. In our investigation, quercetin treatment showed a noticeable decrease in IL6 levels in all the treatment groups (L132Q<sub>120</sub>μM,  $p=0.0006$ ), (HCT116Q<sub>120</sub>μM,  $p=0.0001$ ), (Colo320Q<sub>120</sub>μM,  $p=0.0001$ ) and (Colo205 Q<sub>120</sub>μM,  $p=0.0001$ ) to their relevant control groups, Figure 8b.

#### 3.7.3. Assessment of Human Tumor Necrosis Factor-α

Quercetin treated group showed a significant decrease in TNF-α expression in Q<sub>120</sub>μM HCT116 ( $p=0.0007$ ), Colo 320 ( $p=0.0003$ ), and Colo 205 ( $p=0.0001$ ) cells at a higher dose of quercetin than the

respective control group. In contrast, no significant reduction was observed in L132 ( $p=0.0156$ ) cells to their control group, Figure 8c.

#### 3.7.4. Assessment of Vascular Endothelial Growth Factor

Quercetin significantly reduced VEGF concentrations in HCT116 ( $Q_{120\mu M}$ ,  $p=0.0123$ ) and Colo320 ( $Q_{120\mu M}$ ,  $p=0.0003$ ) and Colo205 ( $Q_{120\mu M}$ ,  $p=0.0084$ ) cancer cells compared to the relevant control groups. Despite this, no considerable difference was detected in the L132 quercetin-treated group, Figure 8d.

## 4. Discussion

Quercetin is a plant pigment and secondary metabolite, acts on a multitude of targets involved in the initiation and promotion/progression phases of colorectal carcinogenesis [20]. Due to disease recurrence, the inadequacy of cancer treatment results in suboptimal outcomes. In our investigation, CCK-8 analysis was carried out, which showed that quercetin has a significant cytotoxic impact on normal epithelial, primary, and metastatic colon cancer cell proliferation. The highly quoted antioxidant activity of quercetin, it is noted that it works directly as a ROS scavenger [21]. The cancer cell regulates ROS and antioxidants precisely to survive with high ROS levels. In contrast, antioxidants protect antioxidant defense systems by scavenging reactive oxygen species [22]. When a cell undergoes stress conditions, it starts producing free radicals, accumulating and increasing the ROS level and generating oxidative stress [23]. Antioxidant enzymes such as LPO, catalase, SOD, ABTS, and DPPH neutralize these free radicals and decrease the level of ROS [24]. Our results demonstrated that, quercetin treatment at  $120\mu M/mL$  enhanced ROS-generated stress by suppressing cancer cells total thiol, catalase, LPO and SOD activity. Oxidative stress can cause cell cycle arrest, senescence, and finally, apoptosis and necrosis [25]. Similarly, the ABTS and DPPH showed the potential ability of quercetin to scavenge free radicals in primary colon cancer cell line. Therefore, the analysis of the antioxidants suggests that targeting antioxidant enzymes might be a promising approach for generating senescence, thereby apoptosis in cancer cells, based on the above outcome. The inhibition of malignant development may be achieved by the induction of cellular senescence in response to pro-tumorigenic stimuli [26]. SA- $\beta$ -Galactosidase, a putative hydrolase enzyme, is a well-known biomarker of cell senescence [27]. The activity is driven by residual lysosomal  $\beta$ -galactosidase at pH 6.0 and reflects increased lysosomal content in senescent cells [28]. This study found a concentration-dependent rise in lysosomal- $\beta$  galactosidase, resulting in a considerable increase in senescent cells in all quercetin-treated cancer cells. Colon cancer commonly undergoes spontaneous apoptosis, possibly due to genetic instability and increased cellular turnover [29]. *Bax* is a pro-apoptotic protein and an essential gateway of the intrinsic apoptotic pathway [30]. The quercetin treatment showed a potential increase in apoptosis by upregulated *Bax* expression and down-regulating an anti-apoptotic gene, *Bcl-2*, in colon cancer cells. Furthermore, the primary tumor suppressor gene *p53* is also known as the guardian of the genome due to its primary role in maintaining genome integrity by detecting mutations and inhibiting cell multiplication with mutated genes or damaged DNA [31]. Here, quercetin significantly upregulated the expression of *p53*. Hence, the reactivation of *p53* in cancer cells might be aiding in inducing cell senescence. Wnt/ $\beta$ -catenin mediated signaling pathway is a critical pathway that facilitates colon cancer cell proliferation [32]. Wnt genes are cysteine-rich glycoproteins secreted by cells into the extracellular matrix that activate receptor-mediated signaling, whereas *CTNNB1* acts as a signal transducer, and the Wnt/ $\beta$ -catenin signaling system regulates cell proliferation, differentiation, migration, genetic stability, apoptosis, and stem cell renewal [33,34]. By analyzing the expression of both *Wnt1* and *CTNNB1* in all the treated colon cancer cells, we demonstrated that quercetin substantially inhibits the *Wnt1* and *CTNNB1* gene expression, thereby Wnt/ $\beta$ -catenin signaling pathway, which has restricted cancer cell proliferation. Epigenetic regulation alludes to the heritable and potentially reversible alterations in the phenotypic variability of the genome that modify gene transcription without altering the DNA sequence [35]. The HDAC protein family is an important regulator of chromatin integrity and governs the consequence of gene expression patterns [36]. In cancer cells, HDAC-mediated gene expression regulation is accomplished by separating the acetyl group from the histone proteins tail to silence the



transcription of tumor suppressor genes. Several studies have reported that overexpressed HDACs may promote tumorigenesis [37]. There are 4 classes of HDACs among them, class I HDACs (*HDAC1*, *HDAC2*, *HDAC8*), class II HDACs (*HDAC4*, *HDAC5*, *HDAC10*), and hTERT are directly involved in tumor-related gene expression [38]. Since HDACs were one of the predicted targets of quercetin, the expression analysis of class I and class II HDAC were performed in quercetin-treated colon cancer cells. Quercetin administration reduced the expression of all HDACs and hTERT in colon cancer in a dose-dependent manner, thereby cell senescence and finally apoptosis. Downregulation of HDACs promotes cytochrome C release by activating the intrinsic apoptosis pathway and the activation of caspases by activating the intrinsic apoptosis pathway [39]. Recent studies have found that HDAC inhibitors significantly impact colon cancer cell proliferation and cell cycle. *HDAC1* and *HDAC2* are reported to be associated with regulating the cell cycle, which is overexpressed in cancer, including colon cancer [40]. Further, the expression of *HDAC5* is anomalous in many cancers and is involved in tumor growth, invasion, and immune response, which have been thoroughly investigated [41]. *HDAC10* contributes significantly to malignancies by regulating various cellular processes via its epigenetic action on specific signaling pathways. Moreover, the downregulated *HDAC10* reduces the Wnt pathway activity, which is also known to promote apoptosis in colon cancer [42]. Hence, the present study signifies that the level of HDACs might be extremely important in revealing their tremendous importance in tumor cells when used with traditional methods [43]. This paradigm is influenced by the abundance of *in vitro* and *in vivo* data indicating that epigenetics play a vital role in colon cancer, and their reversible feature decides the rationale behind the current approach [44]. Chronic inflammation in epithelial cells, such as cancer cells, results in epigenetic alterations, such as DNA methylation modifications [45]. IL10 is a critical immunosuppressive agent and pro-inflammatory cytokine implicated in inflammation that promotes cancer development and immune responses against malignancies [46]. Similarly, TNF- $\alpha$  is a pro-inflammatory multifunctional cytokine that plays crucial roles in cellular processes, including cell viability, proliferation, differentiation, and apoptosis [47,48]. In cancer, the production of VEGF and other growth factors induces the 'angiogenic switch,' which causes new vasculature around the tumor, allowing it to grow tremendously [49]. Hence, by ELISA study, we examined the impact of quercetin over the IL10, IL6, TNF- $\alpha$ , and VEGF levels in colon cancer cells, where we found that quercetin significantly reduced the level of IL10, IL6, TNF- $\alpha$ , and VEGF cytokines in all the quercetin treated colon cancer cell lines. TNF- $\alpha$  is a critical inflammatory cytokine of the tumor microenvironment, facilitating colon cancer invasion [29,51]. Further, the expression of VEGF was determined, a homodimer glycoprotein that promotes angiogenesis in embryonic development and wound healing [52,53]. The result of VEGF expression showed that quercetin effectively inhibited expression levels in all colon cancer cell lines.

## 5. Conclusions

In conclusion, our findings demonstrate that quercetin inhibits proliferation, triggers apoptosis, and suppresses apoptotic genes. The results demonstrated the importance of polyphenols and flavonoids in cancer treatment, and prevention is now widely acknowledged. Based on the gene expression of genetic and epigenetic markers and cytokine analysis, quercetin treatment may significantly prevent inflammation and angiogenesis, which plays a key function in tumor development. Moreover, quercetin shows the potential to modify epigenetic regulation by modulating related signaling pathways like the Wnt/ $\beta$ -catenin signaling pathway, which is a remarkably conserved pathway strongly linked to colon cancer. Hence, a combinational therapy regimen combining quercetin and epigenetic modifiers with current chemotherapeutic medications may assist in slowing the spread of colon cancer and lessen the negative effects of the chemotherapeutic agents.

**Author Contributions:** AB designed and conceptualized the study. MB conducted general experiments, assays, and *in vitro* experiments. AB, MB, SMJ, and SP supported data analysis and wide-ranging aspects of the manuscript preparation and development process. SP and AD supported in crucial editing and statistical analysis. AB, AKD, and SFA critically reviewed the draft manuscript and provided feedback on data and analyses. All authors read and approved the final manuscript.

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