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# Administration of resveratrol enhances cell-cycle arrest followed by apoptosis in DMBA-induced skin carcinogenesis in male Wistar rats

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**Abstract. – OBJECTIVE: Resveratrol (RES), present in fruits and plants, is a natural compound that has been shown various medicinal properties, including protection of cardiovascular disease and cancer risk. However, the effects of RES on skin cancer have not been investigated. The present work was designed to explore the anticancer potential of RES against chemical-induced skin carcinogenesis in rats.**

**MATERIALS AND METHODS: Skin carcinogenesis were induced in male Wistar rats by a single topical application of 7,12-dimethylbenz(a)anthracene (DMBA) and 2 weeks later, 12-O-tetradecanoylphorbol-13-acetate (TPA) were topically applied thrice a week to promote skin carcinogenesis. RES at a dose of 1 or 2 mg/kg body weight/week were administered to DMBA treated rats. The effects of RES on DMBA-modified cell-cycle arrest, apoptosis and protein expressions were analyzed by flow cytometry, immunohistochemistry and Western blot, respectively.**

**RESULTS: RES treatment caused a significant reduction of DMBA-induced tumor occurrence, tumor volume and tumor weight, as compared to DMBA control group. Further, RES treatment increases G2/M arrest and apoptosis by modulating cell-cycle and apoptosis regulated genes such as p53, p21, caspase-3, bax, survivin, cyclin-B and cdc-2 when compared with DMBA control group.**

**CONCLUSIONS: Taken together, the anticancer effect of RES is associated with regulation of cell-cycle and apoptosis in skin cancer, thereby attenuating skin cancer growth. Hence, these findings suggest that RES may be a therapeutic agent for skin cancer treatment.**

*Key Words:*
Skin cancer, Resveratrol, DMBA, Cell-cycle, Apoptosis.

## Introduction

The skin is composed of epidermis, dermis, stratum corneum and subcutaneous tissue. The stratum corneum is the barrier between the host and the physical, chemical and biological agents[1,2]. Several hazardous agents and potential environmental toxins, including industrial chemicals, UV radiation, heavy metals, pesticides, cigarette smoke and pollutants are entered into the skin and that can cause skin-related health problems, including skin cancer[3,4]. Skin cancer is the most common form of cancer and their incidences have been recently increasing as their early detection is easier than other cancers[5]. The most frequent approach for skin cancer treatment is surgery and in later stage, chemotherapeutic agents and radiotherapy are required to completely remove the metastasized cancer[6]. However, long-term exposure of chemotherapeutic drugs leads to various physiological complications and some tumor cells are resistant to specific chemotherapy drugs[7]. Therefore, identifying newer chemopreventive drugs are necessary to protect the skin from both chemical agents and UV radiation that are safe and less toxic.

Over the past few decades, research has been focused on numerous dietary and botanical natural compounds, which have chemopreventive properties that can reduce or prevent the tumorigenesis[8,9]. In this regard, resveratrol (RES), a polyphenol found in various plants, including grapes and *Polygonum cuspidatum* root extracts, an important Chinese folk medicine. In addition to antioxidant, antiinflammatory, cardioprotective properties, RES has been shown to inhibit platelet aggregation and coagulation[10-12]. Several in vitro and in vivo studies suggest that RES is an effective chemopreventive agent in multi-step carcinogenesis in various cancer models[10,13]. Multiple mechanisms have been proposed for the anticancer properties of RES, including regulation of cell-cycle, apoptosis, STAT3 and ERK

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pathway[14-19]. The 7,12-dimethylbenz[a]anthracene (DMBA), a potent polycyclic aromatic hydrocarbon, is an excellent *in vivo* model to induce skin carcinogenesis in rats, followed by administration of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which activates inflammation and epidermal proliferation[20].

Though several earlier studies focused on RES as anti-cancer agents in different cancer models, the role of RES on skin cancer and its mechanism has not yet been examined. Therefore, in the present study, we examined the antiproliferative and apoptotic potential of RES on DMBA-induced skin carcinogenic model in rats. We are the first, to show that RES inhibits tumor growth, incidence and multiplicity by regulating cell-cycle and apoptosis. Further, RES activates signaling cascades that induce apoptosis. Taken together, our data suggests that RES has beneficial effects and potential to be developed as a chemopreventive agent for skin cancer.

## Materials and Methods

### Chemicals

3,5,4'-trihydroxy-tran-stilbene (Resveratrol), 7,12-dimethylbenz[a]anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical (St. Louis, MO, USA). Other chemicals and reagents were purchased locally and were of highest purity grade.

### Animals

Male wistar rats of 10-11 weeks of age were obtained from the Shanghai SLAC Laboratory Animal Co. Ltd and maintained in accordance with Institutional Animal care and use Committee guidelines. The rates were housed at 22°C with a relative humidity of 40-60% and a 12 h light/dark cycle and provided *ad libitum* standard chow with free access of water. The ethical approval for the experiment was obtained from the Institutional Ethical Committee.

### Induction of Tumor by DMBA and Resveratrol Treatment

The dorsal skin of rats (3 × 3 cm area) was shaved using an electrical clipper and divided into four groups, each group consisting of 10 rats, as described in Table I. Fresh DMBA solution was prepared by dissolving 250 µg in 500 µl of acetone. Group II, III and IV rats were received single topical application of DMBA over the shaven area of the skin. After two weeks of DMBA application, 500 µl of TPA (62.5 µg of TPA in 500 µl of acetone) was applied topically three times per week for 16 weeks. Followed by TPA, Group III and IV rats were received intra-peritoneal injection of RES at a concentration of 1 or 2 mg/kg body weight, respectively, every week for 16 weeks. The RES dosage was chosen based on the previous study[21]. The tumor volume and body weight of all the rats were monitored weekly during the experiment period. Tumor volume was determined using Vernier caliper. At the end of 18 weeks, all rats were sacrificed. Tumor tissues were collected and half of tissues were fixed in 10% formalin and the remaining tissues were processed for further biochemical analysis.

### Cell Cycle Analysis

Cell cycle analyses were performed by measuring cellular DNA content with propidium iodide as a dye. Single-cell suspensions were prepared from tumor tissues of control and treated groups as described previously[22]. The single-cell suspensions were fixed with 75% ethanol at –20°C. The ethanol fixed cells were resuspended in PBS containing ribonuclease A (100 µg/ml)

**Table I.** Experimental design.

| Group | Treatment and schedule |
|---|---|
| Group I: Control | Topical application of 500 µl of acetone until the end of experiment. |
| Group II: DMBA | Single application of DMBA, 2 weeks followed by topical application of TPA thrice a week until the end of experiment. |
| Group III: DMBA + RES-1 | Single application of DMBA, 2 weeks followed by topical application of TPA thrice a week and intra-peritoneal (i.p) injection of RES at a concentration of 1 mg/kg body weight every week until the end of experiment. |
| Group III: DMBA + RES-2 | Single application of DMBA, 2 weeks followed by topical application of TPA thrice a week and intraperitoneal (i.p) injection of RES at a concentration of 2 mg/kg body weight every week until the end of experiment. |

and incubated for 1 h at 37°C. The ethanol fixed cells were stained with propidium iodide (50 µg/ml) and incubated for 30 min at room temperature in the dark. The data were acquired and analyzed using a FACS Calibur flow cytometer (Becton-Dickinson LSR II, San Jose, CA, USA) and 'Cell Quest' software.

### Apoptosis Assay

Apoptotic indices were determined using FITC Annexin V kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instruction. Briefly, cells were pelleted from tissues of control and treated groups by centrifugation at 1,000 rpm for 5 min. After resuspending the pellets in binding buffer, add 5 µl of Annexin V-FITC and 5 µl of propidium iodide (50 µg/ml) and incubated at room temperature for 5 min in the dark. Flow cytometry was performed in a FACS Calibur flow cytometer (Becton-Dickinson LSR II, San Jose, CA, USA).

### Immunohistochemistry

The 10 % formalin-fixed tumor tissues were embedded in paraffin and sectioned at 4 µm. The slides were deparaffinized and rehydrated with ethanol. Next, the slides were incubated with anti-Ki-67 primary antibody (Dako, Carpinteria, CA, USA). Followed by HRP/streptavidin conjugated secondary antibody, DAB substrate were added to the slides (Vector Labs, Burlingame, CA, USA) and counterstained with hematoxylin. The slides were then observed under optical microscope. To calculate proliferative indices, 15 randomly selected microscopic (40× objective) fields in each group were calculated by the total number of cells divided by the number of Ki-67-positive cells.

The apoptotic indices were determined *in situ* terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) using the Apoptag peroxidase *in situ a*poptosis detection kit (SerologicAPOPTAG® kit, Millipore, Billerica, MA, USA) according to the manufacturer's instructions. To calculate apoptotic indices, 15 randomly selected microscopic (40× objective) fields in each group were calculated by the total number of cells divided by the number of TUNEL positive cells.

### Western Blot Analysis

Total protein was extracted from tumor tissues of control and treated groups using a lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 1 mM PMSF with protease inhibitor cocktail. The protein concentrations of the supernatant were determined using BCA Protein assay kit (Life Technologies, Grand Island, NY, USA) and stored at –80C. The equal amount proteins (50 µg/well) were separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane. The membranes were incubated overnight at 4°C with primary antibodies specific for p53, p21, bax, caspase-3, cyclin-B, cdc-2, survivin and □-tubulin. The corresponding secondary IgG antibodies with alkaline phosphatase markers were used and incubated for 2 h at room temperature. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The membranes were developed using ECL reagent.

### Statistical Analysis

All data are expressed ad mean ± standard deviation (SD). Significant differences were assessed using Student's *t*-test and $p$ value $< 0.05$ was considered as statistically significant.

## Results

### General Observation

The body weight of control, DMBA and RES treated rats were monitored every week until the end of the experiment period and are summarized in Figure 1A. The average body weight of rats in control, DMBA with different doses of RES treated groups were similar throughout the study. However, the average body weight was reduced after 6 week of DMBA dosed group until the end of the study as compared to other groups, indicating that RES protects the DMBA-decreased body weight.

### Effect of RES on DMBA-Induced Tumor Growth, Multiplicity and Volume

The tumor incidences were significantly reduced in DMBA group treated with 1 or 2 mg/kg b.w of RES (Figure 1B). In addition, inhibition of tumor multiplicity (number and size of tumors/rat) was markedly increased by RES treatment as compared to DMBA treated group (Figure 1C). As we expected, RES treatment showed significant inhibition of tumor volume (Figure 1D) and tumor weight (Figure 1E) comparing to DMBA treated group, suggesting that RES inhibits DMBA-induced tumor growth.

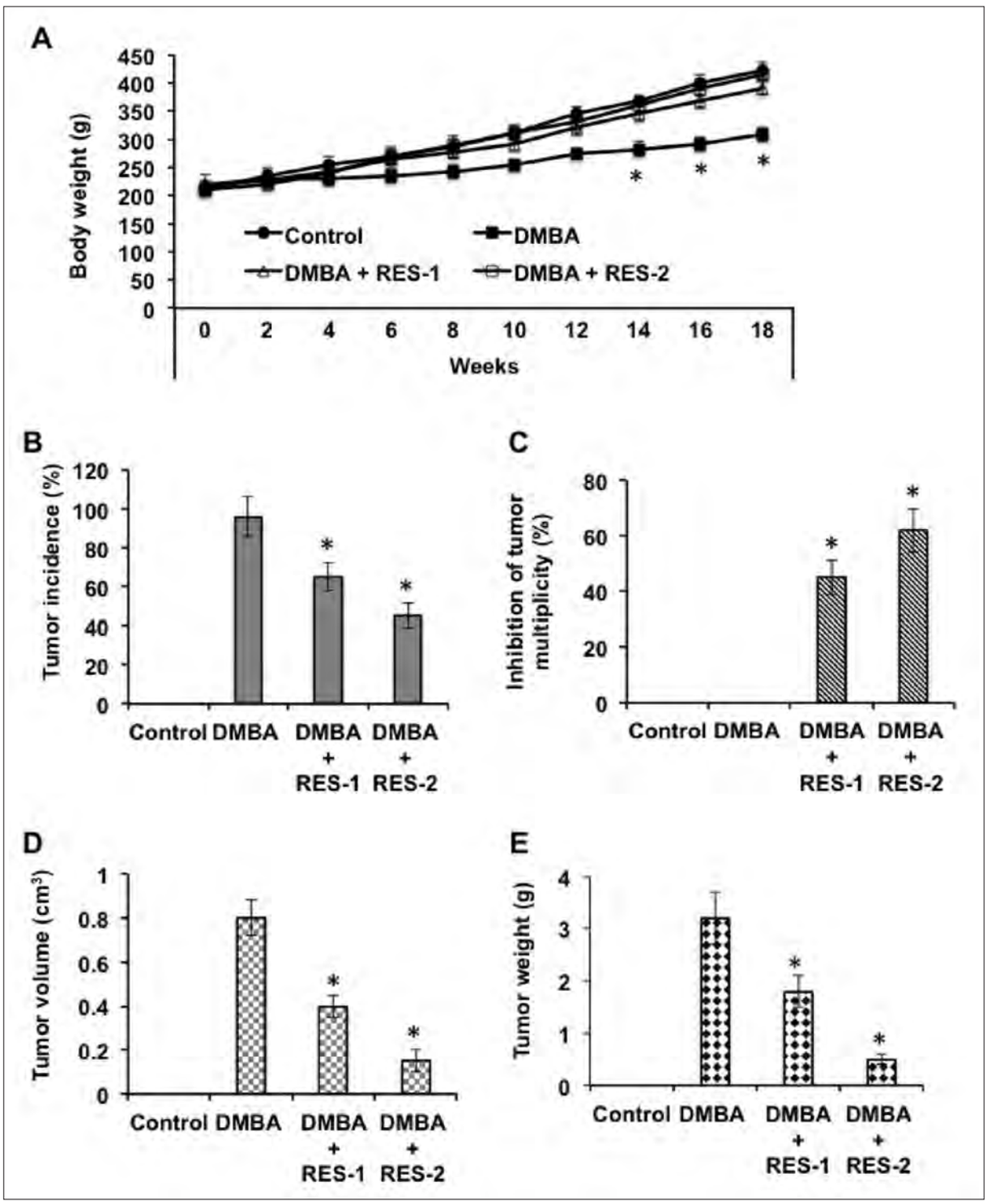


***Figure 1.*** RES inhibits DMBA-induced skin tumor occurrence, multiplicity, volume and weight. Representative bar graph are shown the effect of RES on DMBA-induced skin tumorigenesis; ***(A)*** the change of body weight of rats in every two weeks until the end of experiment period i.e. 18 weeks, ***(B)*** percentage of tumor incidence of rats throughout the duration of experiment, ***(C)*** percent inhibition of tumor multiplicity with respect to DMBA alone treated group, ***(D)*** average tumor volume of all experimental groups, ***(E)*** average tumor weight of all experimental groups. Control, acetone (vehicle) treated group; DMBA, DMBA/TPA treated group; DMBA+RES-1, DMBA/TPA-treated rats with 1 mg/kg body weight of resveratrol; DMBA+RES-2, DMBA/TPA-treated rats with 2 mg/kg body weight of resveratrol. Each data point is represented as mean ± SD of 10 rats in each group. Statistical analyses were performed with Student's *t*-test. $*p < 0.01$ compared with DMBA alone treated group.

### *Effect of RES on DMBA-Altered Cell-Cycle Regulation*

To further evaluate whether RES could affect cell-cycle regulation for its antitumor activity, we measured cell-cycle progression by using flow cytometry. As shown in Figure 2, DMBA treated group resulted in a decrease of $G_2$/M phase 21%, which was accompanied by an increase of $G_1$ phase (64.4%) as compared to control group of 29% and 54.2%, respectively. In-

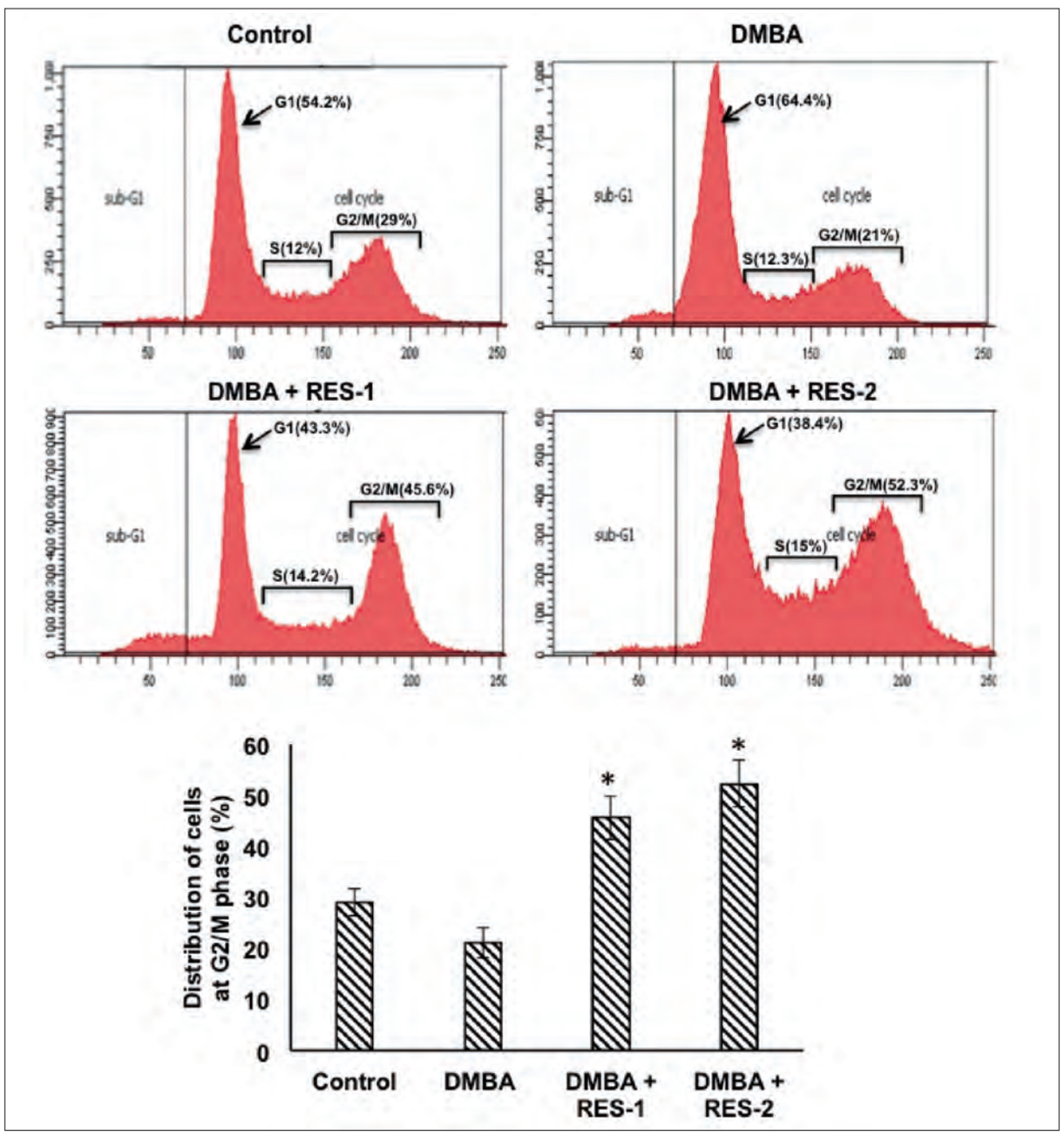


**Figure 2.** RES arrest cells at G2/M phase in DMBA-induced skin tumors. Single-cell suspensions were prepared from tumor tissues of control and treated groups as described in Materials and Methods. Cells were stained with propidium iodide and cell-cycle distribution was assessed by flow cytometry. The flow cytometric analyses are shown as histograms and the percentage of cells at G2/M phase are shown as bar graph. Control, acetone (vehicle) treated group; DMBA, DMBA/TPA treated group; DMBA+RES-1, DMBA/TPA-treated rats with 1 mg/kg body weight of resveratrol; DMBA+RES-2, DMBA/TPA-treated rats with 2 mg/kg body weight of resveratrol. Statistical analyses were performed with Student's *t*-test. $*p < 0.01$ compared with DMBA alone treated group.

terestingly, 1 or 2 mg/kg b.w of RES treated groups were significantly arrest 45.6% and 52.3% of cells at $G_2$/M phase and a concomitant decease of $G_1$ phase 43.3% and 38.4%, respectively, comparing to DMBA treated group, indicating that RES suppresses tumor growth by inhibiting $G_2$/M transition. To authenticate the $G_2$/M transition detected by flow cytometry, we performed Ki-67 immunostaining, as markers of cell proliferation in experimental groups. The percentage of Ki-67 positive cells as seen in dark brown color in the nucleus and quantitation data were significantly increased in DMBA-treated group, whereas the percentage of DMBA-induced Ki-67-positive cells were decreased by RES treatment (Figure 3).

### Effect of RES on DMBA-Suppressed Apoptosis

To examine if the tumor suppression by RES was due to apoptosis, apoptotic indices were determined using FITC Annexin-V kit from respective groups. Upon treatment of RES, significantly increases 2-fold of apoptotic cells when compared with either control or DMBA treated groups (Figure 4). To further confirm the apoptotic indices in DMBA-induced skin tumors, we performed TUNEL assay of tumor tissues in paraffin-embedded sections. As shown in Figure 5, TUNEL positive cells as seen in brown color in the nucleus were significantly increased in tumor sections of RES-treated groups as compared to control or DMBA treated groups. Upon quantification, significant increase of TUNEL positive cells was found in RES-treated groups comparing to DMBA alone treated groups. The results of the experiment confirmed that RES induces cell death through apoptosis in DMBA-induced skin tumor model.

### Effect of RES on Cell-Cycle and Apoptosis Regulated Protein Expressions

To demonstrate the molecular mechanism of observed cell-cycle distribution and apoptosis induction by RES, we measured the protein levels of different cell-cycle and apoptosis regulatory genes by western blot. The protein expressions of p53, p21, bax and caspase-3, which are the regulator of apoptosis, were decreased in DMBA-treated group than control group (Figure 6A). However, higher expression levels of p53, p21, bax and caspase-3 were observed in RES-treated groups comparing to DMBA alone treated group, indicating that RES-induced p53 and its down-regulator have important role in the suppression of DMBA-induced skin tumors.

Furthermore, the cell-cycle regulatory genes, including cyclin-B, cdc-2 and survivin were markedly decreased in RES-treated groups than DMBA-alone treated group (Figure 6B), indicating that reduction of cyclin-B, cdc-2 and survivin due to RES treatment may be responsible for RES-induced $G_2$/M phase cell-cycle arrest.

## Discussion

The incidence of cancers have been increasing around the world with the continually increase in life expectancy, changes in environmental conditions, dietary habits and lifestyle. Among all cancer types, the incidence of skin cancer is apparently increasing and a major public health problem and healthcare expenses. Existing therapies, including chemotherapy and radiotherapy has been the backbone for cancer treatment, however, these therapies is somewhat limited with severe side effects. Therefore, novel therapeutic methods with natural compounds may increase anticancer activities by decreasing the side effects of chemotherapy. Recently, the use of natural dietary supplements have increased significant consideration as chemopreventive agents, which can prevent the process of carcinogenesis[23]. RES is a natural compound, found in grapes as well in other plants and it can reduce the risk cardiovascular disease and cancer risk[24-26]. Moreover, studies reported that RES is an effective chemopreventive agent in three major stages of tumorigenesis. The anticancer activity of RES might be associated with induction of apoptosis of tumor cells but the exact mechanism of anticancer activity is not well understood, particularly in skin carcinogenesis.

In the current study, we demonstrated that rats treated with DMBA/TPA alone exhibited higher tumor incidence, tumor volume and tumor weight along with decreased body weight due to their carcinogenic potential without any treatment. However, DMBA/TPA-induced tumor growth, tumor volume, tumor multiplicity and tumor weight were significantly reduced by either 1 or 2 mg/kg of RES treatment. Further, RES treatment increased $G_2$/M arrest and apoptosis by modulating the cell-cycle and apoptosis regulated genes.

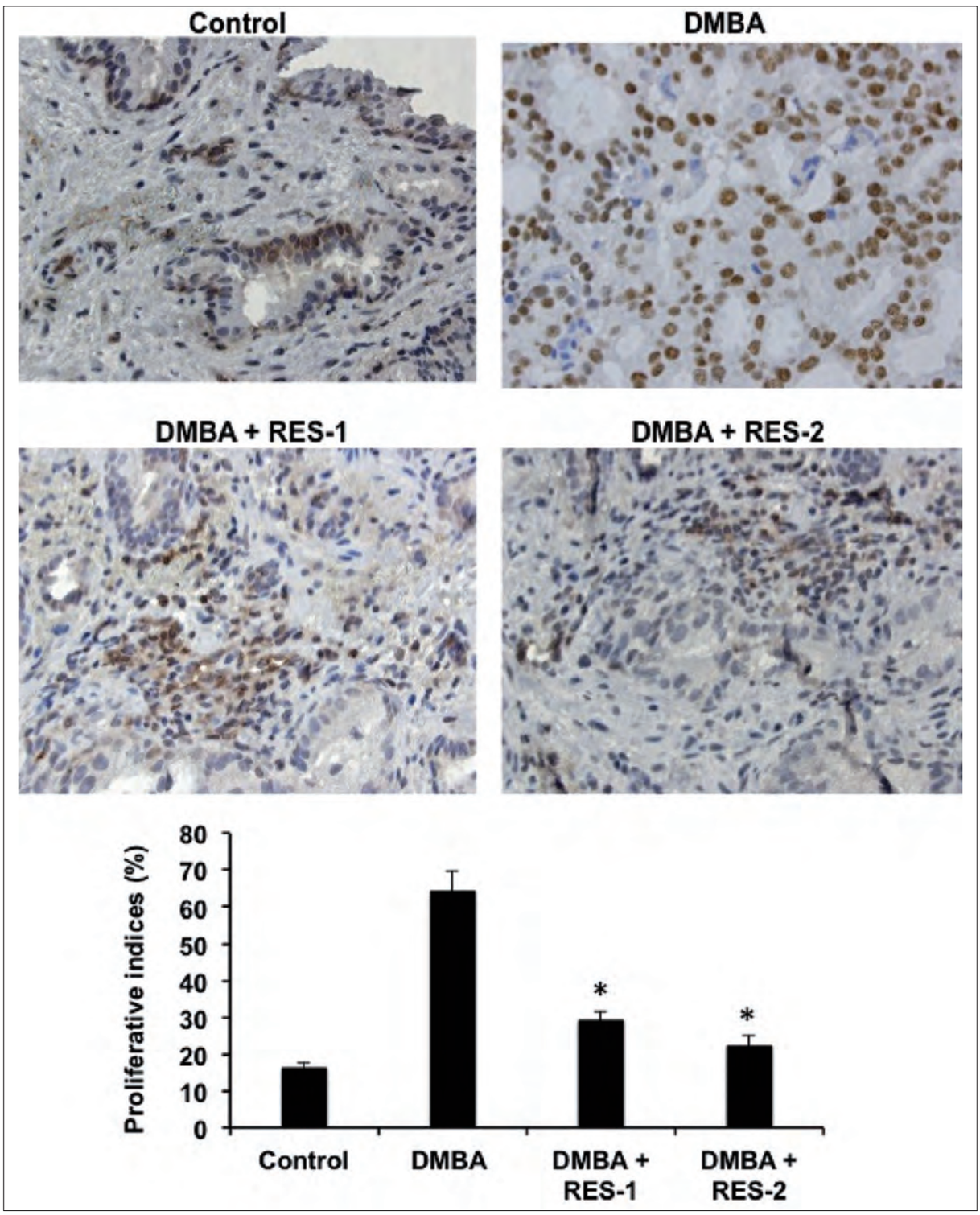


**Figure 3.** ***A,*** RES decreases proliferative cells in the tumor section of DMBA-induced skin tumors. Representative photomicrographs of Ki-67 staining (nuclei stained with brown color) in the tumor section of experimental groups are shown. Bar graph is shown the percentage of proliferative cells by counting Ki-67 positive cells in 15 randomly selected microscopic (40× objective) fields in each group were calculated by the total number of cells divided by the number of Ki-67 positive cells. Control, acetone (vehicle) treated group; DMBA, DMBA/TPA treated group; DMBA+RES-1, DMBA/TPA-treated rats with 1 mg/kg body weight of resveratrol; DMBA+RES-2, DMBA/TPA-treated rats with 2 mg/kg body weight of resveratrol. Statistical analyses were performed with Student's t-test. *$p$ <0 .01 compared with DMBA alone treated group.

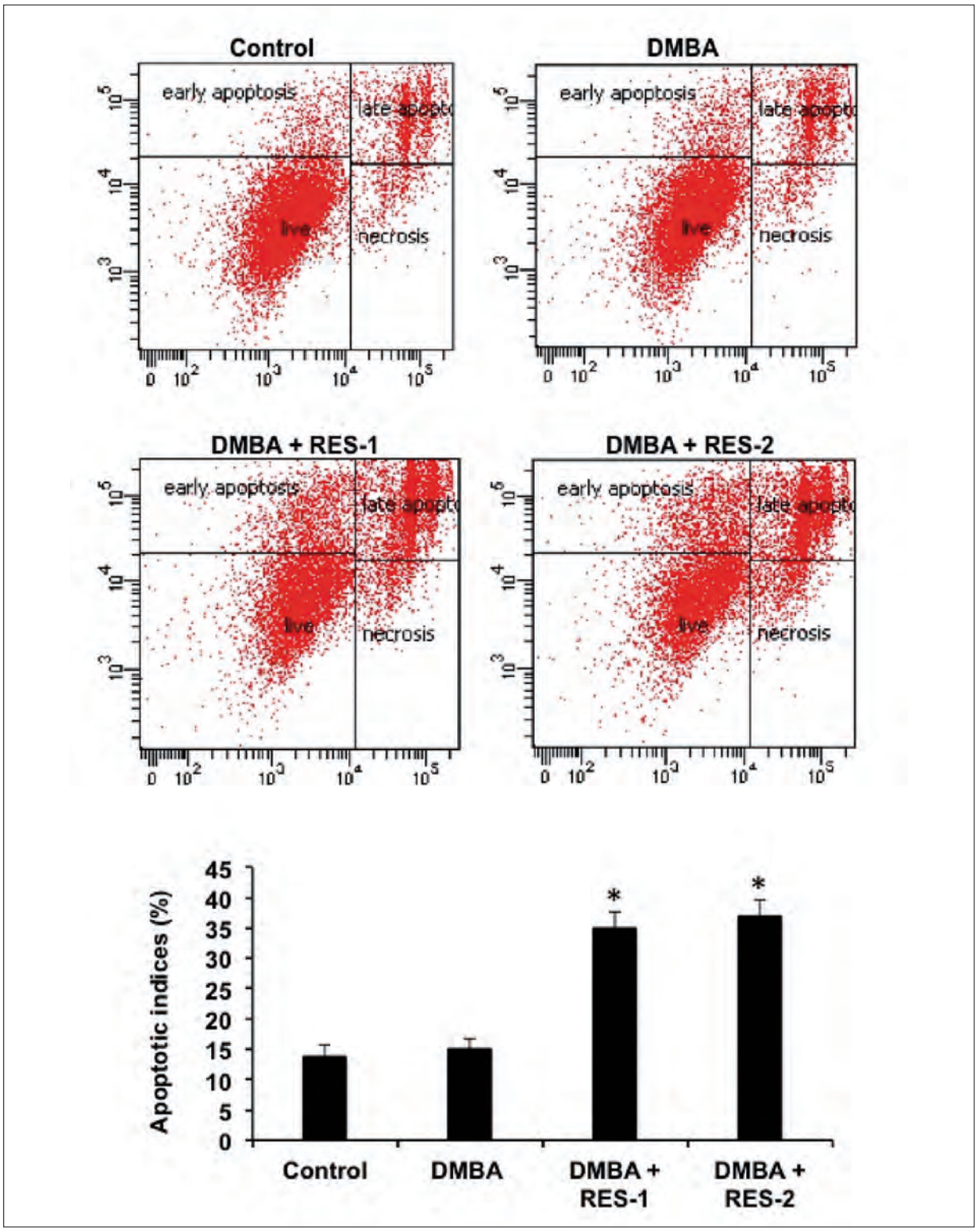


***Figure 4.*** RES induces apoptosis in DMBA-induced skin tumors. Single-cell suspensions were prepared from tumor tissues of control and treated groups as described in materials and methods. Apoptotic cells were determined by FITC Annexin-V staining and flow cytometry. The flow cytometric analyses are shown as histograms and the percentage of apoptotic cells are shown as bar graph. Control, acetone (vehicle) treated group; DMBA, DMBA/TPA treated group; DMBA+RES-1, DMBA/TPA-treated rats with 1 mg/kg body weight of resveratrol; DMBA+RES-2, DMBA/TPA-treated rats with 2 mg/kg body weight of resveratrol. Statistical analyses were performed with Student's *t*-test. $*p < 0.01$ compared with DMBA alone treated group.

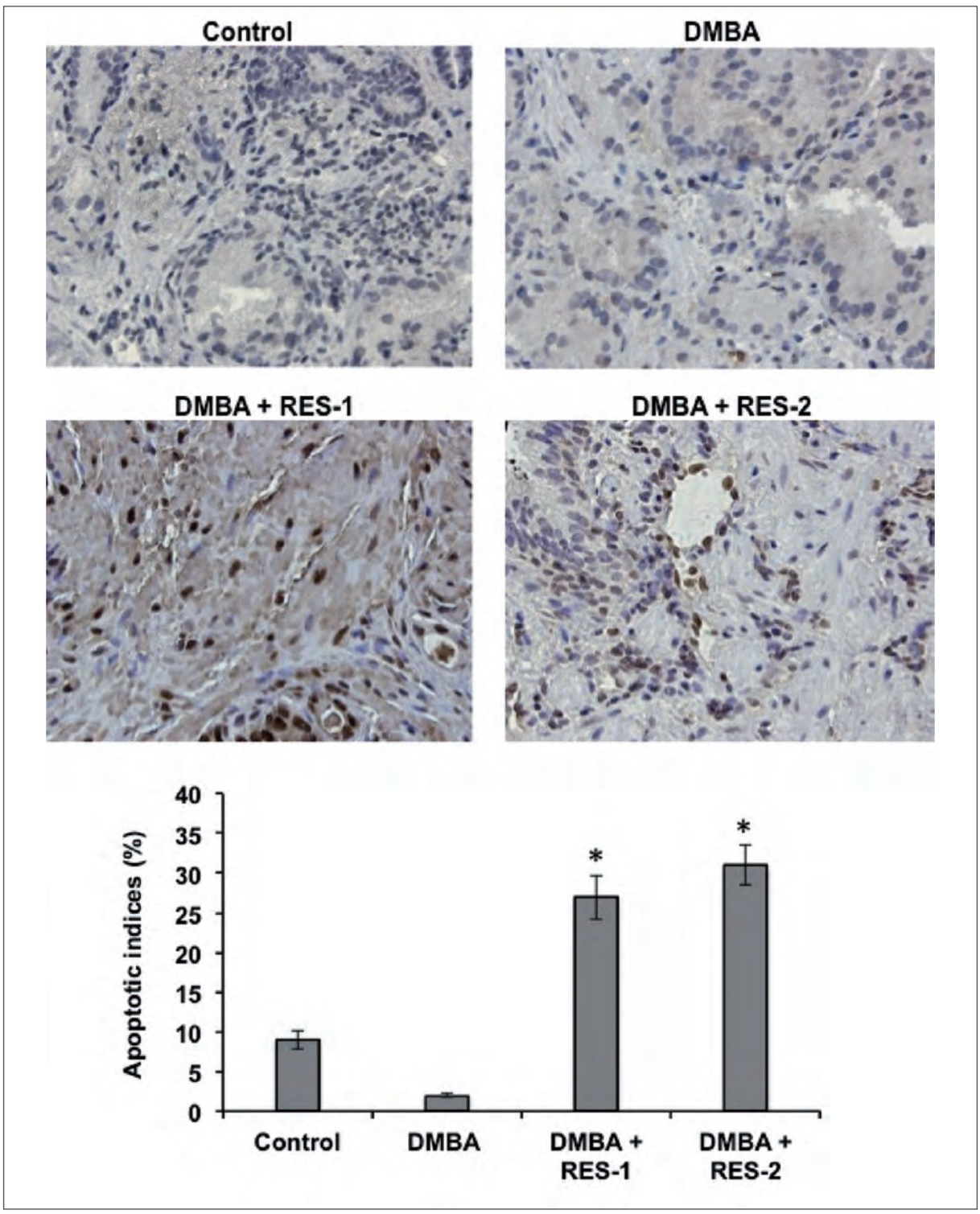


**Figure 5.** RES increases apoptotic cells in the tumor section of DMBA-induced skin tumors. Representative photomicrographs of TUNEL staining (*nuclei stained with brown color*) in the tumor section of experimental groups are shown. Bar graph is shown the percentage of apoptotic cells by counting TUNEL positive cells in 15 randomly selected microscopic (40× objective) fields in each group were calculated by the total number of cells divided by the number of TUNEL positive cells. Control, acetone (*vehicle*) treated group; DMBA, DMBA/TPA treated group; DMBA+RES-1, DMBA/TPA-treated rats with 1 mg/kg body weight of resveratrol; DMBA+RES-2, DMBA/TPA-treated rats with 2 mg/kg body weight of resveratrol. Statistical analyses were performed with Student's *t*-test. *$p < 0.01$ compared with DMBA alone treated group.

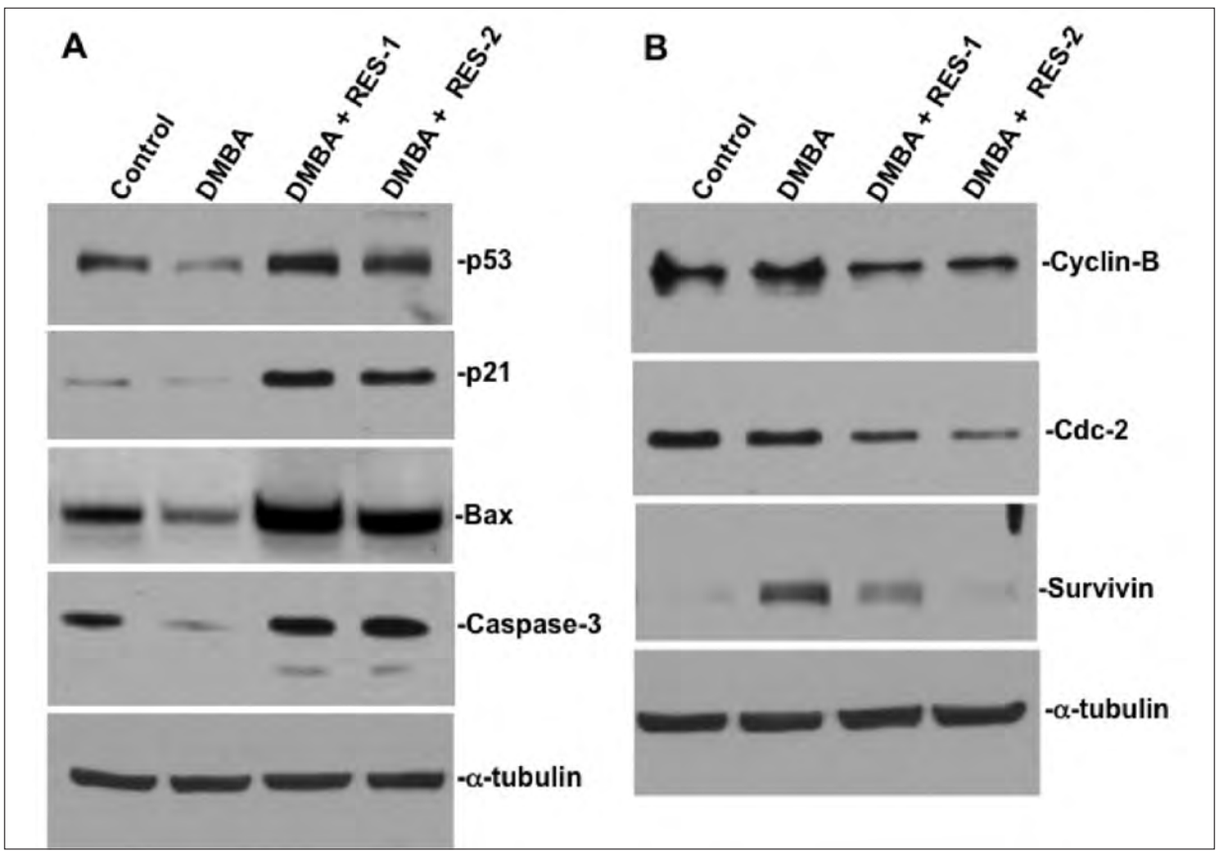


**Figure 6.** RES regulates expression of proteins involved in cell-cycle and apoptosis. ***(A)*** The protein levels of p53, p21, Bax and caspase-3 ***(B)*** cyclin-B, cdc-2 and survivin were determined in tumor tissues from experimental groups by Western blot as described in Materials and Methods. α-tubulin used as a loading control. Control, acetone (vehicle) treated group; DMBA, DMBA/TPA treated group; DMBA+RES-1, DMBA/TPA-treated rats with 1 mg/kg body weight of resveratrol; DMBA+RES-2, DMBA/TPA-treated rats with 2 mg/kg body weight of resveratrol. Statistical analyses were performed with Student's *t*-test. **p* < 0.01 compared with DMBA alone treated group.

Cell cycle checkpoints are paly a major role in protecting genomic DNA against the environmental agents that may cause DNA damage and chromosome segregation[27,28]. The environmental agents-induced these errors transform signals to proteins that affect apoptosis, DNA repair, cell-cycle arrest at different checkpoints[29]. In the present study, RES-increased cell-cycle arrest at $G_2$/M phase may allow cells to complete DNA replication through repair damaged DNA and thereby suppressing the tumor growth process.

The M-phase-promoting factor is an important regulator of the $G_2$ to M transition that consists of regulatory subunit cyclin-B and catalytic subunit cdc-2[30,31]. The regulation of cell-cycle at different checkpoint is activated by these complexes and other several exogenous factors[32]. It has been demonstrated that the p53 tumor suppressor delays $G_2$/M progression in human ovarian cancer cells[33] and rat cell line[34]. Further, study has shown that an increased level of p21, a cyclin-dependent kinase inhibitor, is associated with p53-induced $G_2$/M arrest[35]. Our present study also demonstrates that activation of p53-p21 cascades with RES treatment may cause cell-cycle arrest at $G_2$/M arrest through inhibition of cyclin-B/cdc-2 complex formation. Further, in consistent with our data, Huang et al[36] also reported that RES inhibits cell growth and induces apoptosis through p53-dependent pathway.

The upregulation of proapoptotic and/or downregulation of antiapoptotic proteins are the major biochemical events involved for the induction of apoptosis. The Bcl-2 protein family regulates apoptosis in mitochondria by balancing pro- and anti- apoptotic factors[37], including bax and survivin[38]. Bax is a potent activator of apoptosis and its expression is upregulated by

p53[39]. It has been demonstrated that survivin decreases cell-growth inhibition and apoptosis, which may interrupt cell death[40]. Furthermore, activation of caspase-3 is frequently reflected as point-of-no-return in apoptotic signaling cascade[41]. Several natural compounds and chemopreventive agents, including sulforaphane, resveratrol, curcumin, flavopiridol, nutlin-3 and quinacrine are known to exert their antitumor effects by the induction of apoptosis through p53-dependent mechanisms[14,36,42-45].

## Conclusions

RES has the potential to reduce DMBA-induced skin carcinogenesis by regulating cell-cycle and apoptosis. Thus, our findings suggest that RES might use as a chemopreventive agent for skin cancer treatment. However, further studies are required to determine whether RES can be implemented as a chemopreventive for the treatment of skin cancer.

### Conflict of Interest

The authors declare that there is no conflict of interest to reveal.

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