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RESEARCH ARTICLE

# Quercetin modifies 5′CpG promoter methylation and reactivates various tumor suppressor genes by modulating epigenetic marks in human cervical cancer cells

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## Abstract

The central role of epigenomic alterations in carcinogenesis has been widely acknowledged, particularly the impact of DNA methylation on gene expression across all stages of carcinogenesis is considered vital for both diagnostic and therapeutic strategies. Dietary phytochemicals hold great promise as safe anticancer agents and effective epigenetic modulators. This study was designed to investigate the potential of a phytochemical, quercetin as a modulator of the epigenetic pathways for anticancer strategies. Biochemical activity of DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and global genomic DNA methylation was quantitated by an enzyme-linked immunosorbent assay based assay in quercetin-treated HeLa cells. Molecular docking studies were performed to predict the interaction of quercetin with DNMTs and HDACs. Quantitative methylation array was used to assess quercetin-mediated alterations in the promoter methylation of selected tumor suppressor genes (TSGs). Quercetin induced modulation of chromatin modifiers including DNMTs, HDACs, histone acetyltransferases (HAT) and HMTs, and TSGs were assessed by quantitative reverse transcription PCR (qRT-PCR). It was found that quercetin modulates the expression of various chromatin modifiers and decreases the activity of DNMTs, HDACs, and HMTs in a dose-dependent manner. Molecular docking results suggest that quercetin could function as a competitive inhibitor by interacting with residues in the catalytic cavity of several DNMTs and HDACs. Quercetin downregulated global DNA methylation levels in a dose- and time-dependent manner. The tested TSGs showed steep dose-dependent decline in promoter methylation with the restoration of their expression. Our study provides an understanding of the quercetin's mechanism of action and will aid in its development as a candidate for epigenetic-based anticancer therapy.

**KEYWORDS**

DNMT, epigenetics, HDAC, quercetin, tumor suppressor genes

  

## 1 | INTRODUCTION

Disruption of the epigenome is now accepted as a fundamental mechanism in cancer and studies have documented that epigenetic alterations occur during all stages of carcinogenesis particularly, in the initial stages of onset.[1,2] Epigenetic mechanisms include DNA methylation, histone modification, and RNA-based mechanisms. DNA methylation of CpG islands by DNA methyltransferases (DNMTs), is one of the most well-studied epigenetic events.[3,4] Several studies have documented increased expression of DNMTs and identified aberrantly methylated regions in several cancers.[5,6] Modifications of histone proteins mediated by histone acetyltransferases, histone deacetylases (HDACs), histone phosphorylases, histone methyltransferases (HMTs), histone demethylases, and histone ubiquitinases offer another important regulatory platform for gene transcription.

The equilibrium between the action of opposing enzyme families is central for normal gene expression while disequilibrium has been associated with cancer.[7] HDACs which mediate deacetylation of histones are the best-studied histone modifiers, which cause transcriptional silencing of tumor suppressor genes (TSGs).[8] DNA methylation elevates histone acetylation levels thus demonstrating that DNMT and HDAC activity are interlinked and crucial.[9] Histone phosphorylation also plays a central role in the regulation of genes involved in apoptosis and mitosis.[10] HMTs may exert either repressive or permissive marks depending on the site of methylation. H3K9 (eg, G9A) and H3K27 (eg, EZH2) methyltransferases are overexpressed and lead to aberrant TSG silencing.[11] Many TSGs are silenced via a synergistic series of epigenetic events including aberrant DNA hypermethylation and suppressive chromatin modifications.[12,13] Functional silencing of TSGs can contribute greatly towards cellular dysfunction leading to cancer. Several groups of investigators have shown that numerous genes such as RASSF1A, MLH1, BRCA1, WIF-1, CDH1, MGMT, and APC are hypermethylated and can be used as biomarkers or indicators of prognosis.[14]

Epigenetic processes can be reversed and this principle makes it a potential target for therapeutic intervention.[15] Several studies demonstrate that silenced TSGs in cervical cancer cells are reactivated by the use of epigenetic inhibitors.[16,17] Conventional cancer therapies and existing epigenetic modifiers are characterized by low specificity as well as substantial cellular and clinical toxicity, resulting in side effects and/or poor quality of life for the patient.[18] This clearly indicates the need to identify safe chemopreventive and chemotherapeutic agents that can effectively reverse epigenetic changes with a high degree of specificity.

Extensive epidemiological evidence suggests that a diet of fruit and vegetables can prevent a range of human cancers.[19] A wide range of experimental as well as epidemiological data encourages the use of dietary agents to impede or delay different stages of cancer[19] These have been further validated by in vitro studies that demonstrate the anticancer effect of fruit and vegetables derived phytochemicals including their ability to modulate epigenetic pathways.[16,17] While the synthetic epigenetic modifiers that are currently under trial can be categorized as either DNMT inhibitors or HDAC inhibitors, many studies have shown that dietary agents, by contrast, may be able to modulate both HDAC and DNMT enzymes and could, therefore, be a more potent therapeutic option.[16,17] Natural dietary compounds have a high safety profile and provide an alternate approach to cancer prevention and treatment.

Earlier, we have shown that the ubiquitous phytochemical, quercetin brings about antiproliferative, antimigratory, and proapoptotic effect in human cervical cancer cells, HeLa. This study was designed to investigate the epigenetic modulation mediated by quercetin and to better understand its mechanism of action.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

The human cervical carcinoma cell line, HeLa used in this study was a kind gift from Dr. Tahir Rizvi, UAE University, Al Ain, UAE. The cells were maintained in Dulbecco's modified Eagle's medium (Sigma) to which 10% fetal bovine serum (Sigma) and 100X Pen-strep (Sigma) were added. A humidified atmosphere of 5% $CO_2$ in air at 37°C was maintained.

### 2.2 | Reagent preparation

Quercetin (Sigma) was made into a 66.17 mM stock using dimethyl sulfoxide, aliquoted, and stored at −20°C. The working concentration of 1 mM was made in complete medium. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed and 100 μM of quercetin in 24 hours was identified as the $EC_{50}$ of quercetin in HeLa cells (manuscript submitted). Two sub-lethal doses, (25 and 50 μM) were selected to understand the effect of quercetin on epigenetic mechanisms in HeLa cells. Twenty-five micromolar quercetin has 87% cell viability at 24 hours and 80% viability at 48 hours; whereas, 50 μM quercetin has 77% cell viability at 24 hours and 52% viability at 48 hours (manuscript submitted).

## 2.3 | DNMT activity assay

Nuclear extracts from untreated HeLa cells were prepared using EpiQuik Nuclear Extraction Kit (Epigentek) as per the manufacturer's protocol. The effect of various doses of quercetin (25 and 50 μM) on DNMT activity was measured using the Epiquik DNMT Activity Assay Kit (Epigentek) as per the manufacturer's protocol. Briefly, various doses of quercetin were added to the untreated nuclear extract in substrate-coated assay plate and incubated for 1.5 hours at 37°C to allow the action of the enzyme. The products formed during the incubation were quantitated by an enzyme-linked immunosorbent assay (ELISA) based assay and compared to the untreated control wells. The percentage of inhibition in comparison to control was then calculated following the manufacturer's guidelines and plotted as a graph.

## 2.4 | HDAC activity assay

Nuclear extracts from untreated HeLa cells were prepared using EpiQuik Nuclear Extraction Kit (Epigentek) as per the manufacturer's protocol. The effect of various doses of quercetin (25 and 50 μM) on HDAC activity was measured using the Epiquik HDAC Activity Assay Kit (Epigentek) as per the manufacturer's protocol. Briefly, various doses of quercetin were added to the untreated nuclear extract in substrate-coated assay plate and incubated for 1 hour at 37°C to allow the action of the enzyme. The products formed during the incubation were quantitated by an ELISA based assay and compared to the untreated control wells. The percentage of inhibition in comparison to control was then calculated following the manufacturer's guidelines and plotted as a graph.

## 2.5 | HMT-H3K9 activity assay

Nuclear extracts from untreated HeLa cells were prepared using EpiQuik Nuclear Extraction Kit (Epigentek) as per the manufacturer's protocol. The effect of various doses of quercetin (25 and 50 μM) on HMT-H3K9 activity was measured using the Epiquik HMT-H3K9 Activity Assay Kit (Epigentek) as per the manufacturer's protocol. Briefly, various doses of quercetin were added to the untreated nuclear extract in substrate-coated assay plate and incubated for 1.5 hours at 37°C to allow the action of the enzyme. The products formed during the incubation were quantitated by an ELISA based assay and compared to the untreated control wells. The percentage of inhibition in comparison to control was then calculated following the manufacturer's guidelines and plotted as a graph.

## 2.6 | Molecular modeling studies of DNMT, HDAC, G9A, and EZH2 proteins

Docking of quercetin with DNMT1, DNMT3A, DNMT3B, HDAC1, HDAC2, HDAC3, HDAC4, HDAC7, and HDAC8 was performed as described by us earlier.[20] The interaction of 5-Aza-dC, (a known inhibitor of DNMTs) and TSA (known inhibitor of HDACs) was also previously described by us and used as a reference to compare quercetin's interaction. Likewise, G9A (PDB ID: 5VSC) and EZH2 (PDB ID: 5LS6) structure were retrieved from RCSB and prepared for docking.[21,22] Quercetin was docked to these protein structures using SwissDock server[23] and the least energy model was used for further analysis using UCSF-Chimaera.[24]

## 2.7 | Global DNA methylation quantitation assay

Approximately, $2 \times 10^6$ cells were treated with quercetin (25 and 50 μM for 24 and 48 hours) after which DNA was isolated using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) following manufacturer's protocol. To quantitate the amount of methylated DNA found before and after treatment with quercetin, the Methyl-Flash Methylated DNA Quantification Kit (Epigentek) was used. The kit is based on the detection of methylated DNA by 5-mC antibody, which can be estimated colorimetrically. Optical density values are proportional to the amount of methylated DNA irrespective of its position in the genome. The levels of methylation are represented as percentage of control.

## 2.8 | Quantitation of expression of chromatin modifiers using qRT-PCR

A total of $2 \times 10^6$ cells were plated and treated with 25 and 50 μM quercetin for 48 hours. Cells were then harvested, and the total RNA was isolated using GenElute Mammalian Total RNA Kit (Sigma) as per the manufacturer's protocol. Complementary DNA was prepared by using 2 μg RNA as starting template using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and used as a template for qRT-PCR. Untreated HeLa cells were used as control. Human Epigenetic Chromatin Modification Enzymes RT² Profiler PCR Array (Qiagen) was used to profile the expression of genes that modify DNA and histones thereby altering the structure of chromatin and influencing gene expression. This includes DNMTs, demethylases, histone acetylases, deacetylases, methylases, histone phosphorylases, and ubiquitinases. Similarly, the expression of some of the TSGs whose methylation levels were reduced

were tested for alterations in expression using a custom designed TaqMan-based qRT-PCR array (Thermo Fisher Scientific). Normalization was performed with the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase for the chromatin modifiers array, while global normalization was performed for the custom TSG array. Fold change was calculated by ΔΔCT analysis in comparison to the untreated control, using the DataAssist Software (ThermoFisher Scientific) and expressed as a graph. Fold changes above 1.5 was considered as upregulation, while fold change below 0.5 was considered downregulation in keeping with the current qRT-PCR analysis recommendations. Statistical significance was calculated on the mean of three experiments using two-tailed *t* test with $P \leq .05$.

### 2.9 | Quantitation of promoter methylation status of selected TSGs

EpiTect Methyl II PCR Arrays (Qiagen) facilitate the concurrent detection of the promoter DNA methylation of several TSGs. A total of $2 \times 10^6$ cells were plated and treated with 25 and 50 μM quercetin for 48 hours. Cells were harvested, and genomic DNA was isolated using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) as per the manufacturer's protocol. Untreated cells were used as control. 1 μg of DNA from each sample was then subjected to restriction digestion with the EpiTect II DNA Methylation Enzyme Kit (Qiagen) following the manufacturer's protocol. The MethylScreen technology is based on the differential cleavage of target sequences by using two different restriction endonucleases. These restriction enzymes differ in their dependence on the presence or absence of methylated cytosines in their respective recognition sequences. The restriction digest products were used as the template for the Human Tumor Suppressor Genes EpiTect Methyl II Signature PCR Array (Qiagen). This quantitative polymerase chain reaction array results were analyzed to quantitate the amount of DNA remaining after restriction digest and is used to build the methylation profile for each gene. The promoter methylation levels of the tested TSGs in quercetin-treated cells and untreated HeLa cells was represented as a graph. Statistical significance was calculated on the mean of three experiments using two-tailed *t* test with $P \leq .05$.

### 2.10 | Statistical analysis

All data are expressed as means ± SD of at least three experiments. One-way analysis of variance followed by two-tailed *t* test was used to evaluate the results of all biochemical assays and significance was established at $P \leq .05$.

## 3 | RESULTS

### 3.1 | Quercetin treatment reduces DNMT activity

Quercetin was found to inhibit the activity of DNMTs significantly in a dose-dependent manner. When nuclear extracts were incubated with increasing doses of quercetin (25 and 50 μM) they were found to inhibit the function of the DNMTs by 32% and 49% respectively, in comparison to untreated control as shown in Figure 1A.

### 3.2 | Quercetin treatment reduces HDAC activity

Quercetin was found to reduce the activity of nuclear HDACs significantly in a dose-dependent manner

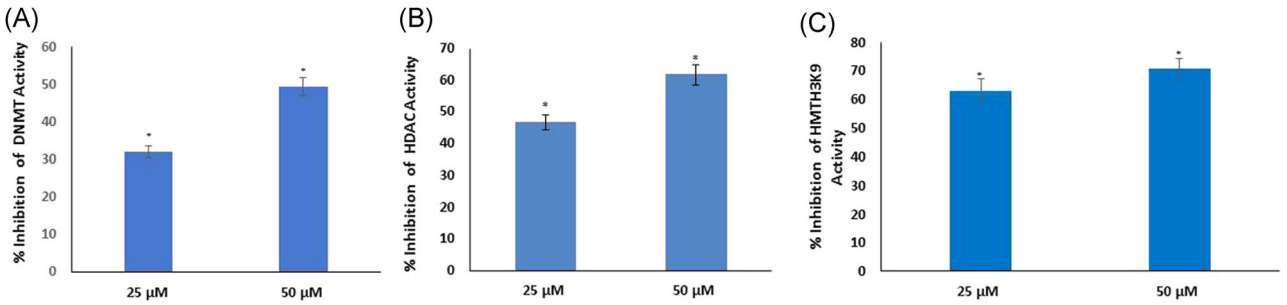


**FIGURE 1** Effect of quercetin on activity of DNMT, HDAC, and HMT H3K9 in HeLa cells. A, Twenty-five and fifty micromolar quercetin-treated HeLa cells demonstrate significant inhibition of DNMT activity in a dose-dependent manner. B, Twenty-five and fifty micromolar quercetin-treated HeLa cells demonstrate significant inhibition of HDAC activity in a dose-dependent manner. C, Twenty-five and fifty micromolar quercetin-treated HeLa cells demonstrate significant inhibition of HMT H3K9 activity in a dose-dependent manner. Values are represented in comparison to untreated control and are means ± SD of three independent experiments. ($P < .05$). DNMT, DNA methyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase

(Figure 1B). When nuclear extracts were incubated with increasing doses of quercetin (25 and 50 μM), they were found to inhibit the function of the HDACs by 47% and 62% in comparison to untreated control.

### 3.3 | Quercetin treatment reduces HMT H3K9 activity

Quercetin is able to reduce the activity of the HMTs that can add between one and three methyl groups to the ninth lysine of histone 3. Nuclear extracts were incubated with increasing doses of quercetin (25 and 50 μM), were found to inhibit the function of the HMT H3K9 by 63% and 71% as shown in Figure 1C.

### 3.4 | Quercetin interacts with the DNMT family and functions as a competitive inhibitor

The docking results strongly suggest that the preferred binding of quercetin on DNMT3A and DNMT3B is within the substrate binding cavity and could competitively inhibit the protein by preventing the entry of the natural ligand into the active site (Figure 2). Docking results of DNMT1 indicates that it may not be competitively inhibited by quercetin. The residues potentially interacting with quercetin are listed in Table 1.

### 3.5 | Quercetin interacts with HDACs and functions as a competitive inhibitor

Docking results indicate that the binding of quercetin is within the substrate binding cavity of various HDAC proteins, namely HDAC2, HDAC8, HDAC4, and HDAC7 and could competitively inhibit their activity (Figure 3 and Table 1). The zinc ion is known to play a crucial catalytic role and in all cases the ligand was found to dock within 5 Å of the zinc ion.

### 3.6 | Quercetin interacts with EZH2 and functions as an inhibitor

Docking results indicate that quercetin is able to mimic the pose of established co-crystallized inhibitor of EZH2 (seen in the PDB structure) (Figure 4A and Table 1). EZH2 plays an important role in DNMT1 recruitment and this interaction could potentially abrogate EZH2 binding with DNMT, as they will compete for binding at the same location.

### 3.7 | Quercetin interacts with G9A and functions as an inhibitor

Docking results indicate that quercetin is able to mimic the pose of established co-crystallized inhibitor of G9A (seen in the PDB structure) (Figure 4B and Table 1). DNMT1 binds to G9A[25]; this binding cavity is the same as the one in which quercetin was observed to dock highlighting its potential to inhibit G9A activity. The decreased HMT H3K9 activity appears to be well correlated with the observed inhibitory action of quercetin

### 3.8 | Quercetin treatment modulates the expression of various enzymes and chromatin modifiers involved in the epigenetic pathway

Quercetin was found to modulate the expression of several genes in the epigenetic pathway. Keeping in mind, the fold change cut-off of 1.5 and 0.5 for downregulation, a shortlist of significant changes was compiled. Quercetin was found to downregulate DNMTs,

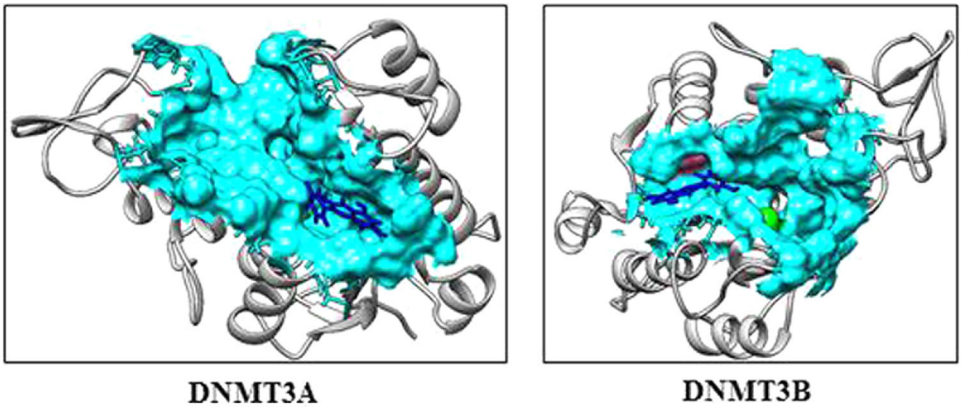


**FIGURE 2** Molecular docking analysis of quercetin with DNMT family of enzymes. The predicted interaction of quercetin (blue) with the active site residues (cyan) in the substrate binding cavity of DNMT3A and DNMT3B is shown. Catalytic residue is represented in green. DNMT, DNA methyltransferase

**TABLE 1** Quercetin interacts with several epigenetic enzymes and may inhibit their action. The energy of the docked model, interacting residues within 5°A of quercetin and its distance from the catalytic residue is listed

| Enzyme | Fullfitness value | Interacting residues | Distance of quercetin from catalytically important residue and hydrogen-bonded interactions |
|---|---|---|---|
| DNMT3A | −1553.7292 | PHE636, ASP637, GLY638, ILE639, SER659, GLU660, VAL661, CYS662, SER665, ASP682, VAL683, ARG684, GLY703, PRO705, CYS706, THR723, LEU726, ARG887, SER888 | 4.58°A from CYS706<br>2.46°A H-bond with VAL683;<br>2.53°A H-bond with PHE636 |
| DNMT3B | −1872.4425 | PHE581, ASP582, GLY583, ILE584, SER604, GLU605, VAL606, CYS607, SER610, ASP627, VAL628, ARG629, GLY648, PRO650, CYS651, ASN652, THR668, LEU671, ARG832, SER833 | 4.47°A from CYS651<br>2.48°A H-bond with VAL628 |
| HDAC2 | −1882.1656 | ASP104, HSD146, GLY154, PHE155, HSE183, GLU208, TYR209, PHE210, LEU276, GLY277, TYR308 | 4.79°A from HSD146 |
| HDAC4 | −1617.528 | PRO676, GLU677, ARG681, SER758, ASP759, PRO800, HIS802, HIS803, MET810, GLY811, PHE812, ASP840, PHE871, PRO942, LEU943, GLY974, GLY975, HIS976 | 2.19°A from HIS803 |
| HDAC7 | −1680.826 | PRO542, ASP626, PRO667, HSD669, HSD670, GLY678, PHE679, CYS680, ASP707, ASN736, PHE737, PHE738, ASP801, PRO809, LEU810, GLU840, GLY841, GLY842, HIS843 | 2.18°A from HIS670 |
| HDAC8 | −1724.0481 | LYS33, ILE34, PRO35, ARG37, TYR100, PRO103, TYR111, TRP141, PHE152, PRO273, MET274, GLY305, TYR306, LEU308 | 3.18°A from TYR306 |
| EZH2 | −4027.2224 | ILE109, MET110, TYR111, SER112, TRP113, ALA622, GLY623, TRP624, GLY625, CYS663, PHE665, THR678, ARG679, ARG685, PHE686, ALA687, ASN688 | 4.13°A from ARG679 |
| G9A | −1475.7151 | LYS1047, MET1048, GLY1049, ASN1112, HIS1113, TYR1154, PHE1158, TRP1159, PHE1166, THR1167, CYS1168, GLN1169, CYS1170 | 2.64°A from TYR1154<br>2.3°A H-bond with HSE1113 |

Abbreviations: DNMT, DNA methyltransferase; HDAC, histone deacetylase

HDACs, and histone phosphorylases. It showed a gene-dependent modulation of HMTs, histone acetylases and ubiquitinases. Twenty-five micromolar quercetin down-regulated the expression of HDAC11, KDM6B, DOT1L, HDAC10, HDAC5, HDAC6, HDAC7, DNMT3A, ESCO1, AURKB, AURKA, DNMT1, AURKC, DNMT3B, NEK6, and KDM5B. It increased the expression of ESCO2 and DZIP3. Whereas, 50 μM quercetin downregulated the expression of AURKC, HDAC5, AURKA, DNMT3A, AURKB, HDAC11, ESCO1, DOT1L, RPS6KA3, KDM5B,

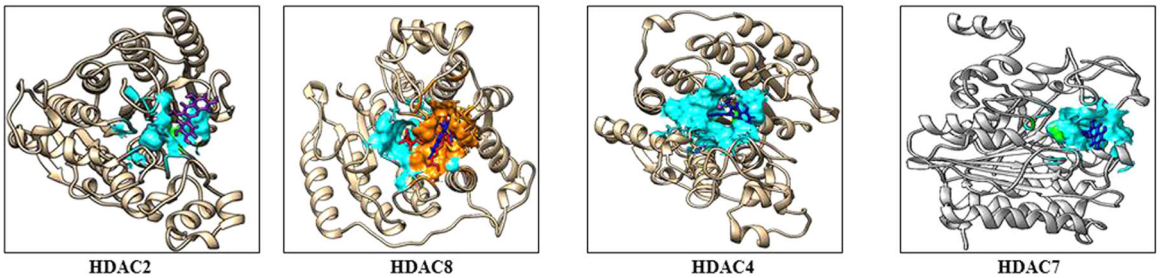


**FIGURE 3** Molecular docking analysis of quercetin with HDAC family of enzymes. The predicted interaction of quercetin (blue) with the active site residues in the substrate binding cavity of HDAC2, HDAC4, and HDAC7. Quercetin docks in the second tunnel (orange) of HDAC8 similar to the co-crystallized inhibitor (red). Catalytic residue is represented in green. HDAC, histone deacetylase

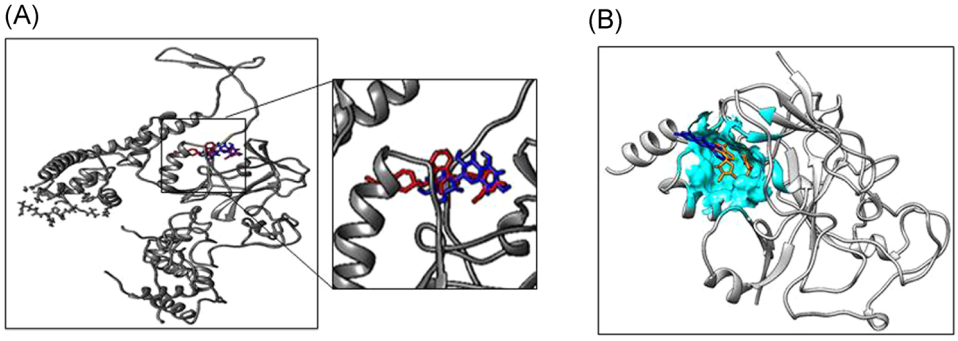


**FIGURE 4** Molecular docking analysis of quercetin with EZH2 and G9A. A, The predicted interaction of quercetin (blue) with EZH2 is represented which overlaps closely with the co-crystallized inhibitor (red). A close-up of the interaction is inset. B, The predicted interaction of quercetin (blue) with G9A is represented which overlaps closely with the co-crystallized inhibitor (orange)

NEK6, HDAC6, EHMT2, HDAC7, HDAC10, DNMT3B, HAT1, HDAC3, DNMT1, HDAC1, and HDAC2. It increased the expression of SETD7, ESCO2, and DZIP3. The graphs are shown in Figure 5.

## 3.9 | Quercetin treatment reduces global DNA methylation

Quercetin mediates a time- and dose-dependent decrease in the global methylation levels of HeLa cells. Twenty-five micromolar treatment in 24 hours brings close to a 50% reduction in methylation, whereas after 48 hours the methylation level is 34% of the control. Fifty- micromolar treatment for 24 hours and 48 hours reduces DNA methylation to 36% and 15% respectively of the control (Figure 6).

## 3.10 | Quercetin treatment reduces the promoter methylation of tested TSGs

A decrease in the activity and expression of DNMTs should reflect in the reduction in promoter CpG methylation. To quantify any changes in the methylation levels, the Methyl II PCR Array was performed after restriction digestion using methylation-sensitive and methylation-dependent enzymes. The methylation percentage of the tested TSGs decreased after 25 and 50 μM quercetin treatment in comparison to untreated control

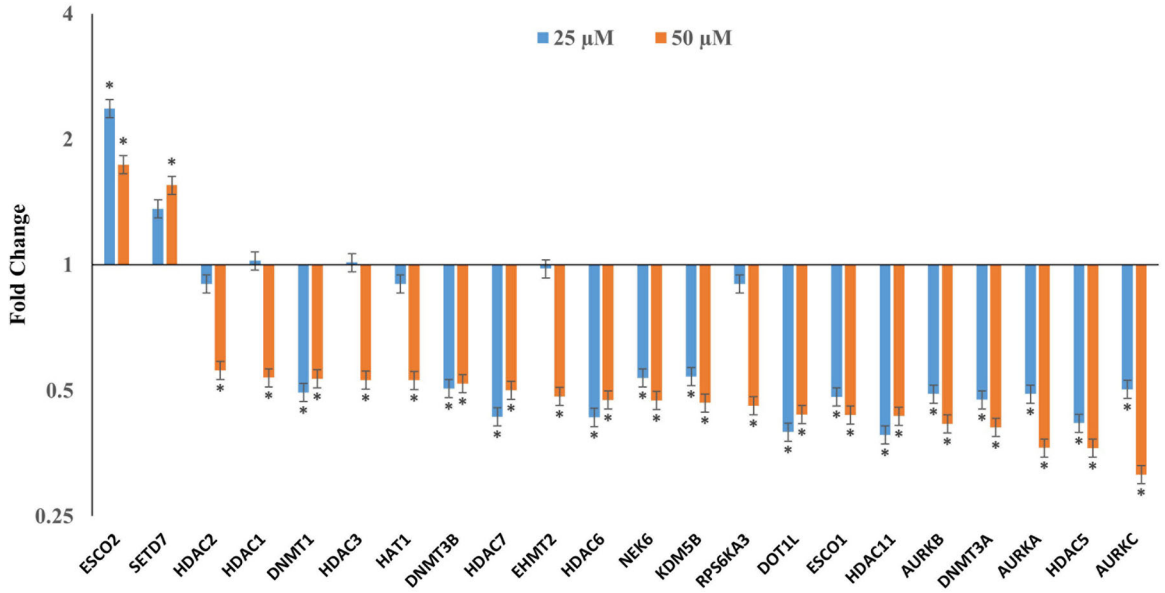


**FIGURE 5** Effect of quercetin on genes involved in chromatin modification. RQ plot of genes involved in the chromatin modification whose expression in HeLa cells is modulated following treatment with 25 and 50 μM quercetin for 48 hours. Fold change was calculated by ΔΔCT analysis in comparison to untreated control after global normalization Values are means ± SD of three independent experiments. ($P \leq .05$). DNMT, DNA methyltransferase; HDAC, histone deacetylase; RQ, relative quantity

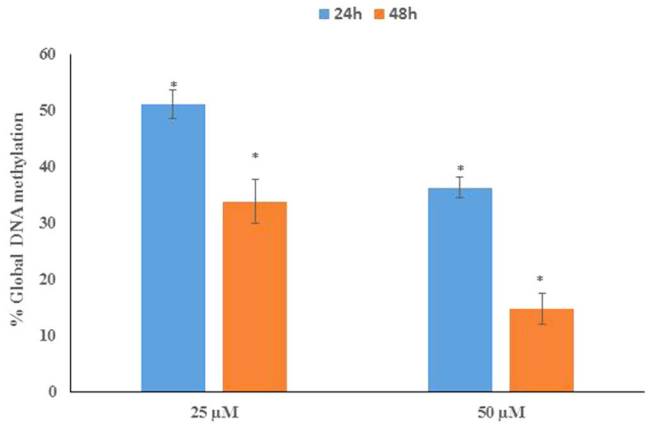


**FIGURE 6** Effect of quercetin on global DNA methylation in HeLa cells. Quercetin (25 and 50 μM) significantly decrease the levels of global DNA methylation in HeLa cells in a time-dependent manner. The decrease is methylation level is represented as a percentage of the untreated control. Values are means ± SD of three independent experiments. ($P \leq .05$)

(Figure 7). Fifty micromolar quercetin treatment decreased the methylation rates of the TSGs to APC (31%), CDH1 (60%), CDH13 (3%), DAPK1 (7%), FHIT (7%), GSTP1 (24%), MGMT (22%), MLH1 (4%), PTEN (11%), RARB (19%), RASSF1 (9%), SOC51 (58%), TIMP3 (11%), and VHL (10%). Likewise, 25 μM quercetin also decreased the methylation rates to APC (31%), CDH1 (62%), CDH13(27%), DAPK1 (31%), FHIT (23%), GSTP1 (38%), MGMT (42%), MLH1 (27%), PTEN (16%), RARB (30%), RASSF1 (20%), SOC51 (64%), TIMP3 (13%),and VHL (21%). In contrast, untreated control showed a higher percentage of promoter methylation for the tested TSGs. (Figure 7).

### 3.11 | Quercetin treatment restores TSG expression fold change

To detect any increase in transcription following the promoter demethylation of TSGs, qRT-PCR was performed. Fifty-micromolar quercetin was found to have increased the transcription of the TSGs, CDH1, MLH1, PTEN, SOC51, TIMP3, and VHL in comparison to untreated control. The fold change or relative quantity (RQ) plot following 25 and 50 μM treatments are shown in Figure 8.

## 4 | DISCUSSION

Aberrant epigenetic chromatin modification, leading to TSG inactivation is recognized as a critical mechanism impacting tumorigenesis. In this study, quercetin was found to modulate the expression and activity of several epigenetic enzymes. MTT assay established the $EC_{50}$ of quercetin in HeLa cells as 100 μM in 24 hours (manuscript submitted); therefore, two sub-lethal doses, 25 and 50 μM quercetin were used in this study.

The central role in epigenetic regulation is played by the DNMT family of enzymes. DNMT1, 3A and 3B are overexpressed in cervical cancer cells when compared with normal cervical epithelium and is correlated to disease progression.[5] Quercetin was found to bring about a significant decrease in the enzymatic activity of DNMTs

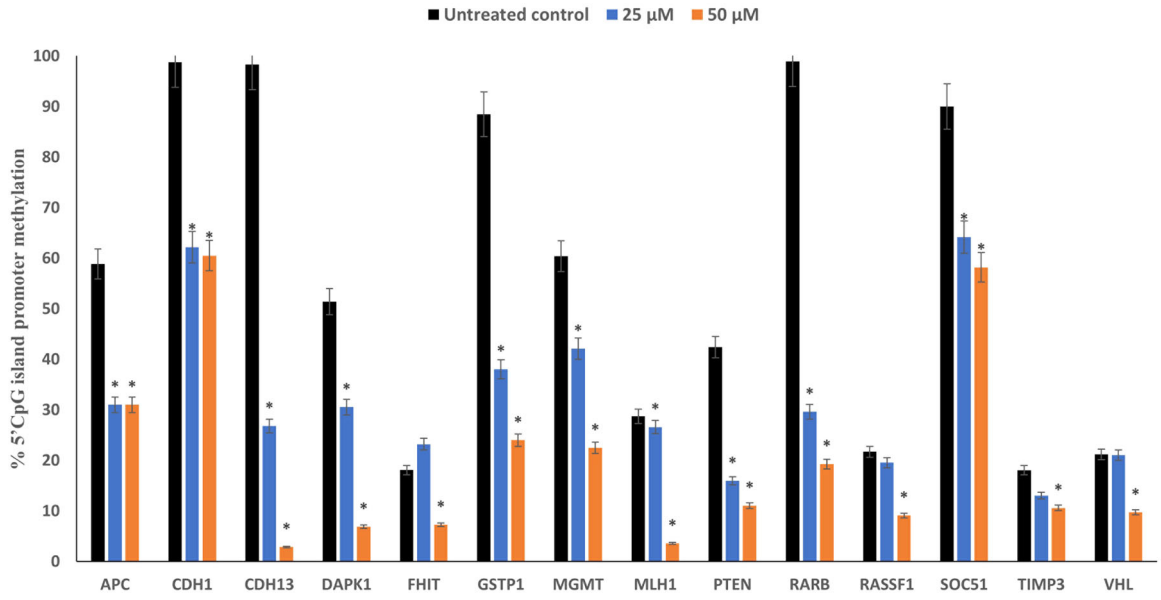


**FIGURE 7** Effect of quercetin on 5′ CpG island promoter methylation of TSGs in HeLa cells using the Human Tumor Suppressor Genes EpiTect Methyl II Signature PCR Array. Quercetin (25 and 50 μM) significantly decreases the promoter methylation levels in HeLa cells in comparison to untreated control. Values are means ± SD of three independent experiments ($P \leq .05$)

(Figure 1A). This decrease correlates well with the downregulation of transcript levels of DNMT1, 3A and 3B (Figure 5). Further, docking studies suggest that quercetin may competitively inhibit DNMT3A and DNMT3B, with the consequent outcome of reduced activity (Figure 2). The polycomb repressor protein, EZH2, which is usually overexpressed in cervical cancer, enables the recruitment of DNMT to target sites.[26] It is interesting, therefore, that docking results suggest that quercetin binding to EZH2 may inhibit its ability to recruit DNMT (Figure 4A). Studies report that the PI3K-AKT pathway and WNT pathway stabilize DNMT1 and contribute to DNA methylation.[27] Remarkably, quercetin promotes a decrease in the PI3K and WNT activity (manuscript submitted). Reduction in the expression and activity of DNMTs has been found to have a positive effect on re-expression of TSGs, loss of cell proliferation, and cell death.[20]

Overexpression of HDAC1, HDAC2, HDAC3, HDAC6, and HDAC7 is found in cervical cancer and is highly correlated to disease stage, progression, angiogenesis, and metastasis.[28,29] Quercetin was able to reduce the activity of class II HDACs significantly, with concomitant downregulation of HDAC1, HDAC2, HDAC6, HDAC7, and HDAC11 expression (Figures 1B and 5). Further, docking results corroborate the reduced activity through direct inhibition of class I HDACs (HDAC2 and HDAC8) and class II HDACs (HDAC4 and HDAC7) (Figure 3). HDAC suppression restores TSG expression, mitigates growth, and induces apoptosis.[20,30]

The expression of histone acetyltransferases is also modulated by quercetin (Figure 5). ESCO1 is required for cell survival and proliferation after DNA damage as well as to control gene expression.[31] The decline in ESCO1 expression after quercetin treatment could explain the cell cycle arrest and cell death seen after quercetin-induced DNA damage (data not shown). Quercetin downregulates HAT1 expression (Figure 5); HAT1 is upregulated in several cancers and in HeLa cells, it is critical for clonogenicity.[32] On the other hand, quercetin upregulates ESCO2; whose function is to repress MMP2 and promote apoptosis.[33]

Histone phosphorylases, AURKA A, B, and C contribute to tumor progression and are overexpressed in cervical cancer.[10] AURKA A, B and C contribute to proliferation, crossing G2-M checkpoint, metastasis, and works co-operatively with HDACs to regulate the protein kinase B pathway.[10] Transcript of all three genes are significantly reduced after quercetin treatment in a dose-dependent manner (Figure 5). RPS6KA3, another phosphorylase which serves as a cancer marker is downregulated after quercetin treatment.[7] NEK6 is overexpressed in cervical cancer and aids in proliferation, metastasis, and helps cross G2-M checkpoint while aiding in DNA damage recovery. It is significant that reduced expression of NEK6 in cancer cells aids apoptosis while normal cells are unaffected by it.[34] Quercetin brings about a dose-dependent reduction in NEK6 expression (Figure 5).

HMTs are modulated by quercetin. SETD7, which functions as a TSG and causes p53 activation; HPV downregulates its expression.[35] SETD7 was found to be overexpressed after 50 μM quercetin treatment. DOT1/KMT4 aids in proliferation, angiogenesis, and G2 stage DNA damage response.[36] DOT1L was also found to decrease with quercetin treatment. G9A/EHMT2 (H3K9 histone methyltransferase) is an oncogene whose overexpression is observed in cervical cancer and together with DNMT causes repression of CDH1 and p53[11] H3K9 methyltransferase activity was significantly reduced by quercetin and this correlates well with the observed downregulation of G9A expression and in silico docking results suggestive of inhibition (Figures 4 and 5). Silencing of G9A has been documented to limit migration and promote apoptosis.[37]

KDM5B, a histone demethylase, particularly demethylates mono-, di- and tri-methylated lysine 4 on histone three. Upregulation of KDM5B is observed in several cancers and represses TSG expression, its downregulation suppresses proliferation, inhibits metastasis, and promotes apoptosis.[38] KDM5B is downregulated by quercetin (Figure 8).

To determine the functional consequence of the vast spectrum of transcriptional changes mediated by quercetin, global DNA methylation, and TSG promoter methylation quantitation assays were performed. Quercetin mediates dose- and time-dependent decrease in global DNA methylation levels (Figure 6). Reversal of promoter methylation of specific TSGs which are aberrantly silenced in cervical cancer is an important therapeutic milestone. Dose-dependent exposure to quercetin resulted in reduced promoter methylation of several TSGs (APC, CDH1, CDH13, DAPK1, FHIT, GSTP1, MGMT, MLH1, PTEN, RARB, RASSF1, SOC51, TIMP3, and VHL) (Figure 7).

DAPK, PTEN, RARβ, RASSF1A, CDH13, MLH1, SOCS1, MGMT, VHL, and FHIT methylation levels are found to be higher in cervical cancer samples than normal and correlate positively with increasing tumor grade.[39-42] APC is a WNT pathway antagonist that regulates migration and apoptosis; it is methylated and silenced in cervical cancer.[43] CDH1 (e-cadherin) methylation was reported to be significantly higher in cervical carcinoma with two-fold increases between CIN lesions.[44] Further, knockdown of EZH2, was found to decrease the H3K27me3 levels in CDH1 promoters and re-establish its expression.[45] Several studies reported the

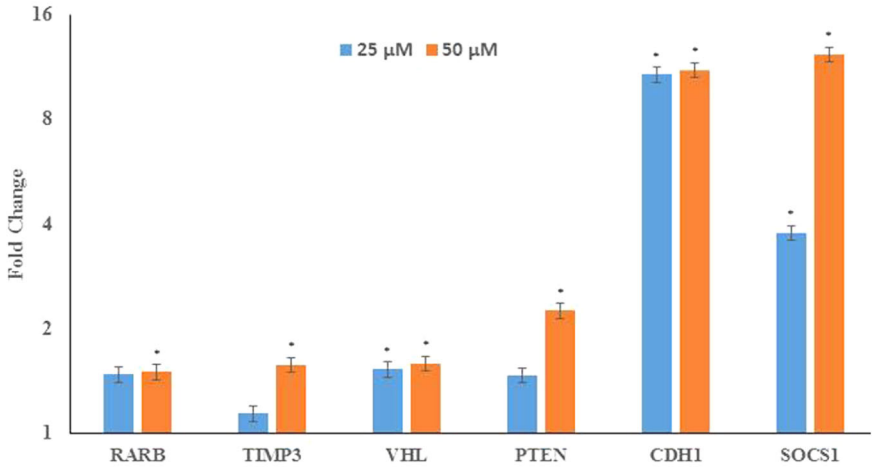


**FIGURE 8** qRT-PCR analysis of the effect of quercetin on TSG expression in HeLa cells. Quercetin (25 and 50 μM) significantly increase the levels of TSG expression in a dose-dependent manner. Fold change was calculated by ΔΔCT analysis in comparison to untreated control after normalization with housekeeping gene. Values are means ± SD of three independent experiments. ($P \leq .05$). qRT-PCR, quantitative reverse transcription PCR; TSG, tumor suppressor gene

decrease in methylation and re-expression of FHIT, DAPK, MGMT, APC, CDH1, and PTEN in cervical cancer cells through the use of demethylating and deacetylating agents.[20,46]

The transcription of these genes was then assessed by qRT-PCR. Quercetin was found to restore the expression of CDH1, MLH1, PTEN, SOC51, TIMP3, and VHL (Figure 8). The restoration of TSG expression following epigenetic modulation explains the mechanism behind the quercetin's anticancer effect, particularly its effect on proliferation, colony formation, migration, and apoptosis. Earlier studies from our lab have shown that inhibition of DNMT and HDAC family by EGCG, sulforaphane, and genistein, promotes anticancer response by lowering promoter methylation and re-expression of TSGs.[20,47,48] These results are supported by the findings of other groups along similar lines.[46,49,50]

Quercetin has been shown to induce apoptosis via inhibition of DNA methylation, HDAC activity, and re-expression of genes in apoptotic pathway in HL60 and U937 leukemia cell lines.[51] Quercetin has been shown to demethylate p16INK4a gene promoter in colon cancer, RKO cell line.[52] Similar epigenetic modulation was also observed in esophageal cancer cell line, Eca9706.[53] These studies effectively highlight the ability of quercetin to modulate epigenetic machinery particularly to reduce promoter methylation and restore expression. However, the bioavailability of quercetin is limited and affected by several factors including gender, source, and form of quercetin (reviewed in).[54-56] Methods to improve bioavailability are actively being sought and techniques such as liposomal and nanoparticle-based delivery and targeted delivery to tumors are finding success.[57-60]

Our study successfully explains the mechanism of action of quercetin, suppressing the expression and activity of epigenetic modulators, with resultant reversal in TSG promoter methylation and attendant restoration of TSG expression. Further, this study comprehensively lists several chromatin modifiers and TSGs, including DNMTs, HDACs, AURKAs, ESCO1/2, NEK6, HAT1, CDH1, MLH1, PTEN, SOC51, TIMP3, and VHL as targets of quercetin action. These results corroborate our earlier investigation showing the antiproliferative, anti-migratory and proapoptotic effects of quercetin (manuscript submitted).

## 5 | CONCLUSION

DNMTs and histone modifiers are the signatory molecules of the epigenetic pathways and are increasingly being studied as roadmaps for cancer treatment. This study allows us to conclude that quercetin may be a powerful arsenal in epigenetic-based chemoprevention strategies. The government and the scientific community have a strong responsibility in ensuring that people are aware of the advantages offered by natural dietary agents and take the right steps in incorporating them into the national public health programs. Incorporation of such dietary agents into our regular diet will go a long way to ensuring apt population-wide chemoprevention strategies. Studies on animal models will further help to substantiate the efficacy of quercetin for therapeutic purposes.

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## CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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