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Phytochemicals induce apoptosis by modulation of nitric oxide signaling pathway in cervical cancer cells

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Abstract. – OBJECTIVE: Nitric Oxide (NO) is produced by NO synthases (NOS) and is a key signaling molecule that regulates tumorigenesis, both aiding and alleviating it. Elevated NO levels are cytotoxic to cancer cells, making NOS an important target for cancer treatment. In the present study, the modulatory effects of the phytochemicals, quercetin, sulforaphane, genistein, and epigallocatechin-3-gallate on NO pathway and apoptosis were shown in HeLa cervical cancer cells.

MATERIALS AND METHODS: Fluorescent microscopy and flow cytometry were used to assess apoptosis. A Griess assay was used to quantitatively measure NO, quantitative PCR array was used to assess the expression levels of genes involved in the NO signaling pathway, and immunocytochemistry was used to determine NOS protein expression. The functional association among the modulated genes was evaluated using network biology analysis, gene set enrichment, and KEGG pathway analysis.

RESULTS: Treatment with the phytochemicals elevated NO levels in HeLa cells and modulated various genes involved in nitric oxide biosynthesis, superoxide metabolism, and oxidative stress, including NOS1, NOS2, NOS3, ALOX12, and SOD2, with a concomitant increase in NOS2 and NOS3 protein expression levels; also, the phytochemicals were found to induce apoptosis.

CONCLUSIONS: These results suggest that the phytochemical-induced cell death is partially attributed to the activation of the NO pathway and upregulation of pro-oxidant ROS generators. Further experimental studies are required to explore this mechanistic association of NO signaling pathway activation and induction of apoptosis in other types of cancer.

Key Words:
Quercetin, Sulforaphane, Genistein, EGCG, Nitric oxide, Phytochemicals, Apoptosis.

Introduction

Nitric oxide (NO) is a free radical that regulates several physiological functions and is formed by the conversion of L-arginine to L-citrulline by nitric oxide synthases (NOS)[1]. There are three known forms of NOS: Neuronal NO synthase (nNOS/*NOS1*), inducible NO synthase (iNOS/*NOS2*) and endothelial NO synthase (eNOS/*NOS3*)[2]. In previous studies, contradictory reports have emerged, describing both the positive and negative effects of NO production on carcinogenesis. Certain reports demonstrated a cytotoxic role of NO; others presented a protective role[3]. The anti-apoptotic effects favor tumor growth and progression and are observed at lower NO concentrations. High NO levels are pro-apoptotic and induce cytotoxicity and high NO levels has been reported to reduce tumor growth and metastasis in several cell lines[1,3-6].

NOS enzymes are key regulators of the redox environment in the cells and initiate production of reactive oxygen species (ROS), which in turn promotes proliferation and tumorigenesis[7]. However, persistently elevated levels of oxidative stress in a cell results in reactive oxygen species-mediated initiation of apoptosis[7]. This may underlie the dual nature of NO in tumor development. Sever-

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al mechanisms for NO-induced apoptosis have been reported previously. The extrinsic pathway of apoptosis has been implicated primarily in NO-mediated apoptosis of tumor cells via sensitization to TRAIL, TNFα, and upregulation of Fas antigen[8]. Upregulation of various pro-apoptotic genes, such as caspase 8, and disruption of mitochondrial membrane potential have been reported in various types of cancer[9,10] molecular mechanisms by which they selectively induce apoptosis are incompletely characterized. We examined the role of nitric oxide (NO). Furthermore, it has been hypothesized that NO influences epigenetic changes of apoptotic genes by regulating the function of DNA methyltransferases[7].

It is well accepted that plant-based diets reduce the risk of cancer and that phytochemicals, owing to their safe toxicity profiles, may hold the key to improved cancer treatment strategies. Several phytochemicals have been shown to modulate NO levels and induce apoptosis in various types of cancer cells, warranting the study of unexplored phytochemicals[9,11-13] molecular mechanisms by which they selectively induce apoptosis are incompletely characterized. We examined the role of nitric oxide (NO). The dietary agent quercetin (Q), sulforaphane (SFN), genistein (GEN), and epigallocatechin-3-gallate (EGCG) have been shown to possess apoptosis-inducing, anti-cancer and anti-inflammatory properties[14-17]. In the present study, the impact of these phytochemicals on NO production, expression of redox and NO pathway-associated enzymes, and apoptosis was studied in cervical cancer cell lines, HeLa.

Materials and Methods

Cell Culture

The human cervical carcinoma cell line, HeLa, was kindly gifted by Dr Tahir Rizvi, (United Arab Emirates University, UAE). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (all from Sigma-Aldrich; S. Louis, MO, USA) and incubated at 37°C in a humidified incubator with 5% CO_2.

Preparation of the Phytochemicals

Q, EGCG, SFN, and GEN were obtained from Sigma-Aldrich; Merck KGaA, S. Louis, MO, USA. A 10 mM stock solution of each phytochemical was prepared; Q and GEN in dimethyl sulfoxide (DMSO), and SFN in serum-free DMEM, and the stock solutions were stored at -20°C. For EGCG, a 10 mM solution was freshly prepared in serum-free DMEM when required. Further dilutions were prepared in complete medium.

Nuclear Morphology Analysis

Nuclear morphology analysis was performed using propidium iodide (PI) staining and was used to determine whether the phytochemicals increased apoptotic death. Briefly, ~$3x10^5$ cells/ml were seeded on glass coverslips and incubated overnight. Subsequently, the cells were treated with 50 µM Q, 5 µM SFN, 50 µM GEN, and 50 µM EGCG. After treatment, cells were fixed using 1:1 of acetone: methanol at -20°C for 10 min, washed with phosphate-buffered saline (PBS), (pH 7.4) twice and stained with PI (10 µg/ml) for 30 sec in the dark at room temperature. The coverslips were then washed with PBS, mounted on a slide, and observed at a wavelength of 515 nm using a Progress Fluorescent Microscope (Olympus Corporation, Tokyo, Japan).

Cell Cycle Analysis Using Flow Cytometry

For cell cycle analysis, ~$2x10^6$ cells were plated in each flask and treated separately with Q (50 µM for 24 or 48 h), SFN (5 µM for 24 or 48 h), GEN (50 µM for 24 or 48 h) and EGCG (50 µM for 24 or 48 h). Following treatment, the cells were harvested and fixed with 70% ethanol. Fixed cells were washed twice with PBS counted and stained using a solution containing propidium iodide (PI; 50 mg/ml), 0.1% Triton X-100 and 100 mg/ml RNase A for 45 min at 37°C. The samples were loaded on a BD-FACS flow cytometry (BD Biosciences, San Jose, CA, USA), and the data were collected and analyzed using FlowJo® (FlowJo LLC, Ashland, OR, USA). Cell cycle analysis was performed on the basis of the quantification of DNA and cells with <2n DNA content (sub-G0) were categorized as the apoptotic population.

Nitric Oxide Detection Assay

To detect the effect of the phytochemicals on NO levels, A Griess Reagent system kit (Promega Corporation, Madison, WI, USA) was used. For detection, ~$1x10^4$ cells were plated and treated separately with 25, 50 or 100 µM Q; 1, 5 or 12 µM SFN; 25, 50 or 100 µM GEN; or 25, 50 or 100 µM EGCG for 24 or 48 h. The assay was performed according to the manufacturer's protocol. For precise NO detection from the treated HeLa cells, a reference curve using nitrite standards was plot-

ted. The absorbance was measured between 520 nm and 550 nm and the concentration was estimated using the curve.

Nitric Oxide Biosynthesis Pathway qPCR Array

To study the modulation of genes controlled by or involved in the signaling of NO, qPCR was performed. The expression of genes involved in NO biosynthesis, superoxide metabolism, oxidative stress response, and genes induced or repressed by NO was assayed. For qPCR, ~$1x10^6$ cells were plated and treated separately with 50 µM Q, 5 µM SFN, 50 µM GEN or 50 µM EGCG for 48 h, and subsequently the RNA was extracted, and cDNA was produced from 2 µg RNA. The RNA was used as the template for the Human Nitric Oxide Signaling Pathway RT² Profiler PCR Array (Qiagen, Inc., Toronto, Ontario, Canada). The array was run on an ABI QuantStudio 3 and ΔΔCq analysis was performed using DataAssist™ software (Thermo Fisher Scientific, Inc. Waltham, MA, USA). The results were compared against an untreated control, with global normalization. Relative quantitation (RQ) indicated the fold change associated with each gene.

Immunocytochemistry of NOS2 and NOS3 Expression

To explain the observed increases in NO levels following treatment, immunocytochemistry analysis was performed using antibodies against NOS2 and NOS3. For analysis, ~$2.5x10^5$ cells were plated on coverslips and treated separately with 50 µM Q, 5 µM SFN, 50 µM GEN or 50 µM EGCG for 48 h. Post-treatment, cells were fixed with ethanol. Fixed cells were washed briefly in PBS, incubated with a primary antibody raised in mouse, washed and incubated with the secondary antibody. Color development was performed using diamino-benzene (DAB) (Sigma-Aldrich, S. Louis, MO, USA) and the cells were imaged using an Olympus camera (Shinjuku, Tokyo, Japan) attached to an inverted microscope (Labomed, Los Angeles, CA, USA). The intensity of color development was directly proportional to the protein levels.

Network Analysis

Identification of functional associations among the assessed pro-oxidants and anti-oxidants was assessed using GeneMANIA (genemania.org/). This analysis identifies the significant regulatory biological processes and associated vital roles of the participating genes[18]. GeneMANIA is a freely accessible platform for the visualization of interactive functional associations among the genes of interest based on genomics, proteomics and transcriptomics data available in public repositories. The prioritization of the interactions was performed based on ranks computed, taking into consideration physical interactions, co-expression, co-localization, published literature and shared protein domains[19]. The webserver provides consensus results of both direct and indirect gene interactions iterated from BioGRID (Biological General Repository for Interaction Datasets), MINT (Molecular INTEraction database), Reactome, Human Protein Reference Database and IntAct[20-24].

Functional and Enrichment Analysis

Gene ontology (GO) analysis of the expressed genes following treatment with the phytochemicals was performed using the PANTHER classification system (http://www.pantherdb.org)[25]. This classification system is based on evolutionary relationships, pathways, experimental and electronic annotations. PANTHER based categorization of the data with respect to the underlying biological processes and cellular functions was performed using the default parameters. Furthermore, KEGG (Kyoto Encyclopedia of Genes and Genomes) based pathway analysis was performed to determine the participation of the queried genes in significant biological pathways[26].

Statistical Analysis

Data are presented as the mean ± standard deviation of at least three experiments. A one-way ANOVA with a two-tailed *t*-test was used to compare the data using GraphPad statistical tools. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Phytochemicals Mediate Apoptosis and Cell Cycle Arrest

Microscopic examination and nuclear morphology assessment of the cells treated with Q (50 µM for 24 and 48 h), SFN (5 µM for 24 and 48 h), GEN (50 µM for 24 and 48 h) and EGCG (50 µM for 24 and 48 h) showed apoptotic changes, including nuclear condensation, fragmentation and formation of apoptotic bodies (Figure 1A and B). Flow cytometry analysis showed that these phytochemicals resulted in cell cycle arrest in the G2-M phase with the accumulation of cells in sub-G0

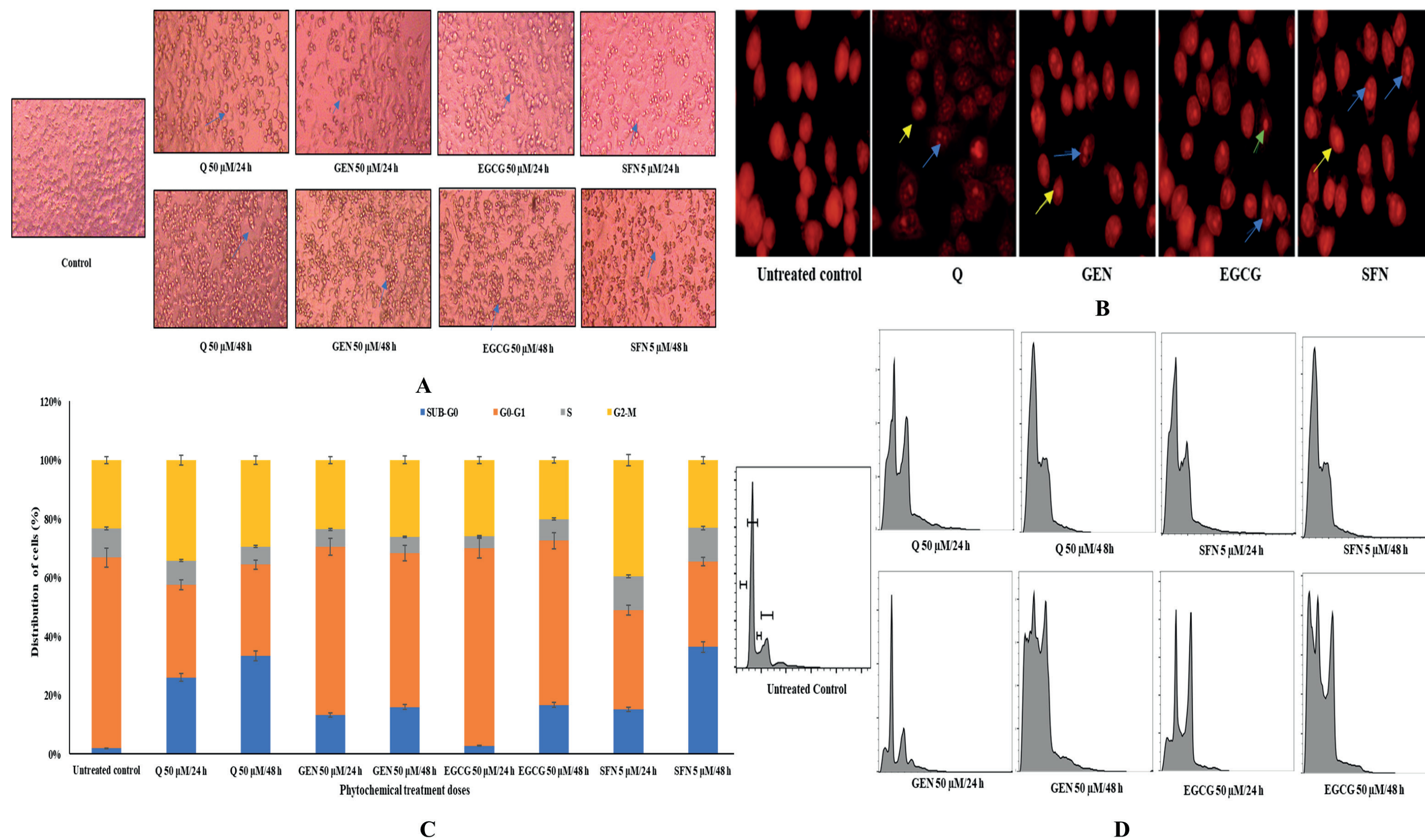

Figure 1. **A,** Microscopic images of HeLa cells following treatment with the selected phytochemicals (Q, SFN, GEN, and EGCG) (10x). In comparison to untreated control, treated cells show a high degree of cell death, rounding off of cells and apoptotic bodies. Blue arrows indicate rounded, dead cells. **B,** Nuclear morphology changes of phytochemicals treated (Q, SFN, GEN, and EGCG), PI-stained HeLa cells assessed by fluorescence microscopy (100x). In comparison to untreated control, which showed a prominent nucleus, treated cells showed chromatin condensation (yellow), fragmentation (blue), and apoptotic bodies (green). **C,** Effect of the phytochemicals (Q, SFN, GEN, and EGCG) on the cell cycle of HeLa cells in comparison to untreated control. Treated cells show an increase in the population of sub-G0 apoptotic population. **D,** Graph showing the distribution of cells in the different phases of the cell cycle.

phases in a dose-dependent manner (Figure 1C). The increase in the proportion of cells in sub-G0 phases compared with the untreated control reflected the occurrence of apoptosis.

NO Levels are Increased Following Treatment with the Phytochemicals in a Dose and Time-Dependent Manner

Following the individual treatments with the four selected phytochemicals, HeLa cells showed a significant increase in NO levels compared with the untreated control group. The levels of NO increased in a dose and time-dependent manner following treatment with the phytochemicals (Figure 2). After treatment with 50 μM Q for 48 h, NO levels increased to 2.23 μM, whereas 5 μM SFN treatment for 48 h increased NO levels to 2.75 μM, 50 μM GEN treatment increased NO levels to 2.37 μM and 50 μM EGCG increased NO levels to 2.58 μM.

NOS2 and NOS3 Protein Expression are Increased Levels Following Treatment with the Phytochemicals

Immunocytochemistry based detection of NOS2 and NOS3 was performed using specific antibodies and color development with DAB. Phytochemical treated cells showed an increase in the intensity of DAB staining compared with the untreated control for both proteins (Figure 3). The increase in signal intensity is associated with the increase in the expression levels of the respective proteins. Elevated NOS2 and NOS3 expression was concurrent with the increase in NO levels and transcript analysis.

Treatment with the Phytochemicals Increases the mRNA Expression Levels of NOS Enzymes

To ascertain the role of NOS enzymes in the detected elevation of NO after treatment with

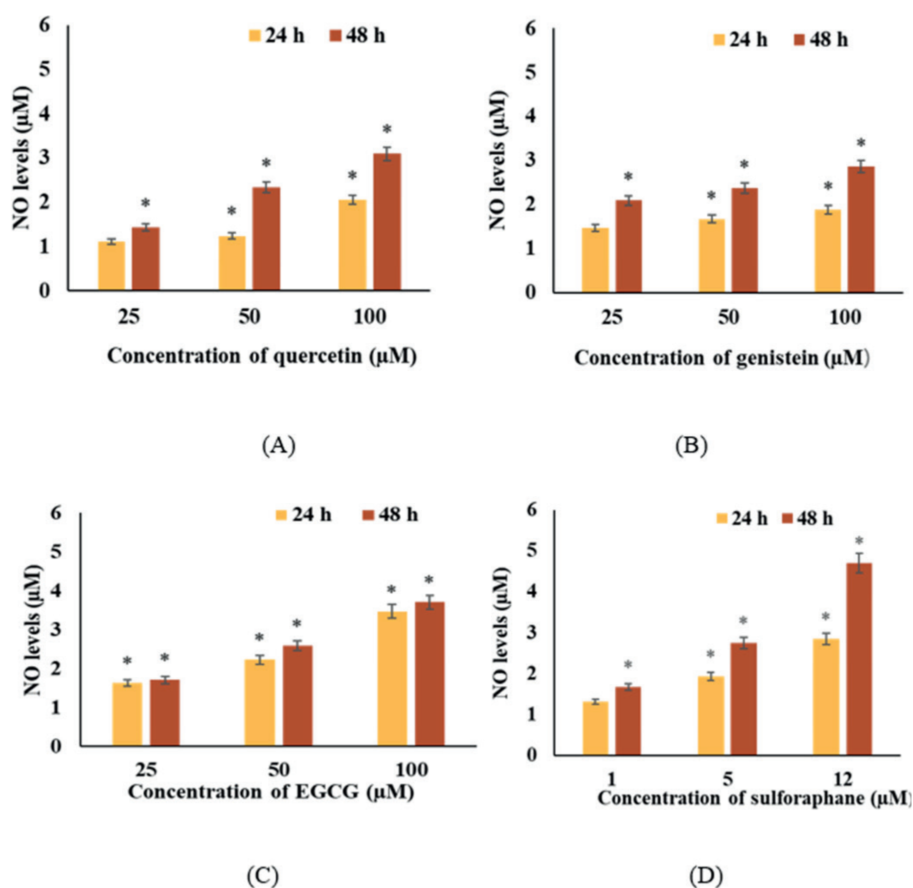

Figure 2. Effect of various concentrations of the selected phytochemicals, Q (A), GEN (B), EGCG (C), and SFN (D), on NO levels in HeLa cells. Treated cells elevated NO levels in a dose and time-dependent manner, in comparison to untreated control cells that showed a value of 1 μM. The significance was established at $p \leq 0.05$.

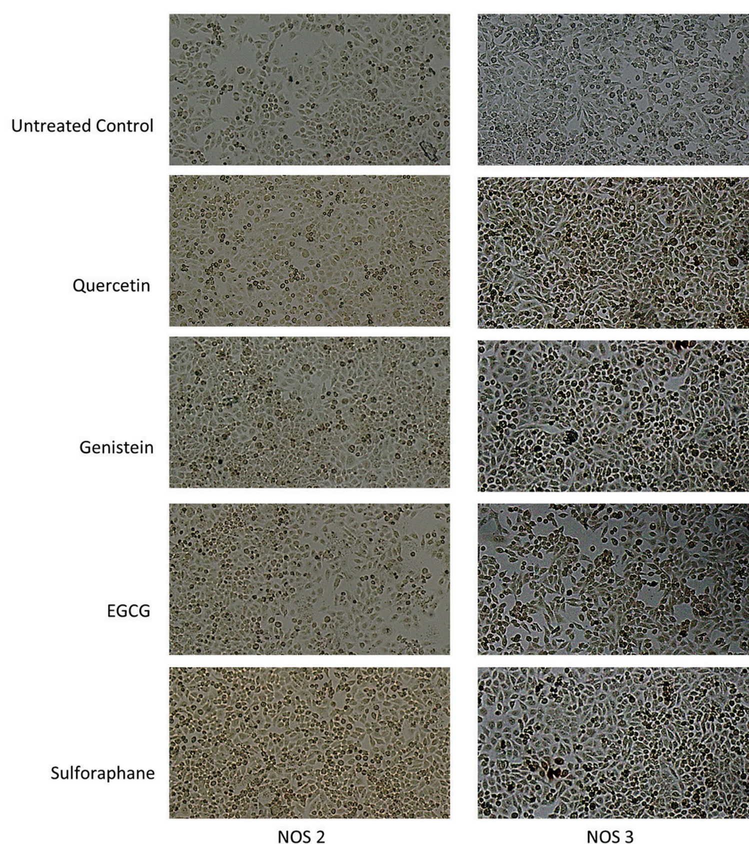

Figure 3. Representative immunocytochemistry images showing the effect of the phytochemicals (Q, SFN, GEN, and EGCG) on *Left*: NOS2 levels in HeLa cells; Right: NOS3 levels in HeLa cells (20x). In comparison to untreated control cells, treated cells showed an increased intensity of DAB staining corresponding to increased protein levels.

the phytochemicals, qPCR-based array was performed. *NOS1* (nNOS) levels were significantly increased following treatment with all the four phytochemicals (Figures 4-7). *NOS3* (eNOS) was found to be upregulated in response to GEN and Q treatment. *NOS2* (iNOS) levels also increased when treated with GEN (RQ 1.7), SFN (RQ 1.9) and EGCG (RQ 2.6). The increase in nNOS, eNOS and iNOS mRNA expression levels were concurrent with the observed increase in NO levels following treatment with the phytochemicals.

Phytochemicals Modulate the Expression of Enzymes Involved in the Maintenance of Redox Balance in the Cells

The expression of various enzymes that impact NO biosynthesis and redox balance in the cells were evaluated. The enzymes with RQ increases ≥1.5 were considered as upregulated whereas, those with RQ values <0.5 were considered downregulated. RQ plots of the genes modulated by the phytochemicals are shown in Figures 4-7 and Table I. The positive regulators of NO biosynthesis were found to be upregulated following treatment with the phytochemicals. The levels of *HSP90AB1*, which is generally regulated in a positive manner by NO, were found to be upregulated following treatment with SFN. *CCNA1* is suppressed by NO and was found to be downregulated following treatment with Q and SFN. Several genes involved in NO signaling pathways were modulated, namely *PRKAR1B*, which was upregulated following treatment with all four phytochemicals. Additionally, *CAMK1* levels were upregulated in cells treated with GEN, and *GRIN2D* expression levels were downregulated in cells treated with GEN and EGCG. Transcriptional elevation of several genes involved in superoxide metabolism was observed. Amongst the genes involved in superoxide release, *ALOX12* and *NOX5* were upregulated following Q treatment, and *ALOX12* expression was also upregulated following SFN treatment. *PRG3* expression was upregulated

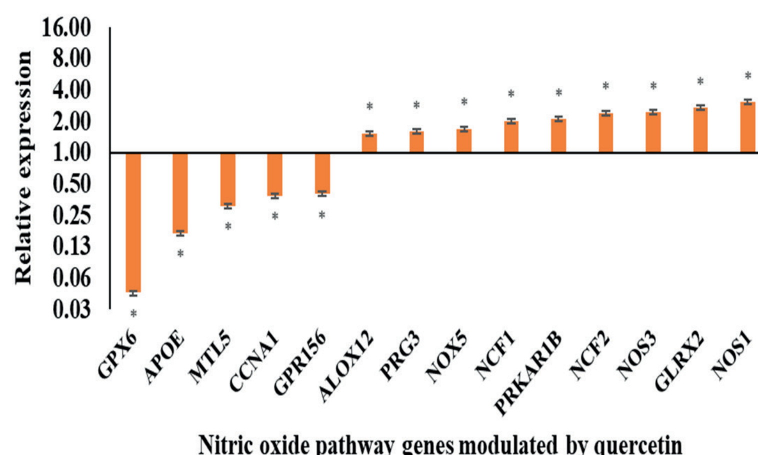

Figure 4. RQ plot of genes involved in the NO pathway whose expression in HeLa cells is modulated following treatment with 50 µM Q for 48 h. ΔΔCT analysis with global normalization was performed. Significance established at $p \leq 0.05$.

following treatment with all of the phytochemical assessed. Expression of *SOD3*, which has oxidoreductase activity, was downregulated following treatment with SFN and EGCG, whereas GEN treatment downregulated *SOD2* expression, which possesses superoxide dismutase activity. *NCF1*, which is also involved in superoxide metabolism, was upregulated in cells treated with Q; whereas, *NCF2* expression was upregulated following treatment with SFN. Amongst the genes with anti-oxidant activity, *ApoE* expression was downregulated following treatment with Q, GEN and EGCG. Amongst the genes with glutathione peroxidase activity, *GPX4* expression was downregulated in cells treated with SFN and EGCG; and Q treatment resulted in downregulation of *GPX6*. *GPR156* expression was downregulated following treatment with Q, GEN, and EGCG. The expression of *GLRX2*, a transcriptional regulator that protects against stress, was upregulated following treatment with Q, SFN, and GEN. A comparative account of the genes modulated by these phytochemicals is presented in Table I.

Network Analysis

Functional associations among the expressed query genes were identified by the generation of related networks (Figures 8-11). The computation of the percentage of physical interactions, co-expression, co-localization, expression, and other interactions was considered to generate genetic networks. The size of the node represents

Table I. Comparative analysis of the modulation of antioxidants and pro-oxidants at transcript level by the phytochemicals, quercetin, genistein, EGCG and sulforaphane to raise the oxidative stress of cancer cells.

Phytochemical	Pro-oxidants upregulated	Antioxidants downregulated
Quercetin	ALOX12, PRG3, NOX5, NCF1, PRKAR1B, NCF2, NOS3, GLRX2, NOS1	GPX6, APOE, MTL5, CCNA1, GPR156
Genistein	PRKAR1B, CAMK1, NOS2, DYNLL1, NOX5, DUOX2, NQO1, GLRX2, SCRT2, NOS3, NOS1, SEPP1, PRG3	MTL5, APOE, SCARA3, GPR156, SOD2, GRIN2D
EGCG	NOS2, PRKAR1B, NOS1, PRG3 GRIN2D, VIMP, SOD2, GPX1, GPR156, SCARA3	NOS1AP, APOE, SOD3, GPX4,
Sulforaphane	DUOX2, CAMK1, HSP90AB1, NOS2, ALOX12, NCF2, NQO1, GLRX2, NOS1, SRXN1, PRKAR1B, SCRT2, PRG3	SOD3, CCNA1, GPX4

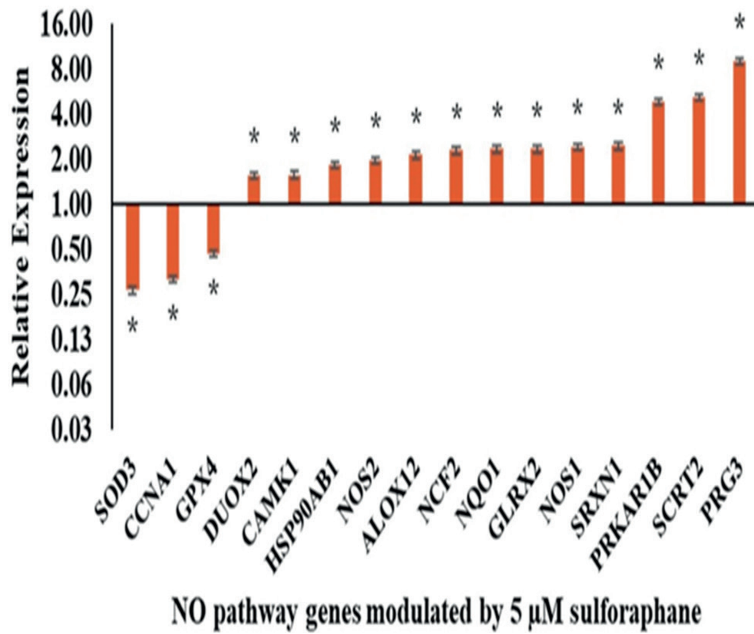

Figure 5. RQ plot of genes involved in the NO pathway whose expression in HeLa cells is modulated following treatment with 5 µM SFN for 48 h. ΔΔCT analysis with global normalization was performed. Significance established at $p \leq 0.05$.

how essential the gene was for the robustness and maintenance of the network (a larger node implies it is relatively more essential). The degree of the essential node represents the importance of the node to maintain the network and the resultant underlying regulatory network of the expressed genes. The color of the edges (interaction) represents the type of the iterated interaction from the databases. For better interactive visualization of the networks, a force-directed layout was used.

Functional and Enrichment Analysis

The modularity of anti-oxidants and pro-oxidants after treatment with phytochemicals was computed based on an average fold change in the expression values. Based on the average fold changes and literature iterations, GO ontology analysis classified the query gene sets amongst the major regulatory processes (Figure 12). Treatment with the phytochemicals significantly affected the genetic regulatory pathways, including

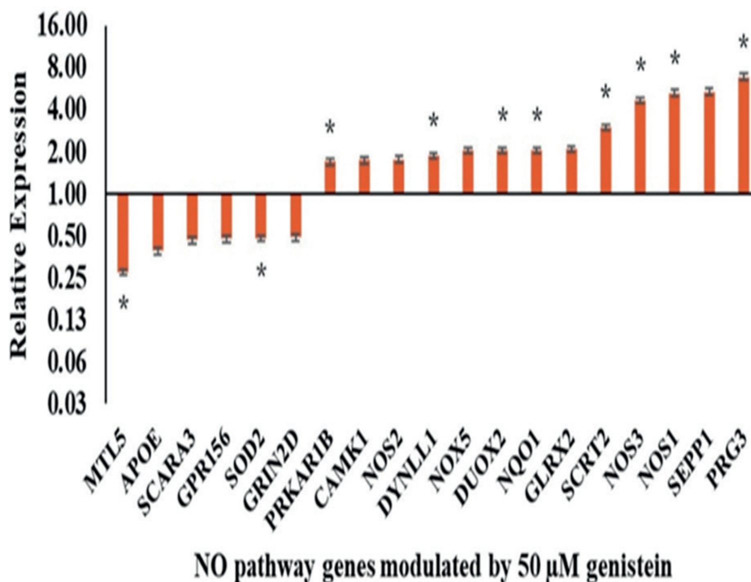

Figure 6. RQ plot of genes involved in the NO pathway whose expression in HeLa cells is modulated following treatment with 50 µM GEN for 48 h. ΔΔCT analysis with global normalization was performed. Significance established at $p \leq 0.05$.

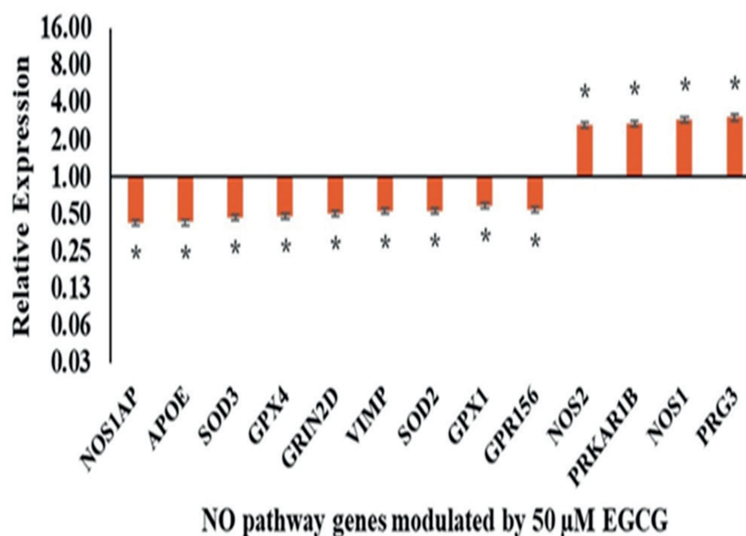

Figure 7. RQ plot of genes involved in the NO pathway whose expression in HeLa cells is modulated following treatment with 50 μM EGCG for 48 h. ΔΔCT analysis with global normalization was performed. Significance established at $p \leq 0.05$.

epigenetic gene regulation, cell proliferation, cholesterol metabolic process, amino acid regulation, regulation of apoptotic process, circadian rhythm, nitric oxide biosynthetic process, and others. Furthermore, KEGG pathway-based analysis showed that treatment with the phytochemicals affected the apoptosis process, calcium signaling pathways, and cancer-related pathways (Table II).

Discussion

Redox-based chemotherapeutic strategies can be geared towards expending anti-oxidant defenses or increasing oxidative damage, resulting in the activation of cell death pathways[27]. As a cancer therapeutic strategy, suppression and elevation of NO have both been attempted. In our

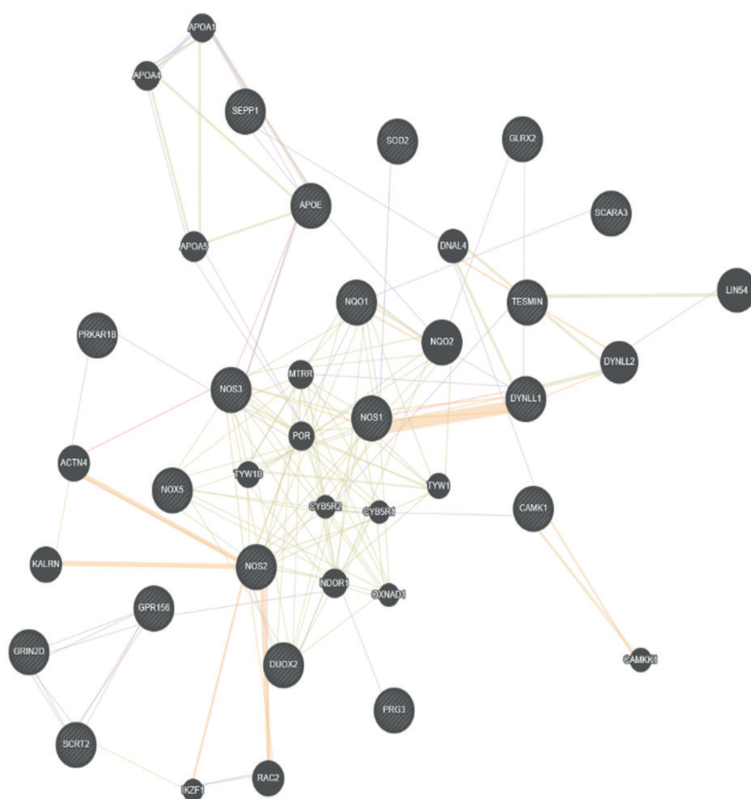

Figure 8. Network based on expression and functional associations of input target genes on treatment with Genistein (Predicted = 62.97%, shared protein domains = 19.62%, co-expression = 11.41%, co-localization = 5.46%, physical interaction = 0.54%).

Table II. KEGG pathway enrichment analysis of target genes under study. Total = total number of genes participating in pathway, Hits=total number of hits in pathway, FDR =adjusted *p*-value.

S. No.	Pathway	Total	Hits	*p*-value	FDR
Genistein					
1	Calcium signaling pathway	188	7	7.89e-07	0.000251
2	Arginine biosynthesis	21	3	2.95e-05	0.00469
3	Fluid shear stress and atherosclerosis	139	5	4.52e-05	0.00479
4	Alzheimer's disease	171	5	0.000121	0.00963
5	HIF-1 signaling pathway	100	4	0.000193	0.0123
6	Arginine and proline metabolism	50	3	0.000411	0.0218
7	Apelin signaling pathway	137	4	0.000641	0.0262
8	Estrogen signaling pathway	138	4	0.000659	0.0262
9	Oxytocin signaling pathway	153	4	0.000971	0.0343
10	Glioma	75	3	0.00135	0.0405
11	Pertussis	76	3	0.0014	0.0405
12	IL-17 signaling pathway	93	3	0.0025	0.0663
13	Circadian entrainment	97	3	0.00282	0.069
14	Pathways in cancer	530	6	0.00371	0.0843
Sulforaphane					
1	Fluid shear stress and atherosclerosis	139	7	7.07e-08	1.12e-05
2	Estrogen signaling pathway	138	5	3.46e-05	0.00258
3	Pancreatic cancer	75	4	5.22e-05	0.00258
4	Glioma	75	4	5.22e-05	0.00258
5	Pertussis	76	4	5.5e-05	0.00258
6	Oxytocin signaling pathway	153	5	5.68e-05	0.00258
7	MAPK signaling pathway	295	6	0.00013	0.0041
8	Prostate cancer	97	4	0.000143	0.0041
9	Transcriptional misregulation in cancer	186	5	0.000144	0.0041
10	Alzheimer's disease	171	4	0.000341	0.0755
Epigallocatechin-3-gallate					
1	Calcium signaling pathway	188	4	0.000489	0.0755
2	Arginine biosynthesis	21	2	0.000823	0.0755
3	Circadian entrainment	97	3	0.00095	0.0755
4	Arginine and proline metabolism	50	2	0.00464	0.255
5	Amyotrophic lateral sclerosis (ALS)	51	2	0.00482	0.255
6	Peroxisome	83	2	0.0124	0.561
7	Endocrine resistance	98	2	0.017	0.561
8	Choline metabolism in cancer	99	2	0.0173	0.561
9	HIF-1 signaling pathway	100	2	0.0176	0.561
10	AMPK signaling pathway	120	2	0.0248	0.718
11	FoxO signaling pathway	132	2	0.0297	0.731
12	Apelin signaling pathway	137	2	0.0318	0.731
Quercetin					
1	Fluid shear stress and atherosclerosis	139	4	2.99e-05	0.00675
2	Phagosome	152	4	4.24e-05	0.00675
3	Arginine biosynthesis	21	2	0.00038	0.0356
4	Leukocyte transendothelial migration	112	3	0.000448	0.0356
5	Platelet activation	124	3	0.000604	0.0384
6	Oxytocin signaling pathway	153	3	0.00111	0.059
7	Arginine and proline metabolism	50	2	0.00217	0.0985
8	VEGF signaling pathway	59	2	0.00301	0.11

previous study, as well as other previous studies, the phytochemicals Q, SFN, GEN, and EGCG were shown to possess anti-cancer and pro-apoptotic properties[14,17,28,29]. In the present study, it was shown that these phytochemicals increased NO levels compared with the untreated samples in a dose-dependent manner. Whilst this appeared to contradict the classical mechanism of NO suppression for anti-cancer effects, in recent years, several effective anti-cancer agents, as well as

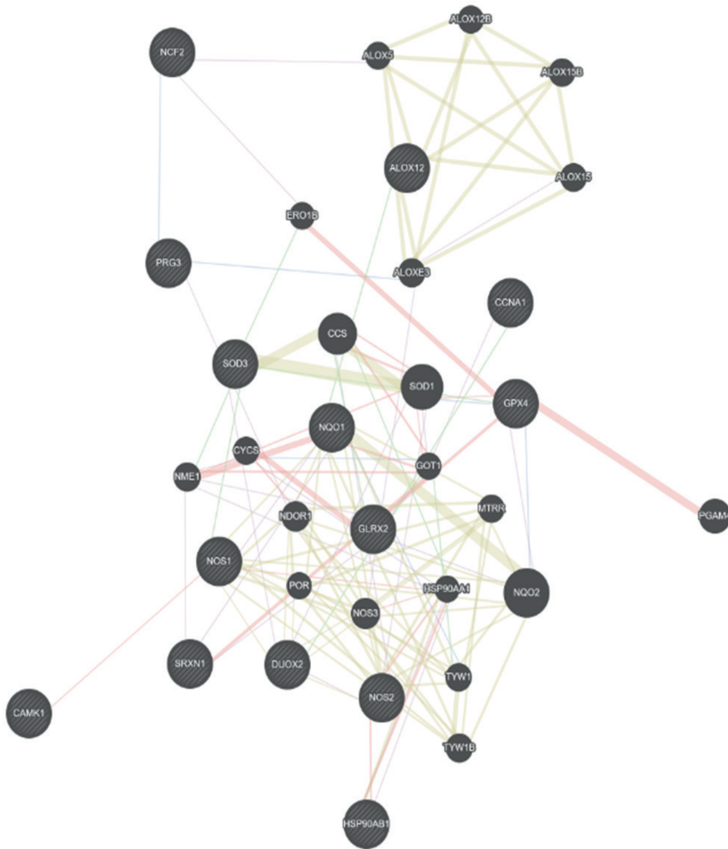

Figure 9. Network based on expression and functional associations of input target genes on treatment with Sulforaphane (Physical interaction = 48.32%, shared protein domains = 31.57%, co-expression = 15.79%, co-localization = 2.28%, genetic interaction = 2.04%).

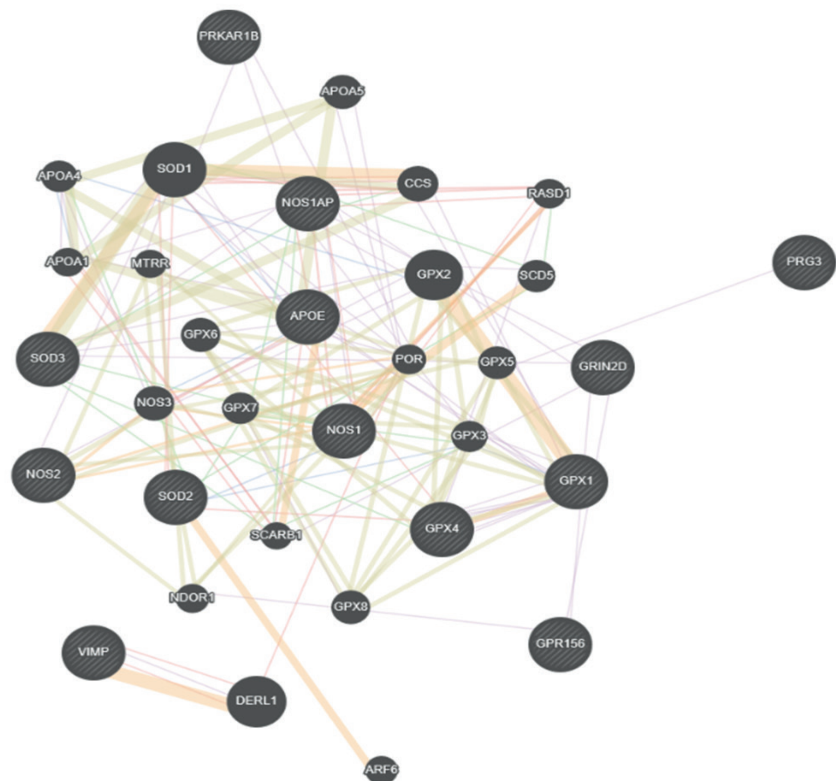

Figure 10. Network based on expression and functional associations of input target genes on treatment with Epigallocatechin-3-gallate (Co-expression = 34.46%, shared protein domains = 22.52%, predicted = 17.91%, physical interactions = 17.52%, co-localization = 7.26%, genetic interaction = 0.33%).

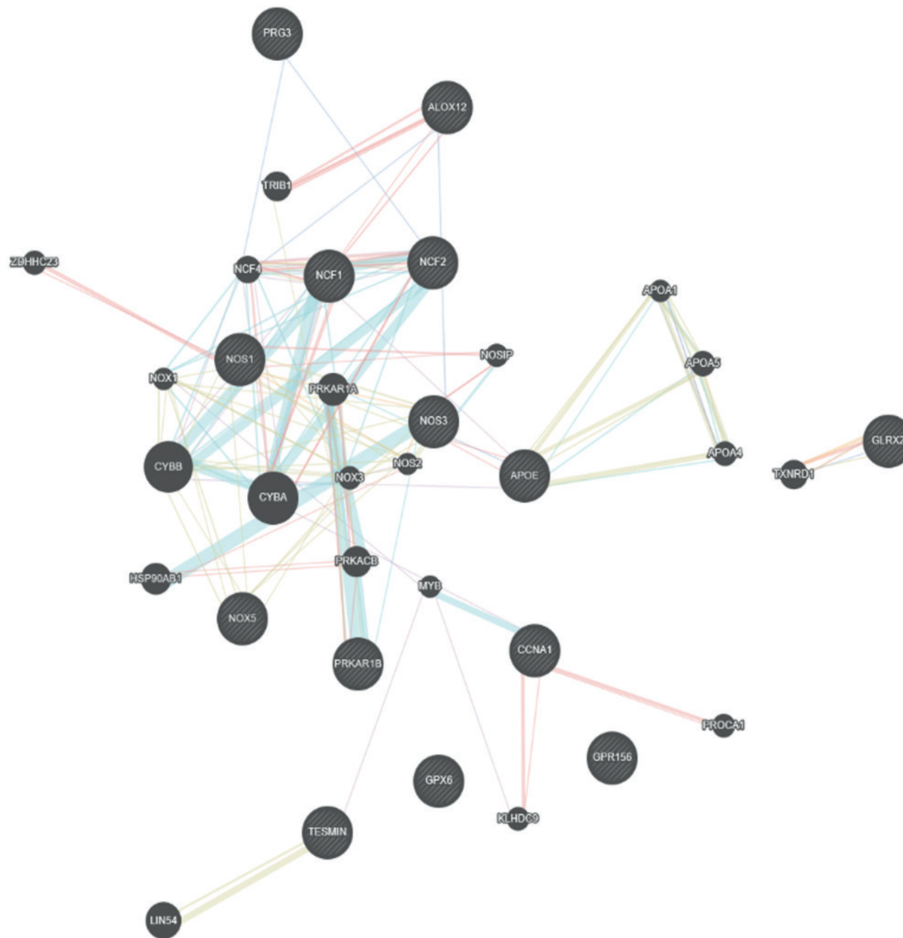

Figure 11. Network based on expression and functional associations of input target genes on treatment with Quercetin (Pathway = 48.18%, Physical interaction = 14.24%, shared protein domains = 13.85%, co-localization = 13.64%, predicted = 5.55%, co-expression = 4.53%).

dietary agents, have been shown to increase NO levels *in vitro*[30-32]. NO is produced by three NOS enzymes. The mRNA transcript levels of the three NOS enzymes following phytochemical treatment were assessed, and it was shown that following treatment with all the phytochemicals, the expression of two or more NOS transcripts was increased. Furthermore, iNOS and eNOS protein levels were found to be significantly increased following treatment with all the four phytochemicals, as shown by immunocytochemistry. Q, GEN, and EGCG have been previously shown to positively affect eNOS expression and protein levels in endothelial cells[30-35]. Dietary agents such as resveratrol and capsaicin have been shown to elevate nNOS levels, whereas the HDAC inhibitor, trichostatin, has been shown to increase eNOS levels in several cell lines[36,37]. Estrogens activate eNOS, and it has been suggested that the ability of these phytochemicals to weakly mimic estrogens may underlie the activation of NOS genes[35]. Several studies[2,6,38] have shown that elevation of nNOS, eNOS and iNOS is associated with caspase-mediated apoptosis in different types of cancer cells. NO produces reactive nitrogen species (RNOS), which produces peroxynitrite that affects macromolecules, leading to apoptotic cell death[39]. High concentrations of NO directly facilitate the release of cytochrome c and activate both mitochondrial and TRAIL-mediated apoptosis[6,38,40]. RNOS can cause direct DNA fragmentation, modulation of transcription factors and activation of p53, affecting the cell cycle checkpoints[41].

Transcriptional modulation of the genes in the NO pathway highlights the role of altered redox balance in inducing apoptosis in HeLa cells. The

treatment of cells with the phytochemicals increased the transcript levels of enzymes required for the production of superoxides and reduced the levels of superoxide dismutases. This is indicative of increased oxidative stress in the cells. Glutathione peroxidases aid in the detoxification of H_2O_2 and reduce ER stress. A steep decline in GPX6 levels with Q treatment was observed, which may have resulted in increased oxidative stress. ALOX12, DUOX2, NCF1, and NOX5 are involved in superoxide production[42-45]. All of these were upregulated following treatment with the phytochemicals. PRG3 is a p53 responsive protein and increases in its expression are associated with apoptosis[46]. Treatment with all the phytochemicals at the concentrations assessed increased PRG3 levels. Increases in the expression of PRKAR1B, PRKCA, and MT3 are associated with an improved cancer response, and expression of these three genes was increased following treatment with the phytochemicals. GRIN2D and APOE are cancer markers, expression of which are increased in cancer cells, and treatment with the phytochemicals decreased expression significantly[47-49]. Additionally, treatment with the phytochemicals increased the expression levels of several anti-oxidant genes, including GSS, MSRA, MPO, TTN and GPX2. MSRA, which was upregulated in response to treatment with the phytochemicals, is a free radical scavenger, but MSRA results in reduced proliferation when upregulated[50]. Whilst the selected phytochemicals produced both pro-oxidant and anti-oxidant responses, it is hypothesized that the upregulated pro-oxidant genes induced oxidative and nitrosative stress result-

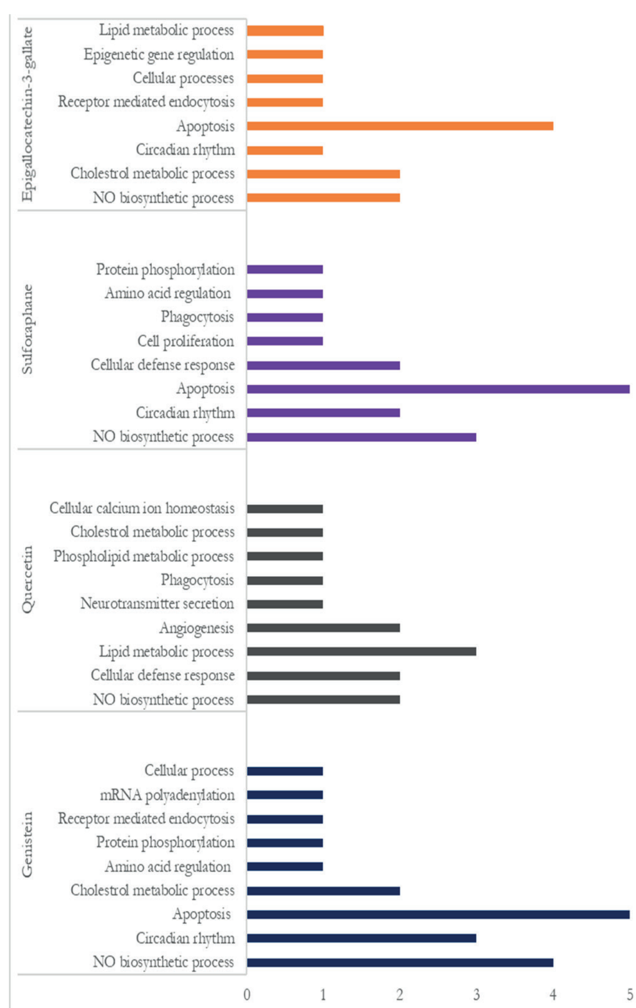

Figure 12. GO ontology analysis of pro-oxidants and anti-oxidants after treatment with phytochemicals.

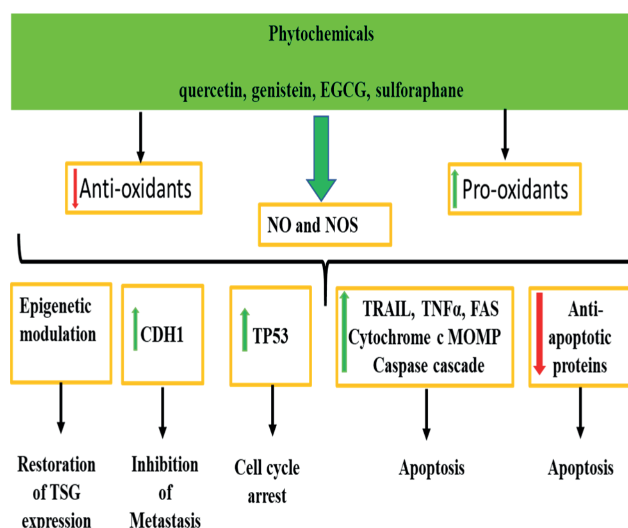

Figure 13. Graphical Abstract: Induction of nitric oxide production and its signaling pathway by the phytochemicals (quercetin, genistein, EGCG, and sulforaphane) aids in apoptotic induction as well as influences several carcinogenic processes.

ing in the observed apoptotic cell death. Other studies have indicated that elevated levels of pro-oxidants overcome the anti-oxidant effects and induce apoptosis[51]. Studies on the selected phytochemicals demonstrating their pro-oxidant properties are discussed below.

Q upregulates p53-inducible gene 3, ROS accumulation and lowers the mitochondrial membrane potential to activate the intrinsic mitochondrial apoptotic pathway in human hepatocarcinoma HepG2 cells[52]. It was also shown to trigger apoptosis via a ROS and AMPK/mTOR pathway in HCT116 colon cancer cells5[3]. SFN has been shown to exhibit anti-cancer activity in T4 human bladder cancer cells via a ROS-mediated intrinsic apoptotic pathway by activating the ER stress and Nrf2 signaling pathways[54]. GEN in combination with 5-flourouracil increases ROS levels and activates the AMPK pathway to initiate apoptosis in HT-29 colon cancer cells[55]. EGCG possesses dual roles as an anti-oxidant and as a pro-oxidant, resulting in cytotoxicity in cancer cells[56]. EGCG reduced proliferation and initiated apoptosis in HT-29 colon cancer cells by increasing ROS and activating the AMPK pathway. Furthermore, it sensitized resistant HT-29 cells to 5-fluorouracil[57]. In Jurkat cells, EGCG mediated G1 cell cycle arrest and apoptosis by increasing ROS levels, and ROS facilitated epigenetic regulation of tumor suppressor genes[58]. Q and EGCG demonstrate the role of redox signaling in epigenetic regulation[59,60]. Similarly, in H1299 lung cancer cells and xenograft tumors, both intracellular and mitochondrial ROS levels were increased by EGCG, resulting in apoptosis and oxidative DNA damage without affecting the normal organs61. Cancer cells are more sensitive to high levels of ROS compared with normal cells. This also shows that the pro-oxidative role of phytochemicals may be selective only for cancer cells, highlighting their importance as therapeutic agents. This thought is in agreement with previous studies[62,63].

The modularity of the induced regulatory mechanisms and varied phenotypic traits were identified by performing network analysis based on average fold-change values following phytochemical treatment. In addition, co-expression, predicted interactions between altered genes based on public databases, and co-localization was considered to generate the related network among the expressed pro-oxidants and anti-oxidants[64]. The strength of the interaction was computed as a percentage (count of interaction type/ total number of the interactions in the network). Such interactions depict the functional associations among the expressed genes by several regulatory mechanisms. In accordance, gene set enrichment and KEGG pathway analysis were performed to identify the effects of the phytochemicals on the underlying biological pathways. Based on KEGG analysis, major participation in query gene sets was identified in the calcium signaling pathway, arginine biosynthesis, glioma, pathways in cancer, choline metabolism, fluid shear stress and atherosclerosis, pancreatic cancer and estrogen signaling pathway amongst others. Furthermore, the functional association amongst the expressed gene sets and apoptosis following phytochemical treatment was clearly reflected in the GO analysis. The upregulation of pro-oxidants (NOX5, NOS1, NOS2, and

NOS3) promotes apoptosis, nitric oxide biosynthesis process, circadian rhythm, epigenetic regulation and cholesterol metabolism[65]. The upregulation of the ALOX12 gene following treatment with Q and SFN predicts the importance of the identification of a novel isoform of the inhibitors to treat colorectal and prostate cancer[66]. The functional association of the expressed genes validated the active participation of the genes in cancer pathways and associated mechanisms. The modulated NO pathway genes and the KEGG analysis reflects the potential of these phytochemicals for treating cancer.

Therefore, based on the results of the present study, cell death was seen as an outcome of phytochemical treatment and may be partly explained by the evident increase in NO levels following the induction of NOS (Figure 13). Additionally, the present study comprehensively listed the effects of these phytochemicals on the genes involved in redox and oxidative pathways. This is the first time to the author's knowledge that a pathway-based analysis of phytochemical-induced transcription data of redox genes has been performed. This study endorses the pro-oxidant activity of phytochemicals to establish their anti-carcinogenic potential. It is important that several pathways associated with redox balance should be explored to understand the impact of treatments on NO regulation, and the contribution of each pathway to the therapeutic potential of the selected phytochemicals. This also ensures that fewer agents will fail at the clinical trial stages. Overall, the present study highlights the need to view NOS and NO pathways as a possible molecular target of chemopreventive agents. Furthermore, it offers a broad spectrum of genes frequently modulated by various phytochemicals, whose functional implications require further study.

Conclusions

The present study showed that the phytochemicals, Q, GEN, EGCG, and SFN induced NO production, altered the redox environment, and thus facilitated apoptosis. These results further highlight the potential of NO inducers as potential chemotherapeutic options, whilst increasing our understanding of the underlying mechanisms. Further, mechanistic studies are required to improve our understanding of the role of NO and its effects on cell physiology to pave the way for clinical applications.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' declaration of personal interests

The authors declare that they have no competing interests.

Declaration of funding interests

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