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Original Research Article

Sanggenon C inhibits proliferation of breast cancer cells and reduces HIF-1α/VEGF pathway activity under hypoxia conditions

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Abstract

Purpose: *To evaluate the function and mechanism of sanggenon C (SC) on breast cancer (BC) cells.*
Methods: *The effect of SC on malignant processes of BC was studied through cell counting kit-8, colony formation, flow cytometry, Transwell, wound-healing and western blot experiments. Besides, the related mechanism of action was explored using western blot assay.*
Results: *SC reduced the cell viability of MDA-MB-231 and MCF-7 cells with half-maximal concentration of (IC_{50}) value of 17.09 and 17.32 μM, respectively. SC also decreased the area ratio of colonies in the plate, but increased the apoptosis and G0/G1 phase arrest in both cell lines. Furthermore, SC decreased the number of invasion cells, but elevated the relative wound width of both cells. Moreover, SC treatment neutralized the hypoxia-induced level of HIF-1α/VEGF signaling.*
Conclusion: *SC suppresses proliferation, mobility and invasion, but induces apoptosis and G0/G1 phase arrest in BC cells, as well as deceased HIF-1α/VEGF pathway activity under hypoxia conditions. The findings of this study reveal that SC is a potential agent for BC management.*

Keywords: *Breast cancer, Sanggenon C, Cell proliferation, Cell cycle arrest, Cell migration and invasion, HIF-1α/VEGF, G0/G1 phase arrest*

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INTRODUCTION

Breast cancer (BC) is the most malignant tumor in women globally, with a steady increase in incidence of the disease. BC is predicted to account for 31 % of all new diagnoses, with 297,790 cases, and 15 % of all the deaths, with 43,170 cases in female cancers in the United States in 2023 [1]. Several therapies have been used for the treatment of BC in clinical practice, containing breast-conserving surgery, mastectomy, adjuvant radiotherapy, chemotherapy, radiation and hormonal therapy [2,3]. However, the 5-year relative survival rate of patients with stage IV BC declined to only 28 % [2]. Hence, it is important to determine or identify potential drugs for the therapy of BC.

Natural bioactive agents have attracted more attention in fighting a variety of cancers due to their unique advantages, such as high anti-tumor efficacy, multi-target inhibition, and the ability to modulate the tumor microenvironment, including BC. Sanggenon C (SC) with a formula of $C_{40}H_{36}O_{12}$ is one of the most affluent flavonoids extracted from the stem bark of *Morus cathayana*, which possesses anti-inflammatory and anti-oxidative properties [4]. Furthermore, an increasing number of researches have reported the potential effect of SC on the treatment of cancers. Tang *et al* [5] found that SC suppresses the proliferation, growth and self-renewal of glioblastoma by targeting the KDM4B-MYC axis. Li *et al* [6] revealed that SC enhances the G0/G1 phase arrest and apoptosis in prostate cancer. Zhou *et al* [7] found that SC promoted apoptosis by activating caspase 3 and 9 pathways in prostate cancer. Chen *et al* [8] stated that SC inhibits the tumorigenesis of gastric cancer through the blockage of mitochondrial fission mediated by ERK-Drp1. Besides, SC evokes apoptosis by inhibiting iNOS expression, NO generation and ROS activation of the mitochondrial pathway in colorectal cancer [9]. Nevertheless, the role and mechanism of action of SC in BC remain unclear. This study intends to examine, *in vitro,* the function of SC in the development of BC, as well as the exploration of its mechanism of action in BC cells.

EXPERIMENTAL

Cell culture

Human BC cell lines, MCF-7 (catalog number: CL-0149) and MDA-MB-231 (catalog number: CL-0150B) were prepared from Procell (Wuhan, China). MCF-7 cells were grown in MEM (PM150410, Procell), while MDA-MB-231 cells were maintained in DMEM (PM150210, Procell) with 0.01 mg/ml insulin (I2643, purity ≥ 98 % (HPLC), Sigma, St. Louis, MO, USA), 10 % fetal bovine serum (FBS, 164210-50, Procell) and 1 % penicillin/streptomycin (P/S, PB180120, Procell) at 37 ℃ with 5 % carbon dioxide (CO_2).

Cell treatment

Both BC cell lines were hatched with different doses of SC (0, 1, 5, 10, 20 and 40 μM) (catalog number: HY-N0617, CAS No.: 80651-76-9, purity: ≥ 97.93 %, MedChemExpress, Monmouth Junction, NJ, USA) for 24 h [9], and then were used for further assays. 0 μM SC referred to cells administered with phosphate buffered saline (PBS, P1020, Solarbio, Beijing, China) for the same timeframe. For hypoxia induction, both cell lines were cultured at 0.5 % O_2 for 4 h, and then hatched with 5, 10 and 20 μM SC for another 4 h, while cells in the control group were continuously cultured at 20 % O_2 for 8 h.

Cell viability examination

Both BC cell lines were sown in 96-well plates with 4×10^4 cells per well. After the cells were exposed to with SC (0, 1, 5, 10, 20 and 40 μM) for 24 h, they were evaluated with cell counting kit-8 (CCK-8) (CA1210, Solarbio) for 2 h at 37 °C, and detected on a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm.

Colony formation test

Both BC cell lines, at 6×10^5 cells/well, were sowed in 6-well plates. Then they were grown at 37 °C for fourteen days, and treated with 4 % paraformaldehyde (P1110, Solarbio) for immobilization, and 0.1 % crystal violet (G1063, Solarbio) for staining for half an hour. The clone numbers were determined manually and imaged.

Flow cytometry

Cells were remixed with 1 mL of binding buffer after they were washed with cold PBS. Next, the cells were dyed with propidium iodide (PI) and FITC-Annexin V (Thermo Fisher Scientific), severally. Apoptotic cells were examined on a FACScan flow cytometry (BD Biosciences, NJ, USA) and assessed with BD CellQuest Pro software (version 5.1, BD Biosciences). Additionally, the cell cycle was detected via the flow cytometry. After being washed with cold PBS, cells were immobilized with ice-cold 100 % ethanol (E7023, Sigma) for 1 h at 4 °C, and rinsed with PBS thrice subsequently. Next, cells were treated with RNase A (100 μg/mL, R1030, Solarbio) for 1 h at 37 ℃, and with propidium iodide (PI, 1000 μg/mL, P3566, Invitrogen) for half an hour without light. The cell population was analyzed using the FACScan flow cytometry.

Transwell assay

The 200 μL of cell suspensions containing 5×10^4 cells were spread into the upper cabinet of 24-well Transwell plates (3422, Corning Company), and coated with Matrigel (356234, Solarbio). MEM (for MCF-7 cells) or DMEM (for MDA-MB-231 cells) with 20 % FBS and SC (5, 10 and 20 μg/ml) were plated into the lower chamber. After 48 h, the Matrigel was abrased, and cells were then fixed with 4 % paraformaldehyde, colored with 0.1 % crystal violet, and then the image captured using an inverted microscope (IX73, (Olympus, Tokyo,

Japan)). Five arbitrary and disparate zones were selected to examine the number of invasion cells.

Wound-healing assay

Cells were inoculated into 6-well plates with a density of 1 × 10^6 cells/well. A scratch wound was performed with a 200-µl pipette tip. Cells were maintained for 24 h, and then photographed with an inverted microscope (Olympus).

Western blot

Total proteins from both cell lines were harvested with RIPA lysis buffer (R0010, Solarbio) and quantified with a BCA kit (PC0020, Solarbio). Protein samples (20 µg) were separated with 10 % SDS-PAGE, and electrically shifted onto PVDF membranes (IPVH00010, EMD Millipore, Billerica, MA, USA). After blocking with 5 % skimmed milk (D8340, Solarbio) at room temperature for 60 min, the membranes were treated with primary antibodies (anti-Cyclin D1 (CCND1) (1:5000, ab226977, Abcam, Cambridge, UK), anti-hypoxia inducible factor-1 subunit alpha (HIF-1α) (1:500, ab216842, Abcam), anti-vascular endothelial growth factor (VEGF) (1:1000, MA1-16629, Thermo Fisher Scientific) and anti-β-actin (1:1000, ab8227, Abcam)) overnight at 4 °C. Next, the membranes were treated with Goat Anti-Mouse IgG H&L (HRP) (ab6789, 1:10000, Abcam) or goat anti-rabbit IgG H&L (HRP) (ab6721, 1:10000, Abcam) at 37 °C for 60 min. Bands were appeared with ECL Western Blotting Detection Kit (Goat IgG) (SW2030, Solarbio), and the intensity of the bands was quantified through ImageJ software (National Institutes of Health, USA).

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical difference was tested by one-way analysis of variance (ANOVA), followed by *post hoc* Bonferroni test, using SPSS 26.0 software (IBM, Armonk, New York, USA). Significant difference was defined as $p < 0.05$.

RESULTS

SC suppressed growth and evoked apoptosis of BC cells

To address the effect of SC in the progression of BC, 0, 1, 5, 10, 20 and 40 µM of SC were incubated with MDA-MB-231 and MCF-7 cells for 24 h. 5, 10, 20 and 40 µM SC caused a significant reduction in the cell viability of MCF-7 cells, with an IC50 value of 17.32 µM (Fig 1 A). The cell viability decreased with an IC50 value of 17.09 µM after MDA-MB-231 cells were cultured with 1, 5, 10, 20 and 40 µM SC (Figure 1 A). Then, three different doses of SC (5, 10 and 20 µM) were chosen for subsequent experiments, and named as the low, middle and high concentrations, severally. Treatment of SC (10 and 20 µM) significantly decreased the area ration of colonies in the plate grown in MCF-7 cells, whereas treatment with SC (5, 10 and 20 µM) all memorably declined the area ratio of colonies in the plate cultured in MDA-MB-231 cells (Figure 1 B). Nevertheless, the administration of all the three different concentrations of SC observably elevated the apoptosis rate of both cells (Fig 1 C). Thus, SC suppressed growth and evoked apoptosis of BC cells.

SC elicited G0/G1 phase arrest in BC cells

Additionally, the effect of SC in cell cycle was evaluated in both BC cell line. The results showed that both the 10 and 20 µM SC treatment significantly elicited G0/G1 phase arrest, and promoted S phase in MCF-7 and MDA-MB-231 cells (Figure 2 A). No significant difference was observed in G2/M phase in both cells treated at all three concentrations of SC (Figure 2 A). Besides, the expression of CCND1 was significantly decreased after both cells were incubated at all the three concentrations of SC (Figure 2 B). Therefore, SC evoked G0/G1 phase arrest in BC cells.

SC suppressed the invasion and mobility of BC cells

To test the action of SC in the invasion and mobility of the BC cell line, transwell and wound-healing experiments were carried out after MCF-7 and MDA-MB-231 cells were incubated with SC (0, 5, 10 and 20 µM) for 24 h. The numbers of invasion cells prominently reduced after both BC cell lines were incubated with 5, 10 and 20 µM SC (Fig 3 A). Additionally, the relative wound width increased after both BC cell lines were administrated with 5, 10 and 20 µM SC (Figure 3 B). Hence, SC inhibited the invasion and mobility of BC cell lines.

SC downregulated HIF-1α/VEGF signaling under hypoxic conditions

To resolve the mechanism of action of SC in the progresses of BC, the HIF-1α/VEGF pathway level was monitored using western blot. The level of HIF-1α and VEGF notably increased after both BC cells were treated with hypoxia, and was neutralized with the introduction of the three

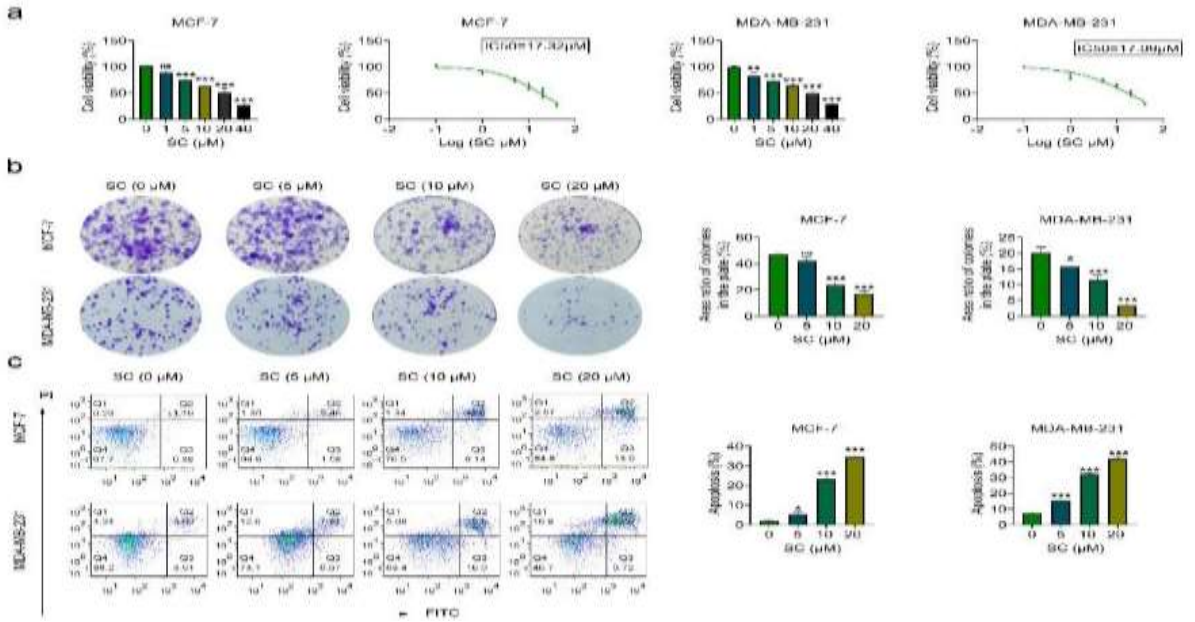

Figure 1: SC suppressed growth and elicited apoptosis of BC cells. (a) Cell viability of MCF-7 and MDA-MB-231 cells. (b) Proliferation of MCF-7 and MDA-MB-231 cells. (c) Apoptosis of MCF-7 and MDA-MB-231 cells. *P< 0.05, **p < 0.01 and ***p < 0.001 *vs.* 0 μM SC; ns = non-significance

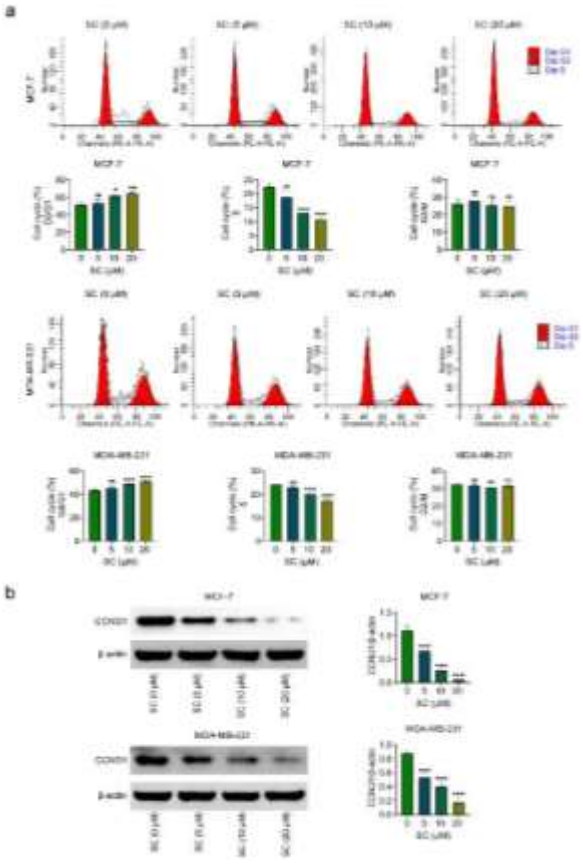

Figure 2: SC elicited G0/G1 phase arrest in BC cells. (a) Cell cycle of the two BC cell lines was analyzed by flow cytometry; (b) Relative protein expression of CCND1. Results were expressed after being normalized with β-actin. *P < 0.05, **p < 0.01 and ***p < 0.001 *vs.* 0 μM SC; ns = no significant

different concentrations of SC (Fig 4). Therefore, SC repressed the HIF-1α/VEGF signaling under hypoxia in BC cells.

DISCUSSION

In the current study, the SC treatment suppressed proliferation, invasion and mobility, but evoked apoptosis and G0/G1 phase arrest in BC cells. Moreover, SC treatment counteracted the hypoxia-induced level of HIF-1α/VEGF signaling. Thus, SC inhibited the malignant progression of BC, and HIF-1α/VEGF signaling under hypoxic conditions. Natural plant compounds have been proven to exert preventive and therapeutic effects on BC. Brazilein, isolated from *Caesalpinia sappan L.*, suppresses the level of programmed death ligand 1 (PD-L1) and epithelial-mesenchymal transition (EMT) in BC cells [10]. Harmine extracted from *Peganum harmala L.*, inhibits the malignant progression involved in the reduction in PI3K activity in BC [11]. Russelioside A, a pregnane glycoside isolated from Caralluma tuberculate, represses the ability of metastasis and cell-intrinsic NF-κB activity in BC [12]. Fisetin decreases the proliferation and migration of BC cells [13]. Recently, Sahoo *et al* [14] summarized the present status and future prospects of flavonoids in the treatment of BC. SC, a type of flavonoid from the stem bark of Morus cathayana [4], has been reported to attenuate the cell viability and clone formation of glioblastoma [5], induce cell apoptosis [6,7] as well as the G0/G1 phase arrest of prostate cancer [6], suppressed

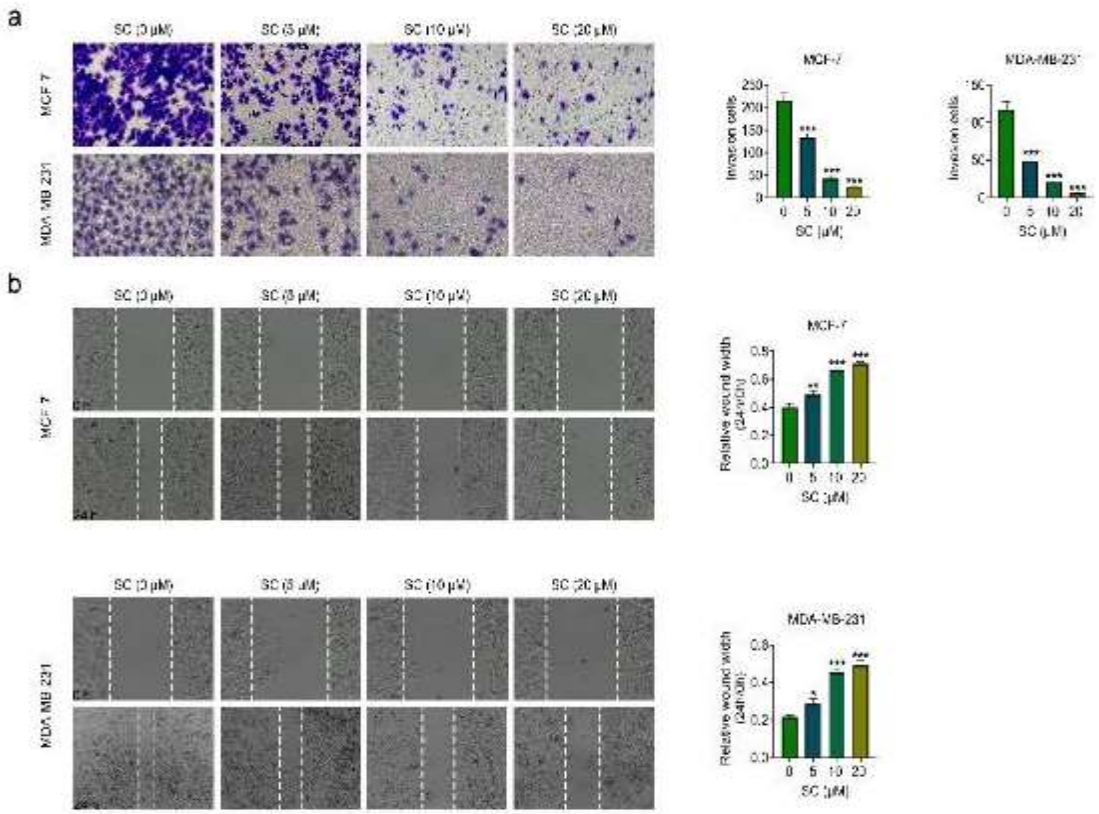

Figure 3: SC repressed invasion and migration of BC cell lines. (a) Number of invasion cells; (b) Relative wound width. *P < 0.05, **p < 0.01 and ***p < 0.001 *vs.* 0 μM SC

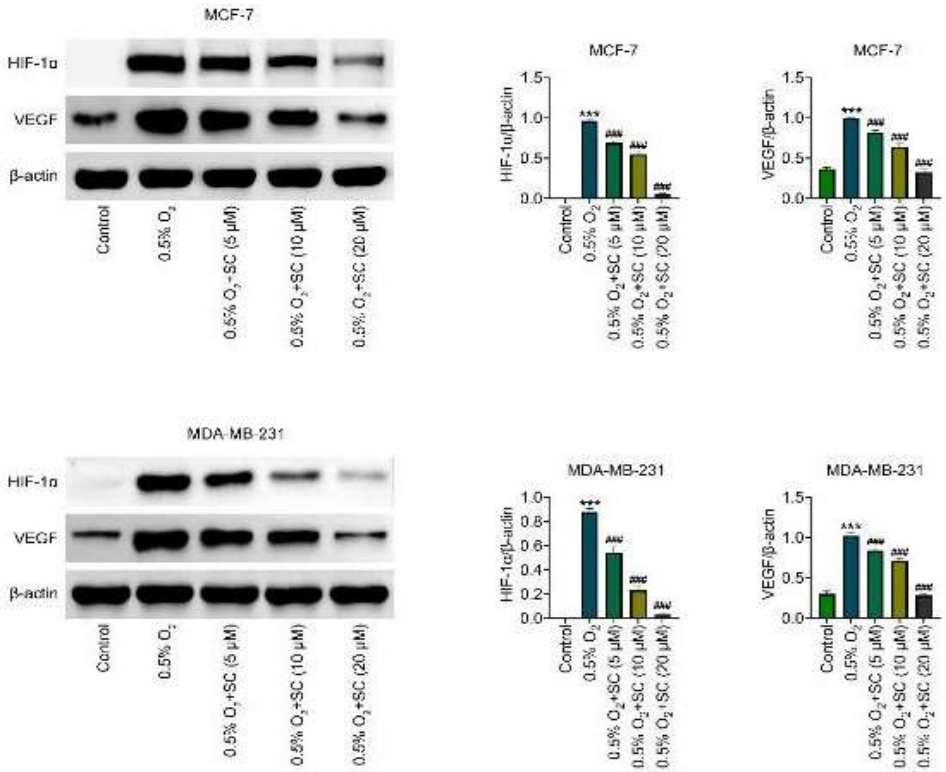

Figure 4: SC repressed HIF-1α/VEGF signaling under hypoxia. Relative protein expression of HIF-1α and VEGF in MCF-7 and MDA-MB-231 cells. The results are presented after normalization with β-actin. ***P < 0.001 *vs.* Control; ###p < 0.001 *vs.* 0.5 % O_2

cell viability and clone formation, but promotes apoptosis and G0/G1 phase arrest of gastric cancer [8], and finally represses growth and evoke apoptosis of colon cancer [9].

Similar to these findings, this study revealed that SC also repressed proliferation, but elicited the apoptosis of both BC cell lines in the present study. Moreover, SC elicited the G0/G1 phase arrest and reduced the relative protein level of CCND1 in BC cells. CCND1 is a key protein in the G1 phase. CCND1 binds to and activates the cyclin-dependent kinase CDK4 unique to the G1 phase. The phosphorylated G1 phase cycle suppressor protein (Rb) is separated from the E2F transcription factor, and the E2F transcription factor initiates transcription so as to activate the gene of the cell cycle, thereby separating the cell cycle from the G1 phase to the S phase [15]. Proliferation, apoptosis, invasion and migration are identified as the primary hallmarks of cancer. Taken together, SC attenuated proliferation, invasion and mobility, but elicited apoptosis and the G0/G1 phase arrest of BC. Hypoxia condition has been proven to facilitate plasticity and heterogeneity, and promotes aggression and metastasis of BC, which is one of the crucial characteristics of the tumor microenvironment of BC. HIF is a critical marker of hypoxia, which acts as a critical role in adaptation to hypoxia by the cells. Among them, HIF-1α is stabilized by hypoxia and is shifted into the nuclei in order to activate the targeted genes, such as VEGF [16]. VEGF is a foregone important angiogenetic factor, which also serves a pivotal role in the cancer processes. Here, the relative protein level of HIF-1α and VEGF was elevated after both BC cell lines were managed with hypoxia, in line with the previous study [17]. However, these elevations were reversed following treatment with SC, which has been revealed to repress the accumulation of HIF-1α and diminish the secretion of VEGF in human hepatocellular carcinoma line Hep3B cells [18]. Thus, SC inhibited the HIF-1α/VEGF signaling under hypoxia in BC.

Limitations of the study

The limitations in this will need to be addressed in further studies on the subject. The *in vivo* effect of SC should be confirmed in xenografted mice. Additionally, more pre-clinical and clinical studies need to be studied in the future.

CONCLUSION

The results reveal that SC suppresses the proliferation, invasion and mobility BC cells, but evokes apoptosis and G0/G1 phase arrest of BC. Furthermore, SC inhibits HIF-1α/VEGF signaling under hypoxia in BC. The findings of this study indicate that SC is a potential agent for BC therapy.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Juanyuan Qu and Jing Li designed the study and carried them out; Juanyuan Qu and Jing Li supervised the data collection, analyzed and interpreted the data; Juanyuan Qu, Jing Li, Yongqiang Ma and Zhihui Wang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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