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Resveratrol and Quercetin in Combination Have Anticancer Activity in Colon Cancer Cells and Repress Oncogenic microRNA-27a

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Resveratrol and quercetin (RQ) in combination (1:1 ratio) previously inhibited growth in human leukemia cells. This study investigated the anticancer activity of the same mixture in HT-29 colon cancer cells. RQ decreased the generation of reactive oxygen species (ROS) by up to 2.25-fold and increased the antioxidant capacity by up to 3-fold in HT-29 cells (3.8–60 μg/mL), whereas IC_{50} values for viability were 18.13, 18.73, and 11.85 μg/mL, respectively. RQ also induced caspase-3-cleavage (2-fold) and increased PARP cleavage. Specificity protein (Sp) transcription factors are overexpressed in colon and other cancers and regulate genes required for cell proliferation survival and angiogenesis. RQ treatment decreased the expression of Sp1, Sp3, and Sp4 mRNA and this was accompanied by decreased protein expression. Moreover, the Sp-dependent antiapoptotic survival gene survivin was also significantly reduced, both at mRNA and protein levels. RQ decreased microRNA-27a (miR-27a) and induced zinc finger protein ZBTB10, an Sp-repressor, suggesting that interactions of RQ with the miR-27a-ZBTB10-axis play a role in Sp downregulation. This was confirmed by transfection of cells with the specific mimic for miR-27a, which partially reversed the effects of RQ. These findings are consistent with previous studies on botanical anticancer agents in colon cancer cells.

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INTRODUCTION

Colon cancer is fifth most common form of cancer in the United States. Diets high in red meats, saturated fat, and low in fiber-rich fruits and vegetables have been identified as important risk factors for cancer initiation and progression (1, 2). A diet rich in fruits and vegetables in colon cancer prevention has been in part attributed to the plant-derived botanicals including polyphenolics which may protect against various chronic degenerative diseases such as cancer or cardiovascular disease (3). Quercetin is a dietary flavonoid found in tea, onion, grapes, wines, and apples (4), and the anticancer activities of quercetin have been explored in several cancer cell line, as well as in vivo (2, 5, 6). Resveratrol is a stilbene analog and a phytoalexin produced by plants as a defense mechanism in response to fungal diseases, stress, and UV radiation; the primary dietary sources of resveratrol are grapes and peanuts (7). Resveratrol exhibited anticancer properties by inhibiting cell proliferation, inducing apoptosis, decreasing angiogenesis, and causing cell cycle arrest in several cancer cell lines (3, 7–9). A combination of resveratrol and quercetin previously exhibited synergistic interactions in their anticancer effects in MOLT-4 leukemia cells (8). In a

study with mouse skin tumors, a combination of resveratrol with tea polyphenolics demonstrated synergistic anticancer effects (10). In pancreatic cancer stem cells, sulforaphane was found to synergize with quercetin in the inhibition of the self-renewal of cells (11). Quercetin and epigallocathechin gallate (EGCG) synergistically inhibited invasion, migration, and epithelial-mesenchymal transition in prostate cancer stem cells (12).

Evidence that specificity protein (Sp) transcriptions factors Sp1, Sp3, and Sp4 are overexpressed in tumors and regulate genes important for cancer cell death and survival is growing (13–19). More recently, the involvement of microRNA27a in the regulation of Sp1 transcription factor through zinc finger protein ZBTB10, an inhibitor of Sp1, has been demonstrated. Additionally, botanical compounds were also found to inhibit Sp transcription factors through the miR-27a-ZBTB10-Sp1 axis (16, 20, 21). Therefore, the aim of this study was to investigate the effects of a mixture of resveratrol and quercetin on Sp1, Sp3, and Sp4 expression and the potential involvement of miR-27a in the downregulation of Sp transcription factors in HT-29 colon carcinoma cells.

MATERIALS AND METHODS

Botanical Extract

Polyphenolics were extracted from a standardized *trans*-resveratrol and quercetin dehydrate supplement (ratio 1:1) in capsule form which was provided by Designs for Health (East Windsor, CT). Polyphenolics were extracted with methanol (50 mg/50 mL), and then centrifuged at room temperature for 10 min at 3000 rpm to remove inactive and insoluble components. Methanol was evaporated in a rotavapor (Büchi Labortechnik AG, Flawil, Switzerland) at 40°C. Residual moisture was evaporated in a speedvac concentrator (Thermo Scientific, Waltham, MA) at 43°C. The final resveratrol-quercetin (RQ) mixture was stored at −80°C and dissolved in dimethyl sulfoxide (DMSO) prior to use.

HPLC-PDA Analysis

The polyphenolic mixture was analyzed and quantified by retention time and Photo Diode Array (PDA) spectra by HPLC-PDA. The chromatographic separation was performed in an Alliance 2695 system (Waters Inc., Milford, MA) and carried out in a Discovery C_{18} column (Supelco Inc., Bellefonte, PA) (250 × 4.6 mm, 5 μm) at room temperature. The chromatographic conditions used were mobile phase A water/acetic acid (98:2), mobile phase B acetonitrile/water/acetic acid (68:30:2). A gradient program with 1 mL/min was used as follow 0 min 100%, A; 20 min 60%, A; 30 min 30%, A; 32 min 0%, A; 35 min 100%, A. The detection wavelengths were set at 306 and 360 nm for *trans*-resveratrol and quercetin, respectively. Standard compounds for the identification and quantitative analysis of quercetin and resveratrol were obtained from Acros Organics (Morris Plains, NJ) and ChromaDex (Irvine, CA), respectively.

Oxygen Radical Absorbance Capacity

The antioxidant capacity was determined using the oxygen radical absorbance capacity assay (ORAC) (22) with fluorescein as fluorescent probe in a FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC). Results were reported in μmol of Trolox equivalents/mL.

Cell Culture

Human adenocarcinoma cells HT-29 [American Type Culture Collection (ATCC), Manassas, VA] were cultured in McCoy's medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (100,000 U/L penicillin and 100 mg/L streptomycin). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For experiments, cells were seeded in DMEM/F-12 medium with 2.5% FBS (stripped with activated charcoal as previously described) (8), and 1% antibiotic (100,000 U/L penicillin and 100 mg/L streptomycin).

Generation of Reactive Oxygen Species

Generation of reactive oxygen species (ROS) was determined using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay (23). In summary, cells (1×10^4 cells/well) were seeded in a clear bottom, 96-well plate and incubated for 24 h, then treated with different concentrations of RQ (0–60 μg/mL). After 24 h, cells were washed twice with phosphate buffer (PBS) and incubated with 200 μM hydrogen peroxide for 2 h at 37°C. Hydrogen peroxide was removed with PBS washes and 10 μM DCFH-DA diluted in PBS was added to cells. Following, cells were incubated for 15 min at 37°C, DCFH-DA was removed, and the fluorescence intensity was measured after 15 min at 37°C using a FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission, BMG Labtech Inc., Durham, NC).

Cell Viability

Cells were seeded (3×10^3 cells/well) in a 96-well plate for 24 h, then the growth medium was replaced with the experimental medium containing various RQ extract concentrations of RQ (0–60 μg/mL). Cell viability was assessed at 48, 72, and 96 h with the Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) according to manufacturer's protocol using a FLUOstar microplate reader at 490 nm (BMG Labtech Inc., Durham, NC). The concentration at which cell viability was inhibited by 50% (IC_{50}) was calculated by sigmoidal nonlinear regression analyses on percentage cell inhibition as a ratio of the control using the Graphpad Prism 5.01 software (Graphpad Software, La Jolla, CA).

Cell Proliferation

HT-29 cells (2×10^4 cells/well) were grown in a 24-well plate for 24 h. Then the growth medium was replaced with experimental medium containing RQ extract concentrations ranging from 0 to 60 μg/mL. After 72-h cell proliferation was determined using a cell counter (Beckman Coulter, Fullerton, CA). Cell counts were expressed as a percentage of control cells.

Cleaved Caspase-3 Activation

Cells were grown (6×10^5 cells/well) for 24 h and then incubated with different RQ concentrations (0–30 μg/mL) for 24 h. Cleaved caspase-3 activation was determined using an ELISA kit (Cell Signaling Technology Inc., Danvers, MA) according to the manufacturer's protocol using a FLUOstar microplate reader (BMG Labtech Inc., Durham, NC) at 450 nm.

Real-Time PCR Analysis of miRNA and mRNA

HT-29 cells were grown (2×10^5 cells/well) in a 6-well plate for 24 h before incubation with different concentrations of RQ (0–30 μg /mL). Total RNA, which contains both mRNA and miRNA, were isolated using the mirVana™miRNA Isolation Kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommended protocol. Extracted nucleic acid was evaluated for quality and quantity using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 and 280 nm.

SuperScript™ III First-Strand (Invitrogen, Carlsbad, CA) was used to reverse-transcribe mRNA. TATA-binding protein (TPB) was used as an mRNA endogenous control. For real time PCR (RT-PCR), proprietary primers for Sp3 and Sp4 (Qiagen, Valencia, CA) were used. The following primers were purchased from Integrated DNA Technologies (Coralville, IA) and used for amplification as follows: TBP (sense 5′-TGC ACA GGA GCC AAG AGT GAA-3′; antisense 5′-CAC ATC ACA GCT CCC CAC CA-3′), caspase-3 (sense 5′-CTG GAC TGT GGC ATT GAG ACA-3′; antisense 5′-CGG CCT CCA CTG GTA TTT TAT G-3′), ZBTB10 (sense 5′-GCT GGA TAG TAG TTA TGT TGC-3′; antisense 5′-CTG AGT GGT TTG ATG GAC AGA G-3′), Sp1 (sense 5′-TCA CCA ATG CCA ATA GCT ACT CA-3′; antisense 5′-GAG TTG GTC CCT GAT GAT CCA-3′), survivin (sense 5′-CCA TGC AAA GGA AAC CAA CAA T-3′; antisense 5′-ATG GCA CGG CGC ACT T-3′). RT-PCR for mRNA was performed using the SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

The TaqMan® MicroRNA Assay for miR-27a and RNU6B (used as control) (Applied Biosystems, Foster City, CA) was used to reverse-transcribe mature miRNA following the manufacturer's protocol in a MasterCycler (Eppendorf, Westbury, NY). RT-PCR for miRNA was carried out with the TaqMan® assay, which contained the forward and reverse primers as well as the TaqMan® probe and TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA). After completion of RT-PCR, relative quantification for both mRNA and miRNA of gene expression was evaluated by using the comparative critical threshold (C_T) method as previously performed (21).

Transfection with miR-27a Mimic

Transfections with 50 nM and 100 nM miR-27a mimic (Dharmacon, Lafayette, CO) were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 6 h as previously described (21). After transfection for 6 h, cells were incubated with 20 μg/mL of RQ for 24 h.

Cell Cycle Kinetics

Cells were seeded (5×10^5 cells/plate) with medium containing 2.5% FBS for 24 h and after cells were treated with RQ (0–20 μg/mL) for 24 h. Cells were fixed with 90% ethanol and stored at -20°C as previously described (8). DNA was stained with propidium iodide containing RNAse (0.2 mg/mL) solution and analysis was carried out at 488 nm excitation and 620 nm emission wavelengths on a FACScan flow cytometer (Becton-Dickinson Immonunocytometry Systems, San Jose, CA). The percentage of cells in each cell cycle phase was analyzed using the ModFit LT version 3.2 for Macintosh by Verity Software House (Topsham, ME).

Western Blotting

Cells were grown (2×10^6 cells/plate) for 24 h and treated with RQ (0–30 μg/mL) for 24 h. Cells were harvested and cell lysates were obtained using a high-salt buffer (1.5 mmol/L $MgCl_2$, 500 mmol/L NaCl, 1 mmol/L EGTA, 50 mmol/L HEPES, 10% glycerol, 1% Triton X-100; adjusted to pH 7.5) supplemented with protease inhibitors (Sigma-Aldrich, St. Louis, MO) (21). Samples were incubated at 100°C for 5 min in 1X Laemmli buffer (0.1% bromophenol blue, 175 mM ß-mercaptoethanol, 50 mM Tris-HCl, 2% SDS). Proteins were separated on a 10% (Sp proteins) or 12% (survivin) SDS-PAGE at 100 V and transferred to PVDF membrane (Bio-Rad, Hercules, CA) as previously performed (21). Membranes were blocked in nonfat milk in PBS-Tween and incubated with primary antibodies overnight at 4°C. After multiple washing steps, membranes were incubated with secondary antibodies. Membranes were washed and incubated with chemiluminescence substrate (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY).

Statistical Analyses

Data from in vitro experiments were analyzed by 1-way analysis of variancewith JMP 8.0 (SAS Institute, Cary, NC). Differences were deemed significant at $P \leq 0.05$ using a Tukey-Cramer HSD comparison for all pairs. For transfections with miR-27a mimic, differences were deemed significant at $P \leq 0.05$ using a *t*-student comparison for all pairs. The analysis of linear (pairwise) correlations was performed where correlations with a P value less than 0.05 were deemed significant. Nonlinear modeling of sigmoidal curves for cell viability were performed using the Graphpad Prism 5.01 software (Graphpad Software, La Jolla, CA).

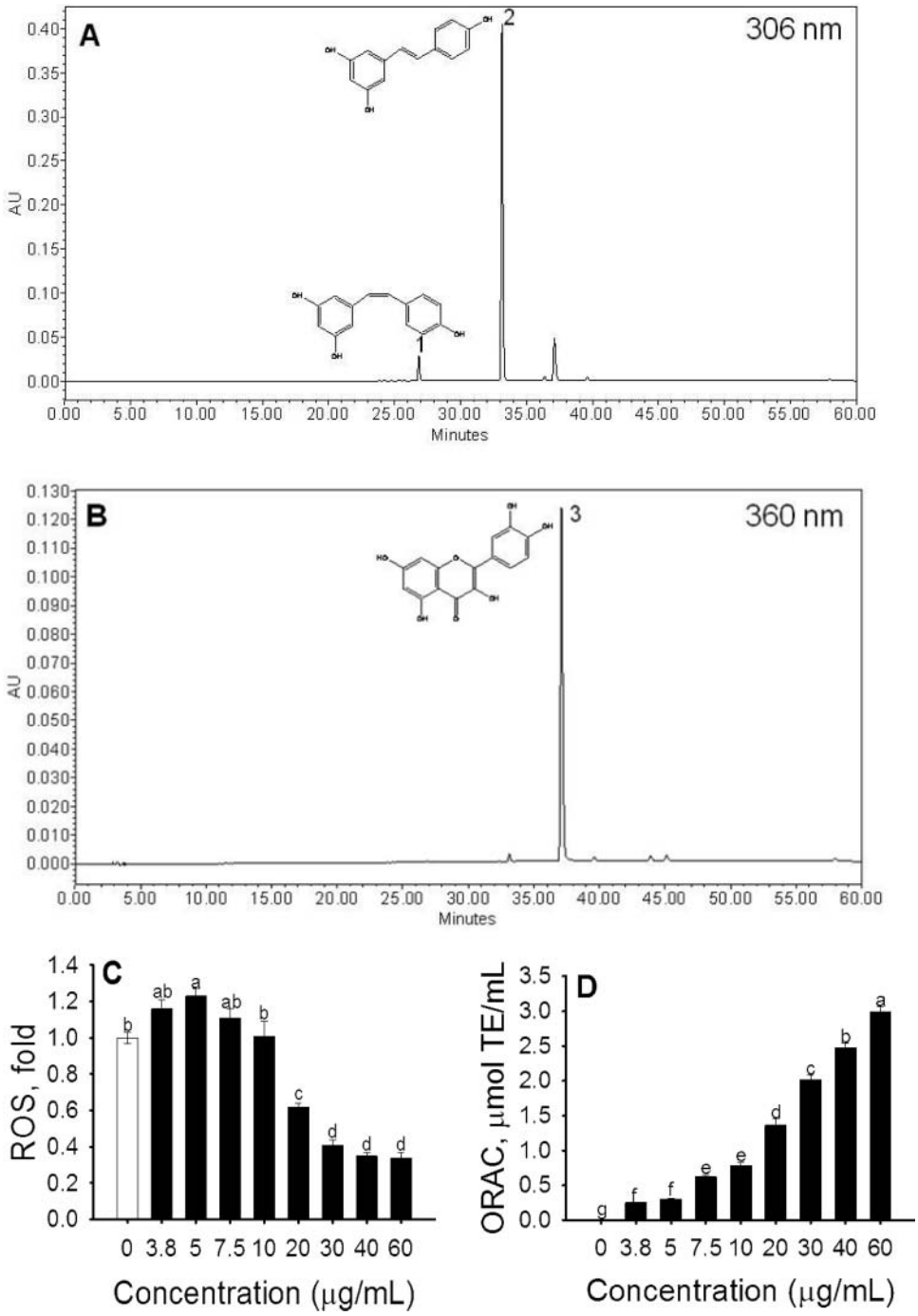

FIG. 1. Chromatographic profile of resveratrol and quercetin in combination. A: stilbenes; B: flavonols. Peak assignments: 1, *cis*-resveratrol; 2, *trans-resveratrol*; 3, quercetin. C: hydrogen peroxide-induced generation of reactive oxygen species (ROS) in HT-29 cells treated with RQ. D: oxygen radical absorbance capacity assay (ORAC)-value of HT-29 cell-supernatant with resveratrol/quercetin after 24 h. Bars represent means ± SEM, $n = 3$. Bars with different letters are significantly different (least significant difference test, $P < 0.05$).

RESULTS

Chemical Composition

The chromatographic profiles (Fig. 1A and 1B) of RQ demonstrate the presence of 2 major polyphenols, *trans*-resveratrol (peak 2) and quercetin (peak 3), respectively, as major ingredients of this botanical supplement. A minor amount of *cis*-resveratrol, an isomer less abundant than *trans*-resveratrol, was also detected (peak 1). The presence of small amounts of *cis*-resveratrol may be produced by photo isomerization and UV-radiation (degradation) of trans resveratrol (24) and might also form during the extraction and concentration process

when exposed to light. However, *trans*-resveratrol is associated with more bioactive properties and represents the major form of resveratrol in grapes, wines, and in the dried roots of *Polygonum cuspidatum* (3, 24).

Generation of Intracellular Reactive Oxygen Species and ORAC

Intracellular generation of ROS was investigated after HT-29 cells were challenged with hydrogen peroxide. RQ slightly induced the generation of ROS at low concentrations (0–10 μg/mL) whereas at concentrations higher than 20 μg/mL generation of ROS was significantly reduced (Fig. 1C) by up to 66% compared to solvent-treated control cells. The antioxidant capacity values (ORAC) (Fig. 1D), were significantly increased by all concentrations of RQ in a dose-dependent manner.

Cell Death and Cell Cycle Kinetics

HT-29 cell viability (Fig. 2A) was significantly decreased by RQ in a dose- and time-dependent manner. The IC_{50} values were 18.13, 18.73, and 11.85 μg/mL, for 48, 72, and 96 h, respectively. RQ also inhibited cell proliferation after treatment for 72 h (Fig. 2B) illustrated by a decrease in cell counts in a dose-dependent manner. The net number of cells remaining was the combined results of cancer cell proliferation as well as the cytotoxicity of RQ. Cell numbers were significantly decreased at all concentrations (3.75–60 μg/mL) and at 60 μg/mL RQ cell proliferation was reduced by up to 73%.

The effects of RQ on cell-cycle progression were determined by fluorescence-activated cell sorting (FACS) analysis (Fig. 2C). In this study, there were minor but significant changes in the percentage of cells in different phases of the cell cycle using 2.5, 5, or 10 μg/mL of extract. There was a significant G0/G1 to S phase block compared to control cells when cells were treated with 20 μg/mL of the mixture (Fig. 2C).

Cleaved capase-3, the activated form, is involved in the execution of apoptosis and was induced up to 1.5-fold even at low concentrations of RQ (5 and 10 μg/mL) (Fig. 2D). At higher concentrations (20 and 30 μg/mL), the induction increased up to 2.3-fold. Poly(ADP-ribose) polymerase 1 (PARP-1) is a substrate for caspase-3 cleavage and produces cleaved PARP-1 (25) and there was an increase in PARP cleavage when cells were treated with RQ, as also shown in the densitometry analysis for PARP-1 cleavage/b-actin (Fig. 2E)

Modulation of Sp1 Transcription Factors and Dependent Genes

Previous studies with several botanical anticancer agents and their synthetic derivatives show that these compounds downregulate Sp transcription factors and Sp-regulated genes (14–19). Results show that RQ (5–30 μg/mL) decreased Sp1, Sp3 and Sp4 mRNA levels and there was also a parallel decrease in Sp1, Sp3, and Sp4 proteins (Fig. 3A, 3B, 3C, and 3E). In addition, RQ also decreased expression of survivin protein and mRNA and these results are consistent with RQ-mediated suppression of Sp transcription factors because survivin is an Sp-regulated gene. We have also measured densitometry for the Sp1, Sp3, Sp4, and Sp-regulated genes as ratio to b-actin (Fig. 3F).

Previous studies demonstrate that the inhibition of ZBTB10 expression by microRNA-27a is at least in part responsible for the increased expression of Sp-transcription factors in cancer cells and tumors (16, 20, 21). In this study, RQ significantly decreased miR-27a (Fig. 4A) and upregulated ZBTB10 mRNA (Fig. 4B), and this was accompanied by decreased Sp proteins and the Sp-regulated gene survivin (Fig. 3D and 3E). These results are consistent with previous studies regarding the effects of other botanicals and their derivatives on the miR-27a-ZBTB10-Sp1-axis in multiple cancer cell lines (14–19,) (21). In this study, the overexpression of miR-27a mimic in HT-29 cells increased miR-27a, decreased ZBTB10 and increased Sp1 mRNA levels and this was consistent with the inactivation of endogenous ZBTB10 expressed in these cells (Fig. 5A–5D). Moreover, the miR-27a mimic also partially reversed RQ-induced downregulation of miR-27a, induction of ZBTB10, and downregulation of Sp1 (Fig. 5). Densitometry has been performed for the Sp-1/b-actin (Fig. 5E).

DISCUSSION

Generation of Intracellular Reactive Oxygen Species and ORAC

The generation of ROS has been associated with oxidative cellular damage that may be involved in the development of various pathological conditions (23, 26). The role of ROS in carcinogenesis is complex. Cancer cells tend to have higher constitutive levels of ROS than normal cells, due in part to mutations in nuclear and mitochondrial genes responsible for the electron transport chain and also due to increased metabolism and mitochondrial activity (27). Elevated levels of ROS may enhance cell proliferation and other events relevant to cancer progression (27). ROS can also cause DNA damage and oxidation of fatty acids in cellular membrane structures, thereby facilitating mutagenesis and cancer development (26, 27). However, many anticancer drugs are mitochondrotoxic and induce ROS, which in turn can lead to cancer cell death. Polyphenolics, such as resveratrol and quercetin, have the ability to scavenge free radicals (23, 26) and also induce activation of antioxidant and detoxifying enzymes, thus protecting cells against oxidative damage from carcinogenic compounds (26, 28). In this study, RQ induced a concentration-dependent increase in intracellular antioxidant capacity (Fig. 1D) and the intracellular concentration of ROS increased at a low RQ concentration (5 μg/mL) but decreased at high concentrations (20–60 μg/mL) (Fig. 1C). One possible explanation for this biphasic effect may be the mitochondrotoxic properties of polyphenolics, which also induce ROS (7, 29), whereas at higher concentrations their antioxidant properties are predominant. Studies with polyphenolics in cancer cells demonstrate their protective effects against oxidative damage in some conditions (23, 30). In this study, RQ induced

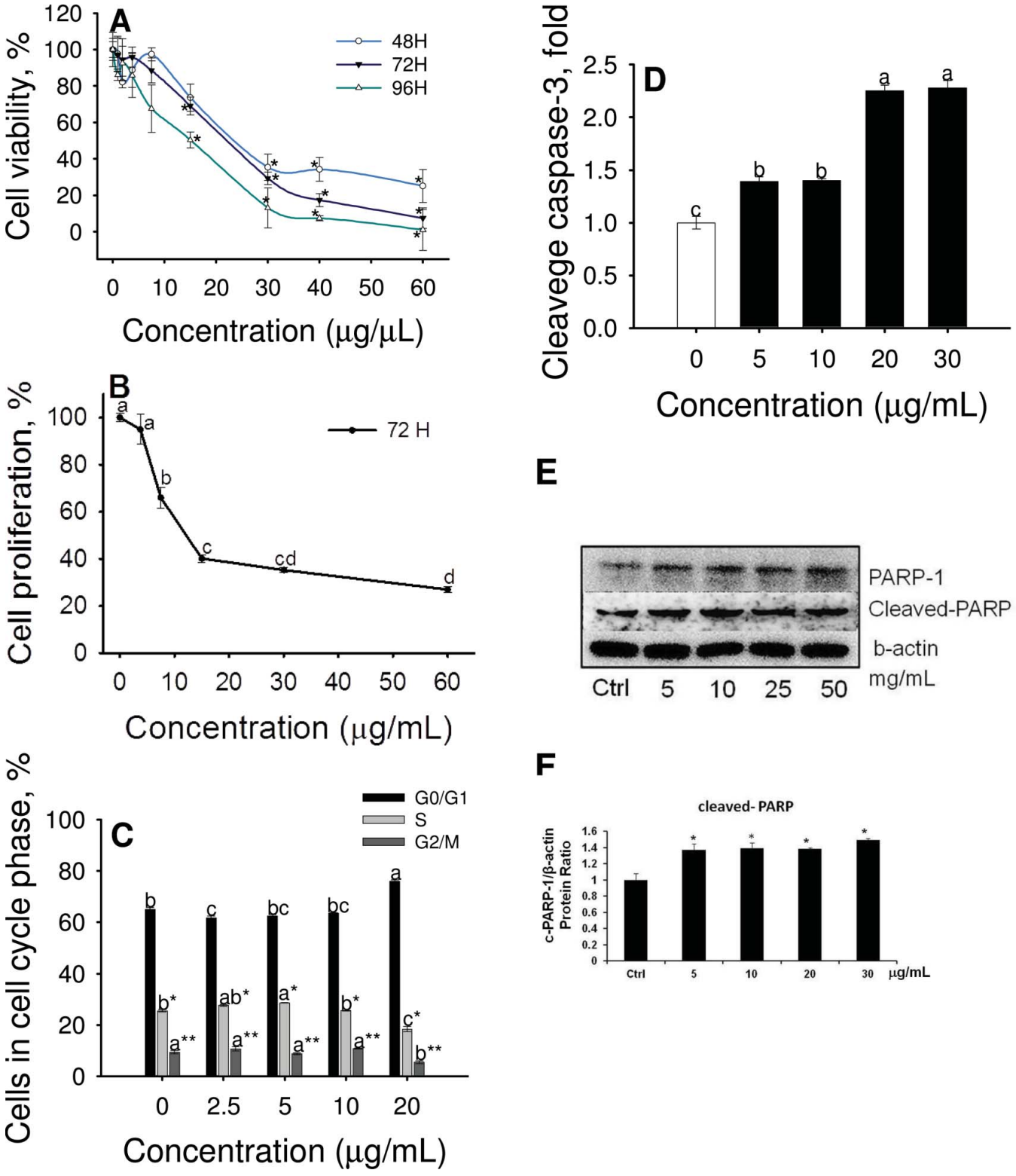

FIG. 2. Effects of (RQ) on cell death in HT-29 Cells. A: Cell viability of HT-29 cells treated with RQ after 48, 72, and 96 h of incubation. B: Cell proliferation of HT-29 cells after 72 h incubation. C: Cell cycle kinetics in HT-29 cells treated with resveratrol/quercetin mixture for 24 h. D: Protein levels of cleaved caspase-3 at 24 h. E: Protein expression of poly(ADP-ribose) polymerase-1 (PARP-1) and cleaved PARP (Asp214) at 24 h and F: protein expression of cleaved PARP normalized to β-actin. Values represent means $\pm$ SEM, $n = 3$. Bars with different letters are significantly different (least significant difference test, $P < 0.05$). (Color figure available online).

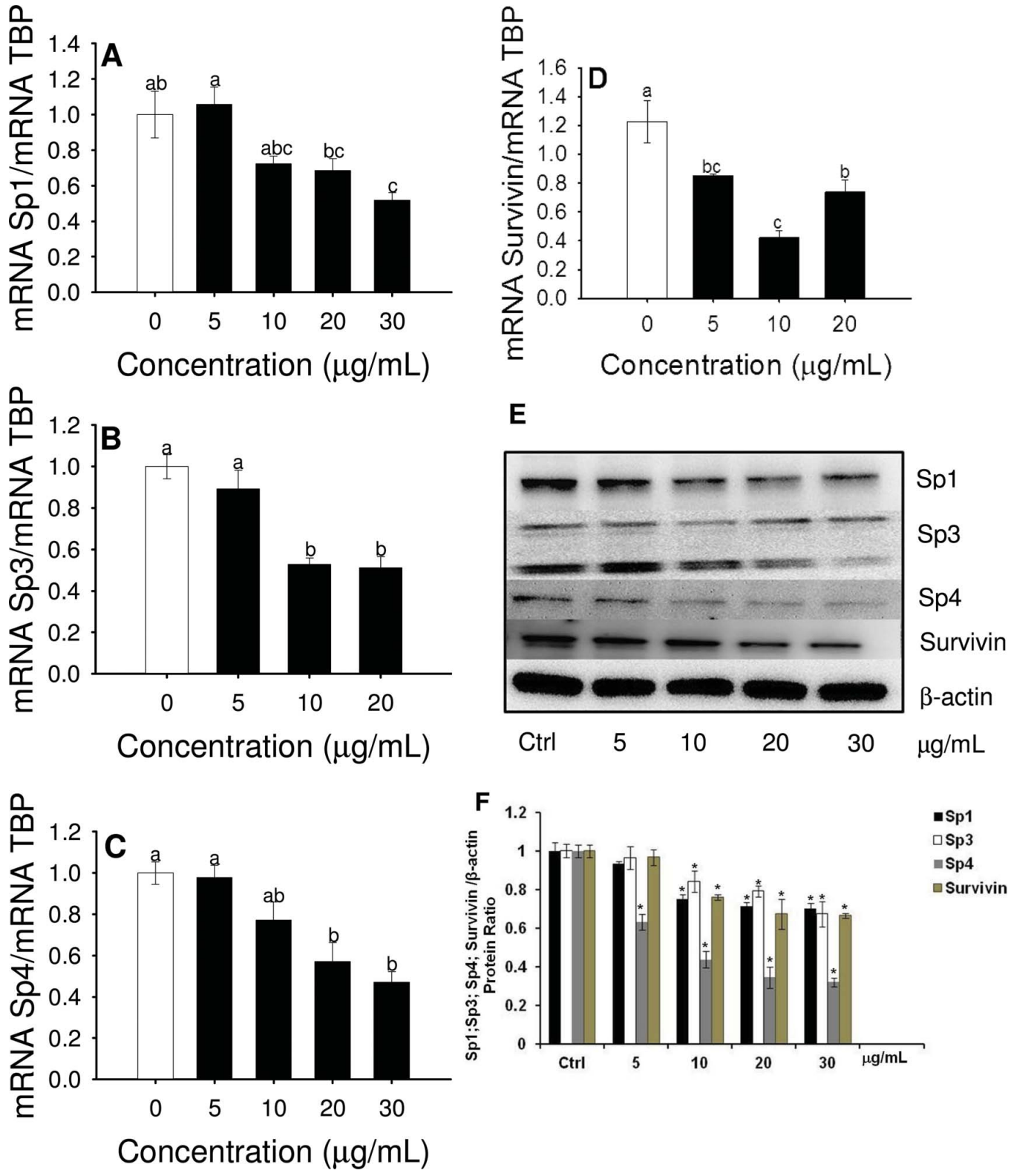

FIG. 3. Expression of specificity protein (Sp)1, Sp3, Sp4, and survivin in HT-29 cells after 24 h of incubation with resveratrol/quercetin. A: mRNA expression of Sp1. B: mRNA expression of Sp3. C: mRNA expression of Sp4. D: mRNA expression of survivin. E: Protein expression of Sp proteins and surviving. F: Protein expression of Sp proteins and surviving normalized to β-actin. Bars represent means $\pm$ SEM, $n = 3$. Bars with different letters are significantly different (least significant difference test, $P < 0.05$). (Color figure available online).

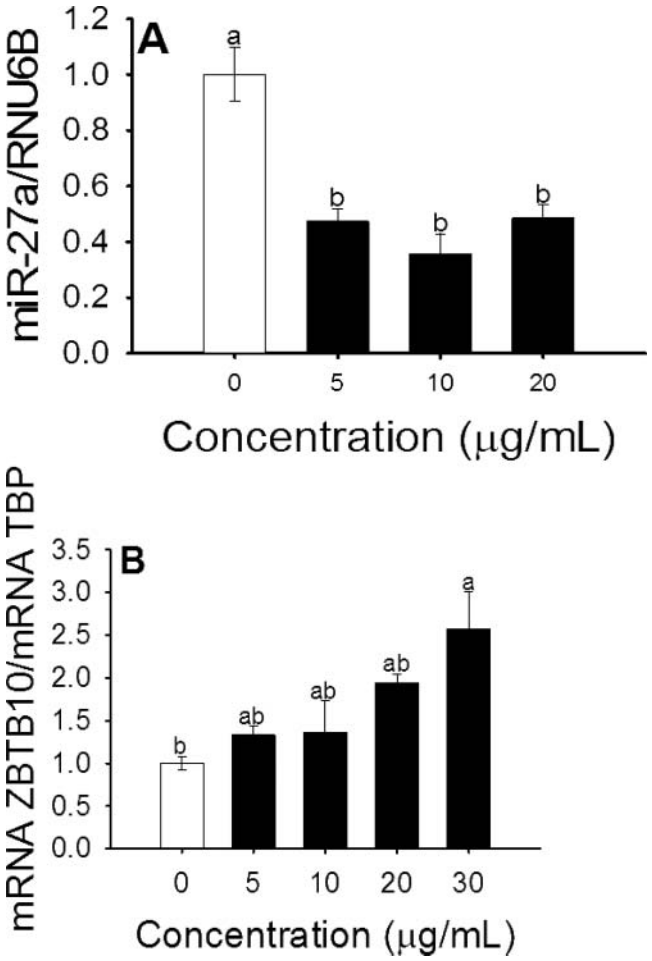

FIG. 4. Expression of miR-27a and ZBTB10 in HT-29 cells 24 h after incubation with resveratrol/quercetin. A: Expression of miR-27a. B: mRNA expression of ZTB10. Bars represent means ± SEM, $n = 3$. Bars with different letters are significantly different (least significant difference test, $P < 0.05$).

ROS at lower concentrations, potentially because of the additional formation of radicals, where RQ concentrations were too low to have a protective effect. At higher concentrations, RQ inhibited the generation of ROS, potentially through scavenging ROS.

Cell Death and Cell Cycle Kinetics

The antiproliferative effects of resveratrol and quercetin were previously demonstrated in MOLT-4 leukemia cells and the combination of resveratrol and quercetin exhibited synergistic effects (8). Based on the previous study, a combination of resveratrol and quercetin at a ratio of 1:1 was investigated in this study, resulting in significant decrease in cell viability and cell proliferation after treatment with RQ (Fig. 2A and 2B). However, it has to be considered that for the chosen cell model, another ratio might have been more effective.

It was previously demonstrated that cell cycle arrest was induced by polyphenolics, including resveratrol and quercetin in several cancer cell lines within different phases (8, 31–33). Tan and others studied the effect of quercetin on HepG2 cells and found that after treatment with quercetin for 48 h, cells were arrested in the G0/G1 phase (34). In MOLT-4 leukemia cells, polyphenolic-mediated cell cycle arrest was influenced by duration of treatment type of polyphenolic (8, 33). In this study, RQ (20 μg/mL) decreased the percentage of cells in the S-phase and increased the percentage of cells in the G_0/G_1 phase, which is consistent with the inhibition of the progression from G_0/G_1 to S-phase (Fig. 2C).

Caspase-3 is a major executive enzyme in apoptosis and a commonly used indicator for the induction of apoptosis (31, 35). PARP-1, an abundant chromatin-associated protein (36), plays an important role in maintaining genome integrity (4, 36) and is cleaved during apoptosis by caspase-3. Previous studies have demonstrated the effects of resveratrol and quercetin, as well as some others polyphenolics on caspase-3 and PARP-1 activity (8, 35, 37). In general, findings from this study are in concordance with previous reports showing that polyphenolics induce apoptosis through the activation of caspase-3 accompanied by cleavage of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP) (35). Previous studies show that resveratrol causes induction of caspase-3 and cleavage of PARP in human articular chondrocytes and myeloid leukemia cells (35).

Modulation of Sp1 Transcription Factors and Dependent Genes

Many botanical compounds have previously been demonstrated to downregulate Sp transcription factors and Sp-regulated genes (14–19). For example, curcumin decreased Sp1, Sp3, and Sp4 levels in bladder (14) and pancreatic (19) cancer cells. The terpenoids betulinic acid also decreased expression of these transcription factors in prostate cancer cells and synthetic analogs of the triterpenoids oleanolic and glycyrrhetinic acid also exhibited comparable effects (14–19). The importance of Sp1 downregulation in terms of the anticancer activity of these compounds is that Sp-regulated genes play an important role in cancer cell and tumor growth (cyclin D1, c-MET, EGFR), survival (NF-kB-p65, survivin, and Bcl-2), and angiogenesis (VEGF and its receptors) (14–19, 38–41).

Sp1 overexpression in gastric and pancreatic cancer patients is a negative prognostic factor (42, 43), as there is evidence that Sp1 exhibits oncogenic properties and plays a role in cell transformation and maintenance of the cancer phenotype (13, 15, 19, 20, 38, 44). The pathways associated with the induction of high levels of Sp1, Sp3, and Sp4 during transformation are not known. However, our studies indicate that at least one mechanism for their elevated expression in cancer cells and tumors is due to inhibition of ZBTB10 expression by microRNA-27a (16, 20, 21). ZBTB10 is a translational repressor and binds GC-rich sites do decrease Sp-dependent transactivation (45). In this study, the effects of RQ were not dose-dependent for miR-27a itself, but for ZBTB10 (Fig. 4A and 4B). Many miRNAs play important roles in carcinogenesis, with either oncogenic or tumor-suppressing activities (46, 47). The overexpression of oncogenic miRNAs

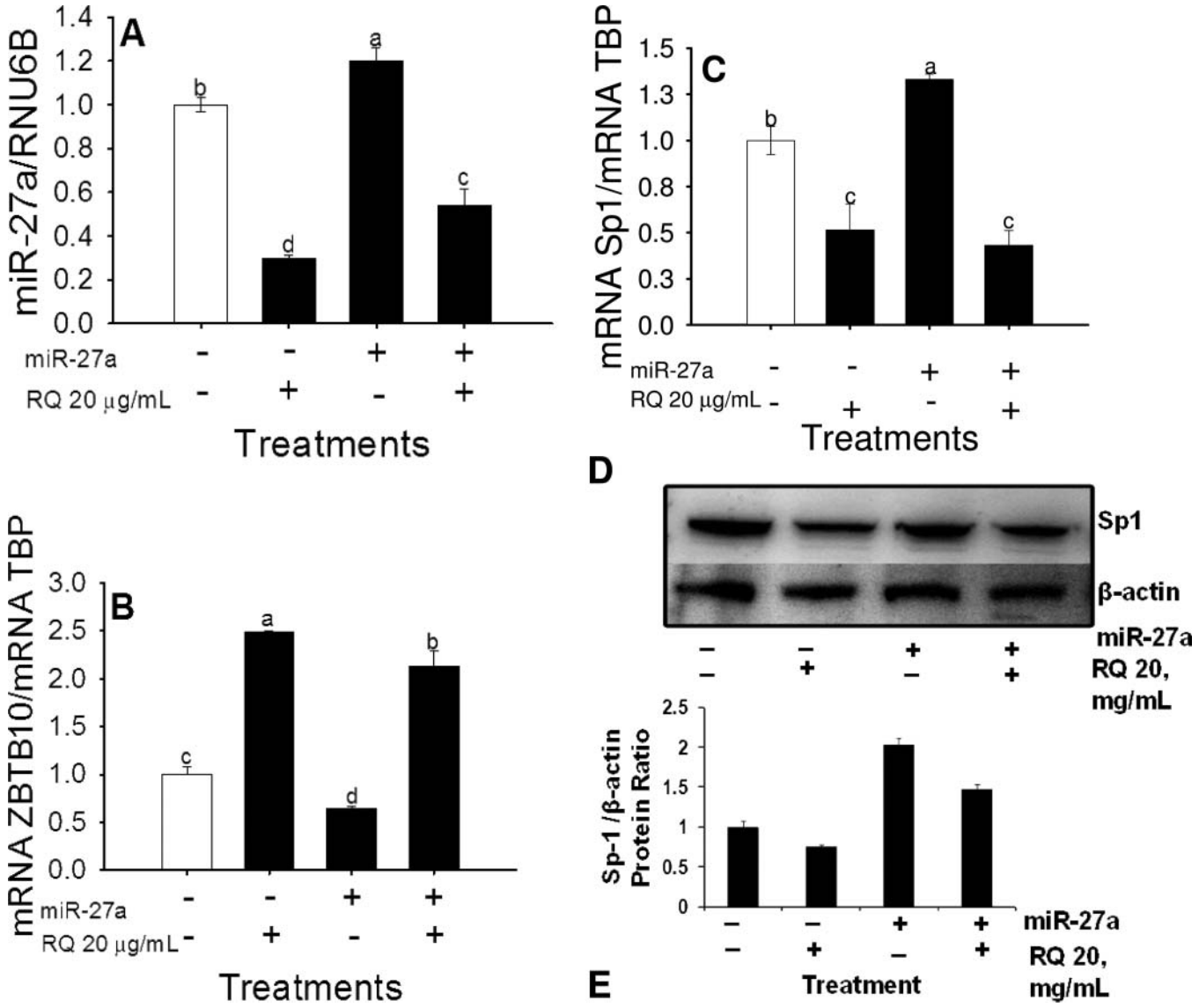

FIG. 5. Effects of miR-27a mimic on the miR-27a-ZBTB10-Sp1 axis after 24 h. A: expression of miR-27a after transfection with miR-27a mimic in HT-29 cells. B: mRNA expression of ZBTB10. C: mRNA expression of specificity protein (Sp)1. D: protein expression of Sp1 protein after transfection with miR-27a mimic. E: Protein expression of Sp1 was normalized to β-actin. Bars represent means $\pm$ SEM, $n = 3$. Bars with different letters are significantly different (least significant difference test, $P < 0.05$).

has been demonstrated in several cancer cell lines (21, 48–50). In a previous study, we showed that miR-27a expression was increased in 6 breast cancer cell lines (21) and also in colon cancer cells (16). In this study, a mimic for miR-27a partially reversed the effects of the extracts on Sp1 protein; however these effects were not significant for Sp1 mRNA.

Results from this study for the first time demonstrate that apoptosis induced by RQ and inhibition of HT-29 cell growth was associated with decreased expression of Sp1, Sp3, Sp4, and Sp-regulated survivin. These latter responses were paralleled by perturbation of the miR-27a-ZBTB10 axis, resulting in the induction of ZBTB10, a potent Sp repressor gene (45). These effects of RQ on mir-27a-ZBTB10 were observed at concentrations of RQ that decrease ROS in HT-29 (Fig. 1C), whereas previous studies with curcurmin and the synthetic triterpenoid methyl 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oate (CDDO-Me) show that their effects on miR-27a-ZBTB10 were dependent on the generation of ROS (19, 20). Current studies in our laboratory focus on mechanisms of ROS-independent downregulation of miR-27a by RQ and other anticancer agents because this pathway plays and important role in the anticancer activity of botanicals and their derivates.

In conclusion, results indicated that a combination of resveratrol and quercetin had cytotoxic effects in colon cancer cells, resulting in apoptosis. Interactions of RQ and the miR-27a-ZBTB10-Sp1 axis were identified as one possible underlying

mechanism. Further studies are needed to assess the role of miRNA-27a and its clinical relevance in the anticancer effects exhibited by botanicals.

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