Combination of quercetin and hyperoside has anticancer effects on renal cancer cells through inhibition of oncogenic microRNA-27a

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Abstract. Quercetin and hyperoside (QH) in combination (1:1 ratio) have previously been shown to inhibit the growth of human leukemia cells. Here, we investigated the anticancer activity of the same mixture in 786-O renal cancer cells. QH 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 786-O cells (3.8-60 μg/ml), whereas IC50 values for viability were 18.2, 18.7 and 11.8 μg/ml, respectively. QH also induced caspase-3 cleavage (2-fold) and increased PARP cleavage. Specificity protein (Sp) transcription factors are overexpressed in cancer cells and regulate genes required for cell proliferation, survival and angiogenesis. QH treatment decreased the expression of Sp1, Sp3 and Sp4 mRNA and this was accompanied by decreased protein expression. Moreover, expression of the Sp-dependent anti-apoptotic survival gene survivin was also significantly reduced, both at the mRNA and protein levels. QH decreased microRNA-27a (miR-27a) and induced the zinc finger protein ZBTB10, an Sp-repressor, suggesting that interactions between QH and the miR-27a-ZBTB10 axis play a role in Sp downregulation. This was confirmed by transfection of cells with a specific mimic for miR-27a, which partially reversed the effects of QH. These findings are consistent with previous studies on botanical anti-cancer agents in colon cancer cells.

Introduction

Renal cell carcinoma (RCC) is the most lethal urologic tumor and the sixth leading cause of cancer-related mortality in Western countries. Each year, approximately 200,000 patients are diagnosed with this malignancy resulting in approximately 100,000 deaths, and its incidence has steadily increased in the last decades (1-3). At present, the most effective treatment for localized RCC is surgical resection; however, 30% of patients develop metastatic disease after surgery, and the median survival for these patients is only 13 months. Therefore, novel therapeutic strategy as well as prophylactic regimens are urgently required.

Prevention of numerous types of cancer through a diet rich in fruits and vegetables has been in part attributed to plant-derived botanicals, including polyphenolics, which may protect against various chronic degenerative diseases such as cancer or cardiovascular disease (4). Quercetin is a dietary flavonoid found in tea, onion, grapes, wines and apples, and the anticancer activities of quercetin have been assessed in several cancer cell lines, as well as in vivo (5,6). Hyperoside 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 hyperoside are Malus micromalus and abelmosk (7). Hyperoside was found to exhibit anticancer properties by inhibiting cell proliferation, inducing apoptosis, decreasing angiogenesis, and causing cell cycle arrest in several cancer cell lines (8). A combination of quercetin and hyperoside (QH) previously exhibited synergistic interactions in their anticancer effects on MOLT-4 leukemia cells (9). In a study with mouse skin tumors, a combination of hyperoside with tea polyphenolics demonstrated synergistic interactions in their anticancer effects on MOLT-4 leukemia cells (9). In a study with mouse skin tumors, a combination of hyperoside with tea polyphenolics demonstrated synergistic interactions in their anticancer effects on MOLT-4 leukemia cells (9). In a study with mouse skin tumors, a combination of hyperoside with tea polyphenolics demonstrated synergistic interactions in their anticancer effects on MOLT-4 leukemia cells (9).

Increasing evidence suggests that specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 are overexpressed in tumors and regulate genes important for cancer cell death and survival (13,14). More recently, the involvement of microRNA-27a (miR-27a) in the regulation of Sp1 transcription factor through zinc finger protein ZBTB10, an inhibitor of Sp1,
has also been demonstrated. Additionally, botanical compounds were also found to inhibit Sp transcription factors through the miR-27a-ZBTB10-Sp1 axis (15-17). Therefore, the aim of this study was to investigate the effects of a mixture of quercetin and hyperoside on Sp1, Sp3 and Sp4 expression and the potential involvement of miR-27a in the downregulation of Sp transcription factors in 786-O renal cell carcinoma cells.

Materials and methods

Botanical extract. Polyphenolics were extracted from a standardized QH dehydrate supplement (ratio 1:1) in capsule form which was provided by Suzhong Pharmaceutical Co., Ltd. (Taizhou, Jiangsu, China). Polyphenolics were extracted with methanol (50 mg/50 ml) and then centrifuged at room temperature for 10 min at 3,000 rpm to remove inactive and insoluble components. Methanol was evaporated in a rotavapor (BUCHI Labortechnik AG, Flawil, Switzerland) at 40 °C. Residual moisture was evaporated in a speedvac concentrator (Thermo Scientific, Waltham, MA, USA) at 43 °C. The final QH 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 photodiode array (PDA) spectra by HPLC-PDA. The chromatographic separation was performed in an Alliance 2695 system (Waters Corporation, Milford, MA, USA) and carried out in a Discovery C18 column (Supelco Inc., Bellefonte, PA, USA) (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 follows: 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 360 and 306 nm for quercetin and hyperoside, respectively. Standard compounds for the identification and quantitative analysis of quercetin and hyperoside were obtained from Acros Organics (Morris Plains, NJ, USA).

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

Cell culture. Human clear cell renal carcinoma 786-O cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured in Mc-Coy’s medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100,000 U/l penicillin and 100 mg/l streptomycin). Cells were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C. For experiments, cells were seeded in DMEM/F-12 medium with 2.5% FBS, and 1% antibiotics (100,000 U/l penicillin and 100 mg/l streptomycin). Generation of reactive oxygen species (ROS) was determined using the 2’,7’-dichlorofluorescein diacetate (DCFH-DA) assay. In summary, 1x10⁴ cells/well were seeded in a clear bottom 96-well plate and incubated for 24 h, and then treated with different concentrations of QH (0-60 μg/ml). After 24 h, cells were washed twice with phosphate-buffered saline (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 the cells. Next, 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 fluorescence microplate reader (485 nm excitation and 538 nm emission).

Cell viability. Cells were seeded (3×10⁴ cells/well) in a 96-well plate for 24 h, and the growth medium was then replaced with the experimental medium containing various extract concentrations of QH (0-60 μg/ml). Cell viability was assessed at 48, 72 and 96 h with the CellTiter 96® AQueous One Solution Cell Proliferation assay (Promega, Madison, WI, USA) according to the manufacturer’s protocol using a FLUOstar microplate reader at 490 nm. The concentration at which cell viability was inhibited by 50% (IC50) was calculated by sigmoidal nonlinear regression analyses of the percentage of cell inhibition, expressed as a ratio to the control, using GraphPad Prism 5.01 software (GraphPad Software Inc., La Jolla, CA, USA).

Cell proliferation. 786-O cells (2x10⁴ cells/well) were grown in a 24-well plate for 24 h. The growth medium was then replaced with experimental medium containing QH extract concentrations ranging from 0 to 60 μg/ml. After 72 h cell proliferation was determined using a cell counter (Beckman Coulter Inc., Fullerton, CA, USA). Cell counts were expressed as a percentage of the control cells.

Cleaved caspase-3 activation. Cells were grown (6x10⁴ cells/well) for 24 h and then incubated with different QH concentrations (0-30 μg/ml) for 24 h. Cleaved caspase-3 activation was determined using an ELISA kit (Cell Signaling Technology Inc., Danvers, MA, USA) according to the manufacturer’s protocol using a FLUOstar microplate reader at 450 nm.

Real-time PCR analysis of miRNA and mRNA. 786-O cells were grown (2x10⁴ cells/well) in a 6-well plate for 24 h before incubation with different concentrations of QH (0-30 μg/ml). Total RNA, which contains both mRNA and miRNA, was isolated using the miVana™ miRNA Isolation kit (Applied Biosystems Inc., Foster City, CA, USA) 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, USA) at 260 and 280 nm.

SuperScript™ III First-Strand (Invitrogen, Carlsbad, CA, USA) was used to reverse-transcribe mRNA. TATA-binding protein (TBP) was used as an mRNA endogenous control. For real time PCR (RT-PCR), proprietary primers for Sp3 and Sp4 (Qiagen Inc., Valencia, CA, USA) were used. The following primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) and used for amplification as follows: TBP (sense, 5’-TGC ACA GGG ACA AAG AGT GAA-3’ and antisense, 5’-CAC ATC ATC ACA GCT CCC CAC CA-3’), caspase-3 (sense, 5’-CTG GAC TGT GGC ATT GAG GAA-3’ and antisense, 5’-CGG CCT CCA CTG GTA TTT
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TAT G-3'), ZBTB10 (sense, 5'-GCT GGA TAG TAG TTA
TGT TGC-3' and antisense, 5'-CTG AGT GGT TTT ATG
GAC AGA G-3'), Sp1 (sense, 5'-TCA CCA ATG CCA ATA
GCT ACT CA-3' and antisense, 5'-GAG TTT GTC CCT GAT
GAT CCA-3'), survivin (sense, 5'-CCA TGC AAA GGA AAC
CAA CAA T-3' and antisense, 5'-ATG GCA CGG CGC
ACT T-3'). RT-PCR for miRNA was performed using the
SYBR GreenER qPCR SuperMix (Invitrogen) on a 7900HT
Fast Real-Time PCR System (Applied Biosystems Inc.).

The TaqMan® MicroRNA Assay for miR-27a and RNU6B
(used as the control; Applied Biosystems Inc.) was used to
reverse-transcribe mature miRNA following the manufac-
turer's protocol in a MasterCycler (Eppendorf North America
Inc., Westbury, NY, USA). 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 Inc.). After completion of RT-PCR, rela-
tive quantification for both miRNA and mRNA gene expression
was evaluated by using the comparative critical threshold (CT)
method. Transfections with 50 and 100 nM miR-27a mimic
(Dharmacon, Lafayette, CO, USA) were performed using
Lipofectamine 2000 (Invitrogen) for 6 h. After transfection,
cells were incubated with 20 μg/ml of QH for 24 h.

Cell cycle kinetics. Cells were seeded (5x10^5 cells/plate) with
medium containing 2.5% FBS for 24 h and were subsequently
treated with QH (0-20 μg/ml) for 24 h. Cells were fixed with
90% ethanol and stored at -20°C. DNA was stained with
propidium iodide containing RNaseA (0.2 mg/ml) solution
and analysis was carried out at 488 nm excitation and 620 nm
emission wavelengths on a FACSscan flow cytometer (BD
Immunocytometry Systems, San José, CA, USA). 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, USA).

Western blotting. Cells were grown (2x10^6 cells/plate) for
24 h and treated with QH (0-30 μg/ml) for 24 h. Cells were
harvested and cell lysates were obtained using a high-salt
buffer (1.5 mmol/l MgCl₂, 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, USA). Samples were incubated at 100°C for
5 min in 1X Laemmli buffer (0.1% bromophenol blue, 175 mM
β-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 membranes (Bio-Rad,
Hercules, CA, USA). 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, USA), and
proteins were visualized with a Kodak Molecular Imaging
System (Carestream Health Inc., Rochester, NY, USA).

Statistical analyses. Data from the in vitro experiments were
analyzed by one-way analysis of variance with JMP 8.0
(SAS Institute, Cary, NC, USA). Differences were deemed
significant at p≤0.05 using a Tukey-Cramer HSD comparison
for all pairs. For transfections with the miR-27a mimic, differences
were deemed significant at p≤0.05 using a Student's
t-test comparison for all pairs. The analysis of linear (pair-
wise) correlations was performed where correlations with a
p-value <0.05 were deemed significant. Nonlinear modeling
of sigmoidal curves for cell viability was performed using
GraphPad Prism 5.01 software (GraphPad Software Inc.).

Results

Chemical composition. The chromatographic profiles (Fig. 1A
and B) of QH demonstrated the presence of 2 major polyphen-
ols, hyperoside (peak 1) and quercetin (peak 2), respectively,
as major ingredients of this botanical supplement. The
chemical structures of quercetin and hyperoside are listed
in Fig. 1C. In fact, both quercetin and hyperoside are
among the most abundant flavonoids in the routine human
diet (4,18).

Generation of intracellular ROS and ORAC. Intracellular
generation of ROS was investigated after 786-O cells were
challenged with hydrogen peroxide. QH slightly induced the
generation of ROS at low concentrations (0-10 μg/ml) whereas
at concentrations >20 μg/ml, generation of ROS was signifi-
cantly reduced (Fig. 1E) by up to 66% compared to that of
solvent-treated control cells. The antioxidant capacity values
(ORAC) (Fig. 1F), were significantly increased by all concen-
trations of QH in a dose-dependent manner.

Cell death and cell cycle kinetics. 786-O cell viability
(Fig. 2A) was significantly decreased by QH in a dose- and
time-dependent manner. The IC₅₀ values were 18.2, 18.7 and
11.8 μg/ml at 48, 72 and 96 h, respectively. QH 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 QH. Cell
numbers were significantly decreased at all concentrations
(3.75-60 μg/ml), and at 60 μg/ml QH cell proliferation was
reduced by up to 73%.

The effects of QH on cell-cycle progression were deter-
mined by fluorescence-activated cell sorting (FACS) analysis
(Fig. 2C). 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 G₂/M to
S-phase arrest when compared to the control cells when cells
were treated with 20 μg/ml of the mixture (Fig. 2C).

Cleaved caspase-3, the activated form, is involved in the
execution of apoptosis and was induced up to 1.5-fold even at
low concentrations of QH (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 (19). There was an increase in PARP cleavage when
cells were treated with QH, as also shown in the densitometry
analysis for PARP-1 cleavage/β-actin (Fig. 2E).

Modulation of Sp1 transcription factors and dependent genes.
Previous studies with several botanical anticancer agents and
their synthetic derivatives have demonstrated that these compounds downregulate Sp transcription factors and Sp-regulated genes (20). The present results showed that QH (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-C and E). In addition, QH also decreased expression of survivin protein and mRNA, and these results are consistent with QH-mediated suppression of Sp transcription factors since survivin is an Sp-regulated gene. We also carried out densitometry for the Sp1, Sp3, Sp4, and Sp-regulated genes, expressed as a ratio to β-actin (Fig. 3F).

Previous studies have demonstrated that the inhibition of ZBTB10 expression by miR-27a is at least in part responsible for the increased expression of Sp transcription factors in cancer cells and tumors (21). In the present study, QH 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 E). 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 (21). In this study, the overexpression of the miR-27a mimic in 786-0 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-D). Moreover, the miR-27a mimic also partially reversed QH-induced downregulation of miR-27a, induction of ZBTB10, and downregulation of Sp1 (Fig. 5). Densitometry was performed for Sp-1/β-actin (Fig. 5E).

### Discussion

**Generation of intracellular ROS and ORAC.** The generation of ROS has been associated with oxidative cellular damage that may be involved in the development of various pathological conditions (22). 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 (23). Elevated levels of ROS may enhance cell proliferation and other events relevant to cancer progression (23). ROS can also cause DNA damage and oxidation of fatty acids in cellular membrane structures, thereby facilitating mutagenesis and cancer development (23). However, many anticancer drugs are mitochondro-toxic and induce ROS, which in turn can lead to cancer cell death. Polyphenolics, such as quercetin and hyperoside, have the ability to scavenge free radicals...
and also induce activation of antioxidant and detoxifying enzymes, thus protecting cells against oxidative damage from carcinogenic compounds (24). In this study, QH induced a concentration-dependent increase in intracellular antioxidant capacity (Fig. 1E) and the intracellular concentration of ROS increased at a low QH concentration (5 μg/ml) but decreased at high concentrations (20-60 μg/ml) (Fig. 1E). One possible explanation for this biphasic effect may be the mitochondriotoxic properties of polyphenolics, which also induce ROS, 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 (25). In this study, QH induced ROS at lower concentrations, potentially because of the additional formation of radicals, where QH concentrations were too low to have a protective effect. At higher concentrations, QH inhibited the generation of ROS, potentially through scavenging ROS.

**Cell death and cell cycle kinetics.** The anti-proliferative effects of quercetin and hyperoside were previously demonstrated in MOLT-4 leukemia cells, and the combination of resveratrol and quercetin exhibited synergistic effects (9). Based on the previous study, a combination of quercetin and hyperoside at a ratio of 1:1 was investigated in the present study, resulting in a significant decrease in cell viability and cell proliferation following treatment with QH (Fig. 2A and B). However, it must be considered that for the chosen cell model, another ratio may have been more effective.

It was previously demonstrated that cell cycle arrest is induced by polyphenolics, including resveratrol and quercetin in several cancer cell lines within different phases (26). Tan et al (27) studied the effect of quercetin on HepG2 cells and found that following treatment with quercetin for 48 h, cells were arrested in the G0/G1 phase. In MOLT-4 leukemia cells, polyphenolic-mediated cell cycle arrest was influenced by the duration of treatment and the type of polyphenolic. In the present study, QH (20 μg/ml) decreased the percentage of cells in the S-phase and increased the percentage of cells in the G0/G1 phase, which is consistent with the inhibition of the progression from G0/G1 to S-phase (Fig. 2C).
Caspase-3 is a major executor enzyme in apoptosis and a commonly used indicator for the induction of apoptosis (28).

**Figure 3.** Expression of specificity protein (Sp)1, Sp3, Sp4 and survivin in 786-O cells after 24 h of incubation with quercetin and hyperoside (QH). (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 survivin. (F) Protein expression of Sp proteins and survivin normalized to β-actin. Bars represent mean ± SEM, n=3. Bars with different letters indicate significantly different results (least significant difference test, p<0.05).

**Figure 4.** Expression of microRNA-27a (miR-27a) and ZBTB10 in 786-O cells 24 h after incubation with quercetin and hyperoside (QH). (A) Expression of miR-27a. (B) mRNA expression of ZBTB10. Bars represent mean ± SEM, n=3. Bars with different letters indicate significantly different results (least significant difference test, p<0.05).

PARP-1, an abundant chromatin-associated protein, plays an important role in maintaining genome integrity and is cleaved
during apoptosis by caspase-3 (29). Previous studies have demonstrated the effects of quercetin, as well as of a number of other polyphenolics, on caspase-3 and PARP-1 activity (30). In general, the findings of this study are in concordance with previous studies that found that polyphenolics induce apoptosis through the activation of caspase-3 accompanied by cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (28). Previous studies have shown that resveratrol causes induction of caspase-3 and cleavage of PARP in human articular chondrocytes and myeloid leukemia cells (28).

**Modulation of Sp1 transcription factors and dependent genes.** Numerous botanical compounds have previously been demonstrated to downregulate Sp transcription factors and Sp-regulated genes (21). For example, curcumin decreased Sp1, Sp3, and Sp4 levels in bladder and pancreatic cancer cells. The terpenoid betulinic acid also decreased expression of these transcription factors in prostate cancer cells, and synthetic analogs of the triterpenoids oleanolic and glycyrhetinic acid also exhibited comparable effects (22). 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 and EGFR), survival (NF-κB-p65, survivin and Bcl-2), and angiogenesis (VEGF and its receptors) (31).

Sp1 overexpression in gastric and pancreatic cancer patients is a negative prognostic factor, as there is evidence that Sp1 exhibits oncogenic properties and plays a role in cell transformation and maintenance of the cancer phenotype (32). The pathways associated with the induction of high levels of Sp1, Sp3 and Sp4 during transformation are not known. However, our study indicated that at least one mechanism for their elevated expression in cancer cells and tumors was due to inhibition of ZBTB10 expression by miR-27a. ZBTB10 is a translational repressor and binds GC-rich sites to decrease Sp-dependent trans-activation (33). In the present study, the effects of QH were not dose-dependent for miR-27a itself, but for ZBTB10 (Fig. 4A and B). Many miRNAs play important roles in carcinogenesis, with either oncogenic or tumor-suppressing activities (34). The overexpression of oncogenic miRNAs has been demonstrated in several cancer cell lines (35). In previous studies, miR-27a expression was increased in 6 breast cancer cell lines and also in colon cancer cells (35,36). In the present 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 demonstrated that apoptosis induced by QH and inhibition of HT-29 cell growth was associated with decreased expression of Sp1, Sp3, Sp4, and Sp-regulated survivin. These latter responses were parallelled by perturbation of the miR-27a-ZBTB10 axis, resulting in the induction of ZBTB10, a potent Sp-repressor gene (36). These effects of QH on miR-27a-ZBTB10 were observed at concentrations of QH that decreased ROS in the HT-29 cells (Fig. 1E), whereas previous studies with curcumin and the synthetic triterpenoid methyl-2-cyano-3, 12-dioxooleana-1, 9-dien-28-oate (CDDOme) showed that their effects on miR-27a-ZBTB10 were dependent on the generation of ROS.

![Figure 5. Effects of microRNA-27a (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) miRNA expression of ZBTB10. (C) miRNA 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 ± SEM; n=3. Bars with different letters indicate significantly different results (least significant difference test, p<0.05).](image-url)
ROS (36). Ongoing studies in our laboratory are focused on mechanisms of ROS-independent downregulation of miR-27a by QH and other anticancer agents as this pathway plays an important role in the anticancer activity of botanicals and their derivatives.

In conclusion, the present results revealed that a combination of quercetin and hydrosyrup had cytotoxic effects on colon cancer cells, resulting in apoptosis. Interactions of QH and the miR-27a-ZBTB10-Sp1 axis were identified as one possible underlying mechanism. Further studies are needed to assess the role of miR-27a and its clinical relevance in the anticancer effects exhibited by botanicals.

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