

## Resveratrol potentiates grape seed extract induced human colon cancer cell apoptosis

Sridhar Radhakrishnan<sup>1</sup>, Lavanya Reddivari<sup>1</sup>, Robert Sclafani<sup>2</sup>, Undurti N Das<sup>3</sup>, Jairam Vanamala<sup>1,4</sup>

<sup>1</sup>Department of Food Science and Human Nutrition, Colorado State University, Fort Collins, Colorado, USA, <sup>2</sup>The Program in Molecular Biology, University of Colorado School of Medicine, Aurora, Colorado, USA, <sup>3</sup>UND Life Sciences, Shaker Heights, Ohio, USA, <sup>4</sup>Cancer Chemoprevention Section, University of Colorado Cancer Center, Aurora, Colorado, USA

### TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
  - 3.1. Chemicals
  - 3.2. LC-MS analysis of GSE
  - 3.3. Cell lines
  - 3.4. Cell proliferation assay
  - 3.5. Cytotoxicity detection
  - 3.6. Apoptosis assay
  - 3.7. Experimental design of combination study
  - 3.8. Fluorescence-activated cell sorting analysis (FACS)
  - 3.9. Western blot analysis
  - 3.10. IGF-1 treatment
  - 3.11. Statistical analysis
4. Results
  - 4.1. Resveratrol (RSV) or grape seed extract (GSE) suppresses cell proliferation of HCT-116 cells
  - 4.2. RSV or GSE induces apoptosis
  - 4.3. RSV-GSE combination suppresses cell proliferation and induces apoptosis
  - 4.4. RSV-GSE combination induces cell cycle arrest in Go/G1 phase in both HCT 116 p53 +/+ and p53 -/- cells
  - 4.5. RSV-GSE combination induces apoptosis via p53-dependent pathway
  - 4.6. RSV-GSE combination suppressed cell proliferation and induced apoptosis via caspase-3-dependent and Bax/Bcl-2 pathways.
  - 4.7. N-acetyl cysteine (NAC) inhibited RSV-GSE combination induced apoptosis in HCT-116 p53 +/+ cells
  - 4.8. RSV-GSE combination is non-toxic to normal cells
  - 4.9. RSV-GSE combination suppressed cell proliferation and induced apoptosis even in the presence of IGF-1
5. Discussion
6. Acknowledgements
7. References

### 1. ABSTRACT

Colon cancer is the third leading cause of cancer deaths in men and women. Grape seed extract (GSE) and resveratrol (RSV) are potent chemopreventive agents against colon cancer both *in vitro* and *in vivo*, at relatively high concentrations. We hypothesized that RSV and GSE may act in concert with each other in potentiating their anti-cancer properties at sub-optimal doses, because they occur as complex mixtures in grapes. In this study, we showed that RSV (~25 micromolar) potentiated GSE ( $\leq 35$  microg/mL) induced colon cancer cell apoptosis via activation of p53 dependent pathways. Elevation of apoptosis was much more pronounced in p53 +/+ cells compared to p53 -/- cells. Apoptosis was strongly correlated with pp53 levels and Bax:Bcl-2 ratio, key players in the mitochondrial apoptotic pathway. Caspase-3 inhibition and reactive oxygen species suppression attenuated apoptosis induced by the combination. RSV-GSE combination suppressed proliferation and induced apoptosis even in the presence of mitogenic growth factor IGF-1, suggesting the importance of understanding the potentiating effects of phytonutrients in combination as they would occur in nature rather than individually.

### 2. INTRODUCTION

In 2010, the American Cancer Society estimated that there were about 102,900 new cases and 51,370 deaths occurred due to colon cancer alone in the United States (1). Since colon cancer has a long latency period before it is detected even in its pre-clinical stage (such as colon polyp), an opportunity exists to institute appropriate dietary preventive strategies to halt its progression (2). Dietary bioactive phytochemicals that have chemopreventive properties, which occur in nature as complex mixtures in fruits, vegetables, grains and herbs, are particularly suited for this purpose (3). However, little information is available on additive, synergistic or antagonistic interactions among these phytochemicals. Resveratrol (RSV), grape seed extract (GSE) and their combination(s) are marketed as popular dietary supplements. Independently, RSV and GSE showed anti-cancer activity both *in vitro* and *in vivo*. GSE lacks resveratrol; however, no studies have been performed or reported on their possible additive or synergistic chemoprevention/protective effects against colon cancer.

*Vitis vinifera* (Grape Vine) is a rich source of several biologically active compounds including anthocyanins, proanthocyanidins, and stilbenes (4). GSE, a mixture containing about 95% standardized proanthocyanidins, is a popular dietary supplement due to its anti-cancer and anti-inflammatory properties (5). *In vitro* studies showed that GSE has significant growth inhibitory action on a variety of colon cancer cells in a dose- and time-dependent manner (6). GSE significantly inhibited cell viability and elevated apoptosis in cancer cells without altering the viability of the normal colon cell lines, thus selectively targeting cancer cells (7). GSE induced G<sub>1</sub> phase arrest and caspase-3 mediated apoptosis in cancer cells (7-9). GSE might thus exert its beneficial effects by elevating apoptosis and suppressing proliferative pathways. Earlier reports suggest that GSE obtained from different commercial vendors can produce comparable biological effects via cell growth suppression in a panel of human colon cancer cell lines (6) indicating that minor differences in proanthocyanin content and composition of GSE may not be very important.

It is well known that GSE typically lacks RSV, except in rare special preparations where RSV is intentionally added (10). RSV, a stilbenoid derived from the skin of red grapes, has been shown to be active against various cancers *in vitro* and *in vivo* (11-14). RSV interferes with all three stages of carcinogenesis: initiation, promotion and progression (15-17). We previously showed that RSV has anti-proliferative and pro-apoptotic properties against HT-29 and/or SW480 human colon cancer cell lines even in the presence of mitogenic insulin like growth factor-1 (IGF-1) by elevating phosphorylated p53 (ser 15) and suppressing IGF-1R/Wnt signaling. However, RSV is effective at relatively high concentrations in *in vitro* studies (RSV > 25-100 micromolar in different cell lines). Though in an occasional instance, RSV was found to be active at ~ 25 micromolar *in vitro* (18). In view of these evidences, we opined that addition of RSV at relatively low concentrations (~ 25 micromolar) to a natural food supplement such as GSE might potentiate the effectiveness of GSE in suppressing colon cancer cell growth, as both of them exist in the fruit matrix in the form of a mixture. Furthermore, the inhibitory efficacy of such RSV-GSE combination on colon carcinogenesis has not yet been reported.

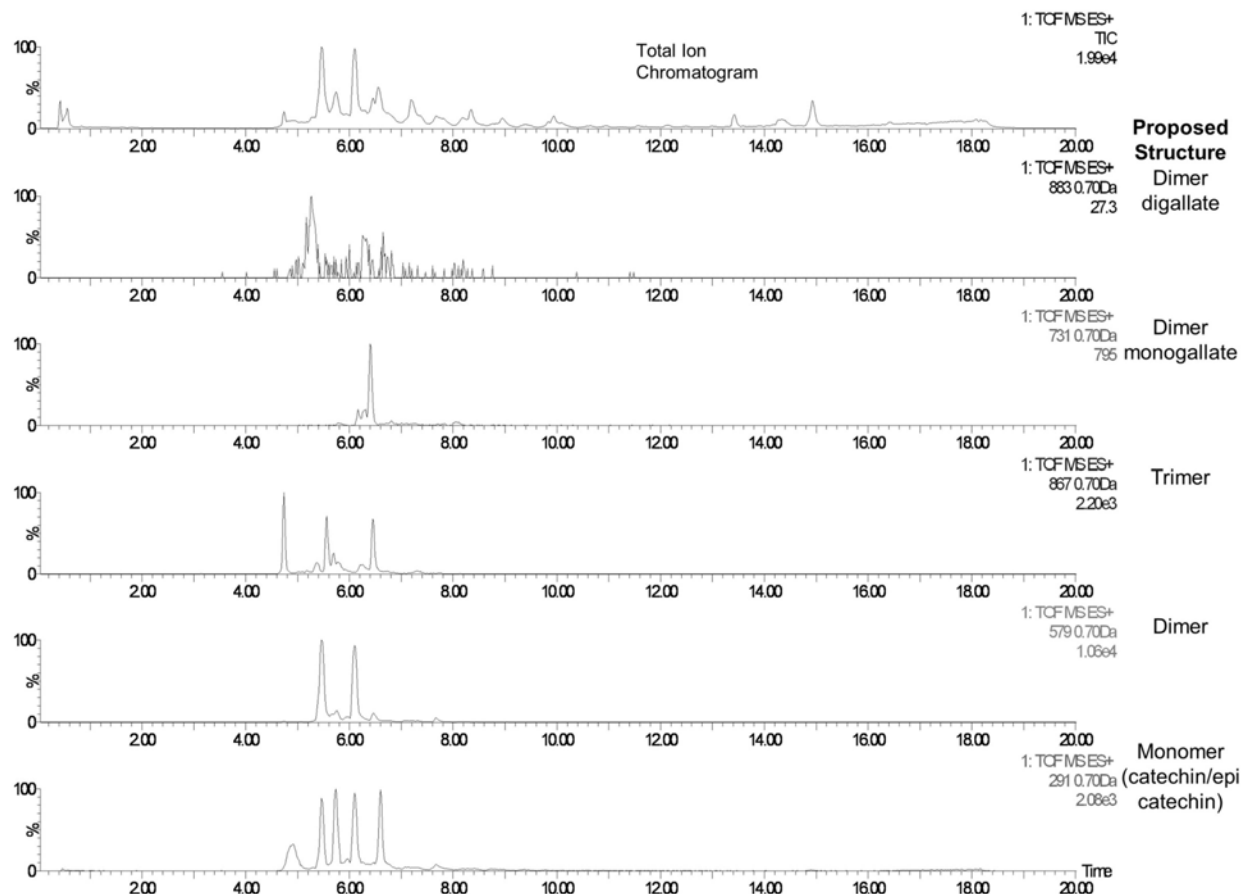
IGF-1 is a growth factor and the IGF axis is frequently activated during obesity and thus, could play a critical role in obesity-promoted colon cancer (19-21). The IGF system includes ligands, receptors, and ligand-binding proteins (IGFBPs). Positive energy balance and chronic hyperinsulinemia observed in obese conditions may deregulate colonocyte growth kinetics, as elevated insulin and suppressed IGFBP-1 and IGFBP-2 levels increase the pool of bioavailable IGF-1. A larger pool of bioavailable IGF-1 activates the IGF-1 receptor (IGF-1R is over expressed in colon cancer cells), and thus may stimulate colonocyte proliferation (19, 22-25). Previously, we showed that RSV suppressed IGF-1R levels in human colon cancer cells (18).

Chemotherapeutic combination approaches have been used to reduce drug toxicity and obtain greater efficacy than the use of single active component (26). In a similar fashion, the phytochemicals in fruits could be used in combination as anti-cancer agents. However, there is little or no evidence for synergistic, additive, or antagonistic effects of grape compounds in combination against human colon cancer cells. We hypothesized that RSV and GSE work together synergistically to suppress colon cancer cell proliferation, at least, in part by inducing apoptosis via p53 dependent mechanisms. P53, a critical tumor suppressor gene, is frequently suppressed in many cancers and is activated upon DNA damage (27). Even though GSE induced apoptosis in a p53 dependent manner in skin cancer fibroblasts, information on GSE's p53 dependent effects on early stage colon cancer cells like HCT 116 with wild type **p53 status** is limited (28). Results of the present study suggest that RSV potentiates the pro-apoptotic properties of GSE. Apoptosis was dependent on ROS/p53/caspase-3 up-regulation and correlated with Bax/Bcl-2 ratio. However, this combination induced apoptosis in p53 <sup>-/-</sup> cells, but to a much lesser extent. Even in the presence of IGF-1, a mitogenic growth factor elevated during obesity, RSV-GSE combination suppressed proliferation and elevated apoptosis in HCT-116 p53 <sup>+/+</sup> cells. The promising results of the RSV-GSE combination in inducing apoptosis of colon cancer cells in a synergistic manner via p53-dependent pathways even in the presence of IGF-1 implies that this combination could be developed as an evidence-based chemopreventive agent against colon cancer.

### 3. MATERIALS AND METHODS

#### 3.1. Chemicals

Fetal bovine serum (FBS) was purchased from Fisher Scientific (Pittsburgh, PA). Grape seed extract (GSE, ORAC value 9000-13000 micromole Trolox equivalents/g, total phenolic content > 85% gallic acid equivalents) was a generous gift from San Joaquin Valley Concentrates (Fresno, CA). It is important to note that GSE has been shown to produce comparable biological actions irrespective of its commercial source (6). GSE is a mixture of mainly dimers, trimers and other oligomers of catechin and epicatechin and their gallate derivatives and was confirmed by UPLC-MS analysis of GSE in this study (Figure 1, (29, 30)). GSE typically lacks resveratrol (RSV) and UPLC-MS analysis of GSE showed either absence of RSV or at best below 0.016% by mass (value obtained by standard curve) even if we assume 90% ionization suppression due to the complex matrix of GSE (data not shown). McCoy's 5A medium (modified, 1X) was purchased from Invitrogen (Carlsbad, CA). The p53 inhibitor Pifithrin- $\alpha$ , the caspase-8 inhibitor z-IETD-FMK, and the caspase-3 inhibitor z-DEVD-FMK were purchased from Calbiochem (San Diego, CA). N-acetylcysteine, catalase enzyme, Dulbecco's Modified Eagle's Medium F-12 (DMEM/F-12), 5-fluorouracil (5-FU) and RSV were from the Sigma Chemical Co. (St. Louis, MO). IGF-1 was obtained from the R&D Systems (Minneapolis, MN).



**Figure 1.** Representative single ion UPLC-MS chromatograms of catechin and epicatechin monomers and their oligomers, and their gallate derivatives present in the grape seed extract used in the study prepared at 5 mg/mL in water-methanol mixture. Detailed protocol for LC/MS is provided in the materials and methods.

### 3.2. LC-MS analysis of GSE

GSE was dissolved in water-methanol mixture to attain a concentration of 5 mg/mL. One microL injections of the solution were performed on a Waters Acquity UPLC system with Acquity UPLC HSS T3 column (1.8 micromolar, 1.0 x 100 mm), using a gradient from solvent A (water + 0.1% formic acid) to solvent B (95% methanol, 5% water, 0.1% formic acid). Injections were made in 100% A, which was held for 2.0 min, a 13 minute linear gradient to 100 % B was applied, and held at 100 % B for 2 minutes, returned to starting conditions over 0.1 minutes, and allowed to reequilibrate at 100% A for 2.9 min. Flow rate was constant at 140 microL/min for the duration of the run. The column was held at 50°C, samples were held at 5°C. Column eluent was infused into a Micromass Q-ToF Micro MS fitted with an electrospray source. Data was collected in positive ion mode, scanning from 50-1200 at a rate of 0.9 scans per second with 0.1 second interscan delay. Calibration was performed prior to sample analysis via infusion of sodium formate solution, with mass accuracy within 5 ppm. The capillary voltage was held at 2200 V, the source temp at 130°C, and the desolvation temperature at 300°C at a nitrogen desolvation gas flow rate of 400 L/hr. The quadrupole was held at collision energy of 7 volts.

### 3.3. Cell lines

The HCT-116 p53 +/- and p53 -/- colon cancer cell lines were obtained from Dr. Bert Vogelstein (School of Medicine, the Johns Hopkins University, Baltimore, MD, USA). Cells were maintained at 37°C with 5% CO<sub>2</sub> and grown in McCoy's 5A medium supplemented with L-glutamine, 25 mM HEPES buffer, 100 mL/L FBS and 10 mL/L Penicillin-Streptomycin mix. CRL-1831 cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained at 37°C with 5% CO<sub>2</sub> and grown in Dulbecco's Modified Eagle's Medium F-12 (DMEM/F-12) supplemented with 2.2 g/L sodium bicarbonate, 0.2 g/L bovine serum albumin, 100 mL/L FBS and 10 mL/L Penicillin-Streptomycin mix. Cell cultures at approximately 75% confluence were used for all experimental treatments.

### 3.4. Cell proliferation assay

Cell viability was assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (WST-1 assay; Roche Diagnostics, Indianapolis, IN) following the supplier's protocol. Briefly, cells were grown in 96-well plates (5000 cells/well), overnight at 37°C, in a 5% CO<sub>2</sub> incubator and treated with RSV and/or GSE for

**Table 1.** Combination index (CI) analysis of HCT-116 p53  $+/+$  and p53  $-/-$  cells treated with different combinations of resveratrol (RSV) and grape seed extract (GSE)

Treatment			P53 $+/+$ cells			P53 $-/-$ cells	
Combinations	RSV (micromolar)	GSE (microg/mL)	Fractional inhibition (%)	CI value	Apoptosis 100 Lumens (X)	Fractional inhibition (%)	CI value
C1	27.0	35.2	70.2	0.668	201.7	74.2	0.687
C2	23.6	50.9	79.0	0.812	245.0	75.2	0.858
C3	15.7	34.0	70.1	0.561	129.4	72.5	0.608
C4	23.60	30.0	59.8	0.610	139.7	60.0	0.792
C5	31.5	68.0	81.6	1.078	383.4	80.4	1.008
C6	31.5	40	54.8	0.846	187.8	68.6	0.884
C7	41.3	24.8	48.2	0.755	99.1	66.9	0.753
C8	45.0	58.8	83.9	1.090	317.0	77.4	1.067
C9	55.0	33.0	63.1	0.896	192.0	72.6	0.893

HCT-116 p53  $+/+$  and  $-/-$  cells were treated with combination of GSE and RSV at different concentrations for 48 h and cell proliferation was determined using WST-1 assay and apoptosis (24 h) was measured using caspase-glo 3/7 assay. Fractional inhibition was calculated as a percentage with respect to control and CI values were calculated based on method of Chou and Talalay (31). C1 and C2 (in bold) were chosen for further experiments based on low concentrations of RSV (~ 25 micromolar) and its ability to induce apoptosis comparable to high doses of RSV and GSE (100 micromolar or 100 microg/mL) in HCT-116 p53  $+/+$  colon cancer cells.

indicated time period. After addition of WST-1 reagent (1:10 dilution in media), cells were incubated for 3 h and absorbance at 450 nm was measured by a micro plate reader. The experiments were repeated three times each time in triplicate and data were expressed as the means  $\pm$  SEs. For experiments using IGF-1, cell proliferation was measured via cell counting using Cellometer (Nexcelom Biosciences, Lawrence, MA). The experiments were repeated three times each time in triplicate and data were expressed as the means  $\pm$  SEs.

### 3.5. Cytotoxicity detection

To validate, if the concentrations of RSV and GSE used in the study are not toxic to the cells (induce necrosis), a dose response cytotoxicity detection assay using different concentrations of RSV and GSE was done. HCT-116 p53  $+/+$  and p53  $-/-$  cells (5000/well) were seeded in 96-well plates and after treatment for 24 h, the Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN) was used to measure the Lactate-dehydrogenase (LDH) activity in supernatants of cells treated with respective treatments, according to the instruction manual protocol. Briefly, after 30 min incubation at 37°C, absorbance was recorded on a micro plate reader at 492 nm. The experiments were done in triplicate and repeated at least three times, and data were expressed as the means  $\pm$  SE. Triton X-100 was the positive control. Cytotoxicity was calculated with the formula: % Cytotoxicity = (experimental value - low control) X 100 / (high control - low control), where low control is the assay medium plus cells and high control is the assay medium plus 2% Triton X-100 plus cells.

### 3.6. Apoptosis assay

Apoptosis induction was measured using 2 different assays; a nucleosomal fragmentation assay (Cell Death Detection Enzyme Linked Immuno Sorbent Assay (ELISA), Roche Diagnostics, Indianapolis, IN) followed by confirmation using caspase-3 cleavage (caspase-glo 3/7 assay, Promega, Madison, WI). Briefly, for the nucleosome ELISA assay, 100,000 cells were plated per well in 12-well plates, and then treated with the respective treatments. After 12 h or 24 h incubation, cells were counted and

assayed for apoptosis as per the manufacturer's protocol. Absorbance was recorded on a micro plate reader at 405 nm. The optical density was normalized to total number of cells in each treatment. For the caspase-glo 3/7 assay, cells were incubated for 12 h or 24 h with respective treatments. After incubation, cells were trypsinized and approximately 20,000 cells from each treatment were incubated with 100 microL of caspase-glo 3/7 reagent for 3 h. The luminescence of each sample was measured using a micro plate reader. DMSO was the solvent control. These experiments were repeated at least twice and each time in triplicate and data were expressed as the means  $\pm$  SEs.

### 3.7. Experimental design of combination study

A two-way combination of RSV and GSE against HCT-116 p53  $+/+$  and p53  $-/-$  cell proliferation was designed (26). The IC 50 values of RSV and GSE were determined on the basis of the dose-response curve (Figure 2A and 2B). Based on IC 50 values of RSV and GSE, a series of concentrations were used to select a few combinations that might demonstrate synergistic, additive or antagonistic response (C1-C9, Table 1). The combination effects were analyzed by the combination index (CI) method. The CI is based on the classic isobologram equation  $CI = D1/d1 + D2/d2$ ; where D1 and D2 are the doses of RSV and GSE, respectively, in the combination system; d1 and d2 are the doses of RSV and GSE alone for the same fractional inhibition, respectively (8). For data analysis of combinations,  $CI < 1$ ,  $CI = 1$ , and  $CI > 1$  indicate synergistic, additive, and antagonistic effects, respectively (8, 26). C1 and C2 were chosen for further experiments based on low concentrations of RSV (~ 25 micromolar) and its ability to induce apoptosis comparable to high doses of RSV and GSE (100 micromolar or 100 microg/mL) in HCT-116 p53  $+/+$  colon cancer cells (Table 1).

### 3.8. Fluorescence-activated cell sorting analysis (FACS)

HCT-116 p53  $+/+$  and p53  $-/-$  cells were plated at a density of  $15 \times 10^6$  cells per 100 mm plate and after 24 h treatment with either the control or the RSV-GSE combinations, cells were trypsinized and centrifuged. The pellet was resuspended with 1 mL of PI staining buffer

containing 4 mM sodium citrate, 0.1% Triton X-100, 50 microg/mL propidium iodide and 200 microg/mL RNase and incubated for 10 min at 37°C in the dark, and the final concentration of sodium chloride was adjusted to 0.15 M. Cells were analyzed using MoFlo (Dako Colorado, Inc.) flow cytometer and high speed cell sorter. Results were reported as *per cent* cells in each phase of the cell cycle.

### 3.9. Western blot analysis

HCT-116 p53  $+/+$  and p53  $-/-$  cells were seeded at a density of  $1.5 \times 10^5$  cells/mL in DMEM F-12 media with 5% charcoal-stripped FBS for 24 h. After treating cells for 24 h, protein was extracted into a high-salt buffer containing 1% protease and phosphatase inhibitor cocktail from Thermo Scientific (Rockford, IL), and protein concentrations were determined by using the BCA Protein Assay kit from Thermo Scientific (Rockford, IL). Cell lysates (45 microg) were incubated at 98°C for 5 min and separated by 10% polyacrylamide gels running at 120 V for 2 h in 1X running buffer, and electrophoretically transferred to Immobilon-FL, low fluorescent PVDF membranes from Millipore (Billerica, MA) at 30 V for 98 min in Tris-glycine transfer buffer. The membranes were blocked with Superblock blocking buffer from Thermo Scientific (Rockford, IL) for 2 h at room temperature. The membranes were incubated with rabbit polyclonal anti-p53 antibody, rabbit polyclonal anti-Bax antibody, both from Cell Signaling (1:1,000; Danvers, MA), and rabbit polyclonal anti-Bcl-2, goat polyclonal anti- $\beta$ -actin both from Santa Cruz Biotechnology (1:500 for Bcl-2, 1: 5000 for  $\beta$ -actin; Santa Cruz, CA) for 2 h at room temperature. Membranes were subsequently probed with anti-goat or anti-rabbit IR Dye secondary antibodies, Licor Biosciences (all 1:10,000, Lincoln, NE). Membranes were scanned and quantified with Odyssey infrared imaging system, Licor Biosciences (Lincoln, NE) using Odyssey software (Licor Biosciences). Membranes were also probed with respective IgG-HRP secondary antibodies from Santa Cruz Biotechnology (1:20,000; Santa Cruz, CA) and scanned using UVP imaging software (UVP BioDoc-It® Imaging System; Upland, CA).  $\beta$ -actin served as a loading control.

### 3.10. IGF-1 treatment

Cells were plated at a density of  $5 \times 10^4$  per well in 12-well plates in DMEM/F-12 containing 5% charcoal-stripped fetal bovine serum. After 24 h, cells were treated with DMSO (solvent control), 25 nM IGF-1 and/or combinations of RSV and GSE (C1 and C2), and anti-proliferative properties of the RSV-GSE combinations were evaluated after 48 h. As colonocytes have greater exposure to the bioavailable IGF-1 in obese condition, in the IGF-1 treatments, the cells were pre-incubated with IGF-1 for 10 minutes followed by the combination treatment to assess the effect of RSV-GSE combination when the cells are already primed to proliferate. Dose response study showed that 25 nM IGF-1 is essential to show a significant elevation of HCT-116 p53 $+/+$  cell proliferation (data not shown). In case of HT-29 and SW 480 advanced colon cancer cell lines even 10 nM IGF-1 was sufficient to prime

the cells to proliferate (18). Thus, we used 25 nM concentration of IGF-1 for subsequent experiments in HCT-116 p53  $+/+$  cells. In the apoptosis experiment, after 24 h of treatment, cells were assayed using the cell death detection ELISA kit according to the manufacturer's protocol.

### 3.11. Statistical analysis

Analysis of variance and Fisher's least square difference at 5% significance level determined the significant differences between treatments. Pearson correlation at 1% determined correlation between apoptosis and Bax/Bcl-2 ratio.

## 4. RESULTS

### 4.1. Resveratrol (RSV) or grape seed extract (GSE) suppresses cell proliferation of HCT-116 cells

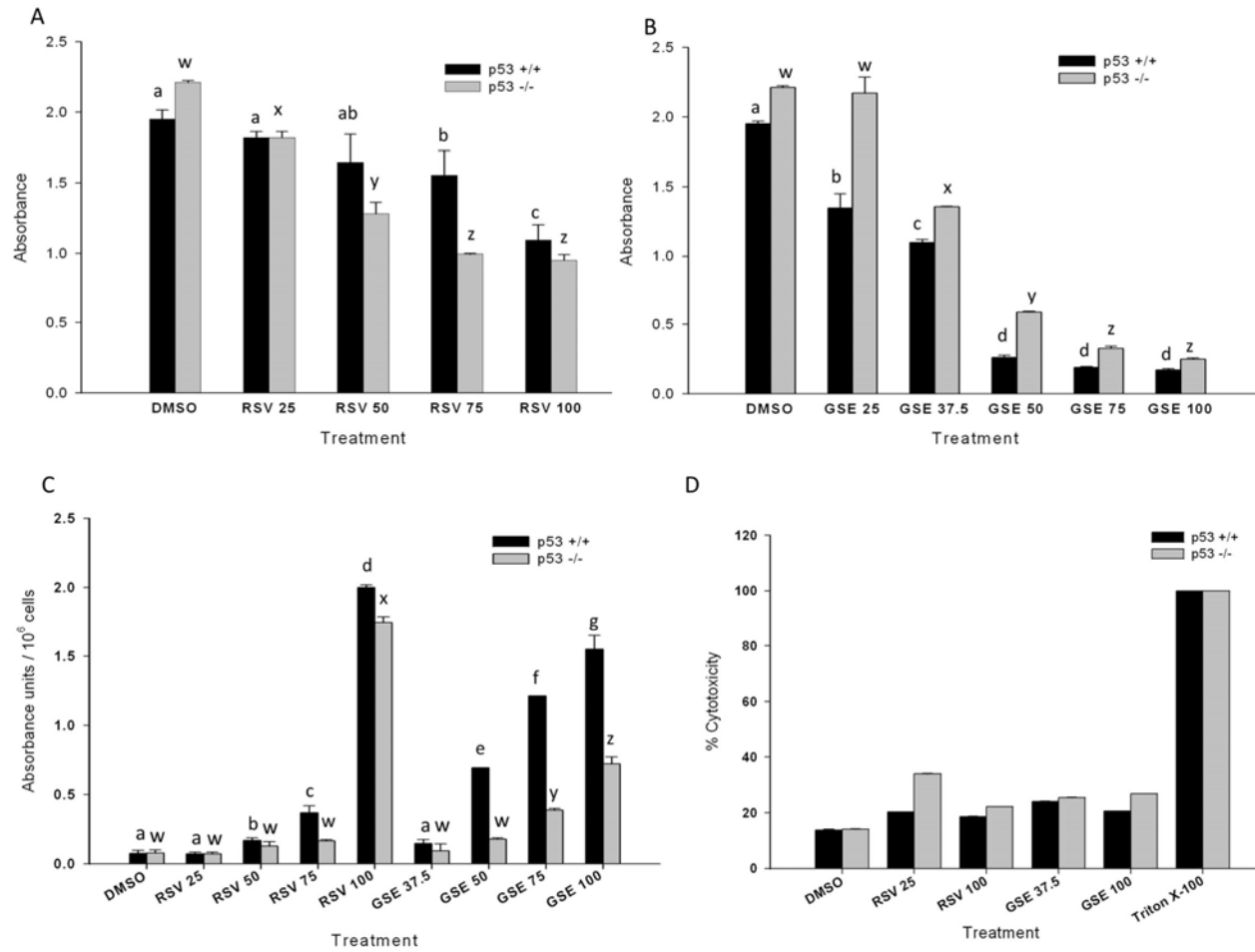
Anti-proliferative and cytotoxic effects of RSV or GSE were investigated using HCT-116 p53  $+/+$  and p53  $-/-$  colon cancer cells. RSV or GSE resulted in suppression of cell proliferation in a dose dependent manner (Figure 2A, 2B). Our results demonstrated that RSV suppresses the colon cancer cell proliferation in both p53  $+/+$  and p53  $-/-$  cells (Figure 2A), however, cell proliferation suppression was more potent in p53  $-/-$  cells (IC 50 of RSV was 72 micromolar in p53  $-/-$  cells compared to 110 micromolar in p53  $+/+$  cells). Treatment of HCT-116 p53  $+/+$  cells with 37.5, 50, 75, 100 and 125 microg/mL of GSE resulted in 31-90% cell proliferation suppression (Figure 2B). Similar growth inhibition was also seen in p53  $-/-$  cells, however the IC 50 for GSE was higher in p53  $-/-$  cells (47 microg/mL compared to 37.5 microg/mL in p53  $+/+$  cells). Together, the data in Figures 2A and 2B clearly demonstrate the efficacy of RSV or GSE to inhibit the growth of human colon cancer cells (HCT-116 p53  $+/+$  and p53  $-/-$ ).

### 4.2. RSV or GSE induces apoptosis

Induction of apoptosis by RSV or GSE in HCT-116 p53  $+/+$  and p53  $-/-$  cells was analyzed using nucleosomal fragmentation assay (Cell Death Detection ELISA). RSV induced apoptosis in both p53  $+/+$  and p53  $-/-$  cells, however, only at higher concentrations close to 100 micromolar (Figure 2C). GSE also induced apoptosis, but mainly in p53  $+/+$  cells (GSE > 75 microg/mL) (Figure 2C). This suggests that low concentrations of RSV and GSE, are unable to induce apoptosis even though they might be able to suppress cell proliferation (Figure 2A, 2B and 2C). Confirmation using a p53 inhibitor (Figure 4E), suggests that RSV induced apoptosis in a p53-independent manner; however, GSE requires a functional p53 to induce apoptosis in HCT-116 cells.

### 4.3. RSV-GSE combination suppresses cell proliferation and induces apoptosis

Based on the anti-proliferative and pro-apoptotic properties of RSV or GSE in the HCT-116 cell lines, the effect of the combination of RSV and GSE on the HCT-116 p53  $+/+$  and p53  $-/-$  cells was analyzed. Possible combinatorial effects (synergy, additive or antagonism)



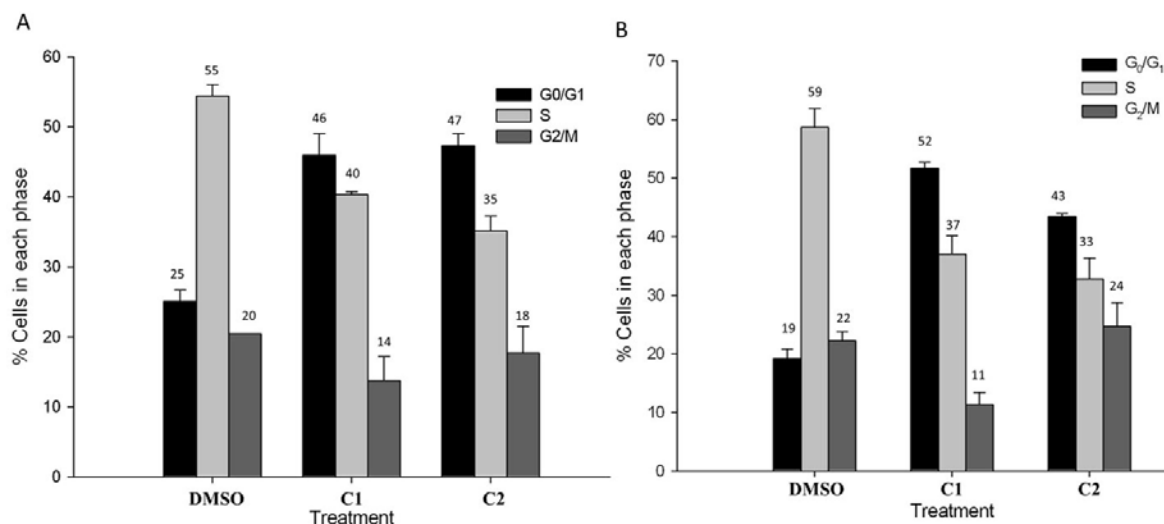
**Figure 2.** Anti-proliferative, cytotoxic and apoptotic effects of resveratrol (RSV) and grape seed extract (GSE). (A) RSV and (B) GSE suppressed HCT-116 colon cancer cell proliferation and (C) induced apoptosis but do not show any (D) cytotoxic effects. HCT-116 p53 +/+ and -/- cells were treated with RSV (25, 50, 75, and 100 micromolar) or GSE (25, 37.5, 50, 75 and 100 microg/mL) for 48 h and cell proliferation was determined using WST-1 assay and cytotoxicity was detected in the supernatants using LDH assay. HCT-116 p53 +/+ and -/- cells were treated with RSV (25, 50, 75 and 100 micromolar) or GSE (37.5, 50, 75 and 100 microg/mL) for 12 h and induction of apoptosis was determined by cell death detection ELISA assay. The rate of apoptosis was expressed as absorbance value measured at 405 nm. Results were expressed as mean  $\pm$  SE for three replicate experiments for each treatment group. DMSO served as a solvent control. Triton X-100 was used as a positive control for LDH assay. Means that differ by a common letter (a, b, c, d, e, f, g for p53 +/+ cells and w, x, y, z for p53 -/- cells) differ ( $p < 0.05$ ).

were assessed using a series of nine different combinations (Table 1). Data were analyzed using combination index (CI) values by the method of Chou and Talalay (31). Results indicated that a combination of RSV and GSE resulted in a greater growth inhibition as compared to either agents alone at the same concentrations (Table 1, Figure 2A and 2B) in HCT-116 p53 +/+ cells after 48 h of treatment. Interestingly, seven out of the nine combinations showed synergism, whereas the other two showed additive effects in p53 +/+ cells (Table 1). Elevated cell proliferation suppression was also seen in p53 -/- cells, wherein the same seven combinations demonstrated synergism as in p53 +/+ cells (Table 1). For the nine combinations, apoptosis was also measured, albeit only in p53 +/+ cells. Out of the nine combinations, two combinations (C1: RSV 27 micromolar and GSE 35 microg/mL and C2: RSV 25 micromolar and

GSE 51 microg/mL) were selected for further experiments based on low CI values, reduced RSV levels, their ability to adequately suppress cell proliferation and induce apoptosis comparable to RSV doses of 100 micromolar. Low doses of RSV and GSE in combinations C1 and C2 are unable to elicit apoptosis individually in p53 +/+ cells (Figure 2C).

#### 4.4. RSV-GSE combination induces cell cycle arrest in Go/G1 phase in both HCT 116 p53 +/+ and p53 -/- cells

Fluorescence-activated cell sorting analysis (FACS) technique was used to determine the effect of RSV-GSE combination on cell cycle progression in HCT-116 cells. Figures 3A and 3B illustrate the distribution of HCT-116 p53 +/+ cells and p53 -/- cells in the G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases after treatment with the RSV-GSE



**Figure 3.** Effect of combination of resveratrol (RSV) and grape seed extract (GSE) on cell cycle progression of HCT 116 colon cancer cells. HCT-116 p53 +/+ (A) and p53 -/- (B) cells were plated at a density of  $15 \times 10^6$  cells per 100 mm plate and after 18 h treatment with the vehicle (DMSO), C1 and C2; they were analyzed by fluorescence-activated cell sorting analysis (FACS). Results were expressed as % of cells in each phase. Numbers on top of the bars represent percent cells in respective phases in each treatment group. C1 and C2 have RSV (27 micromolar and 25 micromolar) and GSE (35 and 51 microg/mL), respectively.

combination, respectively. RSV-GSE combination had higher percentage of cells in the G<sub>0</sub>/G<sub>1</sub> phase compared to control in both p53 +/+ and p53 -/- cells. These results suggest that the RSV-GSE combination targets G<sub>0</sub>/G<sub>1</sub> to S phase progression to arrest HCT-116 cell proliferation.

#### 4.5. RSV-GSE combination induces apoptosis via p53-dependent pathway

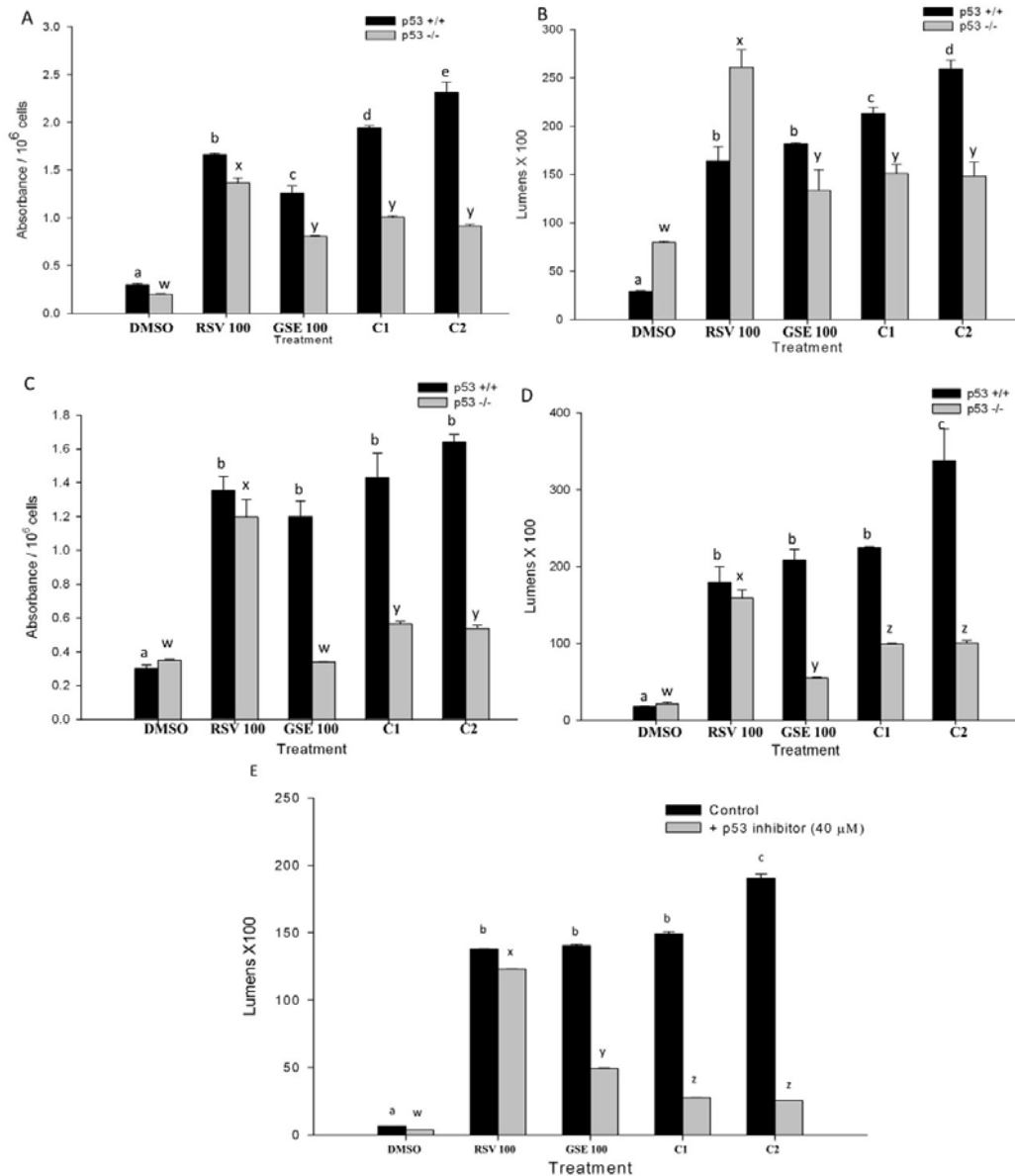
We used nucleosomal fragmentation assay and caspase-glo 3/7 assay to detect whether combination of RSV and GSE induces apoptotic death. There was an increase in apoptosis with the two combination doses (C1 and C2) compared to the control (Figure 4A). This is particularly important, since RSV (~ 25 micromolar) or GSE (~ 37.5 microg/mL) alone do not induce apoptosis at the concentrations present in the combination (Figure 2C and Table 1). Similar results were obtained when apoptosis was measured using the caspase-glo 3/7 assay (Figure 4B).

RSV or GSE alone at high concentrations demonstrated p53 independent and p53 dependent apoptosis respectively, at 24 h time point (Figure 2C), however, at lower concentrations used in C1 or C2 neither RSV nor GSE induced apoptosis in p53 +/+ or p53 -/- cells. RSV-GSE combination showed differences in the induction of apoptosis in p53 +/+ and p53 -/- cells at 12 h (Figure 4A and 4B). These differences were much more pronounced and significant, suggesting that combination treatment requires a functional p53 to induce apoptosis (Figure 4B). Results were confirmed using caspase-glo 3/7 assay (Figure 4D). However, increasing GSE concentration to 50 microg/mL did not elevate apoptosis in C2 compared to C1 indicating that once GSE levels reach 50 microg/mL, RSV had a marginal effect to potentiate GSE actions. This also suggests that the effect of synergy is at best when the two compounds are at low concentrations together.

To confirm the role of p53, transcriptional activity of p53 was inhibited using the p53 inhibitor pifithrin- $\alpha$  (40 micromolar), and treated with the combination treatment for 24 h and then assayed for apoptosis (32). HCT-116 p53 +/+ cells treated with the p53 inhibitor acted like the p53 -/- cells, and the RSV-GSE combination did not induce apoptosis in p53 +/+ cells in the presence of the p53 inhibitor (Figure 4E). This confirms that the RSV-GSE combination induced apoptosis via p53 dependent signaling pathways. Further experiments delineating mechanisms of the pro-apoptotic effects of RSV-GSE combination were only performed in p53 +/+ cells.

#### 4.6. RSV-GSE combination suppressed cell proliferation and induced apoptosis via caspase-3-dependent and Bax/Bcl-2 pathways

To investigate whether the induction of apoptosis by RSV-GSE combination in HCT-116 p53 +/+ cells was through activation of p53 downstream pathways, lysates from cells treated with RSV and GSE, alone and in combination, were subjected to western blot analysis. RSV in combination with GSE elevated pp53 (activated p53, ser 15) in HCT-116 p53 +/+ cell lines (Figure 5A). This is particularly interesting because RSV or GSE could not elevate pp53 levels at lower concentrations present in the combination (Figure 5A). Levels of Bax and Bcl-2, pro and anti-apoptotic members of the BH3 family of apoptotic proteins were measured in the combination treatment in p53 +/+ cells. Combination of RSV and GSE elevated Bax levels and concomitantly suppressed levels of Bcl-2, overall elevating the Bax: Bcl-2 ratio (Figure 5B). Bax: Bcl-2 ratio showed significant correlation with apoptosis in both the cell lines (Pearson correlation coefficient = 0.88 for p53 +/+ cells,  $p < 0.01$ ), indicating the RSV-GSE



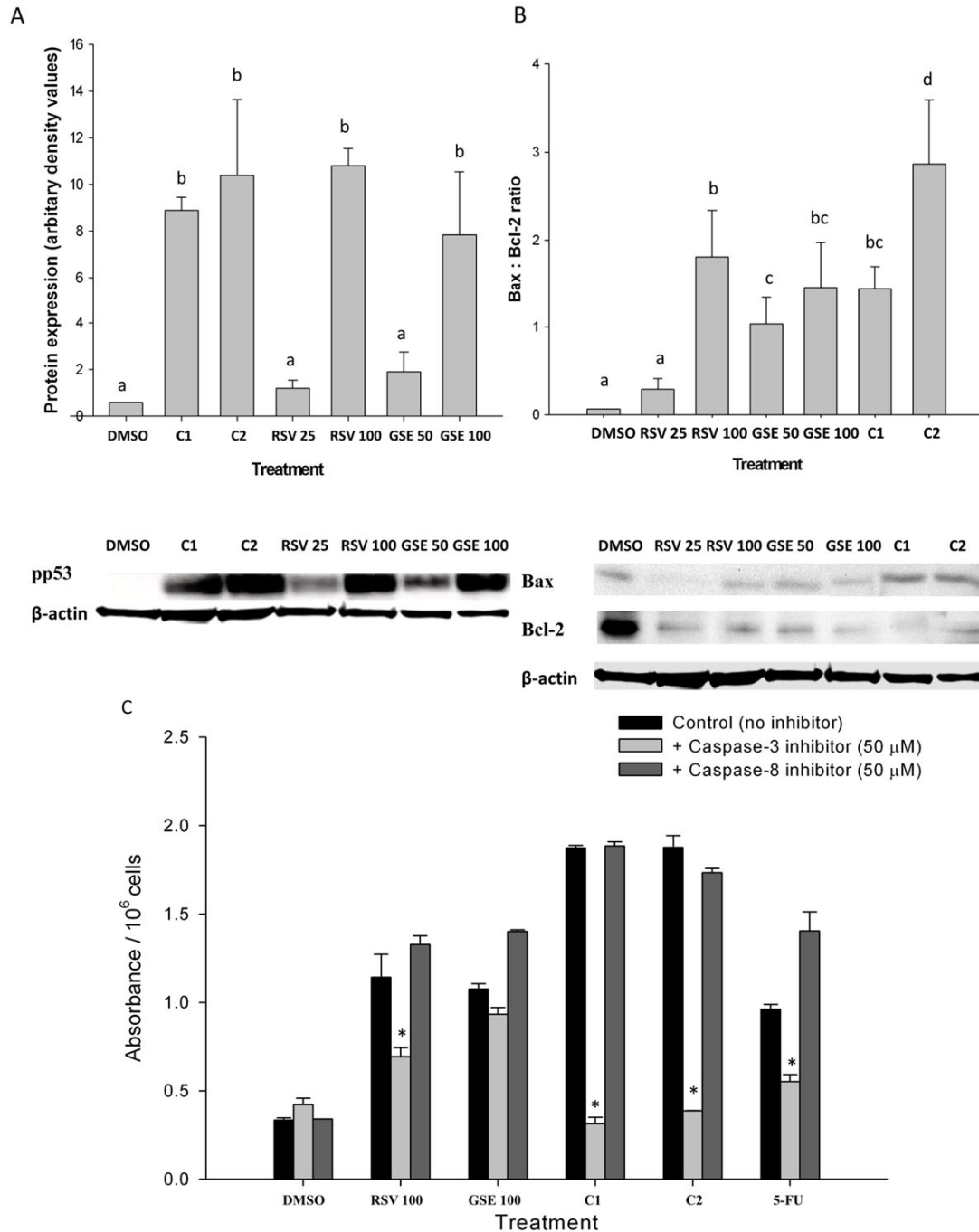
**Figure 4.** Synergistic combination of resveratrol (RSV) and grape seed extract (GSE) induced apoptosis in HCT-116 human colon cancer cell lines. (A, C) HCT-116 p53 +/+ and -/- cells were treated with RSV and/or GSE for 12 and 24 h and induction of apoptosis was determined by cell death detection ELISA assay. The rate of apoptosis was expressed as absorbance value measured at 405 nm, normalized to 10<sup>6</sup> cells. (B, D) HCT-116 p53 +/+ and -/- cells were treated with RSV and GSE alone and in combination for 12 and 24 h and apoptosis was determined using caspase-glo 3/7 assay. E) HCT-116 p53 +/+ cells were pretreated with the p53 inhibitor pifithrin- $\alpha$  (40 micromolar) for 45 minutes followed by RSV and/or GSE for 24 h and apoptosis was detected using caspase-glo 3/7 assay. Results were expressed as mean  $\pm$  SE for three replicate experiments for each treatment group. C1 and C2 have RSV (27 micromolar and 25 micromolar) and GSE (35 and 51 microg/mL), respectively. Means that differ by a common letter (a, b, c, d, e for p53 +/+ cells and w, x, y, z for p53 -/- cells or pifithrin- $\alpha$  treatment) differ ( $p < 0.05$ ).

induced apoptosis might be via the mitochondrial mediated apoptotic pathway (33).

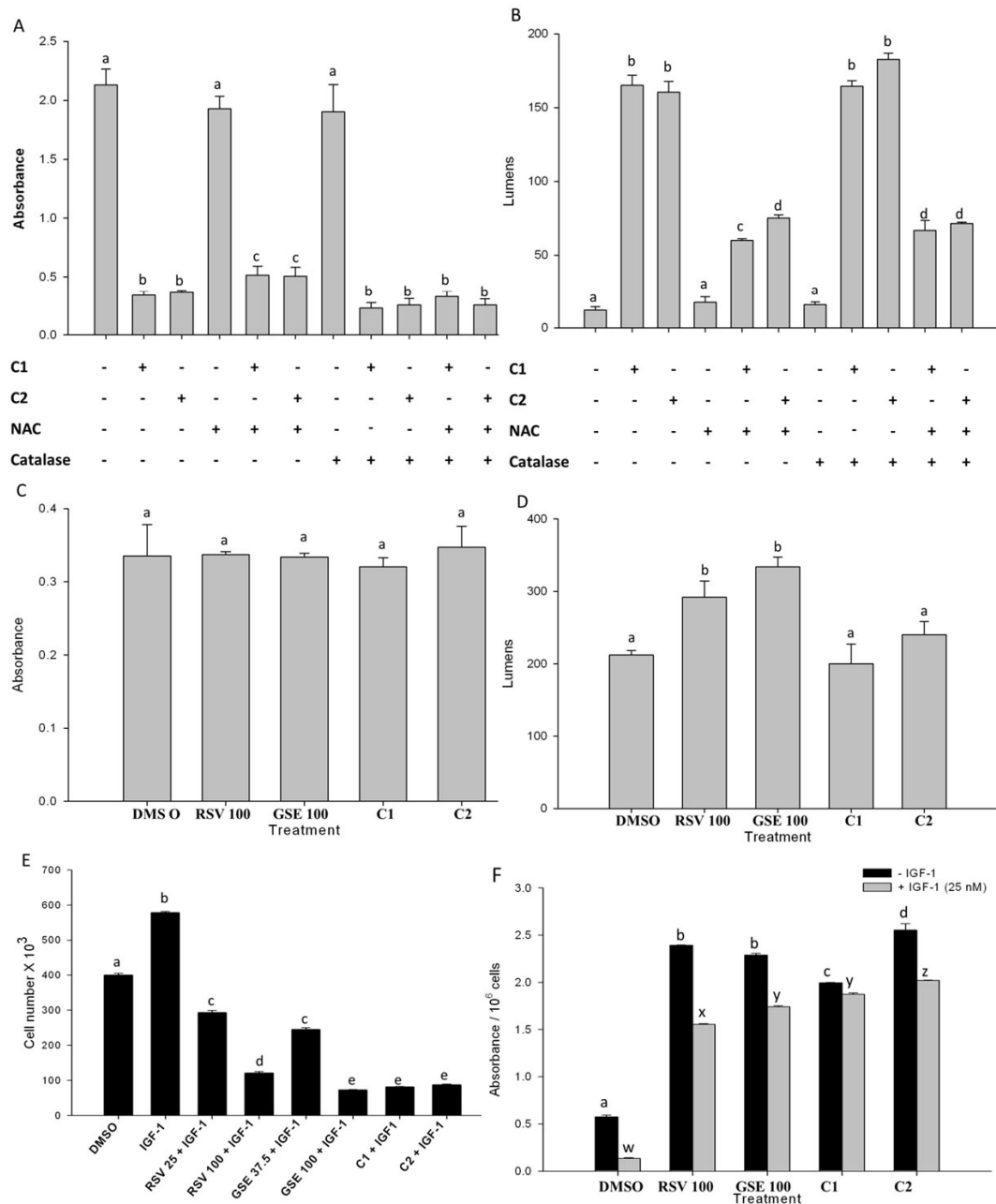
To further confirm the route of apoptosis, caspase-3 and caspase-8 were inhibited separately and then treated with the combination treatment. We measured apoptosis using the nucleosomal fragmentation assay 24 h post treatment. Treatment with the cell-permeable caspase-

3 inhibitor DEVD-CHO (50 micromolar) suppressed RSV-GSE combination induced apoptosis, demonstrating the observed cell death to be apoptotic (Figure 5C). In contrast, incubation of cultures with the caspase-8 inhibitor IETH-CHO (50 micromolar) had no significant effect on apoptosis induction by RSV-GSE combination, indicating that the cell death is not through the death receptor-mediated pathway involving caspase-8 activation (Figure





**Figure 5.** Apoptotic induction by the synergistic combination of resveratrol (RSV) and grape seed extract (GSE) involves pathways downstream of p53. HCT-116 p53  $+/+$  and p53  $-/-$  cells were treated with RSV, GSE and combination of RSV and GSE. C1 and C2 have RSV (27 micromolar and 25 micromolar) and GSE (35 and 51 microg/mL), respectively. Whole cell lysates were analyzed by Western blotting for (A) pp53, (B) Bax, Bcl-2 as described in materials and methods. Blots were incubated with the indicated antibodies. Similar results were obtained in triplicate experiments. (C) HCT-116 p53  $+/+$  cells were treated with cell permeable caspase-3 (50 micromolar) and caspase-8 (50 micromolar) inhibitors for 45 minutes followed by RSV and/or GSE and apoptosis was detected using cell death detection ELISA assay. 5-fluorouracil (5-FU) served as the positive control. RSV 100 and GSE 100 also served as positive control based on earlier reports (28, 60). Results were expressed as mean  $\pm$  SE for three replicate experiments for each treatment group. \* indicates significant suppression compared to control (no inhibitor). Means that differ by a common letter (a, b, c, d) differ ( $p < 0.05$ ).



**Figure 6.** N-acetyl cysteine (NAC) suppresses resveratrol (RSV)-grape seed extract (GSE) combination's anti-proliferative (A) and pro-apoptotic (B) properties. HCT 116 p53<sup>+/+</sup> and p53<sup>-/-</sup> cells were pre-incubated with NAC (2 mM) or catalase (100 U/mL) for 1 h and then subjected to the RSV-GSE combination (24 h treatment in total) and checked for proliferation and apoptosis using WST-1 and caspase-glo 3/7 assay respectively. C-F: RSV-GSE combination is non-toxic to normal cells, but showed anti-proliferative and pro-apoptotic properties even in the presence of insulin like growth factor-1 (IGF-1) in HCT-cells. CRL-1831 normal colonic epithelial cells were treated with RSV, GSE alone and in combination and proliferation (48 h) (C) and apoptosis (24 h) (D) were measured by WST-1 and caspase-glo 3/7 assay respectively. HCT-116 p53<sup>+/+</sup> cells were primed to proliferate by incubation with IGF-1 (25 nM) for 10 minutes followed by RSV and/or GSE. Cell proliferation (E) was measured using WST-1 assay 48 h after treatment. For apoptosis (F), cells were seeded in 12 well plates pre-treated with IGF-1 (25 nM) followed by treatment with RSV and/or GSE and apoptosis was measured using cell death detection ELISA assay 24 h post treatment. Results were expressed as mean  $\pm$  SE for three replicate experiments for each treatment group. C1 and C2 have RSV (27 micromolar and 25 micromolar) and GSE (35 and 51 microg/mL), respectively. Means that differ by a common letter (a, b, c, d, e, f for p53<sup>+/+</sup> cells and w, x, y, z for IGF-1 treatments) differ ( $p < 0.05$ ).

5C). 5-fluorouracil served as the positive control for p53 dependent apoptotic pathway (34).

### 4.7. N-acetyl cysteine (NAC) inhibited RSV-GSE combination induced apoptosis in HCT-116 p53 +/- cells

HCT-116 p53 +/- cells were pre-incubated with NAC (2 mM), a free radical scavenger and/or catalase (CAT; 100 U/mL), a hydrogen peroxide radical scavenger, for 1 h and then treated with RSV-GSE combination for a total time of 24 h. Both proliferation and apoptosis were measured using WST-1 and caspase-glo 3/7 assays, respectively. In the presence of NAC, RSV-GSE combination induced proliferation arrest, however apoptosis was suppressed. CAT could suppress neither proliferation arrest nor apoptosis induced by the combination (Figure 6A, 6B).

### 4.8. RSV-GSE combination is non-toxic *in vitro*

To know the effect of RSV-GSE combination on normal cells CRL-1831 normal colon epithelial cells were treated with the RSV-GSE combination for 24 h and assessed for their proliferation and apoptosis using WST-1 and caspase-glo 3/7 assays, respectively. RSV-GSE combination induced neither proliferation arrest nor apoptosis elevation suggesting that the combination is effective against cancer cells with no adverse action on normal cells (Figure 6C, 6D) *in vitro*. Furthermore, the cytotoxicity of RSV or GSE towards HCT-116 p53 +/- and p53 -/- *in vitro* was evaluated using LDH assay. Minimal cytotoxicity was observed for RSV or GSE even at concentrations up to 100 micromolar and 100 microg/mL, respectively (Figure 2D). Cytotoxicity was compared against the positive control Triton X-100.

### 4.9. RSV-GSE combination suppressed cell proliferation and induced apoptosis even in the presence of IGF-1

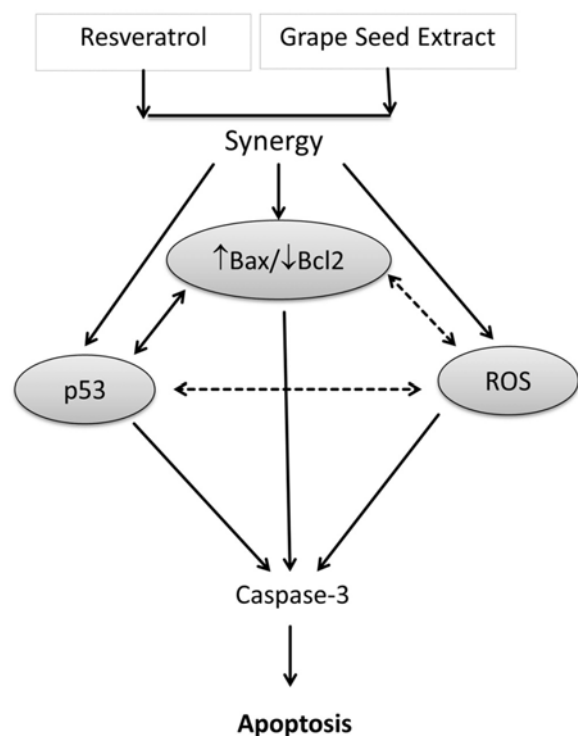
Growth stimulatory effects of IGF-1 and the anti-proliferative effects of RSV-GSE combinations were investigated using HCT-116 p53 +/- cells. Our results demonstrated that RSV-GSE combinations suppressed colon cancer cell proliferation even when the cells were primed to proliferate with IGF-1. After 48 h, IGF-1 (25 nM) treatment elevated HCT-116 cell proliferation confirming its growth stimulatory effects (Figure 6E). Moreover, pre-incubation with IGF-1 (25 nM) for 10 minutes followed by treatment with the combination resulted in suppressed cell proliferation (Figure 6E). Induction of apoptosis by the RSV-GSE combination in the presence of IGF-1 was analyzed using nucleosomal fragmentation assay. The combination treatment induced apoptosis even in the presence of IGF-1 suggesting its role as a potential chemopreventive agent even against IGF-1-promoted colon cancers (Figure 6F).

## 5. DISCUSSION

Current strategy of cancer management includes: surgery, radiation therapy and chemotherapy, and in occasional instances investigational drug use on a case by case basis. However, conventional therapy is associated

with many side-effects. Hence, methods designed to improve the efficacy of these treatment modalities or to devise newer ways to treat or even prevent cancer are needed. However, most chemoprevention trials with single compounds failed to provide satisfactory results. Epidemiological studies showed that plant based diets are associated with reduced risk of developing chronic diseases, such as cancer and cardiovascular disease (3). Phytochemicals possess potent antioxidant and anti-proliferative properties (35). We previously showed that RSV at concentrations > 100 micromolar suppressed colon cancer proliferation even in the presence of IGF-1, a mitogenic growth factor, by inhibiting the critical components in the IGF-1R/Wnt signaling pathway, and elevated apoptosis (18). However, in fruits and vegetables, bioactive compounds exist as a complex mixture that synergize or complement each other's chemopreventive/protective actions. The results of the present study are in support of such belief since the present study showed that suppression of proliferation and elevation of apoptosis in HCT-116 p53 +/- human colon cancer cell lines at 100 micromolar or higher concentrations of RSV could be accomplished at 25 micromolar, when combined with GSE at doses of 35-50 microg/mL (Table 1). These results strongly support our hypothesis that combining bioactive compounds like RSV and GSE could reduce the dose of either compound while providing similar or better anti-cancer properties lending support to the proposal that combinatorial approach towards colon cancer chemoprevention is a feasible approach. These results are important as chemoprevention strategies using single compounds such as beta-carotene, vitamin E, ascorbic acid etc. proved futile (36).

RSV and GSE induced apoptosis in a variety of cancer cell lines (5-9, 11, 13, 14, 16, 18, 37-39). Even though synergistic combination treatments enhanced apoptosis in both HCT-116 p53 +/- and p53 -/- cells, at 12 h post treatment, apoptosis was more pronounced in p53 +/- cells compared to p53 -/- cells, suggesting a significant role for p53. It is noteworthy that at 24 h post treatment both the combinations induced apoptosis in p53 +/- cells. It is remarkable that the involvement of p53 in the enhancement of apoptosis in HCT-116 p53 +/- by RSV-GSE combination is reaffirmed by the use of p53 transcriptional inhibitor pifithrin- $\alpha$ , that is known to block p53-dependent transcriptional activation and apoptosis. RSV and GSE demonstrated p53 independent and p53 dependent apoptosis alone at high concentrations at 24 h time point (Figure 2C). RSV when used at  $\geq$  100 micromolar effectively suppressed proliferation and augmented apoptosis in comparison to the action of GSE when used alone or in combination with RSV in HCT-116 p53 -/- cells. These results might indicate that RSV at higher concentrations works via p53 independent pathways to suppress cell proliferation and induce apoptosis. However, in HCT-116 p53 -/- cells, combination of GSE and RSV did enhance apoptosis to a small extent possibly via p53 independent pathways indicating that RSV might potentiate apoptotic action of GSE independent of p53 status of the cells. Overall, the results are still intriguing since the low concentrations of RSV and GSE in C1 and



**Figure 7.** Summary of the signaling pathways for resveratrol-grape seed extract (RSV-GSE) combination-induced apoptosis. RSV-GSE combination induced apoptosis involves interplay of reactive oxygen species (ROS), p53, Bax and Bcl-2.

C2 did not induce apoptosis individually in p53 +/+ or p53 -/- cells (Figure 2C). It is important to note that C1 and C2 did not differ significantly in inducing apoptosis indicating that the potentiating effect of RSV is marginal once the GSE concentration reaches 50 microg/mL.

In the present study, RSV-GSE combination induced apoptosis via activating p53 (pp53), elevating Bax and suppressing Bcl-2 that leads to an increase in the Bax/Bcl-2 ratio in p53 +/+ cells, which alters mitochondrial membrane permeability. Such altered mitochondrial membrane permeability releases cytochrome C into the cytosol (40, 41) that triggers activation of caspase-9, which accelerates apoptosis by activating other caspases such as caspase-3. It is evident from the results of the present study that caspase-3 inhibitor suppressed apoptosis induced by the combination of RSV-GSE while caspase-8 inhibitor had no significant effect on apoptosis, suggesting that the cell death is not through the major death receptor-mediated pathway involving caspase-8 activation.

Several studies have suggested that cancer chemotherapeutic drugs induce apoptosis of tumor cells, in part, by inducing the formation of reactive oxygen species (ROS) (42, 43). The p53 tumor suppressor gene can induce either apoptosis or senescence in response to cellular stresses (44, 45). ROS accumulation and mitochondrial function can contribute to p53-dependent apoptosis and it has been shown in multiple studies that ROS inducers like

bioactive agents collaborate with p53 to influence apoptosis (42, 46-48). Also, recent articles have pointed out the importance of the Bax, Bcl-2 and ROS production in tumor cells (49). Bcl-2 overexpression, in particular has shown to suppress apoptosis induced by RSV by a pathway that involves ROS production (43). Our results indicated that N-acetylcysteine, a free radical scavenger and glutathione precursor, suppressed apoptosis induced by the RSV-GSE combination. However, catalase, an antioxidant enzyme that brings about its cytoprotective action by suppressing or neutralizing the toxic hydrogen peroxide radicals produced could not inhibit the cytotoxic action of RSV-GSE. These results indicate that this combination (RSV-GSE) does not bring about its actions by augmenting hydrogen peroxide radical formation (50). Since NAC could protect against the combination induced apoptosis, the activation of caspase cascade could be linked to the generation of ROS (42). These results also explain that the pathway that the combination induces ROS and apoptosis could involve interplay between p53, Bax and Bcl-2 (Figure 7) (43, 51). ROS can also act via enhancing mitochondrial membrane permeability and thus can induce apoptosis via the caspase-3 cascade (42). Overall, these results are in support of the proposal that mitochondrial apoptotic pathway is responsible for the observed apoptosis.

Further, RSV-GSE combination did not suppress the proliferation or induce apoptosis of normal colon epithelial cell CRL-1831 line indicating that the RSV-GSE combination preferentially target cancer cells while sparing their normal counterparts. The exact reason for this is not clear but could be due to differential metabolism of bioactive compounds in normal and cancer cells that remains to be established (52-54).

The germline adenomatous polyposis coli mutations in colonocytes are highly predictive of colon cancer. Hence, the use of RSV- GSE combination that selectively activates p53 to induce apoptosis in colon cancer but not in normal cells could be a promising dietary approach in its prevention. This is particularly interesting in the light of the known fact that polyps possess intact p53 and all colon cancers develop from the polyps. Based on these results, it is important to pursue the chemopreventive and chemoprotective properties of RSV-GSE synergistic combination in both rodent and human models of colon cancer in future studies.

Members of IGFs family and the IGF-binding proteins (IGFBPs) play a critical role in the progression of a variety of cancers during obesity including colon cancer (55). Recently we showed that RSV at  $\geq 100$  micromolar concentration could effectively suppress IGF-1 (elevated during obesity) stimulated growth of human colon cancer cell lines via suppression of IGF-1R/PI3K/ $\beta$ -catenin pathway. IGF-1 binding to IGF-1R stimulates downstream proliferating pathways such as the PI3K/Akt and Ras signaling resulting in increased human colon cancer cell proliferation (56, 57). RSV could effectively suppress IGF-1R levels and also downstream PI3K/Akt/ $\beta$ -catenin signaling. This study, for the first time, demonstrated that RSV-GSE combination suppressed IGF-1 stimulated HCT-

116 colon cancer cell proliferation and induced apoptosis even in the presence of IGF-1 suggesting its potential role as a chemopreventive agent against colon cancer even in obese subjects.

In summary, the current study revealed that RSV and GSE work synergistically to suppress proliferation and induce apoptosis in human colon cancer cells via p53 dependent mechanisms, but not in normal CRL-1831 colon epithelial cells. Thus, these results established the molecular basis for the beneficial effect of RSV-GSE combination that is a popular dietary supplement. It is possible that the synergistic combination of RSV-GSE may result in the formation of a variety of potent bioactive metabolites that possess chemoprotective effect in animal/human models at even lower plasma concentrations compared to the present *in vitro* combination used in the study (18, 37, 58, 59).

## 6. ACKNOWLEDGEMENTS

This work was supported by College of Applied Human Sciences Challenge Grant, Colorado State University (2009-2010) and National Research Initiative Grant 2009-55200-05197 from the USDA National Institute for Food and Agriculture (2009-2012). No potential conflicts of interest were disclosed. We are grateful to Drs. Michael Pagliassotti, Robert Zimdahl and Michael Weil, Colorado State University, Fort Collins, CO for their critical feedback on the manuscript. We also thank technical staff at Proteomics and Metabolomics Core, Colorado State University for their assistance with GSE analysis.

## 7. REFERENCES

1. I. American Cancer Society: Cancer Facts and Figures 2010 In: Ed I. American Cancer Society. (2010)
2. W. M. Grady and J. M. Carethers: Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology*, 135(4), 1079-99 (2008)
3. R. H. Liu: Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr*, 134(12 Suppl), 3479S-3485S (2004)
4. M. Nassiri-Asl and H. Hosseinzadeh: Review of the pharmacological effects of *Vitis vinifera* (Grape) and its bioactive compounds. *Phytother Res*, 23(9), 1197-204 (2009)
5. C. Agarwal, R. P. Singh and R. Agarwal: Grape seed extract induces apoptotic death of human prostate carcinoma DU145 cells via caspases activation accompanied by dissipation of mitochondrial membrane potential and cytochrome c release. *Carcinogenesis*, 23(11), 1869-76 (2002)
6. M. Kaur, R. Mandair, R. Agarwal and C. Agarwal: Grape seed extract induces cell cycle arrest and apoptosis

in human colon carcinoma cells. *Nutr Cancer*, 60 Suppl 1, 2-11 (2008)

7. C. Laurent, P. Besancon, C. Auger, J. M. Rouanet and B. Caporiccio: Grape seed extract affects proliferation and differentiation of human intestinal Caco-2 cells. *J Agric Food Chem*, 52(11), 3301-8 (2004)
8. G. Sharma, A. K. Tyagi, R. P. Singh, D. C. Chan and R. Agarwal: Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells. *Breast Cancer Res Treat*, 85(1), 1-12 (2004)
9. M. Kaur, R. Agarwal and C. Agarwal: Grape seed extract induces anoikis and caspase-mediated apoptosis in human prostate carcinoma LNCaP cells: possible role of ataxia telangiectasia mutated-p53 activation. *Mol Cancer Ther*, 5(5), 1265-74 (2006)
10. S. Khanna, S. Roy, D. Bagchi, M. Bagchi and C. K. Sen: Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract. *Free Radic Biol Med*, 31(1), 38-42 (2001)
11. M. Alkhalaf: Resveratrol-induced apoptosis is associated with activation of p53 and inhibition of protein translation in T47D human breast cancer cells. *Pharmacology*, 80(2-3), 134-43 (2007)
12. M. Asensi, I. Medina, A. Ortega, J. Carretero, M. C. Bano, E. Obrador and J. M. Estrela: Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free Radic Biol Med*, 33(3), 387-98 (2002)
13. K. P. Bhat and J. M. Pezzuto: Cancer chemopreventive activity of resveratrol. *Ann N Y Acad Sci*, 957, 210-29 (2002)
14. E. Sexton, C. Van Themsche, K. LeBlanc, S. Parent, P. Lemoine and E. Asselin: Resveratrol interferes with AKT activity and triggers apoptosis in human uterine cancer cells. *Mol Cancer*, 5, 45 (2006)
15. M. Jang and J. M. Pezzuto: Cancer chemopreventive activity of resveratrol. *Drugs Exp Clin Res*, 25(2-3), 65-77 (1999)
16. M. Jang, L. Cai, G. O. Udeani, K. V. Slowing, C. F. Thomas, C. W. Beecher, H. H. Fong, N. R. Farnsworth, A. D. Kinghorn, R. G. Mehta, R. C. Moon and J. M. Pezzuto: Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*, 275(5297), 218-20 (1997)
17. M. H. Aziz, R. Kumar and N. Ahmad: Cancer chemoprevention by resveratrol: in vitro and in vivo studies and the underlying mechanisms (review). *Int J Oncol*, 23(1), 17-28 (2003)
18. J. Vanamala, L. Reddivari, S. Radhakrishnan and C. Tarver: Resveratrol suppresses IGF-1 induced human colon

cancer cell proliferation and elevates apoptosis via suppression of IGF-1R/Wnt and activation of p53 signaling pathways. *BMC Cancer*, 10, 238 (2010)

19. D. LeRoith and C. T. Roberts, Jr.: The insulin-like growth factor system and cancer. *Cancer Lett*, 195(2), 127-37 (2003)

20. E. K. Wei, J. Ma, M. N. Pollak, N. Rifai, C. S. Fuchs, S. E. Hankinson and E. Giovannucci: A prospective study of C-peptide, insulin-like growth factor-I, insulin-like growth factor binding protein-1, and the risk of colorectal cancer in women. *Cancer Epidemiol Biomarkers Prev*, 14(4), 850-5 (2005)

21. J. Ma, M. N. Pollak, E. Giovannucci, J. M. Chan, Y. Tao, C. H. Hennekens and M. J. Stampfer: Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. *J Natl Cancer Inst*, 91(7), 620-5 (1999)

22. J. Vanamala, C. C. Tarver and P. S. Murano: Obesity-enhanced colon cancer: functional food compounds and their mechanisms of action. *Curr Cancer Drug Targets*, 8(7), 611-33 (2008)

23. R. L. Ahmed, W. Thomas and K. H. Schmitz: Interactions between insulin, body fat, and insulin-like growth factor axis proteins. *Cancer Epidemiol Biomarkers Prev*, 16(3), 593-7 (2007)

24. J. Frystyk: Free insulin-like growth factors -- measurements and relationships to growth hormone secretion and glucose homeostasis. *Growth Horm IGF Res*, 14(5), 337-75 (2004)

25. M. Davies, S. Gupta, G. Goldspink and M. Winslet: The insulin-like growth factor system and colorectal cancer: clinical and experimental evidence. *Int J Colorectal Dis*, 21(3), 201-8 (2006)

26. J. Yang and R. H. Liu: Synergistic effect of apple extracts and quercetin 3-beta-d-glucoside combination on antiproliferative activity in MCF-7 human breast cancer cells in vitro. *J Agric Food Chem*, 57(18), 8581-6 (2009)

27. L. Galluzzi, E. Morselli, O. Kepp, N. Tajeddine and G. Kroemer: Targeting p53 to mitochondria for cancer therapy. *Cell Cycle*, 7(13), 1949-55 (2008)

28. A. M. Roy, M. S. Baliga, C. A. Elmetts and S. K. Katiyar: Grape seed proanthocyanidins induce apoptosis through p53, Bax, and caspase 3 pathways. *Neoplasia*, 7(1), 24-36 (2005)

29. H. A. Weber, A. E. Hodges, J. R. Guthrie, B. M. O'Brien, D. Robaugh, A. P. Clark, R. K. Harris, J. W. Algaier and C. S. Smith: Comparison of proanthocyanidins in commercial antioxidants: grape seed and pine bark extracts. *J Agric Food Chem*, 55(1), 148-56 (2007)

30. C. Agarwal, R. Veluri, M. Kaur, S. C. Chou, J. A. Thompson and R. Agarwal: Fractionation of high molecular

weight tannins in grape seed extract and identification of procyanidin B2-3,3'-di-O-gallate as a major active constituent causing growth inhibition and apoptotic death of DU145 human prostate carcinoma cells. *Carcinogenesis*, 28(7), 1478-84 (2007)

31. T. Chou and P. Talalay: Analysis of combined drug effects: a new look at a very old problem. *Trends Pharmacol Sci*, 4, 450-454 (1983)

32. P. J. Murphy, M. D. Galigniana, Y. Morishima, J. M. Harrell, R. P. Kwok, M. Ljungman and W. B. Pratt: Pifithrin-alpha inhibits p53 signaling after interaction of the tumor suppressor protein with hsp90 and its nuclear translocation. *J Biol Chem*, 279(29), 30195-201 (2004)

33. P. M. De Angelis, T. Stokke, L. Thorstensen, R. A. Lothe and O. P. Clausen: Apoptosis and expression of Bax, Bcl-x, and Bcl-2 apoptotic regulatory proteins in colorectal carcinomas, and association with p53 genotype/phenotype. *Mol Pathol*, 51(5), 254-61 (1998)

34. X. X. Sun, M. S. Dai and H. Lu: 5-fluorouracil activation of p53 involves an MDM2-ribosomal protein interaction. *J Biol Chem*, 282(11), 8052-9 (2007)

35. R. H. Liu: Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am J Clin Nutr*, 78(3 Suppl), 517S-520S (2003)

36. J. Lin, N. R. Cook, C. Albert, E. Zaharris, J. M. Gaziano, M. Van Denburgh, J. E. Buring and J. E. Manson: Vitamins C and E and beta carotene supplementation and cancer risk: a randomized controlled trial. *J Natl Cancer Inst*, 101(1), 14-23 (2009)

37. S. Banerjee, C. Bueso-Ramos and B. B. Aggarwal: Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-kappaB, cyclooxygenase 2, and matrix metalloproteinase 9. *Cancer Res*, 62(17), 4945-54 (2002)

38. M. Athar, J. H. Back, L. Kopelovich, D. R. Bickers and A. L. Kim: Multiple molecular targets of resveratrol: Anti-carcinogenic mechanisms. *Arch Biochem Biophys*, 486(2), 95-102 (2009)

39. A. M. Engelbrecht, M. Mattheyse, B. Ellis, B. Loos, M. Thomas, R. Smith, S. Peters, C. Smith and K. Myburgh: Proanthocyanidin from grape seeds inactivates the PI3-kinase/PKB pathway and induces apoptosis in a colon cancer cell line. *Cancer Lett*, 258(1), 144-53 (2007)

40. E. Lomonosova and G. Chinnadurai: BH3-only proteins in apoptosis and beyond: an overview. *Oncogene*, 27 Suppl 1, S2-19 (2008)

41. M. H. Kang and C. P. Reynolds: Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clin Cancer Res*, 15(4), 1126-32 (2009)

42. D. X. Hou, X. Tong, N. Terahara, D. Luo and M. Fujii: Delphinidin 3-sambubioside, a Hibiscus anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway. *Arch Biochem Biophys*, 440(1), 101-9 (2005)
  43. I. C. Low, Z. X. Chen and S. Pervaiz: Bcl-2 modulates resveratrol-induced ROS production by regulating mitochondrial respiration in tumor cells. *Antioxid Redox Signal*, 13(6), 807-19 (2010)
  44. S. Macip, M. Igarashi, P. Berggren, J. Yu, S. W. Lee and S. A. Aaronson: Influence of induced reactive oxygen species in p53-mediated cell fate decisions. *Mol Cell Biol*, 23(23), 8576-85 (2003)
  45. V. A. Shatrov, M. Ameyar, C. Bouquet, Z. Cai, R. Stancou, H. Haddada and S. Chouaib: Adenovirus-mediated wild-type-p53-gene expression sensitizes TNF-resistant tumor cells to TNF-induced cytotoxicity by altering the cellular redox state. *Int J Cancer*, 85(1), 93-7 (2000)
  46. I. Tinhofer, D. Bernhard, M. Senfter, G. Anether, M. Loeffler, G. Kroemer, R. Kofler, A. Csordas and R. Greil: Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by Bcl-2. *FASEB J*, 15(9), 1613-5 (2001)
  47. L. Reddivari, J. Vanamala, S. Chintharlapalli, S. H. Safe and J. C. Miller, Jr.: Anthocyanin fraction from potato extracts is cytotoxic to prostate cancer cells through activation of caspase-dependent and caspase-independent pathways. *Carcinogenesis*, 28(10), 2227-35 (2007)
  48. J. Zhou, G. D. Lu, C. S. Ong, C. N. Ong and H. M. Shen: Andrographolide sensitizes cancer cells to TRAIL-induced apoptosis via p53-mediated death receptor 4 up-regulation. *Mol Cancer Ther*, 7(7), 2170-80 (2008)
  49. Z. X. Chen and S. Pervaiz: Bcl-2 induces pro-oxidant state by engaging mitochondrial respiration in tumor cells. *Cell Death Differ*, 14(9), 1617-27 (2007)
  50. C. M. Aragon, F. Rogan and Z. Amit: Ethanol metabolism in rat brain homogenates by a catalase-H<sub>2</sub>O<sub>2</sub> system. *Biochem Pharmacol*, 44(1), 93-8 (1992)
  51. B. Liu, Y. Chen and D. K. St Clair: ROS and p53: a versatile partnership. *Free Radic Biol Med*, 44(8), 1529-35 (2008)
  52. G. K. Jayaprakasha, Y. Jadegoud, G. A. Nagana Gowda and B. S. Patil: Bioactive compounds from sour orange inhibit colon cancer cell proliferation and induce cell cycle arrest. *J Agric Food Chem*, 58(1), 180-6 (2010)
  53. G. A. Potter, L. H. Patterson, E. Wanogho, P. J. Perry, P. C. Butler, T. Ijaz, K. C. Ruparelia, J. H. Lamb, P. B. Farmer, L. A. Stanley and M. D. Burke: The cancer preventative agent resveratrol is converted to the anticancer agent piceatannol by the cytochrome P450 enzyme CYP1B1. *Br J Cancer*, 86(5), 774-8 (2002)
  54. J. Lu, C. H. Ho, G. Ghai and K. Y. Chen: Resveratrol analog, 3,4,5,4'-tetrahydroxystilbene, differentially induces pro-apoptotic p53/Bax gene expression and inhibits the growth of transformed cells but not their normal counterparts. *Carcinogenesis*, 22(2), 321-8 (2001)
  55. J. Dupont, A. Pierre, P. Froment and C. Moreau: The insulin-like growth factor axis in cell cycle progression. *Horm Metab Res*, 35(11-12), 740-50 (2003)
  56. L. Laurino, X. X. Wang, B. A. de la Houssaye, L. Sosa, S. Dupraz, A. Caceres, K. H. Pfenninger and S. Quiroga: PI3K activation by IGF-1 is essential for the regulation of membrane expansion at the nerve growth cone. *J Cell Sci*, 118(Pt 16), 3653-62 (2005)
  57. C. Desbois-Mouthon, A. Cadoret, M. J. Blivet-Van Eggelpoel, F. Bertrand, G. Cherqui, C. Perret and J. Capeau: Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3 $\beta$  inhibition and Ras activation. *Oncogene*, 20(2), 252-9 (2001)
  58. Z. G. Li, T. Hong, Y. Shimada, I. Komoto, A. Kawabe, Y. Ding, J. Kaganai, Y. Hashimoto and M. Imamura: Suppression of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. *Carcinogenesis*, 23(9), 1531-6 (2002)
  59. L. Tessitore, A. Davit, I. Sarotto and G. Caderni: Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21(CIP) expression. *Carcinogenesis*, 21(8), 1619-22 (2000)
  60. J. W. Park, Y. J. Choi, S. I. Suh, W. K. Baek, M. H. Suh, I. N. Jin, D. S. Min, J. H. Woo, J. S. Chang, A. Passaniti, Y. H. Lee and T. K. Kwon: Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity. *Carcinogenesis*, 22(10), 1633-9 (2001)
- Abbreviations:** RSV: Resveratrol; GSE: Grape Seed Extract; 5-FU: 5-Fluorouracil; IGF-1: Insulin Like Growth Factor 1; FACS: Fluorescence-Activated Cell Sorting
- Key Words:** Resveratrol, Grape seed extract, Synergy, Colon cancer, p53, IGF-1
- Send correspondence to:** Jairam Vanamala, 226 Gifford Building, Campus Mail 1571, Colorado State University, Fort Collins, CO, 80523-1571, Tel: 970-491-7336, Fax: 970-491-7252, E-mail: jairam.vanamala@colostate.edu
- <http://www.bioscience.org/current/volE3.htm>