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EFFECTS OF PROANTHOCYANIDINS IN COMBINATION WITH RESVERATROL AND SULFORAPHANE RESPECTIVELY ON HUMAN BREAST CANCER CELLS

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BIOLOGY

ABSTRACT

Breast cancer is the second most common cancer and the second leading cause of death from cancer among women in the United States. Cancer prevention and therapy through use of phytochemicals that have epigenetic properties has gained considerable popularity during the past few decades. Such dietary components include but are not limited to grape seed proanthocyanidins (GSPs), resveratrol (Res) and sulforaphane (SFN). Here we report for the first time that GSPs and Res in combination dose- and time-dependently inhibited cell viability and posttreatment colony forming ability synergistically in MDA-MB-231 and MCF-7 human breast cancer cells, while GSPs and SFN in combination antagonistically inhibited these two cell lines in a dose- and time-dependent manner. Additional analyses suggest that the synergism between GSPs and Res in MDA-MB-231 cells could be as a result of the enhancement of signal transmission in apoptosis as the combinational treatment with GSPs and Res synergistically up-regulated the expression of proapoptotic protein Bax and down-regulated the expression of antiapoptotic protein Bcl-2. In MCF-7 cells, however, Bax expression was greatly down-regulated by GSPs regardless of the presence of Res or SFN. Furthermore, DNA methyltransferase (DNMT) and histone deacetylase (HDAC) activity assays were conducted. The results show that the combinational treatment with GSPs and Res led to greater inhibition in DNMT and HDAC activities compared with treatment with either GSPs or Res alone in MDA-MB-231 and MCF-7 cells, whereas GSPs and SFN antagonistically inhibited

DNMT and HDAC activities in both cell lines. These findings suggest that the synergism between GSPs and Res in MCF-7 cells and the antagonism between GSPs and SFN in both MDA-MB-231 and MCF-7 cells could be associated with the regulation of DNA methylation and/or histone modifications.

Keywords: grape seed proanthocyanidins, resveratrol, sulforaphane, human breast cancer, synergism/antagonism, epigenetics

DEDICATION

This graduate research is dedicated to my entire family in China, especially to my parents, who supported their only child to study abroad, who have spent countless days and nights in front of a computer screen which was the closest they could get to their son. It is dedicated to my dear aunt, who died from lung cancer two years ago at the age of 53, who had loved me like her own. It is also dedicated to my grandmother, who passed away peacefully last year at the age of 88, who I had spent a great deal of my childhood with.

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LIST OF ABBREVIATIONS

CI	Combination index
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
ER	Estrogen receptor
GSPs	Grape seed proanthocyanidins
HDAC	Histone deacetylase
PR	Progesterone receptor
Res	Resveratrol
SFN	Sulforaphane

INTRODUCTION

Breast Cancer

Breast cancer is the development of tumor in the tissues of the breast. It is the second most common cancer and the most common invasive cancer among women in the United States, exceeded only by skin cancer which is non-invasive. In addition, breast cancer is the second leading cause of death from cancer in women with more than 200,000 new cases and a mortality rate of about 40,000 women per year, preceded only by lung cancer.[1] According to current projections, approximately one in eight women (12.3%) will be diagnosed with breast cancer at some stage of their lives. Breast cancer can also occur in men, though it is a hundred times less likely.

Epigenetics and Cancer

Epigenetics, the study of heritable changes in gene expression without modifying the nucleotide sequence, is among the most important topics in medicinal chemistry and cancer chemoprotection.[2] Such changes include DNA methylation, histone modification

and microRNA expression that have been shown to be associated with various types of cancer, including breast cancers.[3] A number of phytochemicals from fruits and vegetables, which include but are not limited to proanthocyanidins, sulforaphane and resveratrol, have been shown to modulate these epigenetic changes.

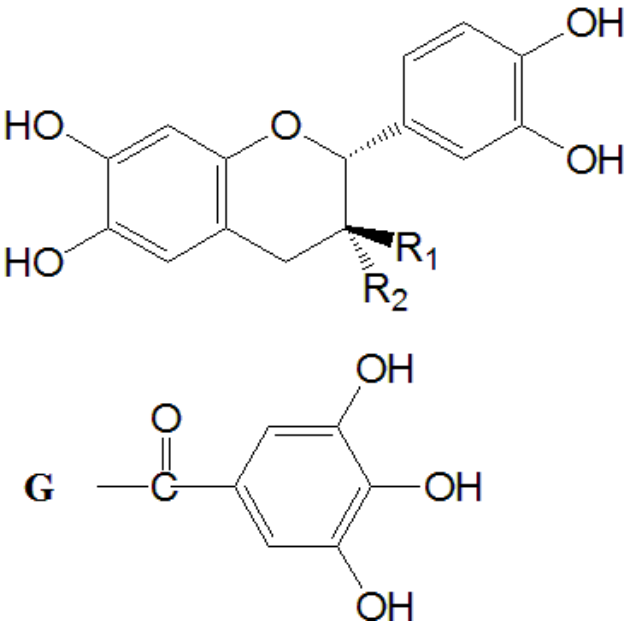
Phytochemicals and Cancer

Breast cancer development varies among different ethnic groups. Apart from genetic factors, another risk factor is believed to be the dietary differences between developed countries and developing countries. In the Western world, people tend to consume a meat- and calorie-rich diet, while people in the Eastern world generally have a plant-based diet. Many of these fruits, vegetables and cereals are rich in anticancer and/or anti-oxidative dietary components that can cause epigenetic changes, such as curcumin, genistein, isothiocyanates (sulforaphane, benzyl isothiocyanate, phenylhexyl isothiocyanate, etc.), proanthocyanidins, resveratrol and epigallocatechin-3-gallate (EGCG). The use of dietary components to regulate epigenetic modifications has gained substantial interest in cancer prevention and therapy during the past few decades.[4-22]

Grape seed proanthocyanidins (GSPs)

Proanthocyanidins refer to a large class of polyphenols called flavanols. Proanthocyanidins can be found in many plants, like apples, cinnamon, aronia fruit, and cocoa

beans, but the powerful compound is most abundant in the bark of the maritime pine and in grapes. Grapes are rich in proanthocyanidins with approximately 60–70% of the proanthocyanidins being contained in the seeds. Grape seed proanthocyanidins predominantly consist of dimers, trimers, tetramers and oligomers of monomeric catechins (Figure 1A).[23] Studies have shown that GSPs are potent antioxidants with many biological properties.[24-26] Chief among them are their anticancer effects which have also been reported in various types of cancer, such as skin cancer and lung cancer as well as breast cancer.[1, 27-31]



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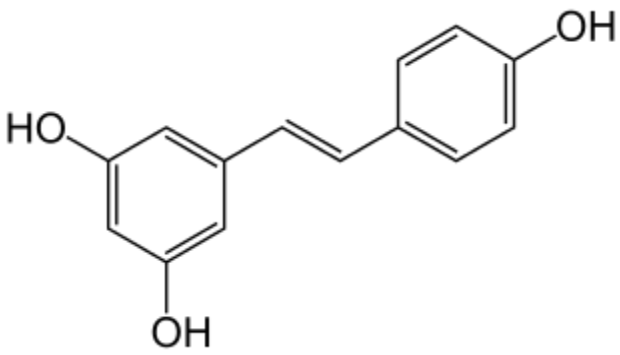

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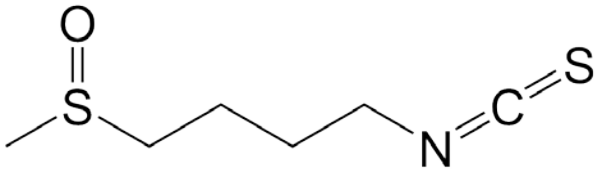


(C)

Figure 1. Chemical Structures. The chemical structures of the building blocks of proanthocyanidins (A), resveratrol (B) and sulforaphane (C). R_1 represents H or OH. R_2 represents H, OH or OG.

Resveratrol (Res)

3,5,4'-trihydroxy-trans-stilbene or resveratrol is a stilbenoid (Figure 1B), a polyphenol as well as a phytoalexin naturally produced by a number of plants such as grapes, berries, peanuts and the roots of Japanese knotweed when under attack by pathogens. However, it is most abundant in the skin of red grapes; thus, it is rich in red wine.[32-33] Like other polyphenols, Res exhibits anticancer properties through a number of epigenetic regulations, among which its ability to inhibit histone deacetylases (HDACs) has been well studied.[34-39] Res and its analogues have also been reported to regulate histone phosphorylation in various cancers.[40-41]

Sulforaphane (SFN)

Sulforaphane, belonging to the isothiocyanate group (Figure 1C), can be found in cruciferous vegetables including but not limited to broccoli, Brussels sprouts, kale, cauliflower and cabbages.[42-44] It is produced from glucoraphanin, which is particularly rich in young sprouts of broccoli and cauliflower, by the enzyme myrosinase upon damage to the plant as a protective mechanism.[42] *In vitro* and *in vivo* studies have shown that SFN exhibits anticancer properties through several mechanisms, such as cell cycle arrest and apoptosis, activating checkpoint kinase 2 and acting as a HDAC inhibitor which increases global and local histone acetylation of a number of genes.[45-51]

GSPs and SFN in Combination

In recent years, effects of dietary components in combination on cancer have gained increasing interest. Previous studies in our lab have shown that phytochemicals acting as DNMT inhibitors as well as those with HDAC-inhibiting properties can work in synergy in inhibiting human cancer.[52-54] Since GSPs have been reported to down-regulate DNMTs and SFN is a well-studied HDAC inhibitor, the combination of them, in theory, should have additive effects at the least, if not synergistic effects, on cancer inhibition.[55-57] Additionally, both GSPs and SFN are heavily consumed by the general population due to their vast availability in fresh produce and products. It is reasonable to assume that they often appear at the same time on people's dining table. Therefore, it is practical and beneficial

to investigate the health benefits of these dietary phytochemicals in combination in human breast cancer.

GSPs and Res in Combination

Dietary phytochemicals are believed to be easier to absorb and exhibit better efficacy in their natural form than in their purified form. It could be due to the fact that there may be other natural compounds acting with the dietary phytochemicals of interest in their natural form in a synergistic manner. We chose GSPs and Res for our second study because they are both abundant in red grapes, which are some of the most consumed fruits by humans and are known to have considerable health benefits. It is worth mentioning that red wine is an excellent source of GSPs and Res because grape seeds and grape skin are preserved and utilized during red wine production. Red wine, which is heavily consumed worldwide, also offers more concentrated GSPs and Res than do red grapes. Collectively, we had decided to investigate the effects of GSPs and Res in combination on human breast cancer cells.

MATERIALS AND METHODS

Cell Culture and Treatment

The estrogen receptor-negative (ER-), progesterone receptor-negative (PR-) and HER2-negative (HER2-) MDA-MB-231 human breast cancer cells and the ER+, PR+ and HER2- MCF-7 human breast cancer cells were obtained from ATCC (Manassas, VA, USA). The immortalized non-cancerous MCF10A human mammary epithelial cells, which were also obtained from ATCC (Manassas, VA, USA), were used as the control.[58] Both MDA-MB-231 and MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech Inc, Manassas, VA, USA) with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA) and 1% penicillin/streptomycin (Corning Cellgro, Manassas, VA, USA). MCF10A control cells were cultured in DMEM/F12 medium (Mediatech) with 5% donor horse serum (Atlanta Biologicals), 20 ng/mL of epidermal growth factor (EGF) (Sigma), 100 ng/mL of cholera endotoxin (Sigma), 0.5 μg/mL of hydrocortisone (Sigma), 2 mM L-glutamine and 1% penicillin/streptomycin (Mediatech). All three cell lines were cultured in a humidity-controlled incubator at 37°C with 5% CO_2, and sub-cultured at 85-90% confluence. After sub-culturing, all cells were

given 24 h to adhere and to recover before they were treated with varying concentrations of GSPs (20, 40, 60 μg/ml), Res (10, 20 μM), SFN (2.5, 5, 7.5 μM) and their combinations (20 μg/ml GSPs with 10 μM Res, 40 μg/ml GSPs with 20 μM Res, 20 μg/ml GSPs with 2.5 μM SFN, 20 μg/ml GSPs with 5 μM SFN, 40 μg/ml GSPs with 2.5 μM SFN, 40 μg/ml GSPs with 5 μM SFN) for 24 h to 72 h. Media and treatment agents were refreshed every 24 h. Dimethyl sulfoxide (DMSO) was used as the vehicle control at the concentration of 0.5% (v/v) in media.

Chemicals

Grape seed proanthocyanidins (GSPs) (> 95% pure) were purchased from Kikkoman Corporation (Tokyo, Japan). Sulforaphane (SFN) (＞98% pure) was purchased from LKT Laboratories (Minneapolis, MN). Resveratrol (Res) (> 99% pure; HPLC) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). GSPs were prepared in DMSO and were stored as a stock at the concentration of 100 mg/ml at -20^{o}C. Resveratrol (Res) was prepared in DMSO and was stored as a stock at the concentration of 100 mM (mmol/L) at -20^{o}C.

MTT Assay

The number of viable cells in each well was estimated by the uptake of the tetrazolium salt, 3-(4, 5-dinethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT). Approximately 4000 cells per well of each cell line were plated in 96-well plates and incubated for 24 h at 37°C with 5% CO_2 to allow the cells to adhere to the bottom. Then the cells were treated with DMSO, GSPs, Res, SFN and their combinations at aforementioned concentrations for 24 h to 72 h. After the treatments, the cells were incubated with 100 μl of 1 mg/ml MTT solution for an additional 3.5 h at 37°C. Thereafter, the MTT solution was aspirated and 150 μl of DMSO was added to each well to dissolve the formazan crystals. Finally, the absorbance was read at 595 nm using a microplate reader (iMark, Bio-Rad). Cellular viability was calculated as a percentage relative to the vehicle control treated by DMSO.

Clonogenic Assay

Cells were treated with DMSO, GSPs, Res, SFN and their combinations at aforementioned concentrations in 6-well plates for 48 h to 72 h at 37°C with 5% CO_2. The cells were then harvested and approximately 500 cells of each treatment were seeded in 6-well plates with fresh media and were incubated undisturbed at 37°C with 5% CO_2 for 7 days, during which time the cells were allowed for proliferation and colony formation.

Afterwards, the media was aspirated, the colonies were washed with cold phosphate buffer saline, fixed with cold 70% methanol and were stained with 0.25% trypan blue solution. Finally, photographs were taken and colonies with over 50 cells were counted.

Apoptosis Assay

Apoptosis of breast cancer cells induced by GSPs, Res, SFN and their combinations were quantitatively determined by flow cytometry using the Annexin V-conjugated Alexafluor 488 (Alexa488) Apoptosis Vybrant Assay Kit (Life Technologies, Carsbald, CA, USA). Approximately 2 x 10^5 cells were seeded in each well of 6-well plates and were left for 24 h at 37°C with 5% CO_2 for adherence and recovery. The cells were then treated using aforementioned method. Thereafter, the cells were harvested by brief tryp-sinization, washed with PBS, and incubated with Alexa488 and propidium iodide for cel-lular staining in Annexin-binding buffer at room temperature for 10 min in the dark. The stained cells were analyzed by fluorescence-activated cell sorting (FACS) using a FACS-caliber instrument (BD Biosciences, San Jose, CA, USA) equipped with cell quest 3.3 software (BD Biosciences).

Western Blot Analysis

Cells were treated as mentioned above. The cells were then harvested, and protein extracts were prepared by RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) following the manufacturer's protocol. Protein concentrations were determined by Bradford using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein extract (50 μg) was loaded into a 4-15% Tris-HCl gel (Bio-Rad) and separated by electrophoresis at 200 V until the dye arrived near to the end of the gel. The separated proteins were transferred to a nitrocellulose membrane at 25 V for 10 min by the Trans-Blot Turbo transfer system (Bio-Rad). Afterwards, the membrane was blocked in Tris-buffered saline (TBS) solution with 0.5% dry milk and 0.5% Tween (TBST) following the SNAP i.d. 2.0 protein detection system protocol (EMD Millipore, Billerica, MA). Primary and secondary antibody incubations were performed according to the manufacturer's protocol. Immunoreactive bands were visualized using Clarity Western ECL Substrate (Bio-Rad).

DNMT Activity Assay

Cells were treated as mentioned above. The cells were then harvested, and nuclear extracts were prepared using EpiQuik Nuclear Extraction Kit (EpiGentek, Farmingdale, NY) following the manufacturer's protocol. DNMT activity assay was performed using

EpiQuik DNA Methyltransferase Activity/Inhibition Colorimetric Assay Kit (EpiGentek) following the manufacturer's protocol.

HDAC Activity Assay

Cells were treated as mentioned above. The cells were then harvested, and nuclear extracts were prepared using EpiQuik Nuclear Extraction Kit (EpiGentek, Farmingdale, NY) following the manufacturer's protocol. HDAC activity assay was performed using EpiQuik HDAC Activity/Inhibition Colorimetric Assay Kit (EpiGentek) according to the manufacturer's protocol.

CompuSyn Analysis

The CompuSyn software version 1.0 (http://www.combosyn.com/) was used to determine synergism/antagonism of combinational treatments. Combination index (*CI*) values were generated by the software. $CI < 1$ indicates synergism, $CI = 1$ indicates additive effect, $CI > 1$ indicates antagonism.[59-60]

Statistical Analysis

All results were generalized from at least three independent experiments with very similar observations. Error bars indicate standard deviation. Significance versus control group (DMSO-treated) was calculated by Student's t-test, in which case, * indicated statistically significant ($P < 0.05$) and ** highly statistically significant ($P < 0.01$).

RESULTS

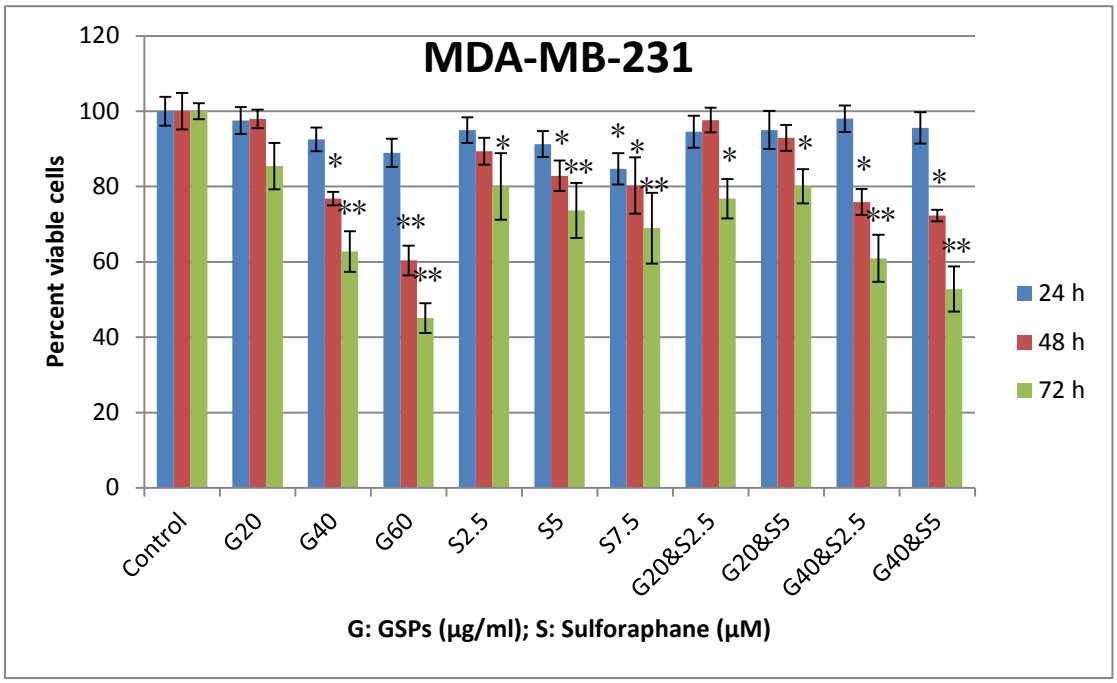
Combinational Treatment with Proanthocyanidins and Sulforaphane Inhibit Human Breast Cancer Cells Antagonistically

GSPs and SFN antagonistically inhibit cell viability and proliferation in MDA-MB-231 and MCF-7 human breast cancer cells with no apparent toxicity in MCF10A human mammary epithelial cells

To determine the anti-carcinogenic effect of GSPs, SFN and their combinations on cell viability in MDA-MB-231 and MCF-7 human breast cancer cells, MTT assays were performed. The cells were treated with 0.5% (v/v) DMSO, GSPs (20, 40 and 60 μg/ml), SFN (2.5, 5 and 7.5 μM) and their combinations (20 μg/ml GSPs with 2.5 μM SFN, 20 μg/ml GSPs with 5 μM SFN, 40 μg/ml GSPs with 2.5 μM SFN, and 40 μg/ml GSPs with 5 μM SFN) for 24 h, 48 h and 72 h. As shown in Figure 2A, treatment with GSPs, SFN and their combinations all resulted in reduction in cell viability in a dose- and time-dependent manner, respectively, in MDA-MB-231 cells. The treatment with GSPs resulted in significant decreases in cell viability in a dose- and time-dependent manner ranging from 2% to 11% after 24 h, 2% to 40% ($P < 0.01$) after 48 h and 15% ($P < 0.05$)

to 55% ($P < 0.01$) after 72 h. The treatment with SFN led to significant decreases in cell viability in a dose- and time-dependent manner ranging from 5% to 15% ($P < 0.05$) after 24 h, 11% to 20% ($P < 0.05$) after 48 h and 20% ($P < 0.05$) to 31% ($P < 0.01$) after 72 h. The treatment with the combinations of GSPs and SFN resulted in significant decreases in cell viability in a dose- (excluding the treatment for 24 h) and time-dependent manner ranging from 2% to 28% ($P < 0.05$) after 48 h and 20% ($P < 0.05$) to 47% ($P < 0.01$) after 72 h. However, each combinational treatment exhibited not much more inhibitory effect than treatment with either GSPs or SFN alone as shown in Figure 2A. Figure 2B shows that GSPs, SFN and their combinations inhibited MCF-7 cells in a similar manner as they did in the MDA-MB-231 cells. The treatment with GSPs resulted in significant decreases in cell viability in a dose- and time-dependent manner ranging from 6% to 31% ($P < 0.01$) after 24 h, 20% ($P < 0.05$) to 50% ($P < 0.01$) after 48 h and 24% ($P < 0.05$) to 62% ($P < 0.01$) after 72 h. The treatment with SFN led to significant decreases in cell viability in a dose- and time-dependent manner ranging from 1% to 10% after 24 h, 0% to 25% ($P < 0.05$) after 48 h and 5% to 44% ($P < 0.01$) after 72 h. The treatment with the combinations of GSPs and SFN resulted in significant decreases in cell viability in a dose- (excluding the treatment for 24h) and time-dependent manner ranging from 0% to 24% ($P < 0.05$) after 24 h, 9% to 45% ($P < 0.01$) after 48 h and 24% ($P < 0.05$) to 51% ($P < 0.01$) after 72 h. However, In MCF-7 cells, some combinational treatments exhibited even less inhibitory effect than treatment with either GSPs or SFN alone as shown in

Figure 2B. Collectively, these findings may suggest an antagonism between GSPs and SFN in inhibiting cell viability in human breast cancer cells.



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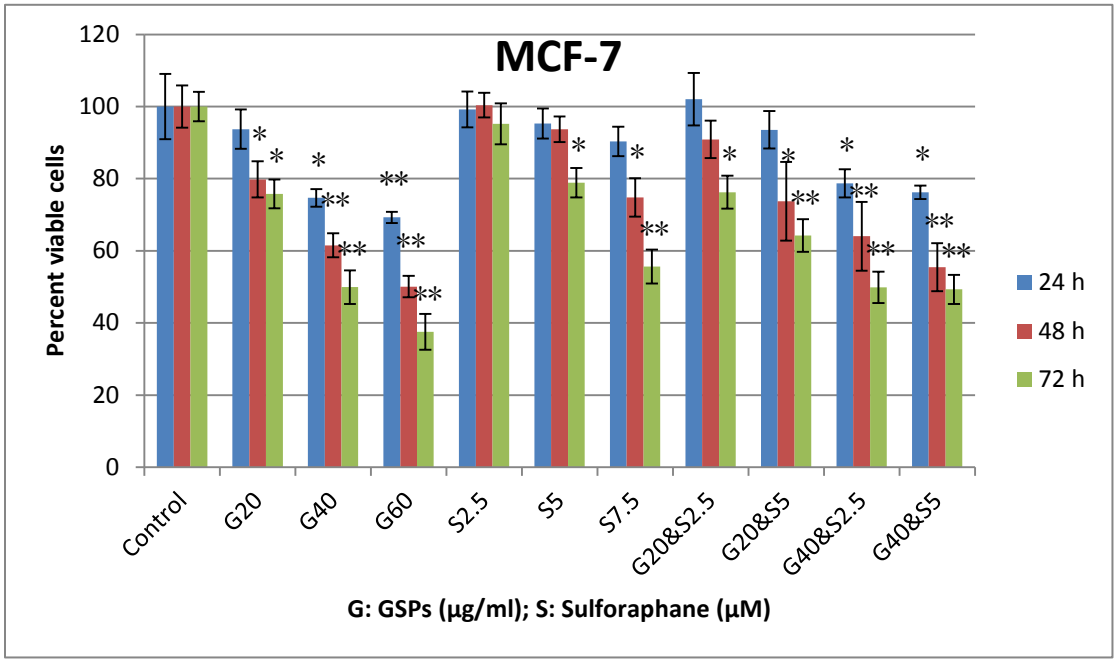

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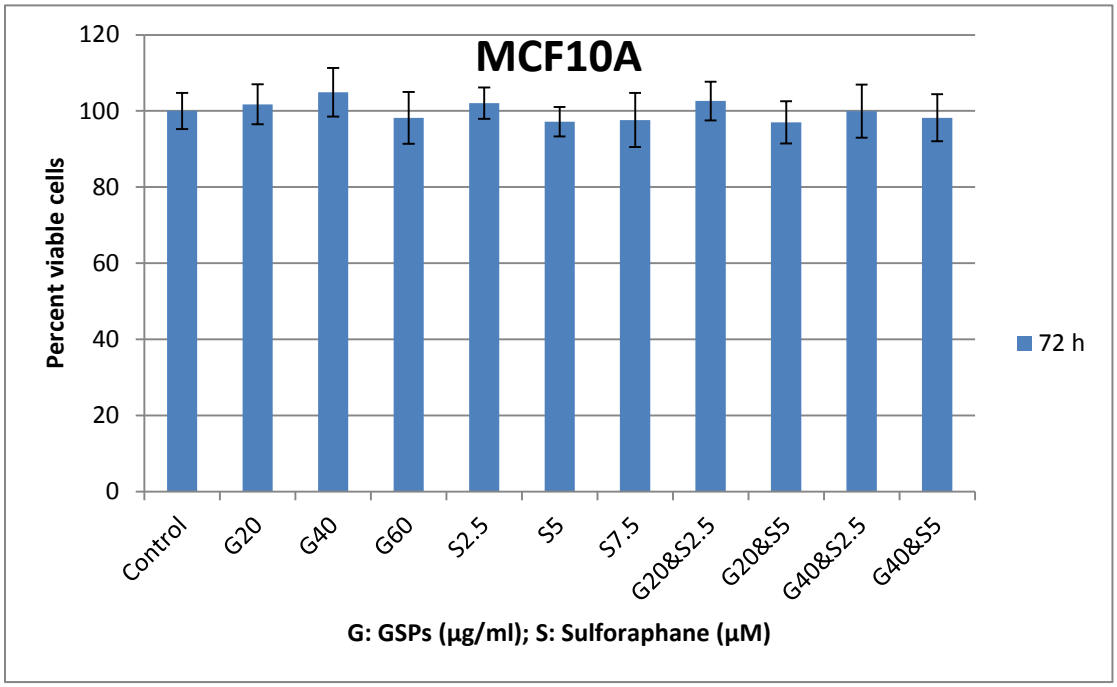


(C)

Figure 2. MTT Assay. Inhibition of cell viability in MDA-MB-231 (A) and MCF-7 (B) human breast cancer cells after treatment with GSPs (20, 40, 60 µg/ml), SFN (2.5, 5, 7.5 µM), and their combinations (20 µg/ml GSPs with 2.5 µM SFN, 20 µg/ml GSPs with 5 µM SFN, 40 µg/ml GSPs with 2.5 µM SFN, and 40 µg/ml GSPs with 5 µM SFN) com-

pared with the DMSO-treated control cells for 24h, 48 h and 72 h. MCF10A human mammary epithelial cells (C) were used as the control cells to determine the toxicity of these phytochemicals of varying concentrations. Results were generalized from three independent experiments with very similar observations. The cell viability of each treatment group is represented in percentage compared with the control group as the mean ± SD. * $P < 0.05$, ** $P < 0.01$

We then conducted MTT assay on the immortalized non-cancerous MCF10A human mammary epithelial cells to investigate the toxicity of GSPs, SFN and their combinations in human mammary cells. The cells were treated with 0.5% (v/v) DMSO, GSPs (20, 40 and 60 μg/ml), SFN (2.5, 5 and 7.5 μM) and their combinations (20 μg/ml GSPs with 2.5 μM SFN, 20 μg/ml GSPs with 5 μM SFN, 40 μg/ml GSPs with 2.5 μM SFN, and 40 μg/ml GSPs with 5 μM SFN) for 24 h, 48 h and 72 h. As shown in Figure 2C, no apparent reduction in cell viability was observed in any treatment group compared with the DMSO-treated control group in MCF10A cells, which demonstrates that GSPs, SFN or in combination post no toxic effect on human mammary cells.

CompuSyn software analysis proves an antagonistic effect of the GSPs and SFN combination treatment on breast cancer cells

To confirm the antagonistic effect on human breast cancer cells between GPSs and SFN, the results from the aforementioned MTT assays were further analyzed by the CompuSyn software version 1.0. Combination index (*CI*) values were generated by the software. $CI < 1$ indicates synergism, $CI = 1$ indicates additive effect, $CI > 1$ indicates antagonism.[59-60] As shown in Table 1, the *CI* values of all combinational treatments of the

MTT assays in both cell lines, except the one treated with the combination of GSPs (40 μg/ml) and SFN (5 μM) for 72 h in MDA-MB-231 cells, exhibited antagonism (*CI* > 1) suggesting antagonism between GSPs and SFN on the inhibition of human breast cancer.

Table 1. Antagonism between GSPs and SFN Indicated by *CI* Values.

Cell Line	Treatment Time (h)	Dose GSPs (μg/ml)	Dose SFN (μM)	Normalized Effect	*CI* Value
MDA-MB-231	24	20.0	2.5	0.94562	1.4719
MDA-MB-231	24	20.0	5.0	0.95000	2.54173
MDA-MB-231	24	40.0	2.5	0.98004	4.65108
MDA-MB-231	24	40.0	5.0	0.95585	3.52299
MDA-MB-231	48	20.0	2.5	0.97647	11.8078
MDA-MB-231	48	20.0	5.0	0.92907	4.6483
MDA-MB-231	48	40.0	2.5	0.75889	1.13195
MDA-MB-231	48	40.0	5.0	0.72304	1.20713
MDA-MB-231	72	20.0	2.5	0.76782	1.41959
MDA-MB-231	72	20.0	5.0	0.80091	2.80654
MDA-MB-231	72	40.0	2.5	0.60931	1.12602
MDA-MB-231	72	40.0	5.0	0.52802	0.97737
MCF-7	24	20.0	2.5	1.00000	25772.6
MCF-7	24	20.0	5.0	0.93561	1.87036
MCF-7	24	40.0	2.5	0.78726	1.20444
MCF-7	24	40.0	5.0	0.76207	1.33563
MCF-7	48	20.0	2.5	0.90874	2.50834
MCF-7	48	20.0	5.0	0.73707	1.51847
MCF-7	48	40.0	2.5	0.64031	1.43417
MCF-7	48	40.0	5.0	0.55448	1.52234
MCF-7	72	20.0	2.5	0.76241	1.51441
MCF-7	72	20.0	5.0	0.64210	1.46536
MCF-7	72	40.0	2.5	0.49904	1.25666
MCF-7	72	40.0	5.0	0.49295	1.53623

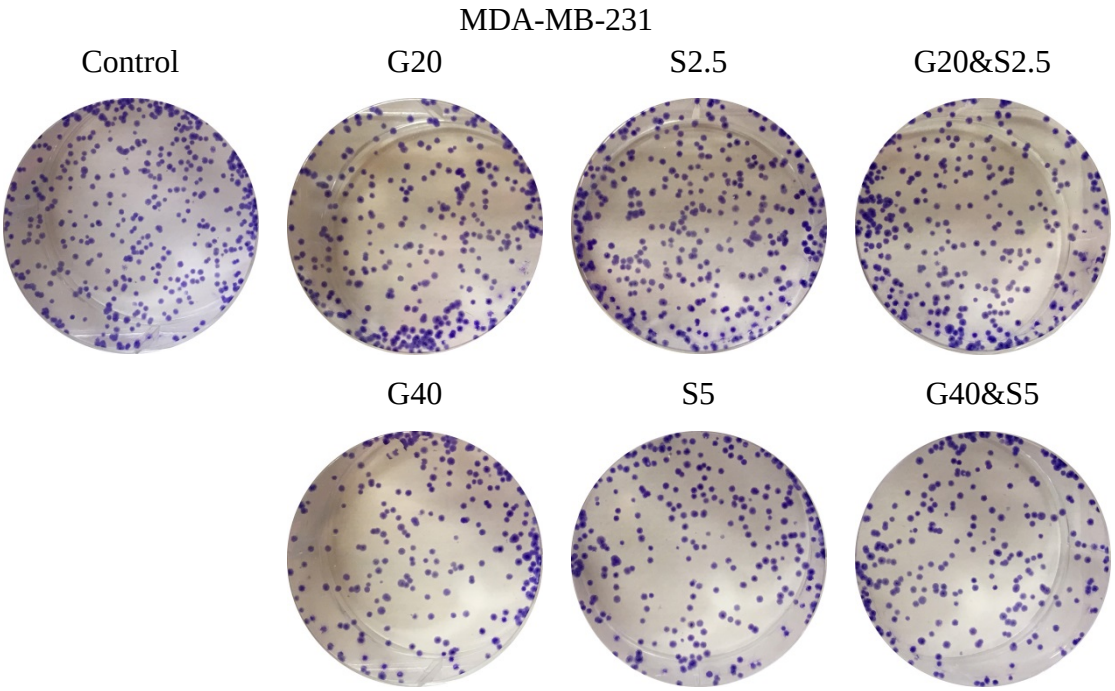
The Combination Index (*CI*) values were generated by the CompuSyn software from calculating the normalized effect (the effect of treatment with phytochemicals compared with that of treatment with DMSO) of the combinational treatments compared with the

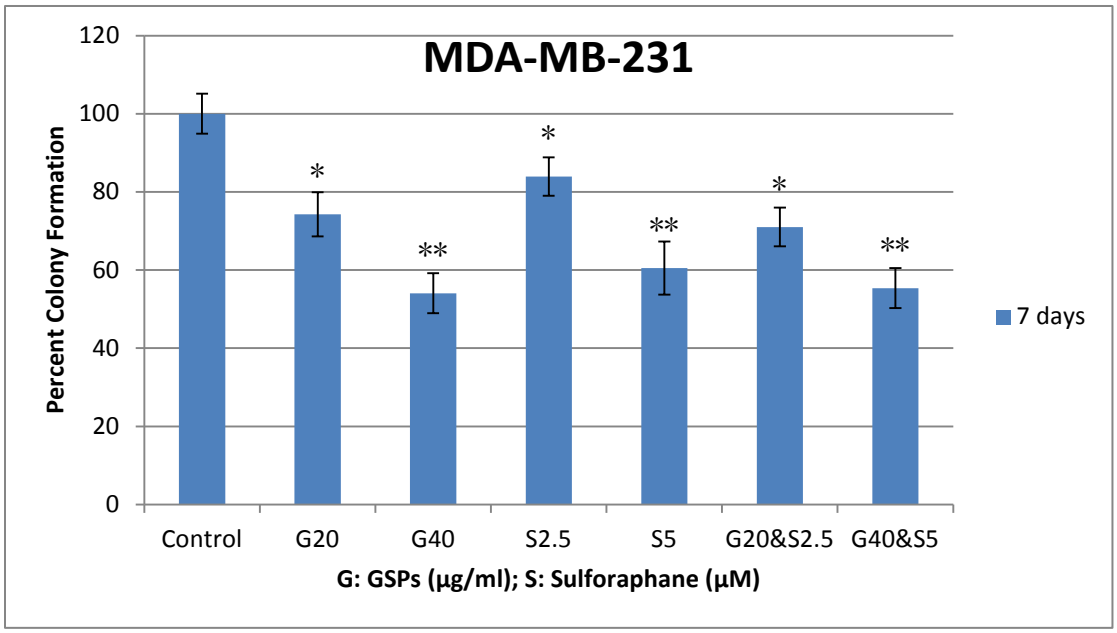
normalized effect of the treatments with GSPs and SFN alone (not shown in this table) from the data of the MTT assays. *CI* < 1 indicates synergism. *CI* = 1 indicates additive effect. *CI* > 1 indicates antagonism.

GSPs and SFN antagonistically reduce posttreatment colony forming ability in MDA-MB-231 and MCF-7 human breast cancer cells but not in MCF10A human mammary epithelial cells

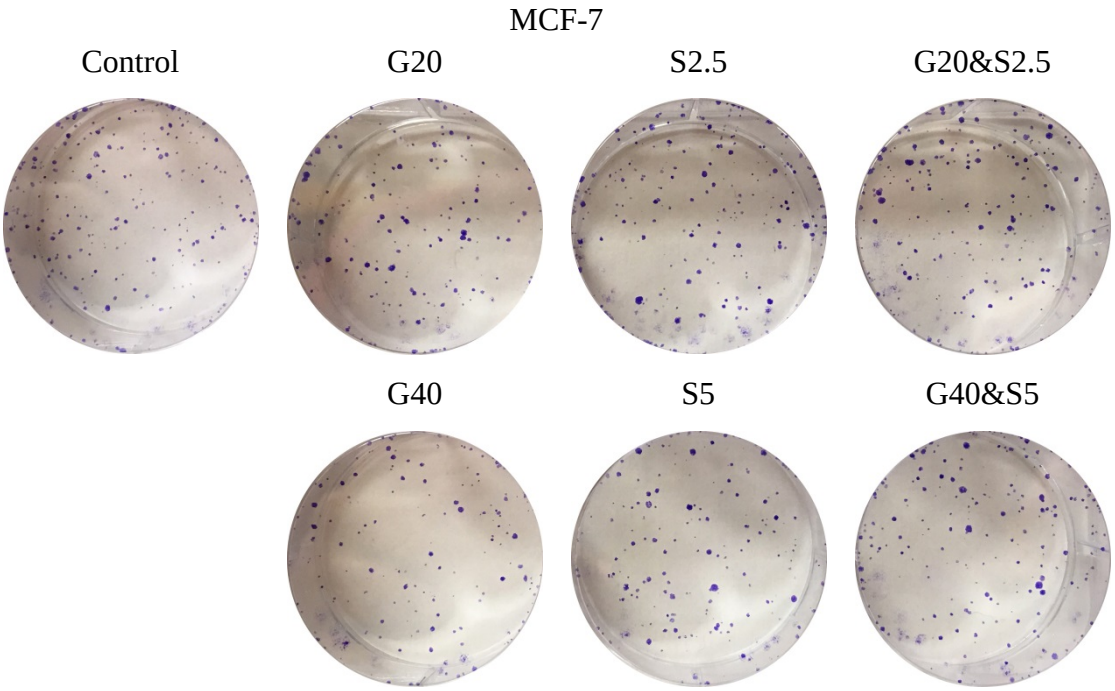
To examine the long term anti-carcinogenic effect of GSPs, SFN and their combinations on posttreatment cell proliferation and colony forming ability in MDA-MB-231 and MCF-7 human breast cancer cells, clonogenic assays were conducted. The cells were treated with 0.5% (v/v) DMSO, GSPs (20, 40 μg/ml), SFN (2.5, 5 μM) and their combinations (20 μg/ml GSPs with 2.5 μM SFN, and 40 μg/ml GSPs with 5 μM SFN) for 72 h before they were trypsinized, counted and the same number of cells were seeded in fresh media to allow adherence, proliferation and colony formation for 7 days. After fixation and staining, photographs were taken and colonies with over 50 cells were counted. As shown in Figure 3A and 3B, GSPs (20, 40 μg/ml) and SFN (2.5, 5 μM) inhibited the posttreatment colony forming abilities of MDA-MB-231 and MCF-7 cells in an antagonistic manner during a 7-day period compared with the DMSO-treated control groups after treatment for 72 h. The groups previously treated with GSPs led to significant decreases in colony formation by 26% ($P < 0.05$) to 46% ($P < 0.01$) in MDA-MB-231 cells and 17% ($P < 0.05$) to 33% ($P < 0.01$) in MCF-7 cells. The groups formerly treated with SFN exhibited significant decreases in colony formation by 16% ($P < 0.05$) to 40% ($P <$

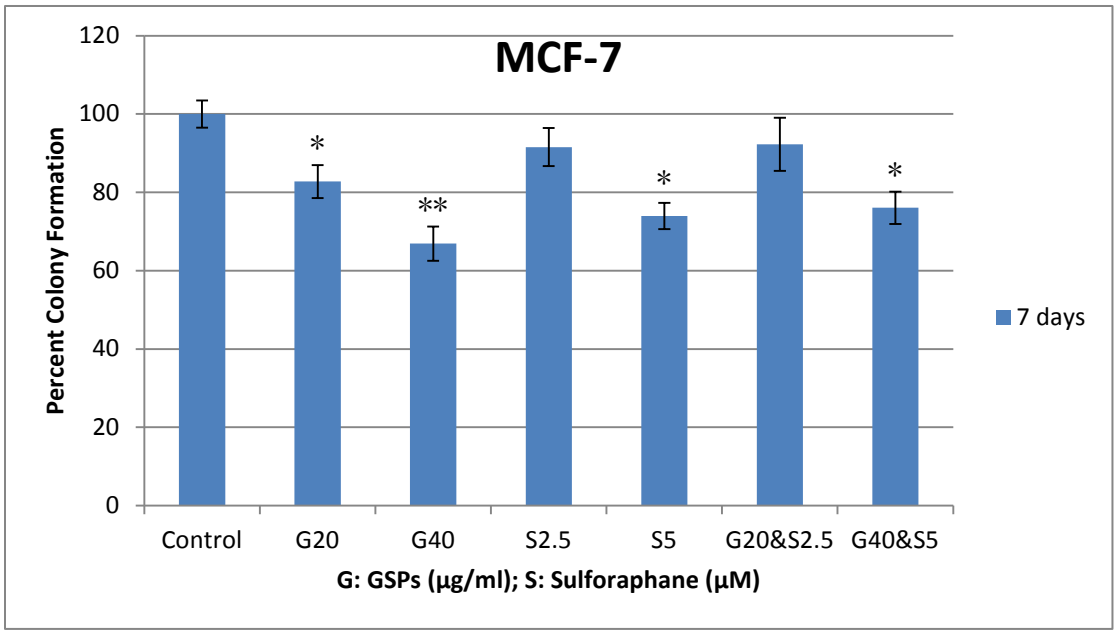
0.01) in MDA-MB-231 cells and 8% to 26% ($P < 0.05$) in MCF-7 cells. However, the pretreatments with GSPs and SFN in combinations led to a less reduction in colony formation by 29% ($P < 0.05$) to 45% ($P < 0.01$) in MDA-MB-231 cells and 8% to 24% ($P < 0.05$) in MCF-7 cells compared with the groups treated with GSPs or SFN alone, which supports the antagonism between the two phytochemicals found in the previous MTT assays.


MDA-MB-231
Control
G20
S2.5
G20&S2.5
G40
S5
G40&S5

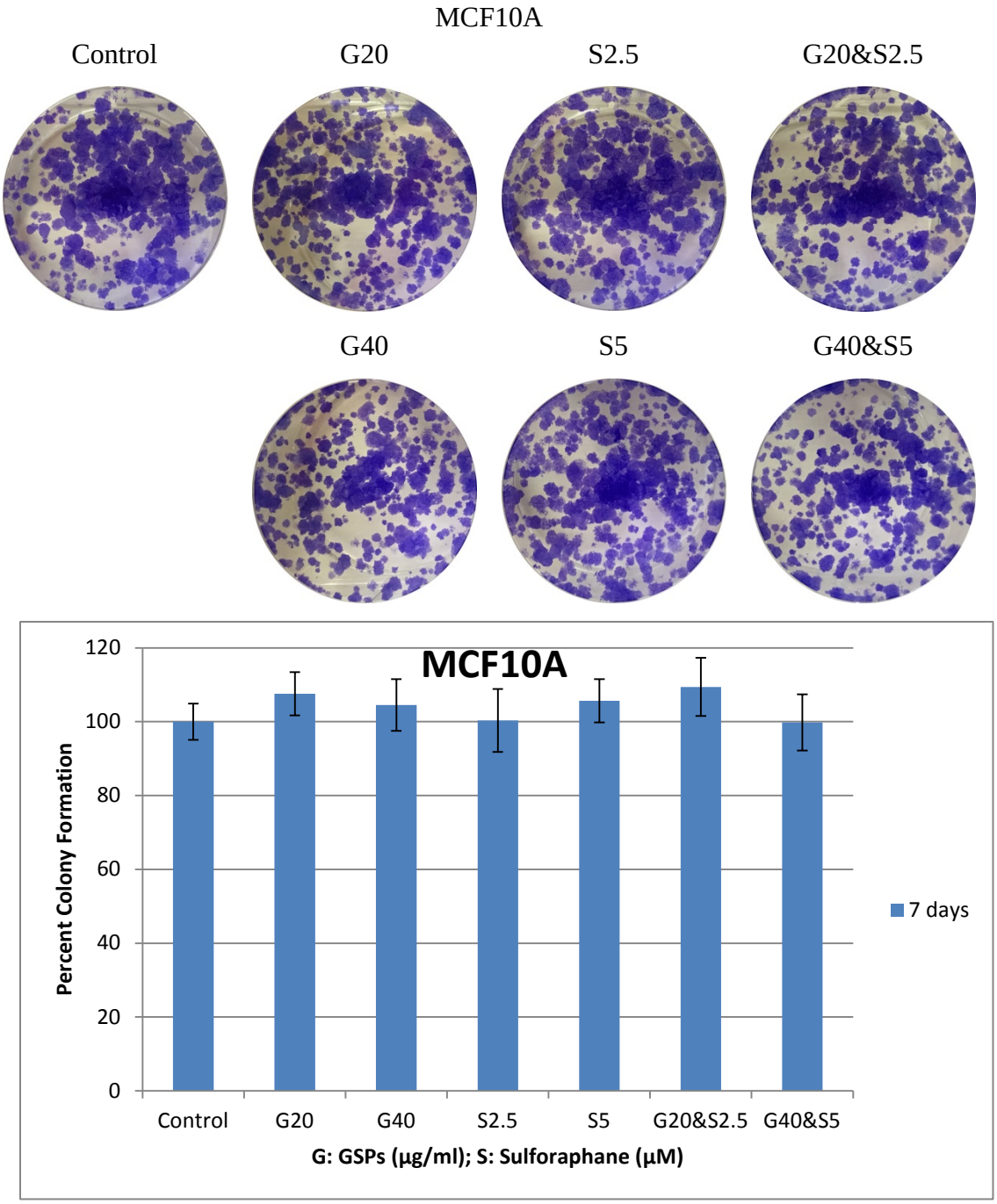

MDA-MB-231
Percent Colony Formation
120
100
80
60
40
20
0
Control
G20
G40
S2.5
S5
G20&S2.5
G40&S5
G: GSPs (μg/ml); S: Sulforaphane (μM)
7 days

(A)


MCF-7
Control
G20
S2.5
G20&S2.5
G40
S5
G40&S5


MCF-7
Percent Colony Formation
120
100
80
60
40
20
0
*
**
*
*
Control
G20
G40
S2.5
S5
G20&S2.5
G40&S5
G: GSPs (μg/ml); S: Sulforaphane (μM)
7 days

(B)



(C)

Figure 3. Clonogenic Assay. Inhibition of colony forming ability in MDA-MB-231 (A) and MCF-7 (B) human breast cancer cells as well as MCF10A (C) human mammary epithelial cells in 7 days after treatment with GSPs (20, 40 µg/ml), SFN (2.5, 5 µM), and their combinations (20 µg/ml GSPs with 2.5 µM SFN, 40 µg/ml GSPs with 5 µM SFN) compared with the DMSO-treated control groups for 72 h. Results were generalized and representative images were selected from three independent experiments with very simi-

lar observations. The colony forming ability of each treatment group is represented in percentage compared with the control group as the mean ± SD. * $P < 0.05$, ** $P < 0.01$

We also conducted clonogenic assay on MCF10A human mammary epithelial cells. As shown in Figure 3C, no apparent reduction in colony formation was observed in any treatment group compared with the DMSO-treated control group in MCF10A cells, which further indicates that GSPs, SFN or in combination post no toxic effect on human mammary cells.

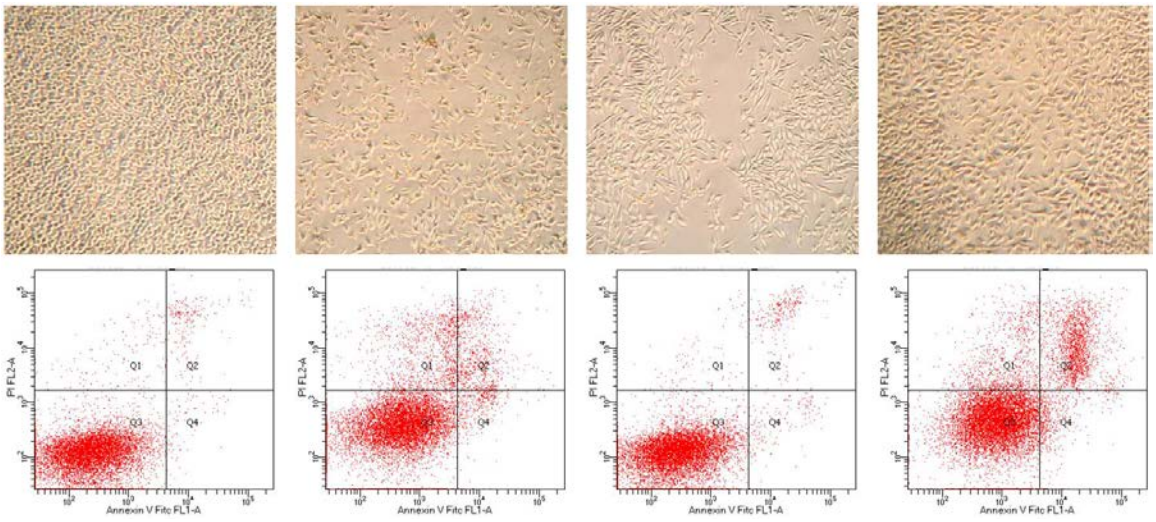
GSPs and SFN induce apoptosis in MDA-MB-231 human breast cancer cells, whereas GSPs and their combination with SFN inhibit apoptosis in MCF-7 human breast cancer cells

To investigate whether or not the antagonistic effects of GSPs and SFN on the inhibition of cell viability and proliferation as well as on the reduction of posttreatment colony forming ability in MDA-MB-231 and MCF-7 human breast cancer cells are associated with the induction of apoptosis, apoptosis analysis was performed by using the Annexin V-conjugated Alexafluor 488 (Alexa488) Apoptosis Vybrant Assay Kit following the manufacture's protocol. Firstly, cell density was reduced in groups treated with GSPs (40 μg/ml) and SFN (5 μM) alone but was reduced less in the group treated with their combination after treatment for 72 h in both MDA-MB-231 and MCF-7 cells as shown in Figure 4A and 4B. Morphological changes were also observed in the phytochemical-treated groups compared with the control group. Secondly, apoptosis was analyzed

using flow cytometry as described above. Cells were counted in four quadrants in the FACS histograms where Q1 (the upper left quadrant) represents dead cells (stained by propidium iodide) that are not associated with apoptosis, Q2 (the upper right quadrant) represents late apoptotic cells (stained by Alexa488 and propidium iodide), Q3 (the lower left quadrant) represents live cells and Q4 (the lower right quadrant) represents early apoptotic cells (stained by Alexa488). Q2 and Q4 were grouped together when the percentage of all four quadrants of cells from each treatment was illustrated in Figure 4C and 4D. Surprisingly, the results show that the combinational treatment of GSPs and SFN significantly induced apoptosis by 20.7% (P <0.05) compared with 10.2% and 4.4% induced by treatment with GSPs and SFN alone respectively in MDA-MB-231 cells (Figure 4A and 4C). However, non-apoptotic cell death (Q1) in the group treated with the combination was less than that treated with GSPs alone. The evidence suggests that the antagonism between GSPs and SFN in MDA-MB-231 cells is not due to the induction of apoptosis. Moreover, Figure 4B and 4D indicate that GSPs and their combination with SFN inhibited apoptosis in MCF-7 cells compared with the groups treated with DMSO and SFN alone. Furthermore, GSPs and their combination with SFN led to no greater than additive effect in non-apoptotic cell death in MCF-7 cells. These findings suggest that the antagonism between GSPs and SFN in MCF-7 cells maybe as a result of the induction of apoptosis.

MDA-MB-231

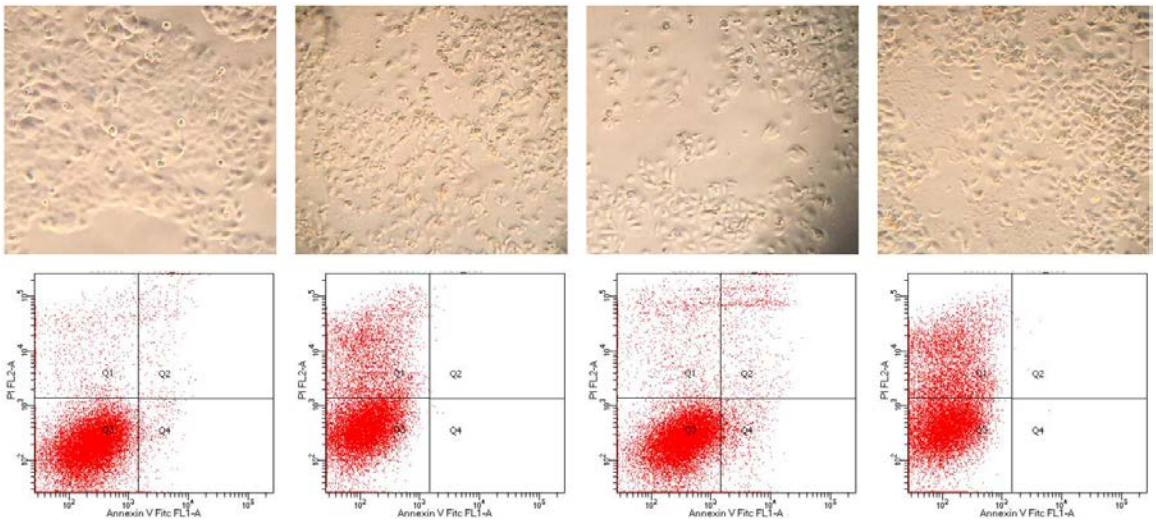
Control G40 S5 G40&S5



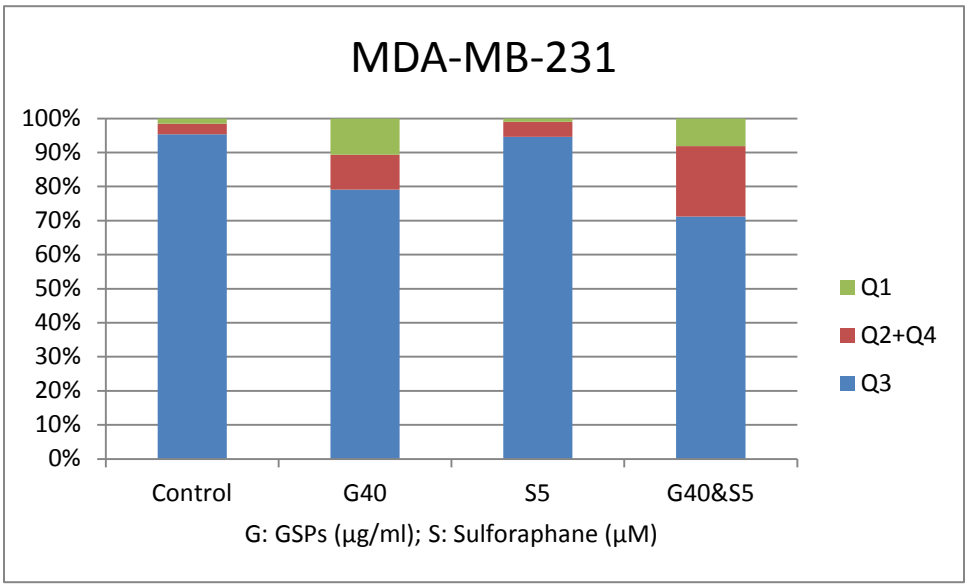
(A)

MCF-7

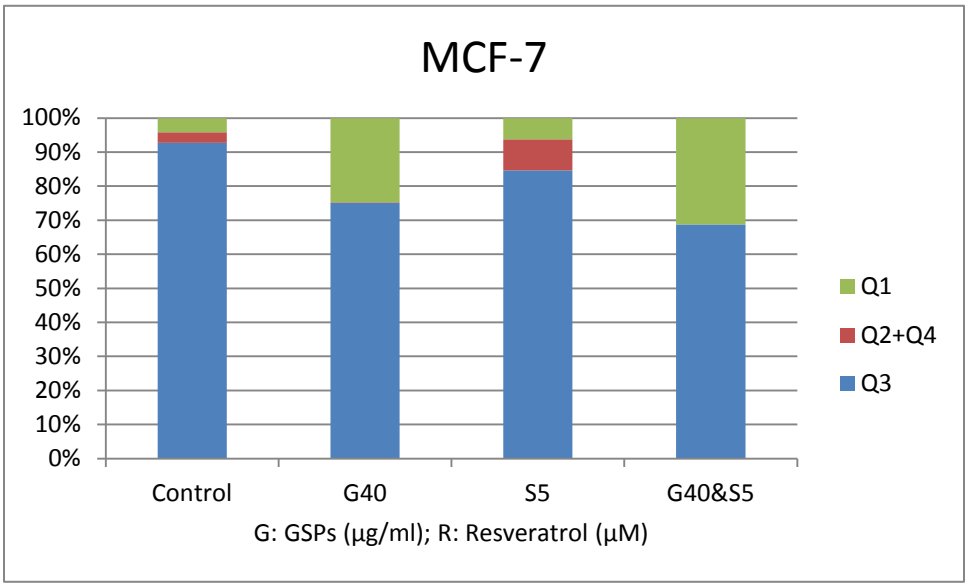
Control G40 S5 G40&S5



(B)



(C)



(D)

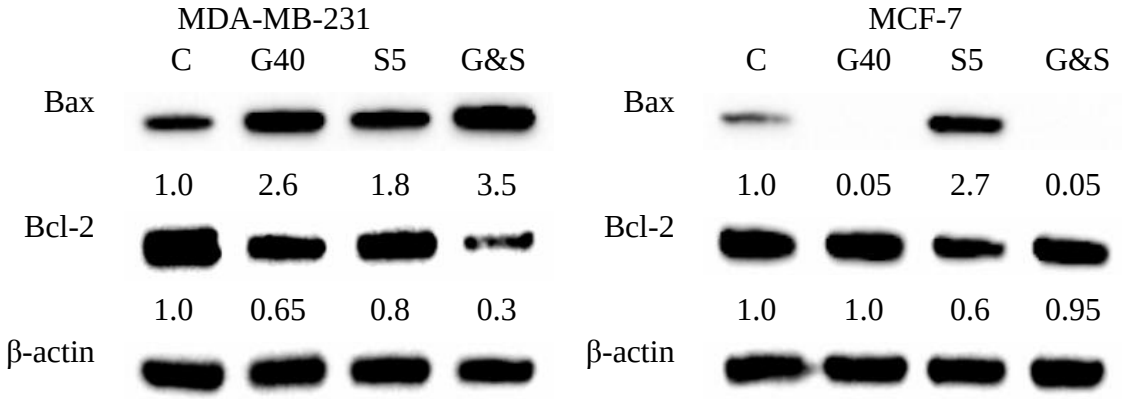
Figure 4. Morphological Change and Apoptosis Assay. Morphological and apoptotic changes of MDA-MB-231 (A and C) and MCF-7 (B and D) human breast cancer cells induced by GSPs (40 μg/ml), SFN (5 μM), and their combination compared with the DMSO-treated control groups after treatment for 72 h. The images were taken at 40X magnification under a microscope after the cells were treated for 72 h. Apoptosis analysis was performed by the Annexin V-conjugated Alexafluor 488 (Alexa488) Apoptosis Vybrant Assay Kit and analyzed by FACS. Q1 (the upper left quadrant) of the FACS histogram represents dead cells (stained by propidium iodide) that are not associated with apoptosis. Q2 (the upper right quadrant) represents late apoptotic cells (stained by

Alexa488 and propidium iodide). Q3 (the lower left quadrant) represents live cells. Q4 (the lower right quadrant) represents early apoptotic cells (stained by Alexa488). The percentage of all four quadrants of cells from each treatment was indicated in C and D. Results were generalized and representative images were selected from three independent experiments with very similar observations.

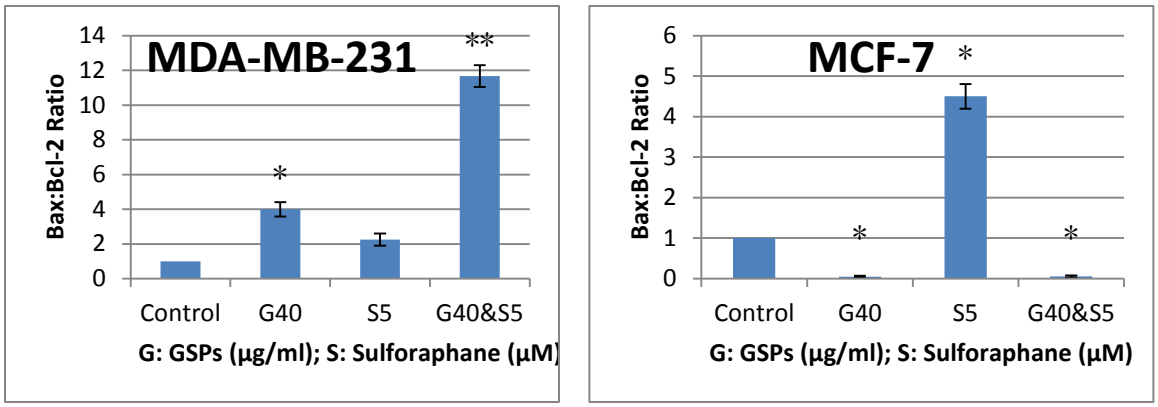
GSPs, SFN and their combination upregulate Bax expression and down-regulate Bcl-2 expression in MDA-MB-231 human breast cancer cells, whereas GSPs and their combination with SFN down-regulate Bax expression in MCF-7 human breast cancer cells

To verify the results of the apoptosis analysis, western blot analysis was performed to determine the expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 in MDA-MB-231 and MCF-7 human breast cancer cells, as the induction of apoptosis is linked to the upregulation of Bax and to the down-regulation of Bcl-2.[61-62] As shown in Figure 5A, GSPs (40 μg/ml) and SFN (5 μM) increased Bax expression by 160% and 80% respectively, and their combination increased Bax expression by 250% compared with the control group treated with DMSO in MDA-MB-231 cells after treatment for 72 h. GSPs and SFN decreased Bcl-2 expression by 35% and 20% respectively and their combination more than additively decreased Bcl-2 expression by 70% in MDA-MB-231 cells. While, in MCF-7 cells, Bax expression was reduced to less than 5% in both GSPs treated group and the group treated with GSPs and SFN in combination compared with the control group. Little or no change in the expression of Bcl-2 was detected in the groups treated with GSPs either alone or in combination with SFN. Although, SFN, as in MDA-MB-231 cells, increased Bax expression by 170% and resulted in a 40%

decrease in Bcl-2 expression compared with the control group in MCF-7 cells. Bax:Bcl-2 protein ratio was further calculated in both cell lines, since the ratio is considered to play a determinant role in signal transmission of apoptosis.[63] As displayed in Figure 5B, the Bax:Bcl-2 protein ratio from the combinational treatment group demonstrated a significant increase ($P < 0.05$) compared with the other groups in MDA-MB-231 cells, while the ratio from the groups treated with GSPs either alone or in combination with SFN expressed a significant decrease ($P < 0.05$) compared with the other groups in MCF-7 cells. These results confirm the findings in the previous apoptosis assays.



(A)



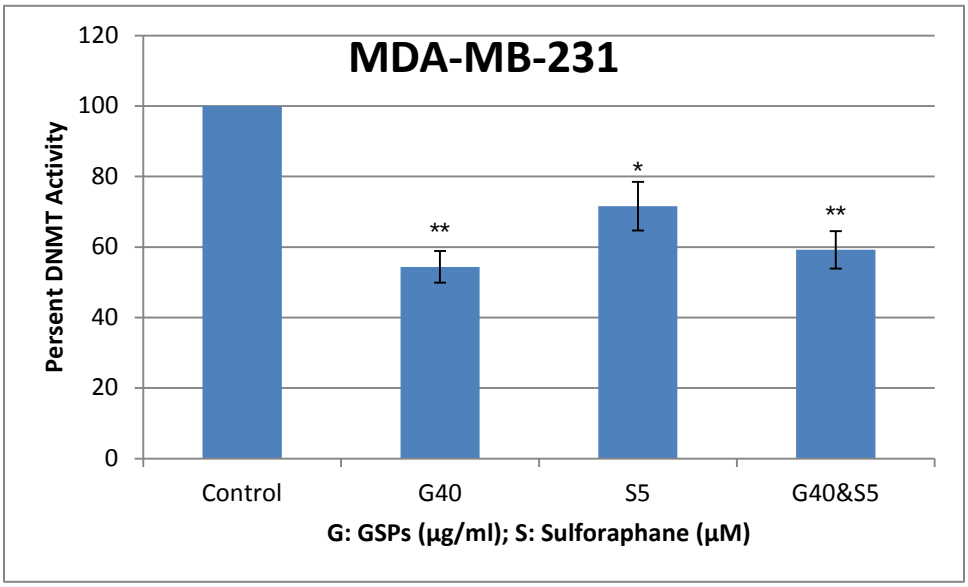
(B)

Figure 5. Western Blot Analysis and Bax:Bcl-2 Ratio. Change of expression of Bax and Bcl-2 in MDA-MB-231 and MCF-7 human breast cancer cells induced by GSPs (40 μg/ml), SFN (5 μM) and their combination compared with the DMSO-treated control groups after treated for 72 h (A). β-actin was used to confirm equivalent loading of the protein samples. The relative density of each band was measured by ImageJ and was shown under each blot of Bax and Bcl-2 after normalization to the control. A representative image was selected from three independent experiments with very similar results. The Bax:Bcl-2 protein ratio is represented as the mean ± SD (B). * $P < 0.05$, ** $P < 0.01$

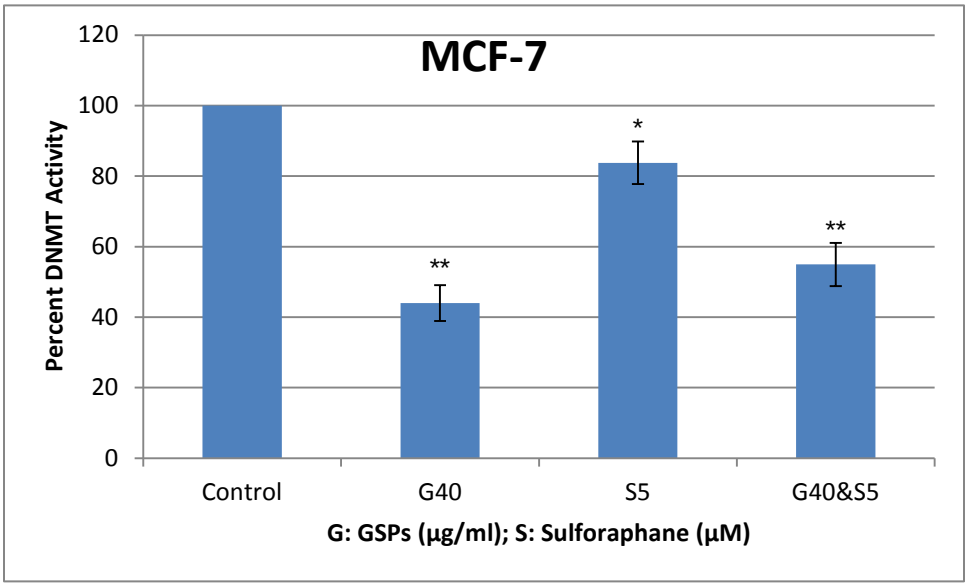
GSPs and SFN antagonistically reduce DNMT activity and HDAC activity in MDA-MB-231 and MCF-7 human breast cancer cells

To further explore the mechanisms of the inhibitory effects of GSPs and SFN on MDA-MB-231 and MCF-7 human breast cancer cells, DNMT and HDAC activity assays were performed. As shown in Figures 6 and 7, GSPs (40 μg/ml) significantly decreased DNMT activity by 46% ($P < 0.01$) in MDA-MB-231 cells and 56% ($P < 0.01$) in MCF-7 cells, and significantly decreased HDAC activity by 48% ($P < 0.01$) in MDA-MB-231 cells and 52% ($P < 0.01$) in MCF-7 cells, compared with the DMSO-treated control groups after treatment for 72 h. SFN (5 μM) significantly decreased DNMT activity by 28% ($P < 0.05$) in MDA-MB-231 cells and 16% ($P < 0.05$) in MCF-7 cells, and significantly decreased HDAC activity by 17% ($P < 0.05$) in both cell lines. The combinational treatment significantly decreased DNMT activity by 41% ($P < 0.01$) in MDA-MB-231 cells and 45% ($P < 0.01$) in MCF-7 cells, and significantly decreased HDAC activity by 46% ($P < 0.01$) in MDA-MB-231 cells and 47% ($P < 0.01$) in MCF-7 cells. The fact that the combinational treatment led to less reduction in DNMT activity and HDAC activity in both cell lines suggests that the antagonism between GSPs and SFN may result from

their combinational effect on regulating DNMTs and HDACs in these human breast cancer cell lines.



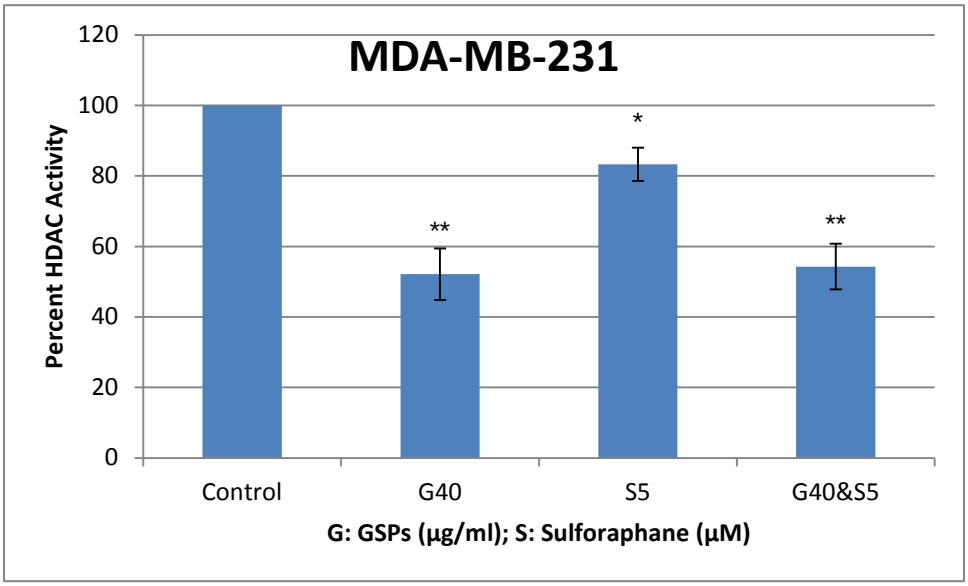
(A)



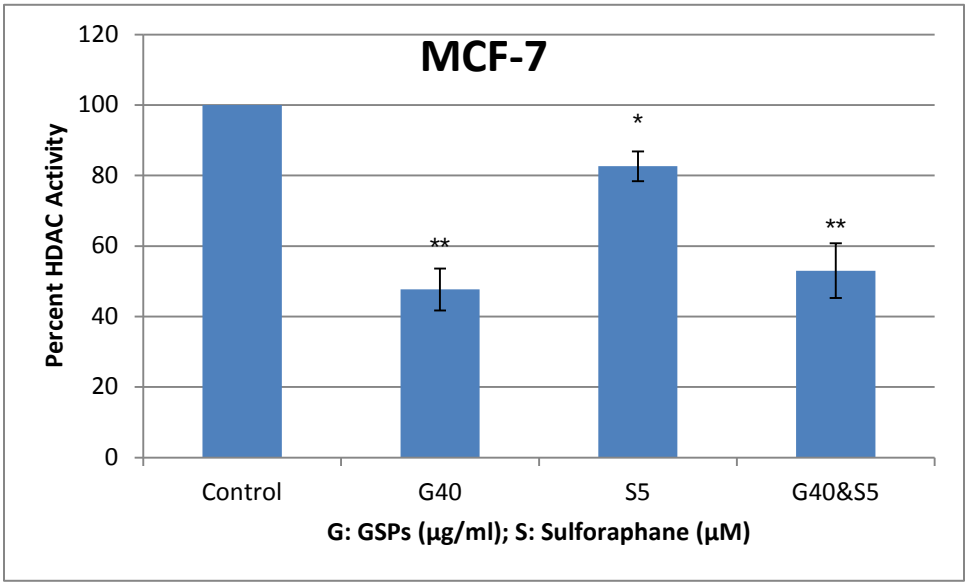
(B)

Figure 6. DNMT Activity Assay. Inhibition of DNMT activity in MDA-MB-231 (A) and MCF-7 (B) human breast cancer cells after treatment with GSPs (40 µg/ml), SFN (5 µM) and their combination compared with the DMSO-treated control groups for 72 h. Results

were generalized from three independent experiments with very similar observations. The DNMT activity of each treatment group is represented in percentage compared with the control group as the mean ± SD. * $P < 0.05$, ** $P < 0.01$



(A)



(B)

Figure 7. HDAC Activity Assay. Inhibition of HDAC activity in MDA-MB-231 (A) and MCF-7 (B) human breast cancer cells after treatment with GSPs (40 μg/ml), SFN (5 μM) and their combination compared with the DMSO-treated control groups for 72 h. Results were generalized from three independent experiments with very similar observations. The

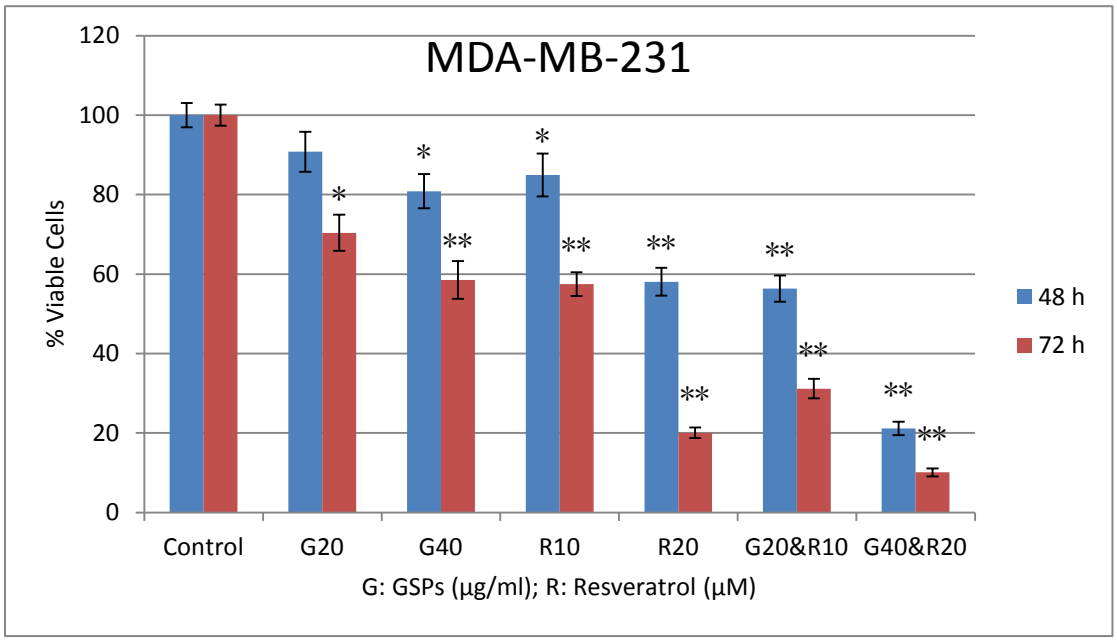
HDAC activity of each treatment group is represented in percentage compared with the control group as the mean ± SD. * $P < 0.05$, ** $P < 0.01$

Combinational Proanthocyanidins and Resveratrol Synergistically Inhibit Human Breast Cancer Cells and Impact Epigenetic-mediating Machinery

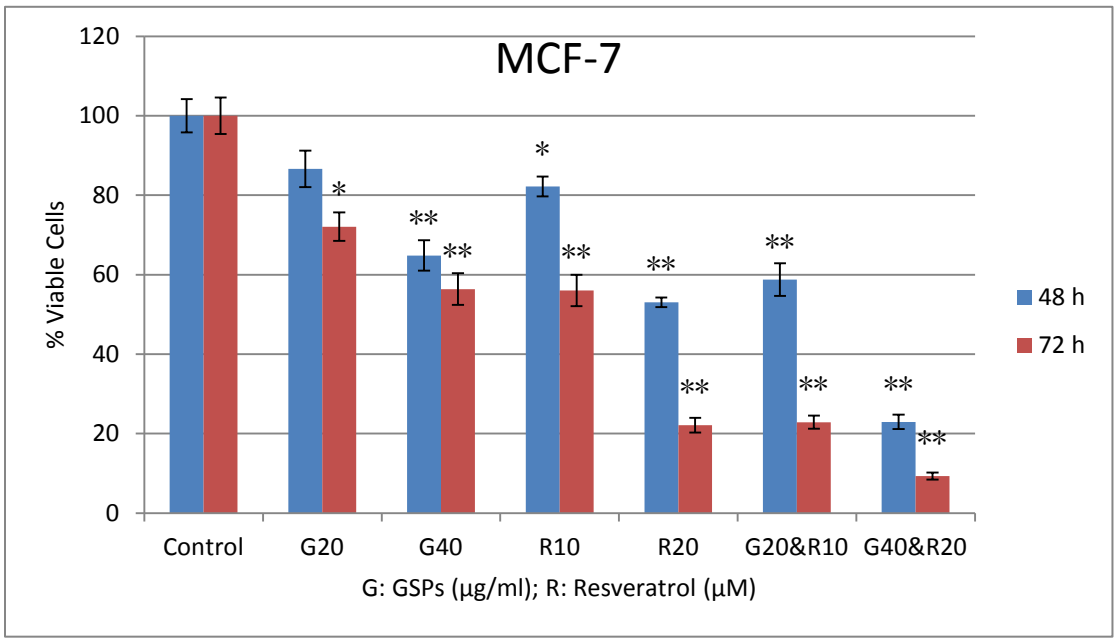
GSPs and Res synergistically inhibit cell viability and proliferation in MDA-MB-231 and MCF-7 human breast cancer cells

To determine the anti-carcinogenic effect of GSPs, Res and their combinations on human breast cancer cells, MTT assay was firstly performed. As shown in Figure 8A and 8B, all treatments with GSPs (20, 40 μg/ml), Res (10, 20 μM) and their combinations (20 μg/ml GSPs with 10 μM Res, and 40 μg/ml GSPs with 20 μM Res) resulted in reduction in cell viability in a dose- and time-dependent manner compared with the DMSO-treated control groups in MDA-MB-231 and MCF-7 cells. The treatments with GSPs resulted in significant decreases in cell viability by 9% to 19% ($P < 0.05$) after 48 h and 30% ($P < 0.05$) to 41% ($P < 0.01$) after 72 h in MDA-MB-231 cells, 13% to 35% ($P < 0.01$) after 48 h and 28% ($P < 0.05$) to 44% ($P < 0.01$) after 72 h in MCF-7 cells. The treatments with Res led to significant decreases in cell viability by 15% ($P < 0.05$) to 42% ($P < 0.01$) after 48 h and 42% ($P < 0.01$) to 80% ($P < 0.01$) after 72 h in MDA-MB-231 cells, 18% ($P < 0.05$) to 47% ($P < 0.01$) after 48 h and 44% ($P < 0.01$) to 78% ($P < 0.01$) after 72 h in MCF-7 cells. The treatments with GSPs and Res in combinations resulted in signifi-

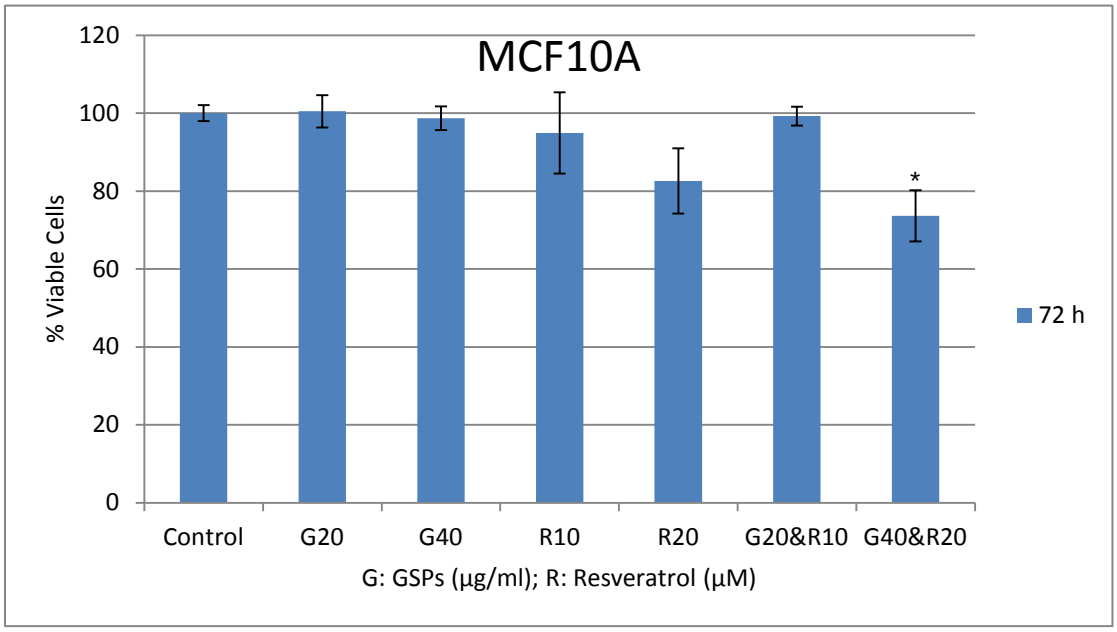
cant decrease in cell viability by 44% ($P < 0.01$) to 79% ($P < 0.01$) after 48 h and 69% ($P < 0.01$) to 90% ($P < 0.01$) after 72 h in MDA-MB-231 cells, 41% ($P < 0.01$) to 77% ($P < 0.01$) after 48 h and 77% ($P < 0.01$) to 91% ($P < 0.01$) after 72 h in MCF-7 cells. Furthermore, each combinational treatment exhibited a more significant ($P < 0.05$) reduction in cell viability than treatment with either GSPs or Res alone in both cell lines suggesting that GSPs and Res inhibited MDA-MB-231 and MCF-7 cells synergistically.



(A)



(B)



(C)

Figure 8. MTT Assay. Inhibition of cell viability in MDA-MB-231 (A) and MCF-7 (B) human breast cancer cells after treatment with GSPs (20, 40 µg/ml), Res (10, 20 µM), and their combinations (20 µg/ml GSPs with 10 µM Res, 40 µg/ml GSPs with 20 µM Res) compared with the DMSO-treated control cells for 48 h and 72 h. MCF10A human mammary epithelial cells (C) were used as the control cells to determine the toxicity of

these phytochemicals of varying concentrations. Results were generalized from three independent experiments with very similar observations. The cell viability of each treatment group is represented in percentage compared with the control group as the mean ± SD. * $P < 0.05$, ** $P < 0.01$

To confirm the synergistic effect on human breast cancer cells between GPSs and SFN, the results from the aforementioned MTT assay were further analyzed by the software CompuSyn version 1.0. Combination index (*CI*) values were generated by the software. $CI < 1$ indicates synergism, $CI = 1$ indicates additive effect, $CI > 1$ indicates antagonism.[59-60] As shown in Table 2, all CI values of the combinational treatments of the MTT assay exhibited synergism ($CI > 1$) in both MDA-MB-231 and MCF-7 cells.

Table 2. Synergism between GSPs and Res Indicated by Combination Index (*CI*) Values.

Cell Line	Treatment Time (h)	Dose GSPs (μg/ml)	Dose Res (μM)	Normalized Effect	*CI* Value
MDA-MB-231	48	20	10	0.56362	0.67285
MDA-MB-231	48	40	20	0.21186	0.54965
MDA-MB-231	72	20	10	0.31165	0.74780
MDA-MB-231	72	40	20	0.10106	0.75330
MCF-7	48	20	10	0.58774	0.99477
MCF-7	48	40	20	0.22958	0.88512
MCF-7	72	20	10	0.22885	0.62425
MCF-7	72	40	20	0.09354	0.70506

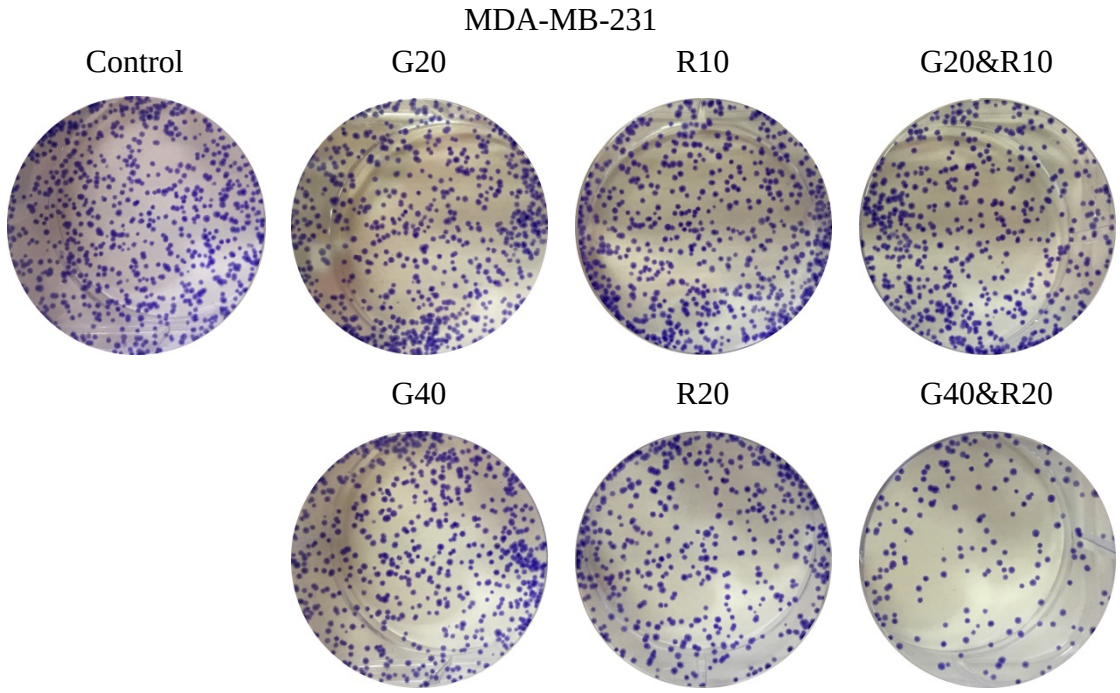
The *CI* values were generated by the CompuSyn software from calculating the normalized effect (the effect of treatment with phytochemicals compared with that of treatment with DMSO) of the combinational treatments compared with the normalized effect of the treatments with GSPs and Res alone (not shown in this table) from the data of the MTT assays. $CI < 1$ indicates synergism. $CI = 1$ indicates additive effect. $CI > 1$ indicates antagonism.

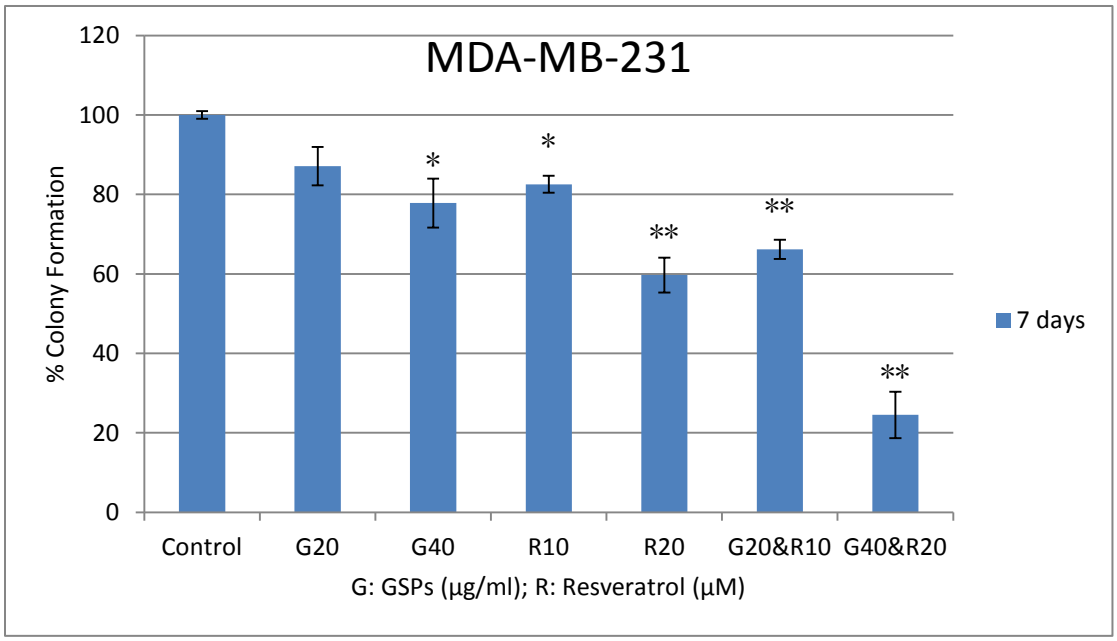
To investigate the toxicity of GSPs, Res and their combinations, MTT assay was performed on the immortalized non-cancerous MCF10A human mammary epithelial cells. The cells were treated with 0.5% (v/v) DMSO, GSPs (20, 40 μg/ml), Res (10, 20 μM) and their combinations (20 μg/ml GSPs with 10 μM Res, and 40 μg/ml GSPs with 20 μM Res) for 72 h. As shown in Figure 8C, the lower dose treatments with GSPs (20 μg/ml), Res (10 μM) and their combination exhibited little to no apparent reduction in cell viability compared with the DMSO-treated control group in MCF10A cells. The higher dose treatment with Res (20 μM) displayed no significant reduction in cell viability, however, the higher dose treatment with GSPs (40 μg/ml) and Res (20 μM) in combination led to a significant decrease in cell viability by 26% ($P < 0.05$), which indicates that GSPs and Res in combination may be toxic to some human mammary cells in higher concentration.

GSPs and Res synergistically inhibit posttreatment colony forming ability in MDA-MB-231 and MCF-7 human breast cancer cells

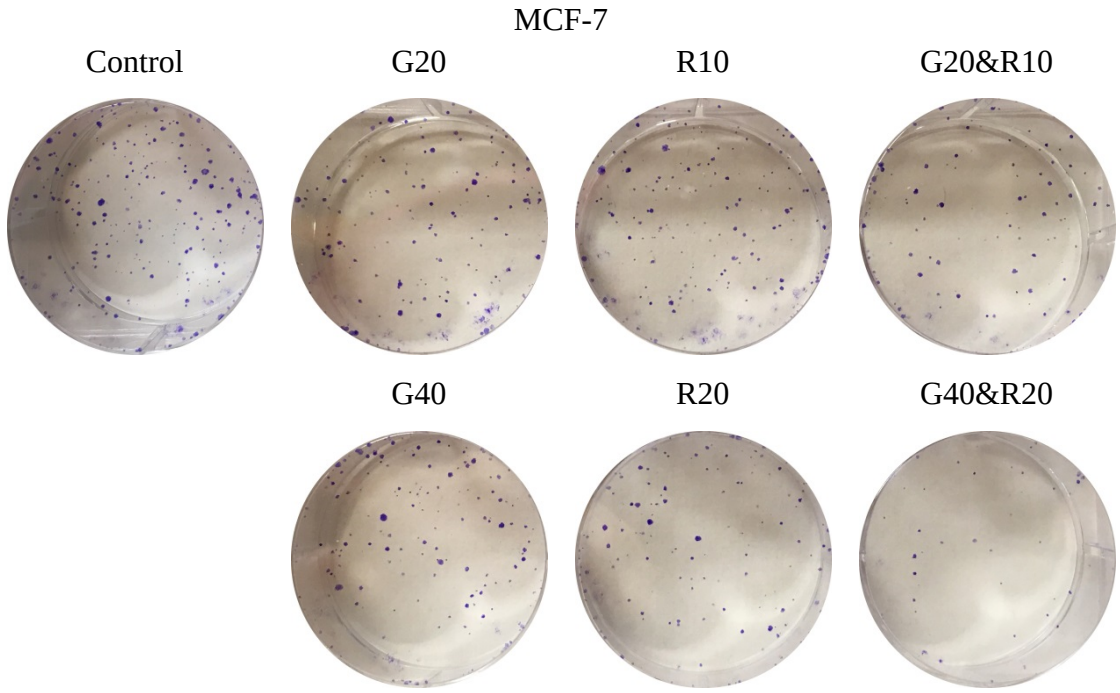
To examine the long-term anti-carcinogenic effect of GSPs, Res and their combinations on cell proliferation in MDA-MB-231 and MCF-7 human breast cancer cells, clonogenic assays were performed. As indicated in Figure 9, GSPs (20, 40 μg/ml) and Res (10, 20 μM) inhibited the posttreatment colony forming abilities of MDA-MB-231 (A) and MCF-7 (B) cells in a synergistic manner during a 7-day period compared with the DMSO-treated control groups after treatment for 48 h. The groups previously treated with GSPs showed significant decreases in colony formation by 13% to 22% ($P < 0.05$) in

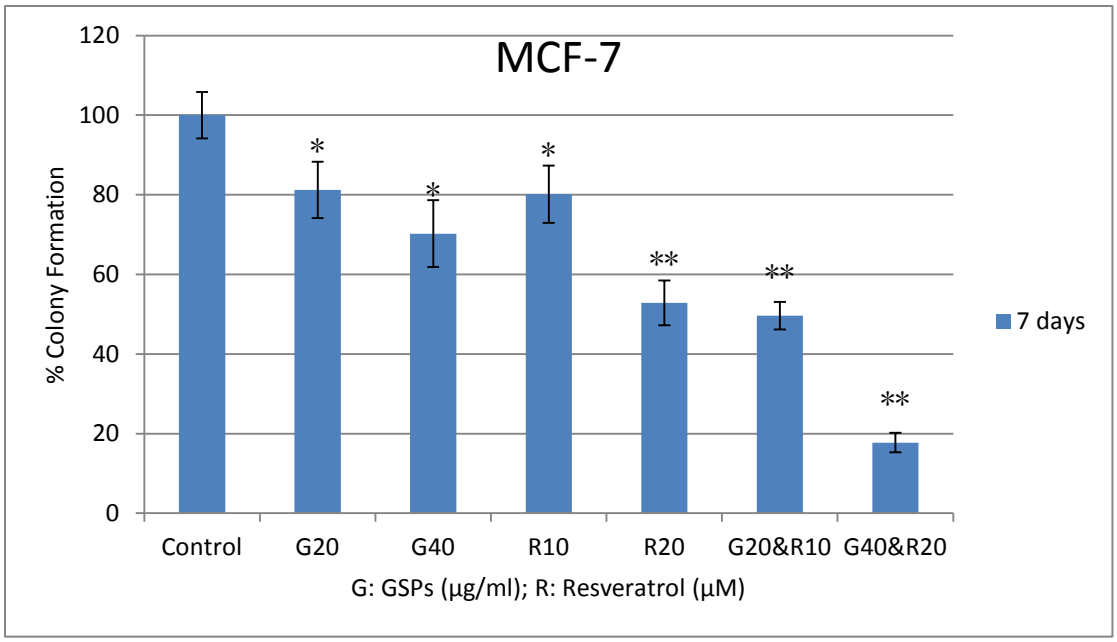
MDA-MB-231 cells and 19% ($P < 0.05$) to 30% ($P < 0.05$) in MCF-7 cells. The groups formerly treated with Res exhibited significant decreases in colony formation by 17% ($P < 0.05$) to 40% ($P < 0.01$) in MDA-MB-231 cells and 20% ($P < 0.05$) to 47% ($P < 0.01$) in MCF-7 cells. And the pretreatments with GSPs and Res in combinations led to significant reductions in colony formation by 34% ($P < 0.01$) to 75% ($P < 0.01$) in MDA-MB-231 cells and 50% ($P < 0.01$) to 82% ($P < 0.01$) in MCF-7 cells.


MDA-MB-231
Control
G20
R10
G20&R10
G40
R20
G40&R20

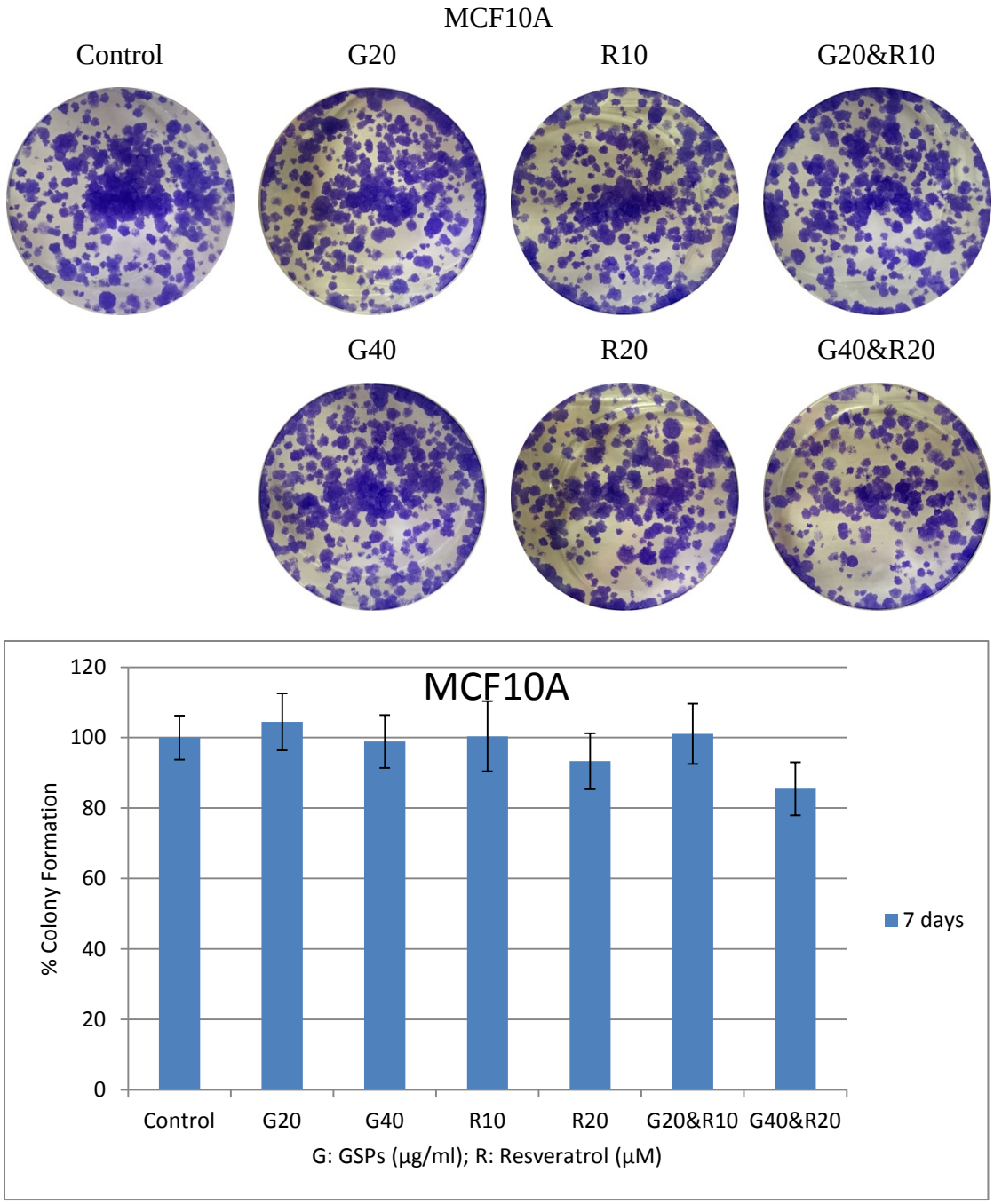

MDA-MB-231
120
100
80
60
40
20
0
% Colony Formation
*
*
**
**
**
Control
G20
G40
R10
R20
G20&R10
G40&R20
G: GSPs (μg/ml); R: Resveratrol (μM)
7 days

(A)


MCF-7
Control
G20
R10
G20&R10
G40
R20
G40&R20


MCF-7
% Colony Formation
120
100
80
60
40
20
0
*
*
*
**
**
**
Control
G20
G40
R10
R20
G20&R10
G40&R20
G: GSPs (μg/ml); R: Resveratrol (μM)
7 days

(B)



(C)

Figure 9. Clonogenic Assay. Inhibition of colony forming ability in MDA-MB-231 (A) and MCF-7 (B) human breast cancer cells as well as MCF10A (C) human mammary epithelial cells in 7 days after treatment with GSPs (20, 40 μg/ml), Res (10, 20 μM), and their combinations (20 μg/ml GSPs with 10 μM Res, 40 μg/ml GSPs with 20 μM Res) compared with the DMSO-treated control groups for 48 h. Results were generalized and representative images were selected from three independent experiments with very simi-

lar observations. The colony forming ability of each treatment group is represented in percentage compared with the control group as the mean ± SD. * $P < 0.05$, ** $P < 0.01$

The posttreatment colony forming ability of MCF10A (C) cells was also accessed using the same method. The results expressed no reduction of colony formation at the lower doses and no significant reduction at the higher ones. Thus, together with the results of the MTT assay, it is safe to conclude that GSPs, Res and their combinations exhibited no toxicity in lower doses which had been chosen for the rest of the experiments in this study. As a consequence, the rest of the study proceeded without the use of MCF10A as control cells.

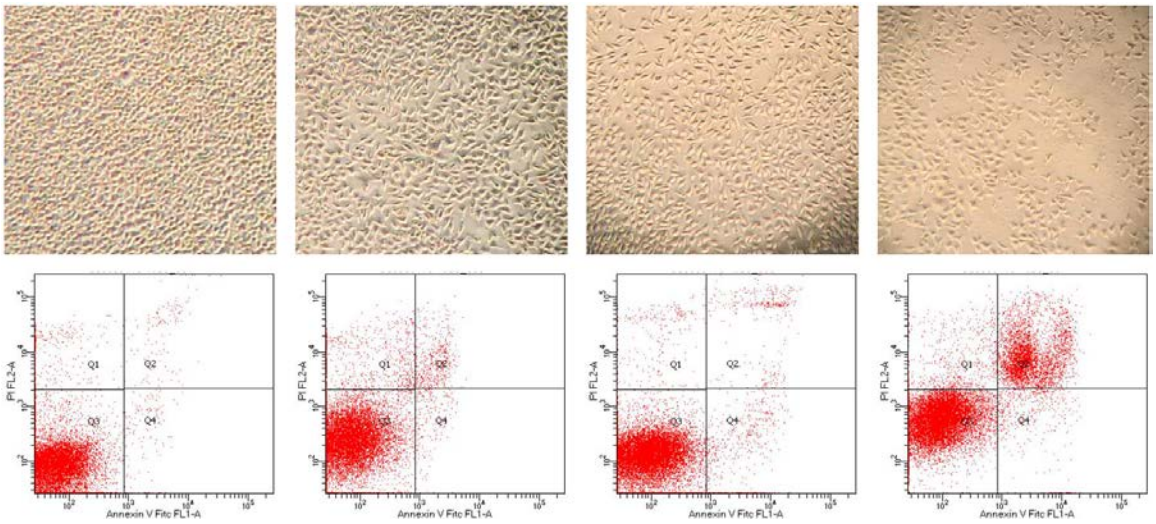
GSPs and Res synergistically induce apoptosis in MDA-MB-231 human breast cancer cells, whereas GSPs and their combination with Res inhibit apoptosis in MCF-7 human breast cancer cells

To investigate whether or not the synergistic effects of GSPs and Res on the inhibition of cell viability and proliferation as well as on the reduction of posttreatment colony forming ability in MDA-MB-231 and MCF-7 human breast cancer cells are associated with the induction of apoptosis, apoptosis analysis was performed by using the Annexin V-conjugated Alexafluor 488 (Alexa488) Apoptosis Vybrant Assay Kit following the manufacture's protocol. Firstly, cell density was reduced in groups treated with GSPs (20 μg/ml) and Res (10 μM) alone and was greatly reduced in the group treated with their combination compared with the DMSO treated control group after treatment for 48 h in

both MDA-MB-231 and MCF-7 cells as shown in Figure 10A and 10B. Morphological changes were also observed in the phytochemical-treated groups compared with the control group. Secondly, apoptosis was analyzed using flow cytometry as described above. Cells were counted in four quadrants in the FACS histograms where Q1 (the upper left quadrant) represents dead cells (stained by propidium iodide) that are not associated with apoptosis, Q2 (the upper right quadrant) represents late apoptotic cells (stained by Alexa488 and propidium iodide), Q3 (the lower left quadrant) represents live cells and Q4 (the lower right quadrant) represents early apoptotic cells (stained by Alexa488). Q2 and Q4 were grouped together when the percentage of all four quadrants of cells from each treatment was illustrated in Figure 10C and 10D. The results show that the combinational treatment of GSPs and Res significantly induced apoptosis by 21.8% (P <0.05) compared with 3.4% and 4.1% induced by treatment with GSPs and Res alone respectively in MDA-MB-231 cells (Figure 10A and 10C). However, such induction of apoptosis was not observed in MCF-7 cells suggested by Figure 10B and 10D. GSPs and their combination with Res inhibited apoptosis in MCF-7 cells compared with the groups treated with DMSO and Res alone. Furthermore, GSPs and their combination with Res increased cell death which was not resulting from apoptosis in MCF-7 cells. The combinational treatment led to 24.6% cell death in MCF-7 cells which is greater than 19.1% caused by GSPs and 5.1% caused by Res combined. All evidence suggests that the synergism between GSPs and Res discovered in previous experiments may be associated with the induction of apoptosis in MDA-MB-231 cells but not in MCF-7 cells.

MDA-MB-231

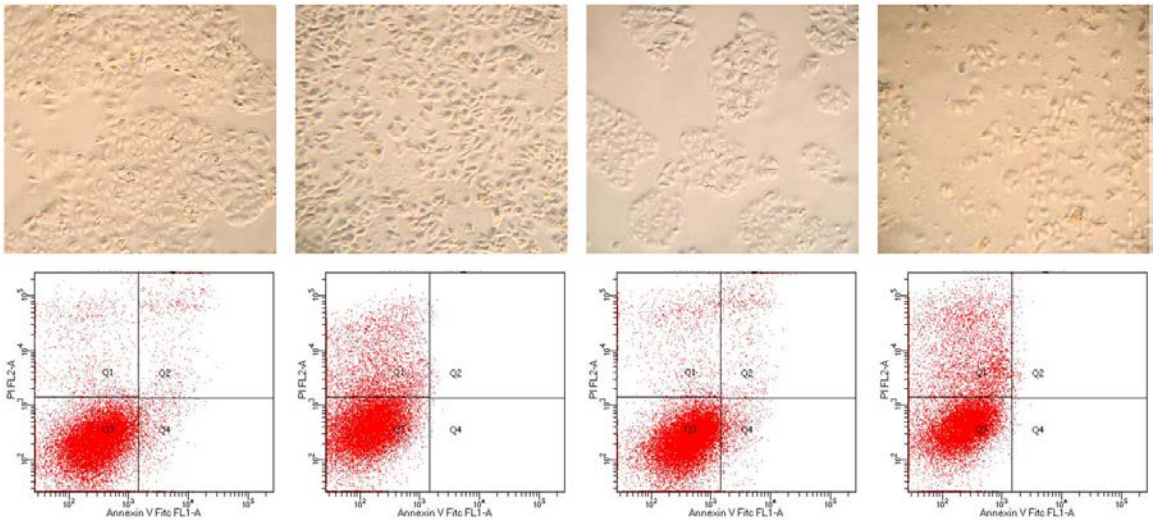
Control G20 R10 G20&R10



(A)

MCF-7

Control G20 R10 G20&R10



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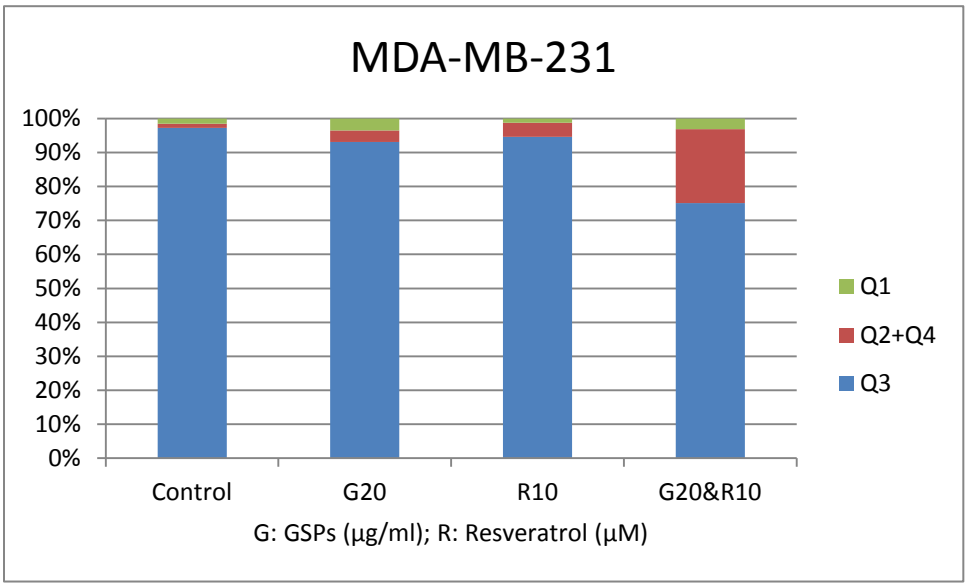
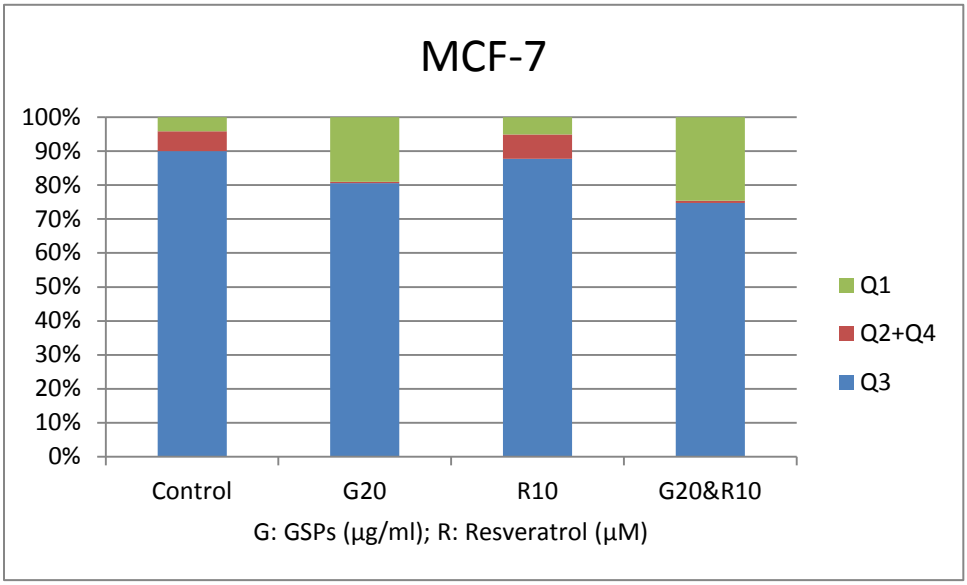


(C)



(D)

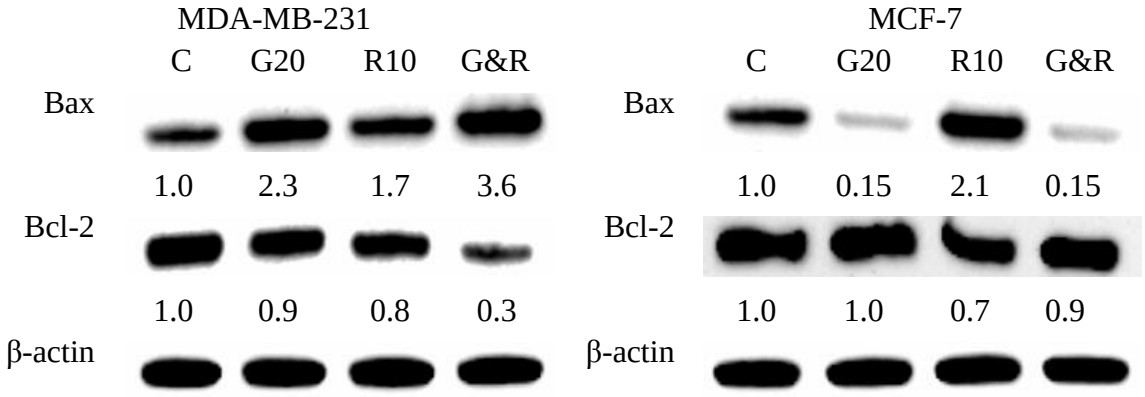
Figure 10. Morphological Change and Apoptosis Assay. Morphological and apoptotic changes of MDA-MB-231 (A and C) and MCF-7 (B and D) human breast cancer cells induced by GSPs (20 μg/ml), Res (10 μM), and their combination compared with the DMSO-treated control groups after treatment for 48 h. The images were taken at 40X magnification under a microscope after the cells were treated for 48 h. Apoptosis analysis was performed by the Annexin V-conjugated Alexafluor 488 (Alexa488) Apoptosis Vybrant Assay Kit and analyzed by FACS. Q1 (the upper left quadrant) of the FACS histogram represents dead cells (stained by propidium iodide) that are not associated with apoptosis. Q2 (the upper right quadrant) represents late apoptotic cells (stained by

Alexa488 and propidium iodide). Q3 (the lower left quadrant) represents live cells. Q4 (the lower right quadrant) represents early apoptotic cells (stained by Alexa488). The percentage of all four quadrants of cells from each treatment was indicated in C and D. Results were generalized and representative images were selected from three independent experiments with very similar observations.

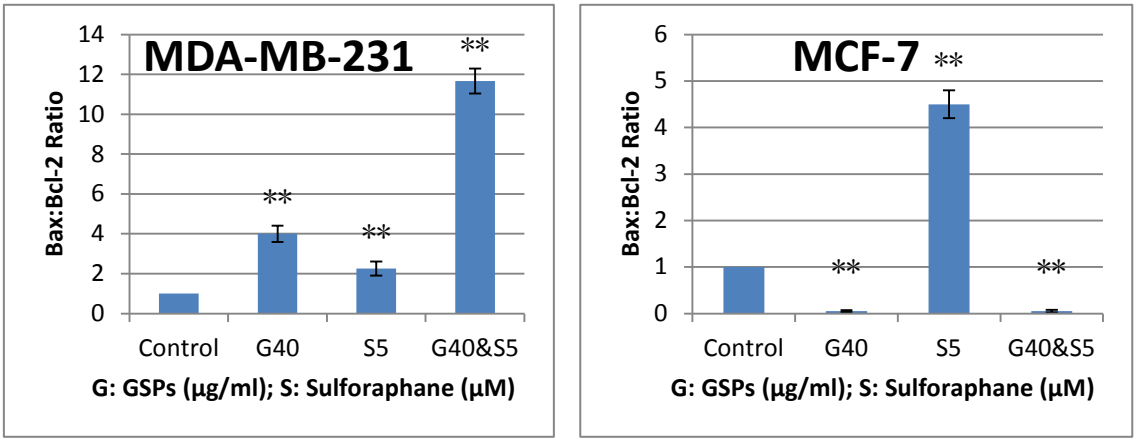
GSPs, Res and their combination upregulate Bax expression and down-regulate Bcl-2 expression in MDA-MB-231 human breast cancer cells, whereas GSPs and their combination with Res down-regulate Bax expression in MCF-7 human breast cancer cells

To verify the results of the apoptosis analysis, western blot analysis was performed to determine the expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 in MDA-MB-231 and MCF-7 human breast cancer cells, as the induction of apoptosis is linked to the upregulation of Bax and to the down-regulation of Bcl-2.[61-62] As shown in Figure 11A, Res (10 μM) increased Bax expression by 70%, GSPs (20 μg/ml) more than doubled Bax expression and their combination nearly quadrupled the expression of Bax compared with the control group treated with DMSO in MDA-MB-231 cells after treatment for 48 h. GSPs and Res decreased Bcl-2 expression by 10% and 20% respectively and their combination synergistically decreased Bcl-2 expression by 70% in MDA-MB-231 cells. While, in MCF-7 cells, Bax expression was reduced to 15% in both GSPs treated group and the group treated with GSPs and Res in combination compared with the control group. Little or no change in the expression of Bcl-2 was detected in the groups treated with GSPs either alone or in combination with Res. Although, Res, as in MDA-MB-231 cells, led to a more-than-two-fold increase in Bax expression and resulted

in a 30% decrease in Bcl-2 expression compared with the control group in MCF-7 cells. Bax:Bcl-2 protein ratio was further calculated in both cell lines, since the ratio is considered to play a determinant role in signal transmission of apoptosis[63]. As displayed in Figure 11B, the Bax:Bcl-2 protein ratio from the combinational treatment group demonstrated a significant increase ($P < 0.05$) compared to the other groups in MDA-MB-231 cells, while the ratio from the groups treated with GSPs either alone or in combination with Res expressed a significant decrease ($P < 0.05$) compared to the other groups in MCF-7 cells. These results suggest that GSPs and Res synergistically induce apoptosis in MDA-MB-231 cells through promoting transmission of apoptotic signals, whereas GSPs either alone or in combination with Res inhibit apoptosis by suppressing transmission of apoptotic signals in MCF-7 cells.



(A)



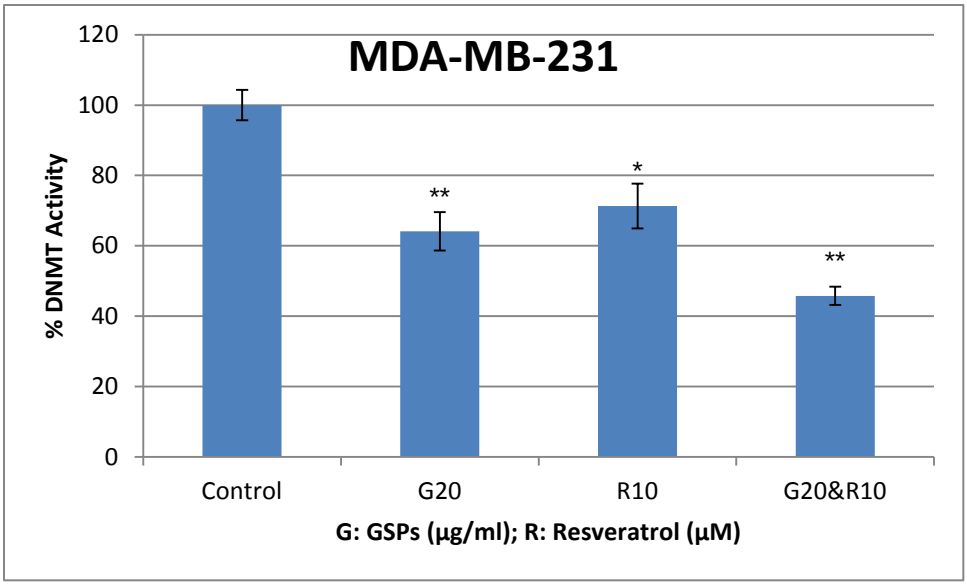
(B)

Figure 11. Western Blot Analysis and Bax:Bcl-2 Ratio. Change of expression of *Bax* and *Bcl-2* in MDA-MB-231 and MCF-7 human breast cancer cells induced by GSPs (20 μg/ml), Res (10 μM) and their combination compared with the DMSO-treated control groups after treatment for 48 h (A). β-actin was used to confirm equivalent loading of the protein samples. The relative density of each band was measured by ImageJ and was shown under each blot of Bax and Bcl-2 after normalization to the control. A representative image was selected from three independent experiments with very similar results. The Bax:Bcl-2 protein ratio is represented as the mean ± SD (B). * $P < 0.05$, ** $P < 0.01$

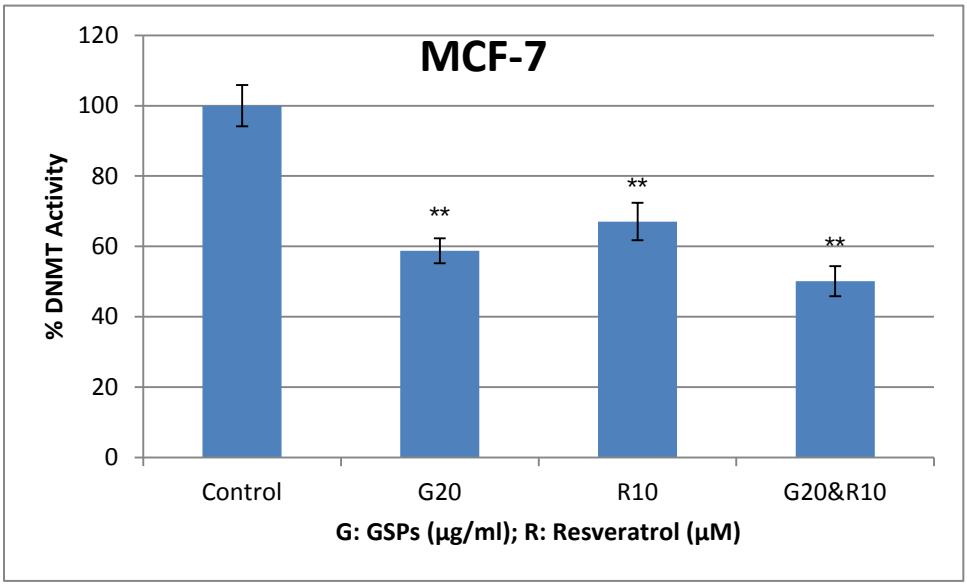
GSPs, Res and their combination decrease DNMT activity as well as HDAC activity in MDA-MB-231 and MCF-7 human breast cancer cells

To further explore the mechanisms of the inhibitory effects of GSPs and Res on MDA-MB-231 and MCF-7 human breast cancer cells, DNMT and HDAC activity assays were performed. As shown in Figures 12 and 13, GSPs (20 μg/ml), Res (10 μM) and their combination significantly decreased DNMT activity and HDAC activity compared with the DMSO-treated control group in both MDA-MB-231 and MCF-7 cells after treatment for 48 h ($P < 0.05$). The combinational treatment resulted in greater decreases in DNMT activity and HDAC activity in both cell lines. The inhibitory effect on HDAC activity in

MCF-7 cells of the combinational treatment is more than additive (Figure 13B), suggesting an epigenetic mechanism at least for HDACs that could be involved in the effects of these compounds.

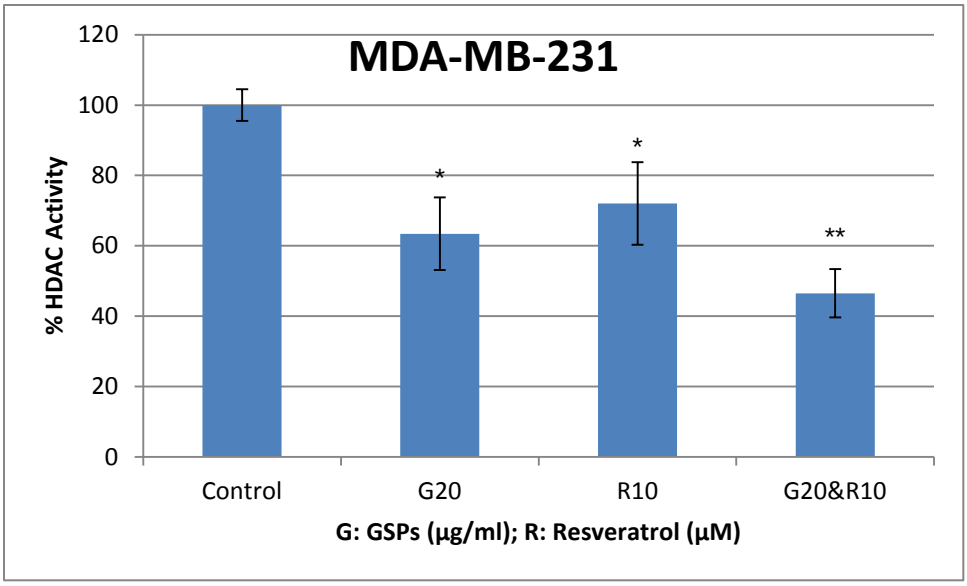


(A)

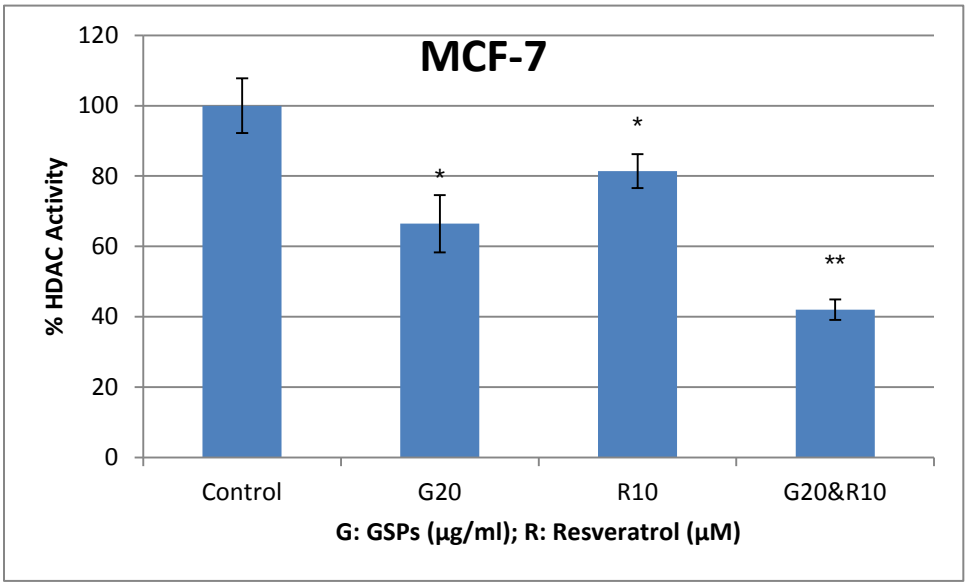


(B)

Figure 12. DNMT Activity Assay. Inhibition of DNMT activity in MDA-MB-231 (A) and MCF-7 (B) human breast cancer cells after treatment with GSPs (20 μg/ml), Res (10 μM) and their combination compared with the DMSO-treated control groups for 48 h. Results were generalized from three independent experiments with very similar observations. The DNMT activity of each treatment group is represented in percentage compared with the control group as the mean ± SD. * $P < 0.05$, ** $P < 0.01$



(A)



(B)

Figure 13. HDAC Activity Assay. Inhibition of HDAC activity in MDA-MB-231 (A) and MCF-7 (B) human breast cancer cells after treatment with GSPs (20 μg/ml), Res (10 μM) and their combination compared with the DMSO-treated control groups for 48 h. Results were generalized from three independent experiments with very similar observations. The HDAC activity of each treatment group is represented in percentage compared with the control group as the mean ± SD. * $P < 0.05$, ** $P < 0.01$

DISCUSSION

In recent years, effects of dietary components in combination on cancer have gained increasing interest. Here we report for the first time the combinational effects of grape seed proanthocyanidins (GSPs) and sulforaphane (SFN) as well as these of GSPs and resveratrol (Res) on MDA-MB-231 and MCF-7 human breast cancer cells.

We chose GSPs and SFN for our study firstly because of their vast availability in fresh produce and products that are heavily consumed by the general population as well as the high chance of their overlapping on people's dining table. Therefore, we sought to investigate the health benefits of these dietary compounds in combination. Secondly, as aforementioned, both GSPs and SFN have exhibited anti-cancer properties in various types of cancers. GSPs have been reported to down-regulate DNMTs, whilst SFN is a well-studied HDAC inhibitor.[55-57] Since both DNA hypermethylation and histone deacetylation have been shown to be associated with many biological processes in cancer development, the combination of DNMT inhibitors and an HDAC inhibitor, in theory, should have additive effects, if not synergistic effects, on cancer inhibition.

We chose GSPs and Res for our study because they are both abundant in grapes, which are some of the most consumed fruits on the planet and are considered to have

considerable health benefits. However, most grapes on the market for direct consumption are seedless due to a natural genetic mutation sometime ago that prevented the young seeds from maturing and developing a hard coat. Since proanthocyanidins are mostly contained in the seeds of grapes, these seedless grapes, as a result, offer little to no proanthocyanidins. Fortunately, the grapes used to produce red wines are seeded and both skin and seeds are preserved and utilized during red wine production. In addition, red wine offers more concentrated GSPs and Res than do red grapes, which makes it more feasible to consume a glass of red wine than a good amount of grapes every day.

Our results from MTT assays indicate that GSPs and SFN inhibited cell viability and proliferation in MDA-MB-231 and MCF-7 human breast cancer cells in a dose- and time-dependent manner. Surprisingly, however, our results also show that GSPs and SFN in combination inhibited both cell lines antagonistically, which means the combinational treatments were less effective than that with GSPs or SFN alone. Their antagonism was further confirmed by the combination index (CI) values generated by the CompuSyn software. To rule out the possibility of measurement errors, we performed MTT assays using MDA-MB-231 and MCF-7 cells treated with epigallocatechin-3-gallate (EGCG), another well-studied dietary component, using the same methods and the same conditions as performed for GSPs and SFN. The results, which were not shown in this report, were consistently similar to those in previous studies. Having validated our MTT results, we further performed clonogenic assays to determine the effect of GSPs, SFN and their combinations on the posttreatment colony forming ability of MDA-MB-231 and MCF-7 cells.

The results coincided with the antagonism between GSPs and SFN found in the previous MTT assays. We observed dose-dependent reduction in colony formation over a 7 day period after treated with GSPs and SFN individually for 72 h in both cell lines as well as a less reduction in the groups treated with the combination. It is evident that GSPs and SFN inhibit MDA-MB-231 and MCF-7 human breast cancer cells antagonistically.

Our MTT assay results also show that the combinational treatments of GSPs and Res reduced cell viability and proliferation in both MDA-MB-231 and MCF-7 cells significantly more than did treatment with either GSPs or Res of the same concentration alone after 48 h and 72 h. The combination index (CI) values generated by the software CompuSyn indicate strong synergism (CI < 1) between GSPs and Res. Additionally, our clonogenic assay results demonstrate that cell proliferation in MDA-MB-231 and MCF-7 cells was reduced not only under the treatment of GSPs, Res and their combinations, but after the treatment as well. The dose-dependent inhibition in the MTT assay was also observed in the clonogenic assay, as the groups treated with the higher doses of GSPs, Res and their combination exhibited fewer colonies than these with the lower dose treatments. Also, the posttreatment effect of GSPs and Res on MDA-MB-231 and MCF-7 cells proved to be synergistic. Collectively, the results of the clonogenic assay support our findings in the MTT assay.

We also used the immortalized non-cancerous MCF10A human mammary epithelial control cells to examine the toxicity of the phytochemicals that we used. In the experiments where the combinational effects of GSPs and SFN were studied, we report no ap-

parent toxicity in cell viability or posttreatment cell proliferation after 72 h treatment at all concentrations we used. Hence, we had chosen the higher concentration of GSPs (40 μg/ml), SFN (5 μM) and their combination which gave rise to greater inhibition in cell viability and posttreatment colony forming ability in MDA-MB-231 and MCF-7 cells, for the rest of the experiments in this study. In the experiments of GSPs and Res in combination, GSPs at 20 μg/ml, Res at 10 μM and their combination demonstrated no toxicity in cell viability or posttreatment cell proliferation after 72 h treatment. However, Res at 20 μM resulted in a decrease, though not significant, in cell viability compared to the DMSO-treated control group in MCF10A cells. Moreover, the combination of GSPs at 40 μg/ml, Res at 20 μM rendered a significant reduction in cell viability and a reduction (not significant) in posttreatment colony formation. Yet, it may be imprudent to conclude that the combination of GSPs and Res at such concentrations could be toxic since MCF10A cells, though non-carcinogenic, are immortalized. Thus, they are considered to exhibit at least some degree of telomerase activity. We have previously reported that Res (15μM) down-regulated *hTERT* (telomerase reverse transcriptase in humans) mRNA levels in HCC1806 human breast cancer cells after 72 h treatment.[60] Therefore, it is reasonable to deduce that the presence of Res may have given rise to the inhibition of MCF10A cells through down-regulating *hTERT* expression rather than toxicity. Regardless, we had chosen the lower concentration of GSPs (20 μg/ml), Res (10 μM) and their combination which led to significant decrease in cell viability and posttreatment colony forming ability in MDA-MB-231 and MCF-7 cells, for the rest of the experiments in this study.

GSPs, SFN and Res have been reported to induce apoptosis in human cancer cells respectively.[30, 54, 60, 63] In this study, we tested their combinational effects on the induction of apoptosis in MDA-MB-231 and MCF-7 human breast cancer cells. Our apoptosis analysis results show that GSPs (20 μg/ml) and Res (10 μM) synergistically induced apoptosis in MDA-MB-231 cells. GSPs (40 μg/ml) and SFN (5 μM) also synergistically induced apoptosis in MDA-MB-231 cells, but antagonistically killed cells through non-apoptotic mechanisms. In MCF-7 cells, treatment with SFN or Res alone induced apoptosis compared to the DMSO-treated control group; however, treatments with GSPs regardless of the presence of SFN or Res almost eliminated both early (Q4) and late (Q2) apoptotic cells. GSPs and SFN exhibit a less than additive effect on non-apoptotic cell deat, while the combinational effect of GSPs and Res on non-apoptotic cell death was more than additive.

We then examined the effects of the phytochemicals on the protein expression of Bax and Bcl-2, since the proteins of the Bcl-2 family are highly associated with the induction of apoptosis. The results of our Western blot analysis show that GSPs, SFN and their combination upregulated Bax expression and down-regulated Bcl-2 expression in MDA-MB-231 cells. So did Res and their combination with GSPs. And the Bax:Bcl-2 protein ratio, which is a determinant role in signal transmission of apoptosis, was significantly higher ($P < 0.01$) after treated with GSPs in combination with either SFN or Res, suggesting that both combinations greatly enhance apoptotic signal transmission in MDA-MB-231 cells. In MCF-7 cells, however, GSPs significantly down-regulated Bax

expression ($P < 0.01$) and caused little to no change in Bcl-2 expression regardless of the presence of SFN or Res. The Bax:Bcl-2 protein ratio was significantly lower ($P < 0.01$) after treated with GSPs alone and in combination with either SFN or Res, which indicates that GSPs inhibit apoptosis via blocking apoptotic signal transmission in MCF-7 cells. These findings from the western blot analysis support the results of our apoptosis analysis.

DNA methylation and histone deacetylation have been recognized to be associated with cancer prevention and therapy through regulating the expression of tumor suppressor genes and oncogenes. DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), which are enzymes that play crucial roles in these processes respectively, have been reported to act in collaboration in cancer development.[64-65] Previous studies in our lab have shown that phytochemicals acting as DNMT inhibitors and ones with HDAC inhibiting properties can work in synergy in inhibiting human cancer.[52-54] Thus, we performed DNMT and HDAC activity assays to further understand the effects of GSPs in combination with SFN and Res respectively on MDA-MB-231 and MCF-7 human breast cancer cells. The results show that GSPs, SFN and Res all acted as strong DNMT inhibitors as well as HDAC inhibitors in MDA-MB-231 and MCF-7 cells. However, the combination of GSPs and SFN resulted in less reduction in DNMT activity and HDAC activity than did GSPs and SFN alone in both cell lines, which may suggest that GSPs and SFN antagonistically inhibit MDA-MB-231 and MCF-7 cells by upregulating cancer suppressor genes through decreasing DNMT and HDAC activities. Whereas, GSPs and

Res in combination led to greater reduction in DNMT activity and HDAC activity than did GSPs and Res alone in both cell lines, indicating that GSPs and Res synergistically inhibit MDA-MB-231 and MCF-7 cells by upregulating cancer suppressor genes through decreasing DNMT and HDAC activities. These findings help illuminate understanding of the enzymatic activities of DNMTs and HDACs in these human breast cancer cell lines. Further analysis of the specific epigenetic modifiers in the DNMT family (DNMT1, DNMT3A and DNMT3B) and HDAC family (HDAC1 *etc.*) is to be conducted in future studies. It would also be interesting in future studies to investigate which cancer suppressor genes these phytochemicals modulate to give rise to such antagonistic/synergistic inhibition on these human breast cancer cell lines.

CONCLUSIONS

Collectively, we report that GSPs and SFN antagonistically inhibited cell viability and proliferation as well as posttreatment colony forming ability in MDA-MB-231 and MCF-7 human breast cancer cells. Further analyses show that GSPs and SFN antagonistically inhibited DNMT and HDAC activities in both cell lines. These findings suggest that the antagonism between GSPs and SFN in MDA-MB-231 and MCF-7 cells could be through the regulation of DNA methylation and/or histone modifications.

We also report that GSPs and Res synergistically inhibited cell viability and proliferation as well as posttreatment colony forming ability in MDA-MB-231 and MCF-7 human breast cancer cells. Further analyses show that GSPs and Res synergistically induced apoptosis in MDA-MB-231 cells by greatly enhancing apoptotic signal transmission. Additional, the combinational treatment with GSPs and Res led to greater inhibition in DNMT and HDAC activities compared with treatment with either GSPs or Res alone in both cell lines suggesting that the synergism between GSPs and Res could be due to the regulation of DNA methylation and/or histone modifications.

Both studies show that GSPs destroyed MCF-7 cells through non-apoptotic mechanisms. GSPs inhibited apoptosis regardless of the presence of SFN or Res in MCF-7 cells by greatly down-regulating Bax expression, thus blocking apoptotic signal transmission.

IMPACT OF EPIGENETIC DIETARY COMPONENTS ON CANCER THROUGH HISTONE MODIFICATIONS

by

YIFENG GAO

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IMPACT OF EPIGENETIC DIETARY COMPONENTS ON CANCER THROUGH HISTONE MODIFICATIONS

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Abstract: Epigenetics, the study of heritable changes in gene expression without modifying the nucleotide sequence, is among the most important topics in medicinal chemistry and cancer prevention and therapy. Among those changes, DNA methylation and histone modification have been shown to be associated with various types of cancers in a number of ways, many of which are regulated by dietary components that are mostly found in plants. Although mechanisms of nutrient components affecting histone acetylation/deacetylation in cancer are widely studied, how those natural compounds affect cancer through other histone modifications, such as methylation, phosphorylation and ubiquitylation, is rarely reviewed. Thus, this review article discusses impacts on histone acetylation as well as other histone modifications by nutrient components, such as genistein, resveratrol, curcumin, epigallocatechin-3-gallate (EGCG), 3,3'-diindolylmethane (DIM), diallyl disulfide, garcinol, procyanidin B3, quercetin, sulforaphane and other isothiocyanates that have been recently reported *in vivo* as well as in various types of cancer cell lines.

Key words: cancer, dietary components, epigenetics, gene expression, histone modifications, phytochemicals

1. Introduction

Epigenetics involves heritable changes in gene expression without altering DNA sequence. The most studied mechanisms of epigenetic regulation that cause such changes are DNA methylation and histone modification which affect gene expression. Histone modification or chromatin remodeling such as histone acetylation and histone deacetylation can change the accessibility of transcription factors to DNA, thereby regulating gene expression. The expression of genes such as *p53*, *p21* and *BAX*, modulates cancer development in a number of ways including cell growth, cell migration, cell cycle arrest, apoptosis and signaling pathways. Thus, manipulating gene expression seems to be the key to cancer therapy. One feasible way to achieve this is through dietary components like sulforaphane, epigallocatechin-3-gallate (EGCG), genistein, resveratrol and many others. These natural phytochemicals have been shown to regulate the chromatin through various epigenetic mechanisms such as histone acetyltransferases (HAT) and histone deacetylases (HDAC) inhibition as well as other histone modifying properties. This review summarizes recent findings on how dietary components affect histone modifications in cancer.

2. Histone and Histone Modifications

In eukaryotic cells, the DNA double helix is packaged into a compact structure called chromatin with the assistance of two major classes of proteins—histones and non-histones. The histones, which are unique to eukaryotes, include H1, H2A, H2B, H3 and H4 [1]. Histone H1 is known as the linker histone, while the rest are considered to be the core histones. Two copies of each of the core histones H2A, H2B, H3, and H4 form a histone octamer—a crystal structure that consists of a "kernel" shaped H3-H4 tetramer interdigitated with two H2A-H2B dimers. Together with 146 bp of core DNA wrapped around it, the histone octamer forms a nucleosome, the basic unit of chromosome [2]. Between two nucleosomes lies the double-stranded linker DNA which associates with histone H1. H1 located in the region of linker DNA sits on the nucleosome, holding the nucleosomes together [3, 4].

Each core histone has a flexible N-terminal tail which consists of amino acids prone to posttranslational modifications including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ribosylation, citrullination, deimination and proline isomerization [3, 5-8]. These histone modifications play an important role in assembling heterochromatin and in maintaining gene boundaries between transcribed and untranscribed genes.

Histone acetylation, catalyzed by histone acetyltransferases (HATs), is a process by which the lysine residues within the protruding N-terminal tail of the histone core are acetylated as part of regulation of gene expression [9]. Using the cofactor acetyl-CoA, HATs transfer acetyl groups to epsilon-amino groups of lysine residues in the N-terminal histone tails, which neutralizes the positive charge. This reduces electrostatic interaction between histones and negatively charged DNA and loosens chromatin structure, which makes it more accessible for transcription factors resulting in promotion of gene expression [10]. HATs, based on sequence homology and structural features as well as functions, can be grouped into four families. The Gcn5-related N-acetyltransferase (GNAT) family, characterized by the presence of a bromodomain, includes Gcn5, PCAF, Hat1, Elp3, Hpa2, Hpa3, ATF-2, and Nut1. These HATs are found to be associated with acetylation of lysine residues on histones H2B, H3, and H4. Named after its four founding members MOZ, Ybf2 (Sas3), Sas2 and Tip60, the MYST family also includes Esa1, MOF, MORF and HBO1. These HATs, characterized by the presence of zinc fingers and chromodomains, are found to be associated with acetylation of lysine residues on histones H2A, H3, and H4 [11]. The p300/CBP family, as its name indicates, consists of p300 and CBP which are metazoan-specific [12]. Characterized by the presence of several zinc finger regions, a bromodomain, a catalytic (HAT) domain, they are found to acetylate histones H2A, H2B, H3 and H4 [13]. The nuclear receptor coactivators (NRC) family, including SRC-1, ACTR (RAC3, AIB1, and TRAM-1), TIF-2 and SRC-3, is found in humans to be associated with acetylation of histone H3 and H4 [14]. SRC-1 and ACTR share consid-

erable sequence homology in that they both interact with p300/CBP and GNAT family member PCAF. TIF-2 is also known to interact with p300/CBP. Several other proteins that exhibit HAT activity include $TAF_{II}250$ (TAF1), TFIIIC (p220, p110, p90), Rtt109 and CLOCK [12, 14-16].

Histone deacetylation, catalysed by enzymes of histone deacetylases (HDACs), is a process by which the lysine residues within the protruding N-terminal tail of the histone core are deacetylated as part of regulation of gene expression [9]. HDACs remove acetyl groups from epsilon-amino groups of lysine residues on a histone, which tightens DNA around histones. This compact conformation makes the DNA less accessible for transcription factors, resulting in the repression of gene expression [17]. HDACs, based on sequence homology to the yeast enzymes and domain organization, can be categorized into four classes [18]. The members of class I, which include HDAC1, HDAC2, HDAC3 and HDAC8, are homologous to the yeast enzyme rpd3 and are primarily nucleus-localized except that HDAC8 can also be found in the cytoplasm [4]. The members of class II, exhibiting a more restricted tissue expression pattern than class I HDACs, are homologous to the yeast protein hda1 and can be divided into two sub-classes—class IIA and class IIB. Class IIA includes HDAC4, HDAC5, HDAC7, and HDAC9, and shuttles between the cytoplasm and the nucleus. Class IIB consists of HDAC6 and HDAC10, and is predominantly located in the cytoplasm [19-21]. Class III HDACs are referred to as sirtuins or the SIR2 family because they are homologous to the yeast-silencing protein

Sir2 [22]. So far, the only known member of class IV is HDAC11 which exhibits characteristics of both class I and class II HDACs [21, 23]. Class I, II and IV are zinc-dependent, whereas class III depends on NAD+ for the deacetylation reaction [24-26].

Histone methylation and demethylation, catalysed by enzymes of histone methyltransferases (HMTs) and histone demethylases (HDMs) respectively, are the processes by which methyl groups are transferred to and removed from amino acids of histone proteins. HMTs act to transfer methyl groups mainly to specific arginine and lysine residues of histone H3 and H4 [27]. There are two major types of HMTs, arginine-specific and lysine-specific [28-30]. Arginine-specific or protein arginine methyltransferases (PRMTs) can be categorized into two types by different restrictions in the arginine binding pocket [31]. The first type of PRMTs, including PRMT1, PRMT3, CARM1/PRMT4 and Rmt1/Hmt1, generates monomethylarginine and asymmetric dimethylarginine [32-34]. The second type, including JBP1/PRMT5 alone, generates monomethyl or symmetric dimethylarginine [31]. Both types of PRMTs result in transcription activation [35, 36]. Lysine-specific PRMTs can also be categorized into two types, SET domain-containing or non-SET domain-containing [28-30]. SET domain-containing lysine-specific PRMTs, targeting the lysine tail region of the histone, can mono-, di-, or tri-methylate lysine residues [37]. Non-SET domain-containing lysine-specific utilizes the enzyme Dot1 which methylates a lysine residue in the globular core of the histone [27]. Histone methylation of lysine residues can lead to either transcription activation or repression, based on the

lysine residue methylated and the number of methyl groups transferred [36]. Both arginine-specific and lysine-specific histone methyltransferases utilize S-Adenosyl methionine (SAM) as a cofactor and methyl donor group [27, 31, 38-40].

HDMs can be categorized into two main classes, flavin adenine dinucleotide (FAD)-dependent amine oxidase and Fe(II) and alpha-ketoglutarate-dependent dioxygenase [41]. These proteins have a number of domains with different functions [42, 43]. SWIRM domain (Swi3, Rsc and Moira domain), found in many chromatin modifying complexes, functions as a proposed anchor site for histone molecules and facilitates demethylase protein and co-factor CoREST. *Jumonji* (N/C terminal domains), connected by a beta-hairpin/mixed domain, act as a binding domain of key cofactors including alpha-ketoglutarate. An amine oxidase domain acts as a catalytic site of LSD proteins. PHD-finger, which binds methylated peptides, is essential to recognition and selectivity for methylated histone residues. Also, zinc-finger is a DNA binding domain contained within HDMs.

3. Histone Modifications and Cancer

Due to the nature of epigenetic modifications in gene regulation, it is no surprise that changes in those modifications such as histone acetylation and histone deacetylation are

found to be associated with cancer development. As aforementioned, histone hyperacetylation induced by either enhanced HAT activity or lack of HDAC activity results in gene expression, whereas histone hypoacetylation caused by either decrease of HAT activity or increase of HDAC activity leads to gene silencing (Figure 1) [44-47]. An aberrant balance between HAT and HDAC activities can lead to carcinogenesis [48-52]. During the past decade, considerable attention has focused on increases of HDAC activity due to its role in transcriptional repression which leads to deregulation of differentiation, cell cycle arrest and apoptosis in many types of cancer [53]. HDACs target many genes such as *p21* and *BAX* and which are frequently repressed in cancer cells and are de-repressed after treatment with HDAC inhibitors [54-56]. *p21,* a cyclin-dependent kinase inhibitor which prevents cell cycle progression from G1 into S phase, expresses defectively in many different cancer cells, leading to uncontrolled cell division. HDAC inhibitors have been shown to inhibit cancer cell proliferation by reactivating *p21* expression [57]. They have also been shown to regulate gene expression through other key regulators of cell cycle and apoptosis, including cyclins (A, E, B1, D1 and D3), transcription factors (GATA-2, c-Myc), apoptosis mediators (CD95, Bax and Bcl-2) and retinoic acid receptors (RAR) [58-65].

There has been growing interest in exploring HDAC inhibitors as cancer prevention and therapeutic agents. This is not only due to their broad spectrum of targets and ability to alter several cellular functions at several stages of carcinogenesis including cell differ-

entiation, cell cycle arrest and apoptosis as mentioned above, but also because cancer cells exhibit more response, 10-fold greater in apoptosis, to increased acetylation induced by HDAC inhibitors than do normal cells [66, 67]. In addition, *in vitro* and *in vivo* studies have revealed that HDAC inhibitors can alter the cell cycle in cancers and modify their ability to undergo mitosis [68-70]. They can activate a G_2 checkpoint during the S phase and G_2 phase of the cell cycle and result in an arrest of the cells in the G_2 phase by inducing accumulation of acetylated histones. In cancer cells, the G_2 checkpoint is frequently lost, which might explain why cancer cells are more sensitive to epigenetic regulations by HDAC inhibitors than normal cells.

A balance between histone methylation regulated by histone methyltransferases (HMTs) and histone demethylation regulated by histone demethylases (HDMs) is necessary. A disrupted balance of regulation can lead to increased susceptibility to disease such as cancer [71]. There are 7 sites on arginine residues that can be mono- or dimethylated and there are 17 sites on lysine residues that can be mono-, di-, or trimethylated. A total of 24 methylation sites on histones have been identified [72]. Many studies have been conducted to demonstrate the functional implications of histone methylation in cancer. Deregulation of H3K4me3 and H3K27me3 have been shown to be closely associated with tumor initiation and progression [73]. In addition, global absence of H4K16 acetylation and H4K20 trimethylation is a common distinguishing characteristic of cancer [56]. Knockout of the enzymes that regulate H3K9 methylation led to 28% of the mice be-

tween the age of 9 and 15 months exhibiting genomic instability and formation of B-cell lymphomas [74]. The dynamic deregulation of histone methylation in cancer development can also be illustrated by the presence of trimethylated H3K27 in the context of hypermethylated tumor suppressor promoters [75].

4. Regulation by Nutrients of Histone Modifications in Cancer

The use of dietary components to regulate histone modifications has gained substantial interest in cancer prevention and therapy. These dietary components include but are not limited to isothiocyanates (sulforaphane, benzyl isothiocyanate, phenylhexyl isothiocyanate, etc.), genistein, resveratrol, curcumin, epigallocatechin-3-gallate (EGCG), 3,3'-diindolylmethane (DIM), diallyl disulfide, garcinol, procyanidin B3 and quercetin. Numerous studies have been conducted to illustrate the mechanisms of the preventive nature of those dietary components as histone modifiers in cancers [76-94].

4.1. Isothiocyanates

Isothiocyanates (ITCs), characterized by a sulfur containing group–N=C=S, are produced by enzymatic conversion of metabolites called glucosinolates and can be found in cruciferous vegetables such as broccoli, cabbage and kale. Studies have shown that

isothiocyanates inhibit cancer cell growth and have proapoptotic properties (Table 1) [95]. Those isothiocyanates include but are not limited to sulforaphane (SFN), benzyl isothiocyanate (BITC) and phenylhexyl isothiocyanate (PHI), among which SFN is the most studied.

4.1.1. Sulforaphane

Sulforaphane (SFN), belonging to the isothiocyanate group, can be found in cruciferous vegetables such as broccoli, Brussels sprouts and cabbages [96-98]. It is produced from glucoraphanin, which is particularly rich in young sprouts of broccoli and cauliflower, by the enzyme myrosinase upon damage to the plant as a protective mechanism [96]. *In vitro* and *in vivo* studies have shown that SFN exhibits anticancer properties through several mechanisms, such as cell cycle arrest and apoptosis, activating checkpoint kinase 2 (CHEK2) and acting as a HDAC inhibitor which increases global and local histone acetylation of a number of genes (Table 1) [99-105]. Treated with SFN, HCT116 human colorectal cancer cells showed a dose-dependent increase in TOPflash reporter activity, in inhibited HDAC activity and in $p21^{Cip1/Waf1}$ [106]. In the same study, molecular modeling showed a likely interaction for SFN-cysteine, a conjugate of SFN and cysteine which is an effective HDAC inhibitor *in vitro*, within the active site of the HDAC-like protein, with the carboxylate group of SFN- cysteine arranged as a bidentate Zn ligand. Another study on human colon cancer cells showed that SFN and some other

ITCs inhibited HDAC activity and increased HDAC protein turnover with the potency proportional to alkyl chain length [107]. Moreover, continuous exposure to SFN results in the release of HDAC3 from 14-3-3 followed by nuclear import to compete with a peptidyl-prolyl cis/trans isomerase 1 (Pin1) pathway that directs HDAC3 for degradation in the cytoplasm in colon cancer cells. Protein kinase CK2 phosphorylates SMRT and HDAC3 in the nucleus to dissociate of the corepressor complex and to enhance binding of HDAC3 to 14-3-3 or Pin1 when SFN has been removed within 24 h, allowing recovery from cell cycle arrest [108].

In prostate cancer cells, HDAC6 is known to affect the acetylation state of the heat shock protein Hsp90, a key androgen receptor (AR) chaperone which is the central signaling pathway in prostate cancer. SFN has been found to enhance Hsp90 acetylation by inhibiting HDAC6 deacetylase activity, thereby attenuating AR signaling [109]. SFN also exhibited inhibitory effects on HDAC activity by 40, 30 and 40% in benign hyperplasia (BPH-1), and cancerous LNCaP and PC-3 prostate epithelial cells, respectively, at the concentration of 15 μM. A 50–100% increase in acetylated histones was also observed in all three cell lines treated with SFN, and in BPH-1 cells the interaction of acetylated histone H4 with the promoter region of the *p21* gene and the *bax* gene was enhanced [110]. A more recent study on these cell lines indicated that, at the concentration of 15 μM, SFN selectively induced cell cycle arrest and apoptosis in BPH-1, LNCaP and PC-3 prostate epithelial cells but not in normal PrEC cells. The same study also showed that SFN selec-

tively decreased HDAC activity, and Class I and II HDAC proteins, increased acetylated histone H3 at the promoter for *p21*, induced p21 expression and increased tubulin acetylation in prostate cancer cells [111].

An *in vivo* study demonstrated that SFN reduced the growth of human PC-3 prostate cancer cells by 40% in male nude mice. This was accompanied by a significant decrease in HDAC activity in the xenografts, as well as in the prostates and mononuclear blood cells (MBC), compared to control mice, when consumed at a daily dose of 7.5 μM per animal in the diet for 21 days. Another *in vivo* study revealed that SFN suppressed tumor development in Apc^{min} mice and triggered an increase in acetylated histones that specifically associated with the promoter region of the *p21* and *bax* genes in the polyps [113]. Pledgie-Tracy *et al.* demonstrated that SFN inhibited HDAC activity and decreased the expression of estrogen receptor alpha (ER-α), epidermal growth factor receptor (EGFR), and human epidermal growth factor receptor 2 (HER2) in four human breast cancer cell lines, MDA-MB-231, MDA-MB-468, MCF-7 and T47D cells [114].

Apart from its HDAC inhibitory properties, SFN can also regulate other histone modifications such as histone methylation and histone phosphorylation in different types of cancer (Table 1). Balasubramanian *et al.* showed that SFN reduced trimethylation of lysine 27 of histone H3 (H3K27me3) in SCC-13 skin cancer cells [115]. In human colon cancer cells, SFN as well as some other isothiocyanates (ITCs) were found to cause DNA

double-strand breaks and increase phosphorylation of histone H2AX, ataxia telangiectasia and Rad3-related protein (ATR) and CHEK2 [108]. Similar regulations were revealed in PC-3 human prostate cancer cells, as well [116]. In LNCaP human prostate cancer cells, SFN induced cell arrest in mitosis and increased Ser^{10} phosphorylation of histone H3 which is a sensitive marker for mitotic cells [117]. In addition, SFN, at the concentration of 15 μmol/L, significantly enhanced phosphorylation of histone H1 by 67% in MCF-7 human breast cancer cells and increased cell cycle arrest in mitosis by 10 times compared to control cells [118].

A few human clinical trials have also been conducted to evaluate the effects of SFN. The first study was designed to determine the toxicity of SFN by using healthy volunteers that used glucoraphanin or isothiocyanate as the SFN source [193]. It was a randomized, placebo-controlled, double-blind phase 1 clinical trial consisting of three study groups; 25 μmol of glucosinolate, 100 μmol of glucosinolate, or 25 μmol ITC for 7 days, and evaluated parameters of safety, tolerance, and pharmacokinetics. Notably, no significant toxicities were observed at the doses employed. A second study was a randomized placebo-controlled chemoprevention trial performed in Qidong, People's Republic of China where high levels of airborne toxin phenanthrene and aflatoxin-contaminated foods were present [194]. Residents of Qidong were administered hot drinking water infused with 3-day old broccoli sprouts. An inverse correlation between SFN treatment and excretion of carcinogens was observed. In another study, HDAC activity was significantly de-

creased in peripheral blood mononuclear cells by more than 50% in normal healthy volunteers after 3–6 h from ingestion of 68 g of broccoli sprouts which was accompanied by an increase in acetylated histones H3 and H4 [112]. These clinical trials suggest the significance of SFN as an epigenetic anticancer agent for humans.

4.1.2. Benzyl Isothiocyanate and Phenylhexyl Isothiocyanate

Other isothiocyanates, such as benzyl isothiocyanate (BITC) and phenylhexyl isothiocyanate (PHI) have also been found to be able to regulate the development of different types of cancer through histone modifications. BITC significantly decreased the expression and activity of HDAC1 and HDAC3 in BxPC-3 human pancreatic cancer cells as well as HDAC3 in Capan-2 human pancreatic cancer cells, whereas HDAC expression in normal HPDE-6 cells was unaffected [119]. PHI was shown to suppress HDAC1 and HDAC2 activity in LNCaP cells. The resulting histone hyperacetylation enhanced accessibility to the p21 promoter for transcription, thereby leading to G1 arrest and apoptosis [120]. In acute lymphoid leukemia cell line Molt-4, PHI increased acetylation of histone H3 and H4 markedly [121]. Moreover, Lu *et al.* discovered that PHI induced histone H3 hyperacetylation and p16 hypomethylation in a concentration-dependent manner in myeloma cell line RPMI8226 [122]. In addition, PHI not only can act as a HDAC inhibitor, but can regulate histone methylation in cancer cells as well. Zou *et al.* revealed for the first time that PHI had opposite effects on the methyltransferases for H3K4 and H3K9 in

primary acute leukemia cells [123]. They examined the methylation status at H3K4 and H3K9 and found that PHI increased the methyltransferase activity of H3K4 and decreased the methyltransferase activity of H3K9 in both myeloid and lymphoid leukemia cells.

4.2. Curcumin

Curcumin, a diarylheptanoid, is the principal curcuminoid (approximately 80%) of the South Asian spice turmeric (*Curcuma longa*) (Table 1) [124, 125]. It is a natural polyphenol with a bright-yellow color that is responsible for the yellow color of turmeric and can be used as a food additive for coloring. Curcumin can exist in several tautomeric forms, including a 1,3-diketo form and two equivalent enol forms. Numerous publications have shown curcumin's bioactive properties including anti-inflammatory, anti-angiogenic and wound-healing, anti-oxidant, anti-angiogenic and anti-cancer. However, reports on its epigenetic activities in cancers have only emerged during the past decade [126-129].

Recent reports have indicated curcumin's epigenetic modifying properties as a HDAC inhibitor. Lee *et al.* delineated that curcumin, in medulloblastoma cells, prompted apoptosis and cell cycle arrest at the G2/M phase by decreasing HDAC4 expression and activity and by increasing tubulin acetylation. Their *in vivo* study on medulloblastoma

xenografts showed that curcumin suppressed tumor growth and markedly enhanced the survival of the Smo/Smo transgenic medulloblastoma mice [130]. The role of curcumin as a HDAC inhibitor can also be seen in other types of cancer. In human breast cancer cell line MCF-7, curcumin was found to increase global levels of acetylated H3K18 and H4K16 [131]. In cervical cancer cells, HDAC1 and HDAC2, and viral onco-proteins (E6/E7) are commonly overexpressed. Curcumin was reported to inhibit the expression of HDAC1, HDAC2 and HPV E6/E7 and to differentially enhance the acetylation and up-regulation of p53 in the cervical cancer cell line SiHa and SiHaR which is a drug resistant clone derived from SiHa, thereby resulting in cell cycle arrest at G1/S phase [132].

Interestingly, not only does curcumin exhibit HDAC inhibitory abilities, it has been reported to have HAT inhibitory properties as well. *In vitro* and *in vivo* studies on several prostate cancer cell lines conducted by Shah *et al.* suggested that curcumin suppressed p300 and CBP occupancy at androgen receptor (AR) functional sites by decreasing the association of histone acetylation and pioneer factors, which resulted in the inhibition of AR residence and downstream target gene expression. They further verified the role of curcumin as a HAT inhibitor by using HDAC inhibitors, finding that the effects of cur-cumin on AR activity were reversed [133]. Another study on the human prostate cancer cell line LNCaP indicated that although the total HDAC activity was suppressed upon treatment with curcumin probably due to the decrease of HDAC8 expression, the expres-sion of HDAC1, HDAC4, HDAC5 and HDAC8 were increased [134]. The same study

also showed that curcumin decreased H3K27me3 at the *Neurog1* promoter region as well as at the global level, suggesting that apart from its abilities to inhibit HDAC and HAT activity, curcumin can also affect histone methylation.

4.3. (-)-Epigallocatechin-3-gallate

(-)-Epigallocatechin-3-gallate (EGCG) is a type of catechin that is abundant in tea, green tea in particular, as well as other vegetables and nuts (Table 1). It is a well-known antioxidant that has therapeutic applications in the treatment of many disorders such as cancer. Other catechins include (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG) and (−)-epigallocatechin (EGC) [135]. Although, all of them share similar bioactive properties with EGCG, studies show that EGCG is the most abundant (more than 50%) anti-tumorigenic component in green tea and is the most potent epigenetic agent in regulating histone modifications in various types of cancer [136-139]. In our studies, EGCG was found to inhibit the proliferation of human breast cancer MCF-7 and MDA-MB-231 cells in a dose- and time-dependent manner but to cause no damage to the control MCF10A cells. We found that EGCG inhibited the transcription of human telomerase reverse transcriptase (*hTERT*) through *hTERT* promoter hypomethylation and histone deacetylations. The level of acetyl-H3, acetyl-H3K9 and acetyl-H4 were decreased, resulting in the remodeling of the chromatin structures of the *hTERT* promoter [140].

In addition, EGCG also acts as a HDAC inhibitor in cancer cells. In LNCaP human prostate cancer cells, EGCG (5-20 μM) dose- and time-dependently inhibited class I HDACs (HDAC1, 2, 3 and 8), resulting in the acetylation of p53 [141]. Furthermore, reports have shown that EGCG can regulate histone methylation, as well. Li *et al.* discovered that EGCG can alter histone acetylation and methylation status leading to reactivation of ERα expression in ERα-negative MDA-MB-231 breast cancer cells [142]. In skin cancer cells, EGCG has been shown to affect histone acetylation and histone methylation as well as histone ubiquitination. Choudhury *et al.* showed that EGCG reduced the level of polycomb group (PcG) proteins (Ezh2, eed, Suz12, Mel18 and Bmi-1) following a decrease of H3K27me3 and H2AK119ub formation and HDAC1 activity and an increase of acetylated H3 formation [143]. Moreover, EGCG dose-dependently suppressed global DNA methylation levels in human epidermoid carcinoma A431 cells, accompanied by a decrease of HDAC activity as well as levels of methylated H3K9 and an increase of levels of acetylated H3K9 and H3K14 and acetylated H4K5, H4K12 and H4K16 [144]. Also, EGCG reduced Bmi-1 and Ezh2 level in SCC-13 cells as well as cell survival which is associated with a global reduction in H3K27me3 [145]. Modification of histone phosphorylation by EGCG was also observed in human lung cancer cells both *in vitro* and *in vivo*. Li *et al.* reported that EGCG inhibited tumor growth of H1299 human non-small cell lung carcinoma cell line both in culture and in xenografts in a dose-dependent manner. The level of tumor cell apoptosis and oxidative DNA damage was proportional to the

formation of 8-hydroxy-2'-deoxyguanosine (8-OhdG) and phosphorylated histone 2A variant X (gamma-H2AX) [146].

4.4. Genistein

Genistein is a phytoestrogen belonging to the category of isoflavones and can be found in numerous plants as food source including lupin, fava beans, soybeans, kudzu, and psoralea, as well as in medicinal plants *Flemingia vestita* and *F. macrophylla*, and in coffee (Table 1) [147-149]. Many investigations have shown genistein's anti-cancer and anti-angiogenic properties in a variety of cancers as a result of its estrogen-like behavior that interacts with animal and human estrogen receptors, as well as its ability to modulate epigenetic activities such as DNA methylation and chromatin remodeling [46, 150-154]. In prostate cancer, genistein was first reported to induce the expression of *p21* (WAF1/CIP1/KIP1) and *p16* (INK4a) by upregulating the acetylation of histone 3, 4, and H3K4 at the *p21* and *p16* transcription start sites by genistein treatment in androgen-sensitive (LNCaP) and androgen-insensitive (DuPro) human prostate cancer cell lines [47]. Similar mechanisms and results can be seen in a later study on breast cancer cells conducted by Li *et al.* [155]. Just like sulforaphane mentioned above, genistein was also reported to inhibit HDAC6-Hsp90 cochaperone function leading to down-regulation of AR protein in LNCaP cells [156]. In addition, Majid *et al.* demonstrated that, in LNCaP and PC-3 cell lines as well as in A498, ACHN and HEK-293 renal cell carcinoma

(RCC) cell lines, genistein induced mRNA expression of *BTG3*, a tumor suppressor gene which is usually transcriptionally down-regulated in prostate cancer, by decreasing methylation of *BTG3* promoter and by increasing the levels of acetylated histones H3 and H4, H3K4me3, and RNA polymerase II at the *BTG3* promoter [157, 158].

Uncontrolled tumor growth in colon cancer can be inhibited by blocking the WNT signaling pathway or by enhancing the expression of its antagonist DKK1. Wang *et al.* illustrated that genistein increased DKK1 expression in SW480 and HCT15 cells by inducing histone H3 acetylation of the DKK1 promoter region, thereby resulting in a dose-dependent G2 phase arrest and reduction of cell proliferation [159]. In another human colon cancer cell line, HT29, inhibition of HDAC activity was observed by both genistein and EGCG treatment, causing a significant decrease of the HDAC1 protein level [160]. Coxsackievirus and adenovirus receptor (CAR) is often down-regulated in cancer. Pong *et al.* suggested that the combination of genistein and histone deacetylase inhibitor FK228 synergistically increase CAR expression in bladder cancer by increasing histone acetylation in the CAR promoter gene [161].

4.5. 3,5,4’-trihydroxy-trans-stilbene

3,5,4’-trihydroxy-trans-stilbene (resveratrol) is a stilbenoid, a polyphenol, as well as a phytoalexin naturally produced by a number of plants such as grapes, berries, peanuts

and the roots of Japanese knotweed (*Polygonum cuspidatum*) when under attack by pathogens (Table 1). But, it is most abundant in the skin of red grapes, thus, it is rich in red wine [162, 163]. Like other polyphenols, resveratrol exhibits anticancer properties through a number of epigenetic regulations. The most well studied regulation pathway on cancer by resveratrol is, perhaps, its effects on SIRT1 (mammalian ortholog of the yeast silent information regulator 2) which is a type of sirtuin belonging to class III HDAC as aforementioned. A number of studies both *in vitro* and *in vivo* have supported resveratrol's role in up-regulating SIRT1 in various types of cancer [164-167]. In addition, resveratrol has recently been reported to dose-dependently inhibit all eleven human HDACs of class I, II and IV in hepatoma cell lines HepG2, Hep3B and HuH7, resulting in a dose-dependent antiproliferative effect on all cell lines with no toxic effect on primary human hepatocytes. In the same study, resveratrol also exhibited HDAC inhibitor activity in human blood samples [168]. Moreover, Kim *et al.* showed that LYR71 (a derivative of trimeric resveratrol) inhibited RANTES-stimulated breast cancer cells via inhibition of STAT3 activation. STAT3 is responsible for tumor progression caused by up-regulation of MMP-9. LYR71 was found to decrease STAT3 activation and to suppress the expression and activity of MMP-9 by segregating p300 and deacetylating histone H3 and H4 on the *MMP9* promoter [169]. Resveratrol has also been reported to act as a histone demethylase inhibitor. Abdulla *et al.* indicated that resveratrol showed a potent inhibitory effect on LSD1 activity in HEK293, HepG2 and FAO cell lines [170].

Furthermore, resveratrol and its analogues has been reported to regulate histone phosphorylation in various cancers [171, 172].

4.6. Other Nutrient Components Regulating Histone Modifications

4.6.1. Selenium

Selenium (Se) is a chemical element that can be found in Brazil nuts, chicken and beef as a nutrient (Table 1) [173]. Se is vital for human well-being and exhibits antioxidant as well as anti-cancer properties. It was reported that the combination of selenium and green tea resulted in a greater inhibition of tumor development than either selenium or green tea alone in a rat colorectal cancer model, accompanied by marked reduction of DNMT1 expression and induction of histone H3 acetylation [174]. Another study showed that selenite dose- and time-dependently triggered partial promoter DNA demethylation and re-expression of the pi-class glutathione-S-transferase (*GSTP1*) in LNCaP cells, accompanied by decrease of histone deacetylase activity and increase of levels of acetylated H3K9, and decrease of levels of methylated H3K9 [175]. Additional publications supported the HDAC inhibitor ability of the selenium analogs of SAHA (SelSA-1 and SelSA-2) [176, 177]. Moreover, selenomethionine treatment resulted in statistically significant expression changes for 50 genes with the increase of phosphorylated histone H3 on serine 10 bound to the *GJB2* promoter [178].

4.6.2. Quercetin

Quercetin, a plant pigment that can be found in fruits, vegetables, leaves and grains, is a polyphenolic bioflavonoid which possesses anti-inflammatory and anti-cancer properties (Table 1). It was reported to dose-dependently inhibit COX-2-mediated angiogenesis in human endothelial cells and to effectively suppress p300 HAT activity, leading to attenuation of p300-mediated acetylation of NF-κB [179]. A study mentioned previously indicated that quercetin effectively inhibited LSD1 activity in HEK293, HepG2 and FAO cell lines [170], suggesting that quercetin has histone demethylase inhibitor ability.

4.6.3. Diallyl disulfide

Diallyl disulfide (DADS or 4,5-dithia-1,7-octadiene) is an organosulfur compound produced from garlic when crushed (Table 1). In human colon cancer cells, DADS was found to inhibit cell proliferation by suppressing HDAC activity and increasing histone H3 and H4 acetylation as well as $p21^{waf1/cip1}$ expression [180]. Similarly, the expression of acetylated histone H3, H4 and $p21^{waf1}$were increased by DADS treatment both *in vitro* and *in vivo* in human promyelocytic leukemia cell line HL-60, resulting in significant inhibition of tumor growth [181].

4.6.4. 3,3’-Diindolylmethane

3,3’-Diindolylmethane (DIM), found in cruciferous vegetables such as broccoli, Brussels sprouts, cabbage and kale, is a phytochemical with various anti-cancer properties (Table 1) [182, 183]. In PC-3 and LNCaP cells, DIM markedly reduced HDAC2 activity causing increase expression of *p21* [184]. In human colon cancer cells, DIM selectively down-regulated class I HDACs both *in vitro* and *in vivo*, which led to increased expression of *p21* and *p27*, and induction of DNA damage, resulting in cell cycle arrest in G2 phase and apoptosis, respectively [185].

4.6.5. Garcinol

Garcinol, a polyisoprenylated benzophenone derivative that can be isolated from the rind of *Garcinia indica* fruit, has been reported to inhibit carcinogenesis via histone modifications (Table 1). Collins *et al.* explained that garcinol can trigger cell cycle arrest at S phase by inhibiting H3K18 acetylation in the breast cancer cell line MCF-7 as well as in the osteosarcoma cells lines U2OS and SaOS2. In contrast, garcinol treatment increased global levels of acetylated H4K16 and trimethylated H4K20 in MCF-7 breast cancer cells, upregulating DNA damage 88lavin88ng markers [131]. This study suggests that garcinol is a pleiotropic bioactive agent. And its HAT inhibitory effect can also be seen in

HeLa cervical carcinoma cells. Balasubramanyam *et al.* found that garcinol inhibited HAT p300 and PCAF both *in vitro* and *in vivo* [186].

4.6.6. Procyanidin B3

Procyanidin B3 (Pro-B3), a B type proanthocyanidinas as well as a catechin dimer (catechin-(4α→8)-catechin) that can be found in red wine, barley, beer, peach and *Jatropha macrantha*, is identified as a HAT inhibitor (Table 1) [187-189]. Pro-B3 was reported to suppress cell proliferation through inhibition of p300-mediated AR acetylation both *in vitro* and *in vivo* in prostate cancer cells [190].

5. Discussion

Dietary components have been shown to interfere with a number of epigenetic mechanisms sustaining cancer development such as HAT or HDAC aberrations as well as other histone modifying properties in various types of cancers both in culture and in animal studies. While some of them are well developed such as genistein, resveratrol, curcumin, EGCG and sulforaphane, others like DIM, diallyl disulfide, garcinol, procyanidin B3 and quercetin remain to be further explored. What is also worth mentioning is histone *O*-GlcNAcylation which has recently been added to the growing list of histone modifica-

tions. O-GlcNAc transferase, an enzyme encoded by the *OGT* gene in humans, glycosylates many proteins including histones [191, 192]. Consistently, *O*-GlcNAc modifications on histones may regulate cancer development. However, despite of the growing evidence suggesting the involvement of *O*-GlcNAcylation in carcinogenesis, comprehension of the underlying mechanism is poorly understood. Few studies, if any, have been conducted on the role of dietary components in cancer development through histone *O*-GlcNAcylation. Further studies are required to understand the impact of these dietary components in carcinogenesis and to identify new epigenetic agents that could be used for cancer prevention and therapy.

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Abbreviations

8-OhdG	8-hydroxy-2’-deoxyguanosine
AR	Androgen receptor
ATR	Ataxia telangiectasia and Rad3-related protein
BITC	Benzyl isothiocyanate
BPH	Benign prostatic hyperplasia
CAR	Coxsackievirus and adenovirus receptor
CHEK2	Checkpoint kinase 2
DADS	Diallyl disulfide
DIM	3,3’-diindolylmethane
EC	(−)-epicatechin
ECG	(−)-epicatechin-3-gallate
EGC	(−)-epigallocatechin
EGCG	(-)-epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor
ER-α	Estrogen receptor alpha
FAD	Flavin adenine dinucleotide
GNAT	Gcn5-related N-acetyltransferase

GSTP	Glutathione-S-transferase
H3K14	Histone H3 lysine 14
H3K27me3	Trimethylation of histone 3 at lysine 27
H3K4	Histone H3 lysine 4
H3K4me3	Histone H3 lysine 4 trimethylation
H3K9	Histone H3 lysine 9
H3K27me3	Histone H3 lysine 27 trimethylation
H4K12	Histone H4 lysine 12
H4K16	Histone H4 lysine 16
H4K5	Histone H4 lysine 5
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HER2	Human epidermal growth factor receptor 2
HMT	Histone methyltransferase
hTERT	Human telomerase reverse transcriptase
ITC	Isothiocyanate
LYR71	6-methyl-2-propylimino-6, 7-dihydro-5H-benzo [1, 3]-oxathiol- 4-one
MBC	Mononuclear blood cell
NRC	Nuclear receptor coactivator

PBMC	Peripheral blood mononuclear cell
PcG	Polycomb group
PHI	Phenylhexyl isothiocyanate
Pin1	Peptidyl-prolyl cis/trans isomerase 1
PRMT	Protein arginine methyltransferase
Pro-B3	Procyanidin
RAR	Retinoic acid receptor
RCC	Renal cell carcinoma
SAM	S-Adenosyl methionine
Se	Selenium
SFN	Sulforaphane

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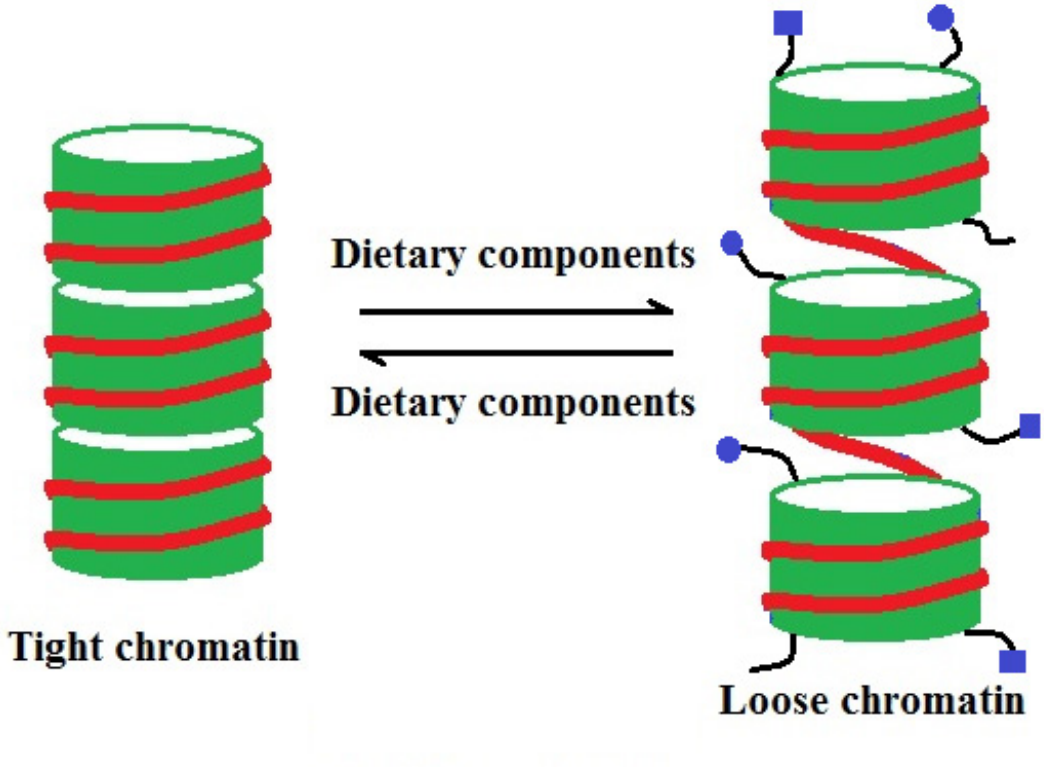
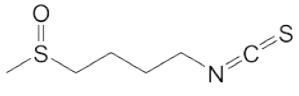
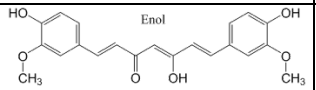
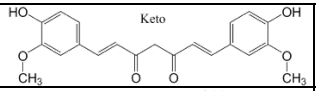
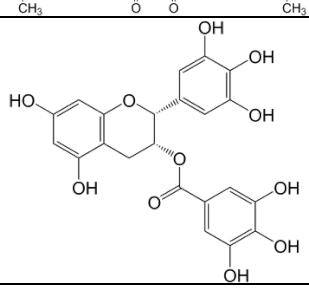
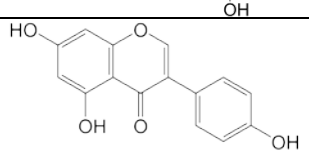
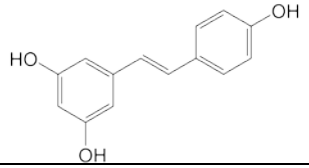
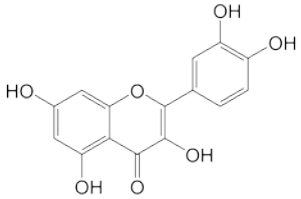
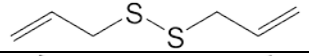
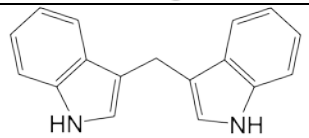
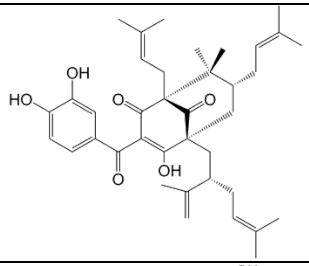
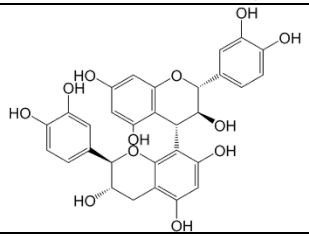


Figure 1. Chromatin conformational changes affected by dietary components. Dietary components can affect gene expression by changing chromatin conformation through enzymes, such as HAT/HDAC and HMT/HDM. HAT enhancers and HDAC inhibitors, such as diallyl disulfide, genistein and sulforaphane, trigger a loose chromatin which allows DNA accessibility to transcriptional factors, leading to gene expression. In contrast, HAT inhibitors, such as curcumin, EGCG and quercetin, cause a tight chromatin which makes DNA inaccessible to transcriptional factors, resulting in gene silencing. Also, many dietary components, such as EGCG, genistein and resveratrol, modify histone methylation through HMT or HDM, which can either increase or decrease gene transcription, depending upon which amino acids in the histones are methylated, and how many methyl groups are attached. Green cylinder represents histone octamer. Red line represents DNA. Blue dot represents acetyl group. Blue square represents methyl group.

Table 1. Dietary components and their epigenetic effects on histone modifications.

Dietary Components	Source	Structure	Epigenetic Effect(s) on Histone Modifications
Sulforaphane and other isothiocyanates like benzyl isothiocyanate and phenylhexyl isothiocyanate	Cruciferous vegetables such as broccoli, Brussels sprouts and cabbages		Inhibited HDAC activity, increased HDAC protein turnover; released HDAC3 from 14-3-3, increased phosphorylation of histone H2AX, ATR and CHEK2 in HCT116 colon carcinoma cells [106-108]; enhanced Hsp90 acetylation by inhibiting HDAC6 deacetylase activity, inhibited HDAC activity, selectively induced cell cycle arrest and apoptosis, selectively decreased Class I and II HDAC proteins, increased acetylated histone H3 at the promoter for *p21*, increased Ser^{10} phosphorylation of histone H3 in LNCaP prostate cancer cells [109-111, 117]; enhanced Hsp90 acetylation by inhibiting HDAC6 deacetylase activity in VCaP prostate cancer cells [109]; inhibited HDAC activity, selectively induced cell cycle arrest and apoptosis, selectively decreased Class I and II HDAC proteins, increased acetylated histone H3 at the promoter for *p21*, reduced tumor growth *in vivo* accompanied by a significant decrease in HDAC activity, increased phosphorylation of histone H2AX, ATR and CHEK2 in PC-3 prostate cancer cells [108, 110-112, 116]; enhanced interaction of acetylated histone H4 with the promoter region of *p21* and *bax* by inhibition of HDAC activity, selectively induced cell cycle arrest and apoptosis in BPH-1 benign prostatic hyperplasia cells [110, 111]; inhibited HDAC activity and decreased the expression of ER-α, EGFR, and HER2 in MDA-MB-231, MDA-MB-468 and T47D breast cancer cells [114]; inhibited HDAC activity and decreased the expression of ER-α, EGFR, and HER2, enhanced phosphorylation of histone H1 in MCF-7 [114, 118]; reduced trimethylation of lysine 27 of histone H3 in SCC-13 squamous carcinoma cells [115]
Curcumin	South Asian spice turmeric		HDAC inhibitor [130-132], HAT inhibitor [133, 134], down-regulating histone methylation [134]

			
(-)-Epigallocatechin-3-gallate	Tea, vegetables and nuts		HAT inhibitor [140], HDAC inhibitor [141, 143, 144], down-regulating histone methylation [144, 143-145], affecting histone ubiquitination [143], up-regulating histone phosphorylation [146]
Genistein	Lupin, fava beans, soybeans, kudzu, psoralea, coffee, *Flemingia vestita* and *F. macrophylla*		HAT enhancer [47, 155, 157-159, 161], HDAC inhibitor [161, 165], upregulating histone methylation [157, 158]
Resveratrol	Grapes, berries, peanuts and Japanese knotweed		HDAC inhibitor [168], HDM inhibitor [170], regulating histone phosphorylation [171, 172]
Selenium	Brazil nuts, chicken and beef	N/A	HDAC inhibitor [174-177], down-regulating histone methylation [175], up-regulating histone phosphorylation [178]

Quercetin	A plant pigment found in fruits, vegetables, leaves and grains		HAT inhibitor [179], down-regulating histone demethylation [170]
Diallyl disulfide	Garlic		HDAC inhibitor and HAT enhancer [180, 181]
3,3'-Diindolylmethane	Cruciferous vegetables such as broccoli, Brussels sprouts, cabbage and kale		HDAC inhibitor [184, 185]
Garcinol	The rind of *Garcinia indica* fruit		HAT inhibitor [131, 186], upregulating histone acetylation [131], upregulating histone methylation [131]
Procyanidin B3	Red wine, barley, beer, peach and *Jatropha macrantha*		HAT inhibitor [187-189]

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