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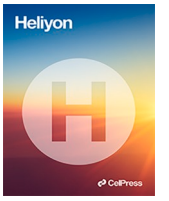

Brevilin A exerts anti-colorectal cancer effects and potently inhibits STAT3 signaling *in vitro*

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A R T I C L E I N F O

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A B S T R A C T

Colorectal cancer (CRC) is the third most common cause of cancer-related morbidity worldwide, with an estimated of 1.85 million new cases and 850,000 deaths every year. Nevertheless, the current treatment regimens for CRC have many disadvantages, including toxicities and off-targeted side effects. STAT3 (signal transducer and activator of transcription 3) has been considered as a promising molecular target for CRC therapy. Brevilin A, a sesquiterpene lactone compound rich in Centipedae Herba has potent anticancer effects in nasopharyngeal, prostate and breast cancer cells by inhibiting the STAT3 signaling. However, the anti-CRC effect of brevilin A and the underlying mechanism of action have not been fully elucidated. In this study, we aimed to investigate the involvement of STAT3 signaling in the anti-CRC action of brevilin A. Here, HCT-116 and CT26 cell models were used to investigate the anti-CRC effects of brevilin A *in vitro*. HCT-116 cells overespressing with STAT3 were used to evaluate the involvement of STAT3 signaling in the anti-CRC effect of brevilin A. Screening of 49 phosphorylated tyrosine kinases in the HCT-116 cells after brevilin A treatment was performed by using the human phospho-receptor tyrosine kinase (phospho-RTK) array. Results showed that brevilin A inhibited cell proliferation and cell viability, induced apoptosis, reduced cell migration and invasion, inhibited angiogenesis, lowered the protein expression levels of phospho-Src (Tyr416), phospho-JAK2 (Y1007/1008) and phospho-STAT3 (Tyr705), and inhibited STAT3 activation and nuclear localization. Brevilin A also significantly reduced the protein expression levels of STAT3 target genes, such as MMP-2, VEGF and Bcl-xL. More importantly, over-activation of STAT3 diminished brevilin A's effects on cell viability. All these results suggest that brevilin A exerts potent anti-CRC effects, at least in part, by inhibiting STAT3 signaling. Our findings provide a strong pharmacological basis for the future exploration and development of brevilin A as a novel STAT3-targeting phytotherapeutic agent for CRC treatment.

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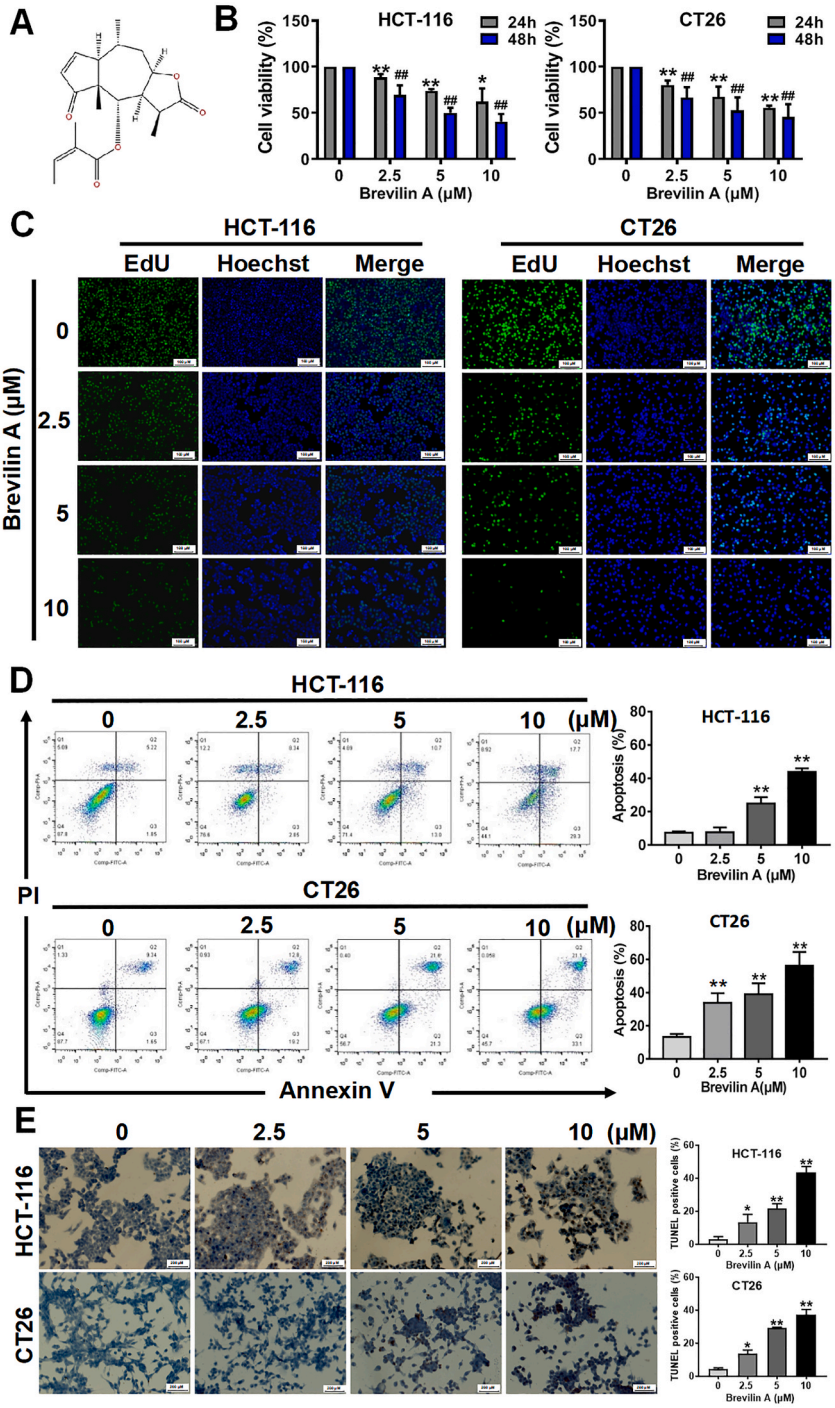

Fig. 1. Brevilin A inhibited viability and proliferation, induced apoptosis in colorectal cancer (CRC) cells. (A) Chemical structure of brevilin A. (B) Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cytotoxic effects of brevilin A against HCT-116 and CT26 cells were incubated with various concentrations of brevilin A (2.5, 5, 10 μM) for 24 or 48 h. (C) Cell proliferation was assessed by the EdU (5-Ethynyl-2′-deoxyuridine) assay. The scale bar represents 100 μm. (D) Representative flow cytometry plots of cell apoptosis. Apoptosis was analyzed using the Annexin V/PI double staining assay. (E) Analysis of apoptosis by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay in both HCT-116 and CT26 cells. The quantitative results were analyzed by using Image J software (right panel). The scale bar represents 200 μm. Data are shown as mean ± SD from three independent experiments. For B: $^{**}P < 0.01$ *vs.* vehicle (24 h); $^{\#\#}P < 0.01$ *vs.* vehicle (48 h); For D–E: $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the corresponding control.

1. Introduction

Among the most common malignant tumors, colorectal cancer (CRC) has higher morbidity and mortality rates [1]. Currently, surgery, chemotherapy, radiotherapy and immune-targeted therapy have been utilized in the treatment of CRC [2,3]. Although these treatment strategies for CRC have made great progress, the overall situation is not optimistic. There are several limitations in the current CRC therapeutic modalities, such as toxicities, gene mutation and low response rate [4]. It is imperative to develop novel, effective, and safe therapeutic agents for CRC treatment.

STAT family proteins are closely related to cancer and are considered as cancer therapeutic targets. This family includes seven members, they are STAT1, STAT2, STAT3, STAT4, STAT5α, STAT5β and STAT6 [5]. CRC is characterized by continuous activation of STAT3 [6]. STAT3 regulates multiple downstream target genes that control cell cycle and apoptosis, thereby regulating cancer cell proliferation and survival [7]. In addition, abnormal activation of STAT3 can induce the expressions of matrix metalloproteinases and other STAT3 target genes that promote tumor invasion and metastasis [8]. Therefore, inhibiting STAT3 activity is suggested as a therapeutic strategy for CRC. Up to now, many natural compounds, for example, luteolin, leonurine, and andrographolide, which were isolated from Chinese medicinal herbals were explored as STAT3 inhibitors to treat different types of tumors [9–12].

Centipedae Herba, the dried whole plant of *Centipeda minima* (L.) A. Br. et Aschers., has many bioactivities, including anti-rhinitis, anti-sinusitis, anti-inflammation, anti-cough and anti-asthma [13]. It is also traditionally used in China to treat cancers. It contains polyphenolic acids, flavones, sesquiterpene lactones. Studies have shown that sesquiterpene lactones have anti-inflammatory, anti--oxidative and anti-cancer properties [13,14]. Brevilin A, a sesquiterpene lactone in Centipedae Herba, has been proved to possess anti-fibrotic [15], anti-virus [16] and anti-cancer effects, including anti-breast cancer [17], anti-lung cancer [18,19], anti-hepatoma [20], anti-glioblastoma [21], anti-nasopharyngeal carcinoma, anti-melanoma [22] and anti-gastric cancer [23] effects, which stimulates researchers' interest in developing brevilin A or its derivatives as novel anti-cancer drug(s) [24]. In the future, whether it can be developed into a drug for clinical treatment of tumors needs further study. In addition, a study also suggests that brevilin A could induce apoptosis in CT26 cells [25], however, the underlying mechanism of action is not well understood. In this study, we aimed to explore the anti-CRC effects of brevilin A in different CRC cell lines, and examined whether STAT3 signaling was involved.

2. Materials and methods

2.1. Cell culture and reagents

All cell lines were purchased from EK-Biosciences (Shanghai, China). HUVECs (Human umbilical vein endothelial cells) were cultured in McCoy's 5A. CT26 and HCT-116 cells were cultured in DMEM supplement with 1% penicillin/streptomycin (GIBCO, USA) containing 10% fetal bovine serum (GIBCO, USA). Cells were cultured in a humidified environment at 37 °C with 5% CO_2. Brevilin A (purity >98%, chemical structure was shown in Fig. 1A) was purchased from Chengdu Desite Biotechnology Co. Ltd. (Sichuan Provience, China). SPF eggs (fertile) were obtained from Guangdong Dahuanong Animal Health Products Co. Ltd. (Guangdong Provience, China). Antibodies against GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PCNA (proliferating cell nuclear antigen), MMP-2 (matrix metalloproteinase-2), Bcl-xL (B-cell lymphoma-extra large) and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against VEGF (vascular endothelial growth factor) was obtained from Affinity Biosciences (Jiangsu Provience, China). Antibodies against phospho-STAT3 (Tyr705), STAT3, phospho-JAK2 (janus kinase 2) (Y1007/1008), JAK2, phospho-Src (proto-oncogene tyrosine-protein kinase Src) (Tyr 416) and Src were purchased from the Cell Signaling Technology (Beverly, MA, USA). Recombinant human $VEGF_{165}$ was obtained from PeproTech Inc. (Rocky Hill, NJ, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma (St. Louis, MO, USA). Human Phospho-RTK array kit was obtained from R&D Systems (Minneapolis, USA).

2.2. Cell viability

Based on previously described methods [22], HCT-116 and CT26 cells were used to test the cytotoxicity of brevilin A by the MTT assay. Cells (4000 cells/well) were seeded in 96-well plates and treated with various concentrations of brevilin A (2.5, 5, 10 μM) or vehicle for 24 or 48 h. In each well, 20 μL of MTT solution (5 mg/ml) were added and incubated at 37 °C for another 4 h, and the medium was then removed, the crystals were dissolved in 200 μL of dimethylsulfoxide (DMSO). Finally, the absorbance in each well was conducted at 570 nm using a microplate spectrophotometer (BD Biosciences, USA).

2.3. Annexin V-FITC/PI apoptosis assay

HCT-116 and CT26 cells were exposed to brevilin A (2.5, 5, 10 μM) for 24 h, and then harvested and stained with Annexin V-FTIC/PI as directed by the manufacturer. FACS (fluorescence-activated cell sorting) flow cytometers (BD Biosciences, USA) was used for the flow cytometric analysis.

2.4. EdU (5-Ethynyl-2′- deoxyuridine) staining

The indicated concentrations of brevilin A (2.5, 5, 10 μM) were added to treat HCT-116 and CT26 cells for 24 h. Cells were stained with EdU according to the experimental protocol (RiboBio), which has been described previously [26]. The EdU positive cells were

counted using Image J software V1.44.

2.5. Preparation of nuclear and cytoplasmic fractions

The indicated concentrations of brevilin A (2.5, 5, 10 μM) were added to treat HCT-116 and CT26 cells for 24h. After that, cells were collected. Nuclear Extraction Kit (Abcam) was used to prepare cytoplasmic and nuclear extracts.

2.6. Plasmid transient transfection

pcDNA3.1 EV and pcDNA3.1-STAT3 plasmids were obtained from GenePharma Co., Ltd (Shanghai, China). The manufacturer's protocol was followed to transfect plasmids into HCT-116 cells using Lipofectamine 3000 (ThermoFisher Scientific, USA). Plasmid transfection method was prepared as described previously [27]. To perform functional assays, cells were transfected with indicated plasmids for 48 h.

2.7. Cell migration and invasion assays

24-well Transwell chambers (8 μm pores, Corning, USA) were used to determine cell migration and invasion. Matrigel solution (80 μL) was placed in the wells of an ice-cold 24-well plate. Then the plates were placed at 37 °C for 4 h. In both assays, cells were seeded in the upper chamber at 0.2 mL/well in DMEM with or without brevilin A (1.25, 2.5 μM), the lower chamber was filled with 800 μL of 10% FBS in DMEM. Invaded or migrated cells were imaged and counted by a microscope (Leica, Germany).

2.8. Endothelial cell tube formation

HUVECs tube formation assay is a common approach to evaluate the angiogenesis *in vitro* [28]. 100 μL of Matrigel was placed in 96-well plate and incubated at 37 °C for 2 h. HUVECs (3 0000 cells/well) were resuspended in 100 μL of medium supplemented with 2% FBS, containing with recombinant $VEGF_{165}$ (20 ng/mL) or not, as well as different concentrations of brevilin A (2.5, 5, 10 μM). After 3 h of incubation, the formation of lumen-like structures in HUVECs were photographed under a microscope (Leica, Germany).

2.9. Chick embryo chorioallantoic membrane (CAM) assay

CAM assay is a model for studying angiogenesis *in vivo* [29]. Fertilized chicken eggs were incubated at 37 °C and under 70% humidity. A window was opened in each shell after Day 10 of incubation. Brevilin A at 5 μM was added into the region between preexisting vessels, in the embryos and incubated for 6 h. After incubation, the embryos in each treatment group were photographed by Olympus digital camera (Tokyo, Japan).

2.10. TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining

HCT-116 and CT26 cells were treated with brevilin A (2.5, 5, 10 μM) for 24 h, after that, cells were fixed in 4% paraformaldehyde and stained with TUNEL kit according to the manufacturer's instructions (Elabscience). The TUNEL-positive cells were viewed under optical microscope (Zeiss, Germany), and counted as a percentage of the total cells by using the Image J software V1.44.

2.11. Western blot analysis

The indicated concentrations of brevilin A (2.5, 5, 10 μM) were added to treat HCT-116 and CT26 cells for 24h. Western blotting was used to analyze cell extracts after treatment as previously described [22]. Equal amount of each protein sample was transferred from the gel onto polyvinylidene fluoride (PVDF) membranes. Then, the PDVF membranes were directly blocked in TBS supplement with 1% (v/v) Tween-20 and 5% (v/v) BSA for 2h. The membranes were washed in TBST (TBS supplement with 1% (v/v) Tween-20) and then incubated with specific antibodies (1:1000) at overnight 4 °C. After that, the PDVF membranes were washed in TBST and then incubated with secondary antibodies (1:3000, Cell Signaling Technology, USA). Finally, protein signals were determined by ECL detection reagents (Genebase Bioscience Co., Ltd, Guangzhou, China).

2.12. Human Phospho-RTK array

Proteins were extracted from treated cells after 24 h, then the RTK array was performed according to the experimental procedure. First, we collected the cells after 5 μM brevilin A treatment, rinsed and lysed the cells, and measured their protein concentration using the protein assay. Second, the array membranes were incubated in the blocking buffer at room temperature for 2 h. Then, they were incubated at 4 °C overnight. Following that, the membrane was washed in washing buffer and incubated with the anti-phosphotyrosine-HRP detection antibody for 2 h at room temperature. Finally, the membranes were washed in washing buffer, incubated with detection reagents and exposed.

2.13. Statistical analysis

All data are presented as mean ± SD. Dunnett's multiple comparisons were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA) and statistical significance was determined by one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Brevilin A reduced viability, inhibited proliferation and induced apoptosis of CRC cells

To explore the effects of brevilin A on CRC cell viability, we performed the MTT assay. Three concentrations of brevilin A (2.5, 5, 10 μM) were added to HCT-116 and CT26 cells for 24 or 48 h respectively. Results showed that brevilin A time- and dose-dependently decreased the viability of HCT-116 and CT26 cells (Fig. 1B). 10 μM of brevilin A caused 37.7% and 59.3% reduction in the cell viability after 24 h and 48 h treatment in HCT-116 cells, respectively; 10 μM of brevilin A caused 44.4% and 54.1% reduction in the cell viability after 24 h and 48 h treatment in CT26 cells, respectively. The EdU assay showed that brevilin A inhibited the proliferation of CRC cells (Fig. 1C). Moreover, flow cytometry and TUNEL assay results demonstrated that brevilin A dose-dependently induced apoptosis in both HCT-116 and CT26 cells (Fig. 1D-E).

3.2. Brevilin A inhibited CRC cell migration and invasion

The migration or invasion of CRC cells treated with or without brevilin A for 48 h were evaluated by using the Transwell assay. As

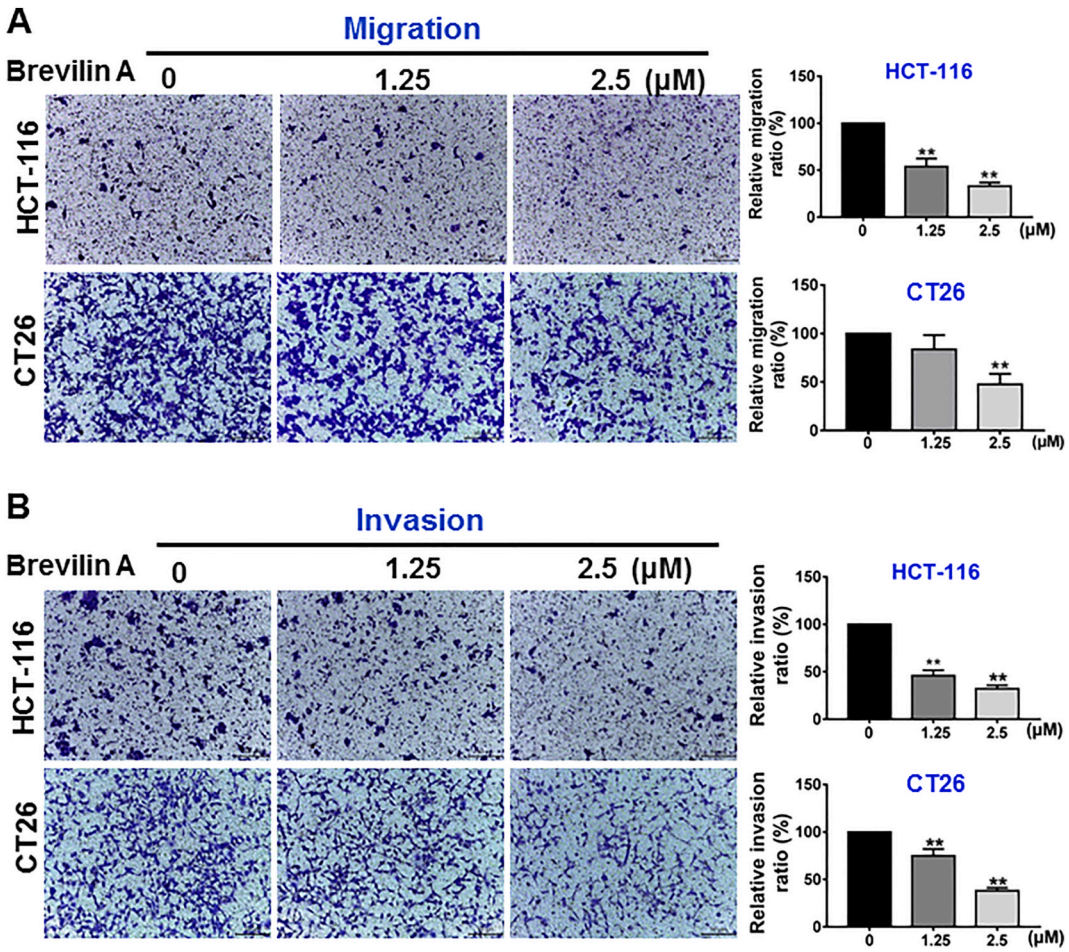

Fig. 2. Inhibitory effects of brevilin A on colorectal cancer (CRC) cells migration and invasion. (A) Representative images of cell migration after brevilin A (1.25, 2.5 μM) treatments. (B) Representative images of invasive cells after brevilin A (1.25, 2.5 μM) treatments. The quantification of migrated and invasive cells were shown in the right panel. The scale bar represents 50 μm. Data are presented as mean ± SD from three independent experiments, $^{**}P < 0.01$ *vs.* the corresponding control.

shown in Fig. 2A and B, brevilin A at the concentrations of 1.25 and 2.5 μM significantly inhibited HCT-116 and CT26 cells migration and invasion.

3.3. Brevilin A exerted anti-angiogenic activity in HUVECs tube formation and CAM assays

Tumor growth, proliferation and metastasis are closely related to angiogenesis [30]. Here, we determined the effect of brevilin A on angiogenesis by using HUVECs tube formation and CAM assays. The number of tube connections was measured to assess the tube formation ability of HUVECs [31]. As shown in Fig. 3A, we found that recombinant $VEGF_{165}$ (20 ng/mL) treated HUVECs showed higher number of tube formation when compared to that of the 2% serum medium treated HUVECs control cells. Interestingly, brevilin A significantly reduced the number of the tube formation that were induced by recombinant $VEGF_{165}$.

CAM assay is another approach to explore the vascular function [32]. There was a significant reduction in angiogenesis in CAM areas after treating with 5 μM of brevilin A. As a result, affected areas presents an avascular CAM structure with only a few small capillaries. These small vessels were thinner, and showed fewer branches than those in the unaffected areas (Fig. 3B). All these results suggest that brevilin A exerts anti-angiogenesis activity both *in vitro* and *in vivo*.

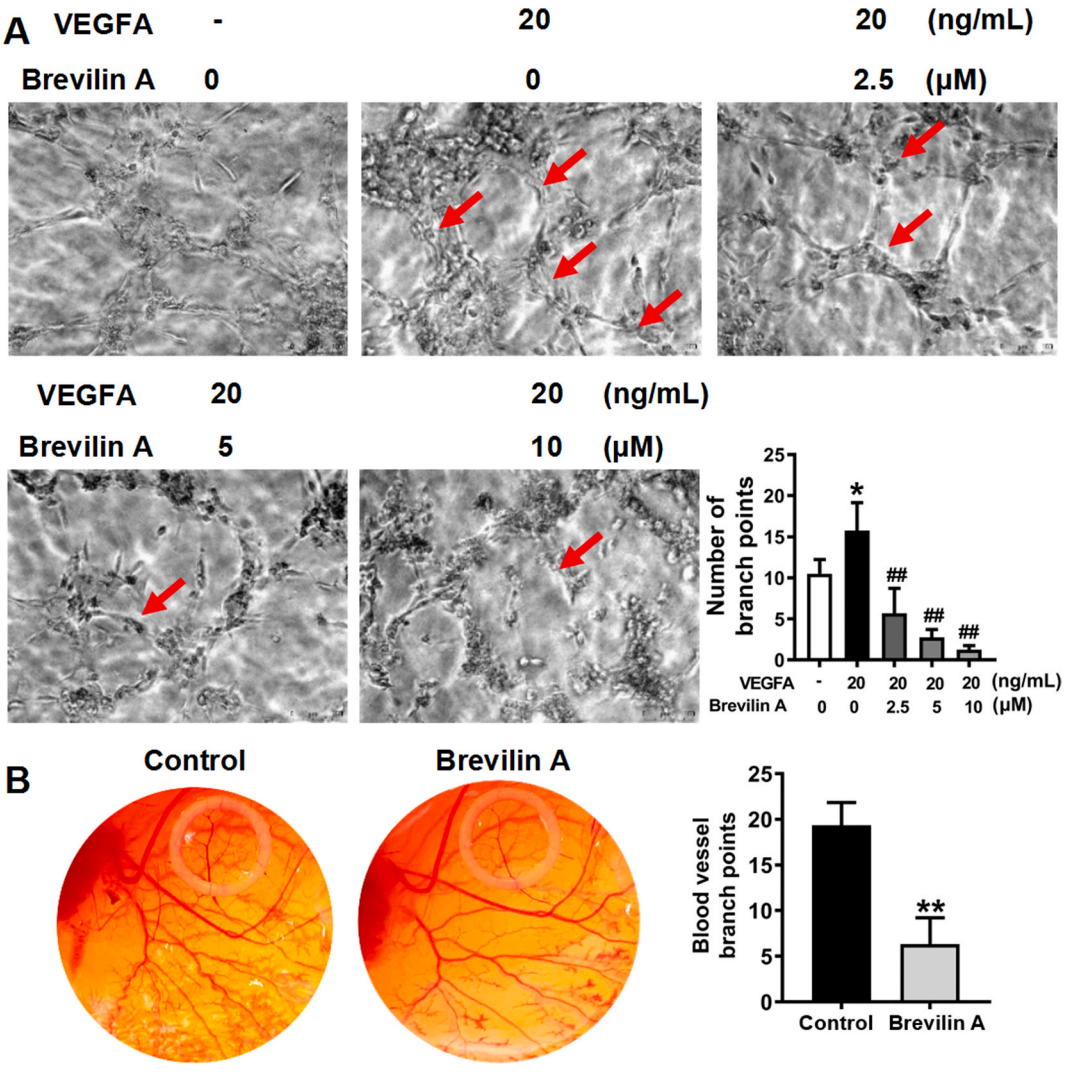

Fig. 3. Effects of brevilin A on angiogenesis both *in vivo* and *in vitro*. (A) Effect of brevilin A (2.5, 5, 10 μM) on capillary tube structures in human umbilical vein endothelial cells (HUVECs). HUVECs suspended in different concentrations of brevilin A containing media were seeded onto a growth factor-reduced Matrigel. Recombinant vascular endothelial growth factor (VEGF, 20 ng/mL) was added to stimulate capillary-like tube formation. Representative images after brevilin A treatment were captured by using Leica microscope. Red arrow represents the tube-like structure(s). The scale bar represents 100 μm. $^{*}P < 0.05$ *vs.* vehicle (Control). $^{\#\#}P < 0.01$ *vs.* Recombinant $VEGF_{165}$-treated cells (VEGF). (B) Effect of brevilin A on angiogenesis in chorioallantoic membrane (CAM) assay. CAM was photographed with a camera. Data are presented as mean ± SD from three independent experiments, $^{**}P < 0.01$ *vs.* the corresponding control.

3.4. Brevilin A inhibited STAT3 activity and reduced nuclear expression in CRC cells

It was found that brevilin A mainly affected the STAT3 signaling. Hence, Western blot analysis was performed to investigate whether brevilin A modulated STAT3 activation. In cultured HCT-116 and CT26 cells, brevilin A reduced STAT3 phosphorylation, but not the total STAT3 levels (Fig. 4A). In HCT-116 cells, when compared with control, the levels of p-STAT3/STAT3 were decreased about 24.7%, 72.7% and 79.7% after treated with 2.5, 5 and 10 μM brevilin A, respectively. While, in CT26 cells, the inhibition rates of 2.5, 5 and 10 μM of brevilin A were 34.8%, 37.2% and 53.3%, respectively. Src and JAK2 are upstream tyrosine kinases of STAT3 [33]. As shown in Fig. 4C and D, brevilin A significantly inhibited the protein levels of phospho-JAK2 (Y1007/1008) and phospho-Src (Tyr416), but not JAK2 and Src in CRC cells (Fig. 4B). Take the phospho-JAK2 (Y1007/1008)/JAK2 level for example, in HCT-116 cells, the inhibition rates of brevilin A (2.5, 5 and 10 μM) were 29.9%, 26.2% and 57.5%, respectively; while, in CT26 cells, the inhibition rates of brevilin A (2.5, 5 and 10 μM) were 23.1%, 44.9%, 41.4%, respectively. After phosphorylation, STAT3 homodimersafter phosphorylation and translocates into the nucleus to regulate the transcription of target genes. Here, the effect of brevilin A on STAT3 nuclear localization was also examined. As expected, protein levels of STAT3 in the nuclear fractions of CRC cells were significantly down-regulated 31.2% in HCT-116 cells, and 34.1% in CT26 cells after 10 μM of brevilin A treatment, respectively (Fig. 4C), suggesting that brevilin A inhibits the STAT3 signaling in CRC cells.

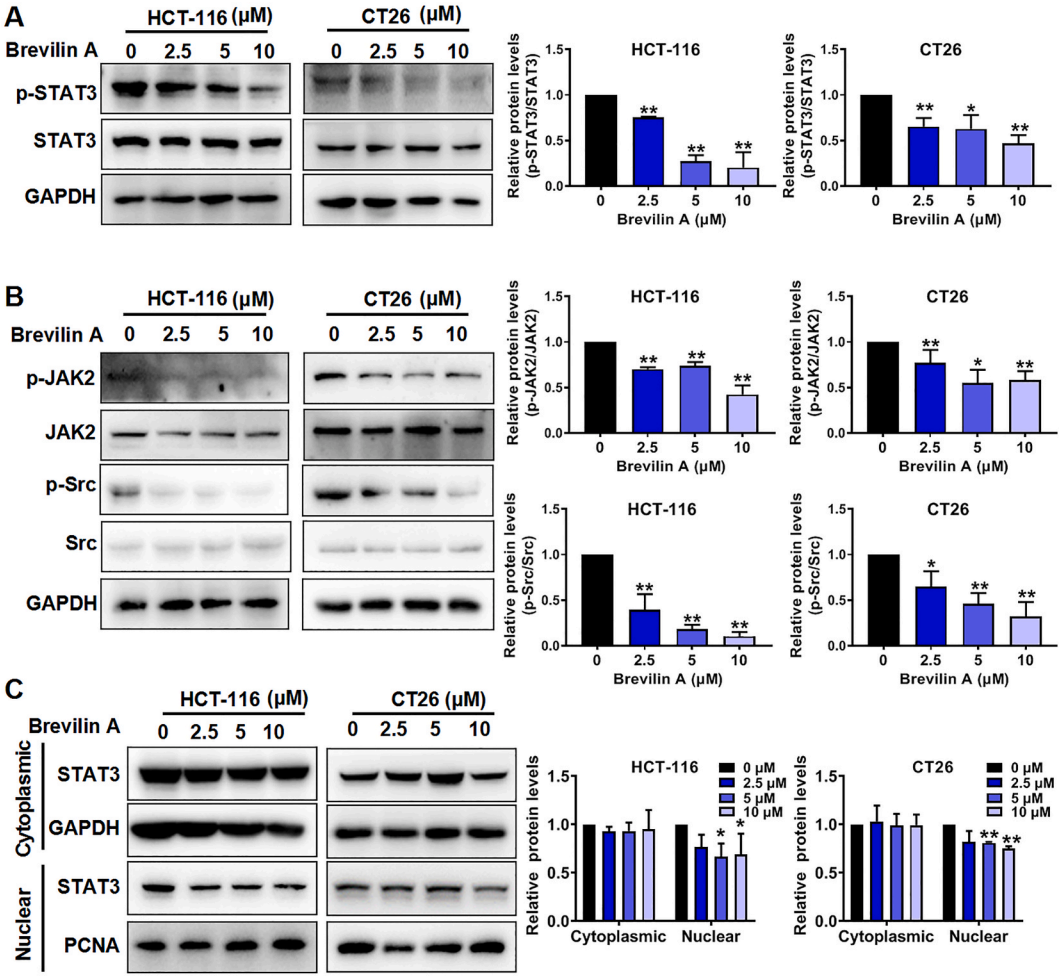

Fig. 4. Brevilin A inhibited signal transducer and activator of transcription 3 (STAT3) signaling in colorectal cancer (CRC) cells. HCT-116 and CT26 cells were treated with indicated concentrations of brevilin A (2.5, 5, 10 μM) or vehicle for 24 h. Total cell lysates were extracted for Western blot analyses using antibodies specific to phospho-STAT3 (Tyr705) and STAT3 (A), phospho-JAK2 (Tyr1007/1008) and JAK2 (B), phospho-Src (Tyr 416) and Src (C). (D) Brevilin A decreased STAT3 nuclear localization in CRC cells. Protein levels of STAT3 in cytoplasmic and nuclear extracts were examined by immunoblotting. The representative results (left panel); and quantitative results analyzed using Image J software (right panel) were shown. Data are presented as mean ± SD from three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the corresponding control.

3.5. Brevilin A reduced STAT3 downstream related protein levels

Bcl-xL, MMP-2 and VEGF are the downstream target genes of STAT3, which contribute to cell survival, cell migration, invasion, and angiogenesis, respectively [34,35]. Here, we found that 10 μM of brevilin A decreased the protein levels of Bcl-xL about 63.2% in HCT-116 cells and 45.8% in CT26 cells, respectively (Fig. 5A); it also down-regulated the protein levels of MMP-2 about 47.2% in HCT-116 cells and 43.2% in CT26 cells (Fig. 5B); down-regulated the protein levels of VEGF about 54.7% in HCT-116 cells and 74.6% in CT26 cells, respectively (Fig. 5C). All these results suggested that brevilin A reduces STAT3 downstream survival-, migration- and angiogenesis-related protein levels.

3.6. Overexpression of STAT3 in HCT-116 cells diminished the inhibitory effects of brevilin A on cell viability

To confirm that brevilin A's anti-CRC effects are STAT3-dependent, HCT-116 cells were overexpressed with STAT3. Overexpression of STAT3 in HCT-116 cells was validated by Western blotting (Fig. 6A). More importantly, overexpression of STAT3 significantly diminished the cytotoxic effect of brevilin A (Fig. 6B), and the inhibition rate was changed from 62.3% to 78.8% at 10 μM of brevilin A, suggesting that STAT3 signaling is involved in the anti-CRC effects of brevilin A.

4. Discussion

CRC is one of the most common malignancies worldwide. The current treatment options in CRC including surgery, chemotherapy, targeted therapy and immunotherapy, etc [36]. However, their limitations and adverse events have been reported, such as anaphylaxis, toxicity and asthenia, etc. The active compounds in traditional Chinese herbal medicines possess many advantages, such as diverse structural features and biological activities, availability of in a wide range of sources, and low toxicity and side effects. Therefore, they are the important natural sources for the discovery of new anticancer drugs [37,38]. It is reported that many active components have potent anti-cancer activities. For example, garcinol, a natural compound derived from *Gambogic genera*, inhibits the metastasis of esophageal cancer by inhibiting p300 and TGF-β1 signaling pathways [39]. Icariin, a natural compound isolated from Epimedii Folium could induce apoptosis and inhibit the migration of breast cancer cells by inhibiting the SIRT6/NF-κB signaling pathway [40].

Studies have reported that there are abundant sesquiterpene lactones in Centipedae Herba, which possess strong anticancer properties [41–43]. Brevilin A is an abundant constituent in the medicinal herb *Centipeda minima* (L.) A. Br. et Aschers. In this study, brevilin A was obtained by commercial products from Chengdu Desite Biotechnology Co. Ltd. (Sichuan Provience, China). Previous

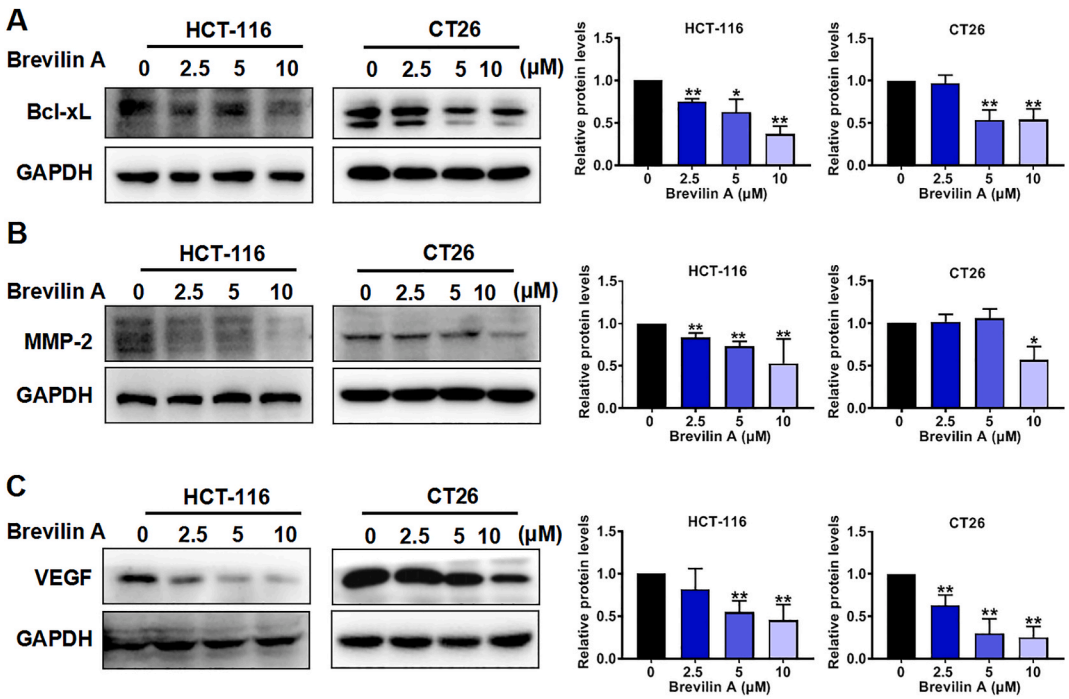

Fig. 5. Brevilin A downregulated protein levels of signal transducer and activator of transcription 3 (STAT3) target genes. Total cell lysates were extracted for Western blot analyses using antibodies specific to Bcl-xL (A), MMP-2 (B) and VEGF (C). The representative results (left panel); and quantitative results analyzed using Image J software (right panel) were shown. Data are shown as mean ± SD from three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the corresponding control.

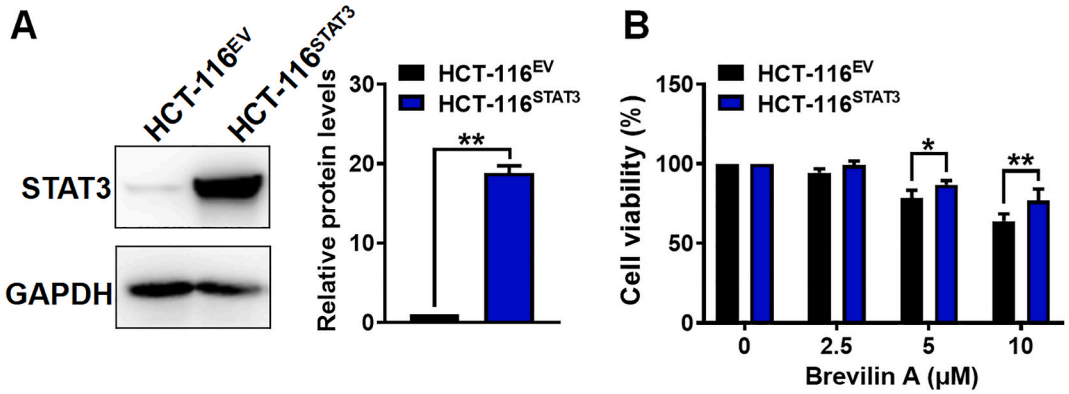

Fig. 6. Overexpression of STAT3 in HCT-116 cells diminished the effects of brevilin A on cell viability. (A) Protein levels of STAT3 in HCT-116 transfected with pcDNA3.1-EV or pcDNA3.1-STAT3 plasmid. (B) HCT-116^{EV} or HCT-116^{STAT3} cells were treated with brevilin A for 24 h. Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Data are shown as mean ± SD from three independent experiments. *$P < 0.05$, **$P < 0.01$ *vs.* the corresponding HCT-116^{EV}. EV: empty vector.

studies have reported the isolation and purification methods [44,45]. In this study, the purity of brevilin A is more than 98%.

STATs play a vital role in inflammation, cellular responses to cytokines involved in carcinogenesis [46]. Studies has demonstrated that STAT3 is a key transcription factor, which plays a key role in CRC occurrence and development [47]. One of the major obstacles in the targeted treatment for cancer is the serious side effects caused by on- and off-target reactions [48]. On-target side effects are associated with pharmacological activity on normal tissues, while off-target side effects are characterized as toxicity due to unexpected or unknown functions [49]. For cancer therapy, some STAT3 inhibitors have been approved in clinical trials, such as WP1066, TTI-101, and OPB-51602 [50]. However, several clinical trials were stopped due to their adverse events, for example, nausea, fatigue and elevated blood pressure have been reported in these clinical studies, which limit their clinical translation [51]. Increasing studies have demonstrated that several low-toxicity, high-potency natural compounds can effectively target STAT3 and treat tumors, such as artesunate and curcumin [10].

In this study, we found that brevilin A inhibited CRC cell viability, suppressed cell proliferation, reduced cell migration and invasion, induced apoptosis in both HCT-116 and CT26 cells. Moreover, we observed that there was a decrease in the phosphorylation of

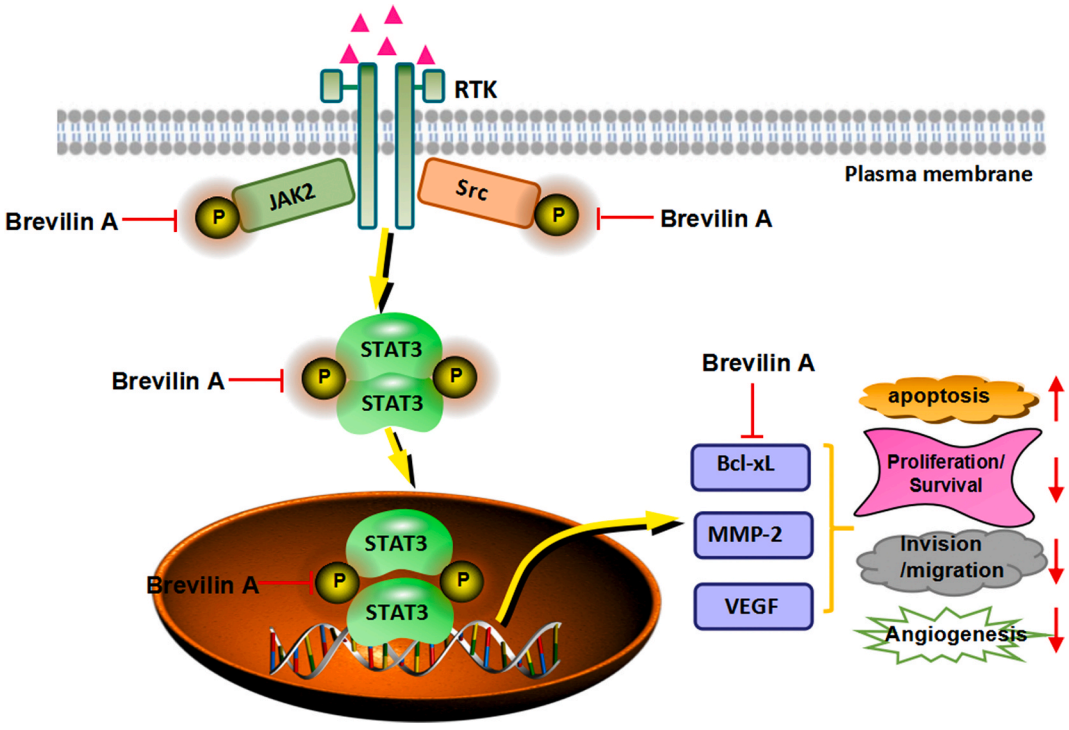

Fig. 7. Schematic diagram of this study.

STAT3 in CRC cells after brevilin A treatment. There was also a dose-dependent reduction in STAT3 nuclear localization in CRC cells after brevilin A treatment. Moreover, activation of STAT3 significantly diminished brevilin A's cytotoxic effects. Fig. 7 shows the schematic diagram of this study. At present, one study showed that brevilin A also induced apoptosis of CRC cells by increasing reactive oxygen species (ROS) levels [25]. It is known that each active compound has multi-target properties. In the future, we will further explore whether there is a relationship between ROS and STAT3 activity, as well as the mechanism underlying the enhanced ROS levels after brevilin A treatment with both *in vitro* and *in vivo* studies.

It has been demonstrated that receptor tyrosine kinases (RTKs) and their downstream signaling pathways play important roles in the development of CRC [52,53]. STAT3 can also be activated by a variety of RTKs besides Src and JAK2 [33,54]. In order to examine whether brevilin A inhibited STAT3 phosphorylation via RTKs, the brevilin A-treated HCT-116 cells were examined using a Phospho-RTK array kit. Results showed that brevilin A down-regulated 14 phospho-RTKs in HCT-116 cells. Among these, brevilin A exerted the most potent inhibitory effect on epidermal growth factor receptor (EGFR) (Supplementary Fig. S1). In CRC proliferation and metastasis, EGFR plays an important role as an upstream regulator of STAT3. In future studies, it will be interesting to explore whether brevilin A inhibits the EGF (a ligand of EGFR)-induced activation of STAT3 signaling and reduces the proliferation and invasion of CRC cells under EGF stimulation.

In summary, we found that brevilin A reduced CRC cells proliferation and viability, induced apoptosis, reduced cells migration and invasion, inhibited angiogenesis. Moreover, brevilin A suppressed STAT3 nuclear localization, reduced the phosphorylation of STAT3 and its two upstream Src and JAK2, down-regulated STAT3 target genes. More importantly, activation of STAT3 significantly diminished the cytotoxic effects of brevilin A. Taken together, our study suggests that the anti-CRC action of brevilin A is at least in part, attributed to the inhibition of STAT3 signaling. These findings of this study provide a pharmacological basis for developing brevilin A as a novel STAT3-targeting phytotherapeutic agent for the treatment of CRC.

Author contributions

Mingjing Meng; Jincheng Tan; Hui Chen; Zhiqiang Shi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hiu-Yee Kwan; Tao Su: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18488.

A list of abbreviations

Bcl-xL	B-cell lymphoma-extra large
CRC	Colorectal cancer
CAM	Chorioallantoic membrane
DMSO	Dimethylsulfoxide
EV	Empty vector
EGFR	Epidermal growth factor receptor
FACS	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

HUVECs Human umbilical vein endothelial cells
JAK2 Janus kinase 2
MMP-2 Matrix metalloproteinase-2
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Phospho-RTK Phospho-receptor tyrosine kinase
PCNA Proliferating cell nuclear antigen
PVDF Polyvinylidene fluoride
RTKs Receptor tyrosine kinases
ROS Reactive oxygen species
STAT3 Signal transducer and activator of transcription 3
Src Proto-oncogene tyrosine-protein kinase Src
TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF Vascular endothelial growth factor

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