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Research Article

Effects and Mechanisms of Fucoxanthin from *Hizikia fusiforme* on Inhibiting Tongue Squamous Cell Carcinoma Proliferation via AKT/mTOR-Mediated Glycolysis

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Fucoxanthin, a natural carotenoid, contains special ene bonds and 5, 6-mono-epoxide compounds in its molecule, which has physiological activities such as anticancer, antioxidant, anti-inflammatory, antiobesity, and antidiabetic. However, the anticancer actions and underlying mechanisms of fucoxanthin on oral cancer remain to be assessed. In this study, we used tongue carcinoma (CAL-27) cells to examine the effects and underlying mechanisms of fucoxanthin derived from *Hizikia fusiforme* on CAL-27 proliferation. MTT assays were used to estimate fucoxanthin's effect on the proliferative capacity of CAL-27 cells. Flow cytometry was used to examine how fucoxanthin affects apoptosis initiation and alters cell cycle progression in CAL-27 cells. Spectrophotometry was used to assess the impact of fucoxanthin on adenosine triphosphate (ATP) generation, glucose uptake, lactate production, and the enzymatic activities of pyruvate kinase and hexokinase in CAL-27 cells. Western blotting was used to investigate fucoxanthin's impact on the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway and glycolysis-related protein expression levels in CAL-27 cells. Fucoxanthin significantly inhibited CAL-27 cell viability time- and dose-dependently ($p < 0.01$). Administration of fucoxanthin induced CAL-27 cell apoptosis and cell cycle arrest at the G1 phase ($p < 0.05$). Furthermore, fucoxanthin significantly decreased glucose uptake, lactate production, and ATP production in CAL-27 cells ($p < 0.01$) and significantly inhibited glycolysis-related enzyme activities and protein expression levels ($p < 0.05$). Fucoxanthin also substantially decreased the expression levels of phosphorylated ribosomal protein S6, phosphorylated AKT, and phosphorylated mTOR ($p < 0.05$). In conclusion, fucoxanthin inhibited CAL-27 cell proliferation, and this mechanism may be associated with the AKT/mTOR-mediated glycolysis pathway.

1. Introduction

Oral cancer is a threat to global public health and accounts for 1%–2% of all cancers [1–3]. It is closely related to factors such as frequent consumption of alcohol and hot foods, smoking, and a history of oral diseases. Tongue squamous cell carcinoma (TSCC) is the most prevalent oral cancer. It can be transferred to organs such as the lungs in late-stage TSCC, and lymph node metastasis is common [4]. Currently, tongue cancer is treated mainly via comprehensive

surgical sequences [5]. Although treatment methods and technologies have improved, the mortality rate has not decreased [6].

Many Chinese monomers inhibit tumor growth [7, 8]. Drug components in natural plants cause few adverse reactions owing to their single components and thus allow for a wide range of treatments. Hence, they can be used as auxiliary or alternative methods for targeted treatment of tongue cancer. Therefore, finding high-efficiency traditional Chinese medicine monomers to treat tongue cancer has become a research focus.

Aberrant modifications in energy metabolism are a prominent characteristic of tumor cells, pushing them towards aerobic glycolysis as a primary means of energy generation [9]. Research has demonstrated that inhibiting the tumor glycolysis pathway effectively impedes tumor cell proliferation and induces tumor cell death. Additionally, studies have confirmed that the protein kinase B (AKT)/mammalian target of the rapamycin (mTOR) pathway facilitates the crucial expression of enzymes involved in the glycolysis pathway, resulting in enhanced glucose uptake by tumor cells and dysregulated glycolytic activity [10–12]. This phenomenon contributes to increased energy supply, tumor growth promotion, and uncontrolled tumor cell proliferation. These studies indicate that the AKT/mTOR signaling pathway is significantly associated with glycolytic metabolism. Hence, a potential therapeutic strategy for treating TSCC may involve targeting the glycolytic pathway, controlled via the AKT/mTOR signaling pathway, as well as attenuating tumor cell energy supply.

Hizikia fusiforme is a plant of the Sargassum family of the phylum Ochrophyta, which has been used as a drug to treat diseases for thousands of years in China and Japan [13]. Research has demonstrated that *Hizikia* extract exhibits diverse effects, including antitumor, antifatigue, antiradiation, antiviral, antiaging, and immunomodulatory properties [14–16]. Fucoxanthin is the main active ingredient in hijiki. Numerous studies have highlighted the ability of fucoxanthin to impede tumor cell growth by promoting apoptosis, inhibiting DNA synthesis, and arresting the cell cycle. Notably, fucoxanthin significantly inhibits various cancers, including cervical, breast, gastric, liver, and lung cancers. Bae et al. found that fucoxanthin partially attenuated alterations in gene expression associated with glycolysis and mitochondrial respiration involving hexokinase, peroxisome proliferator-activated receptor γ coactivator 1β, and pyruvate dehydrogenase kinase 3 [17]. Nevertheless, insufficient data exist on the regulatory effects of fucoxanthin, specifically on TSCC. Our findings will contribute to theoretical and experimental foundations for potential applications of fucoxanthin isolated from hijiki (Figure 1) for preventing and treating TSCC.

2. Materials and Methods

2.1. Cell Cultures. The human TSCC cell line, CAL-27, was supplied by Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). CAL-27 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Solaribo, Beijing, China). CAL-27 cells were cultured in a carbon dioxide (CO_2) incubator at 37°C with 5% CO_2.

2.2. Effect of Fucoxanthin on CAL-27 Cell Viability. CAL-27 cells in the logarithmic growth phase were aseptically cultured by seeding into 96-well plates at 5000 cells/well. The cells were divided into the drug, control (no drug), and blank (blank medium only) groups. After a 24-hour

incubation, the drug group was treated with various concentrations of fucoxanthin, and the control group received an equivalent volume of untreated medium. Six replicate wells were prepared per dose. After further incubation for 0, 6, 12, 18, and 24 hours, each well was supplemented with 10 μL of MTT solution (Solaribo, Beijing, China). The cells were then incubated for 4 hours, and the absorbance (A) at 570 nm was estimated using a microplate reader.

2.3. Effects of Fucoxanthin on CAL-27 Cell Ability to Form Colonies. CAL-27 cells in the logarithmic growth phase were aseptically cultured by seeding into 6-well plates at 5000 cells/well and then incubated for 24 hours. The experiment consisted of a control group (untreated) and low-, medium-, and high-dose administration groups (3, 6, and 9 μg/mL, respectively), with three replicate wells per group. After incubating for 24 hours, the cell culture media were replenished every other day, and the cells were cultured for 14 days until visible colonies formed. After removing the cell culture medium, the wells were washed three times with phosphate-buffered saline (PBS). Subsequently, the cells were fixed in a 4% paraformaldehyde solution for 15 minutes and then stained with a 0.5% crystal violet solution for 15 minutes. After discarding the staining solution, the wells were rinsed with distilled water, and the excess water was drained. Photographs of the colonies in each well were captured using a camera and counted under a microscope.

2.4. Effect of Fucoxanthin on CAL-27 Apoptosis. CAL-27 cells in the logarithmic growth phase were aseptically cultured by seeding into 12-well plates at 5×10^4 cells/mL and then incubated for 24 hours. After a 24-hour incubation with 0, 3, 6, or 9 μg/mL fucoxanthin, the cells were collected and suspended in 100 μL double-staining incubation solution. Next, 5 μL of annexin V-FITC staining solution and 5 μL of propidium iodide (PI) staining solution (BD, Tokyo, Japan) were added to the cell suspension. Subsequently, the cells were incubated in the dark at room temperature for 15 minutes before detection. Apoptosis was assessed using flow cytometry (BD FACSVerse, Franklin Lakes, NJ, USA), and the proportions of early- and late-stage apoptotic cells were quantified.

2.5. Effect of Fucoxanthin on CAL-27 Cell Cycle. CAL-27 cells in the logarithmic growth phase were aseptically cultured by seeding into 6-well culture plates at 5×10^4 cells/mL and then incubated for 24 hours. After incubation with 0, 3, 6, or 9 μg/mL fucoxanthin for 24 hours, the cells were rinsed twice with cold PBS. Subsequently, cells were detached and fixed by adding 1 mL of 70% ethanol at 4°C for at least 18 hours. After two washes with precooled PBS, 0.5 mL of RNAseA/PI dye solution (BD, Tokyo, Japan) was applied and left for 20 minutes at room temperature. The cell cycle distribution in each group was investigated using flow cytometry.
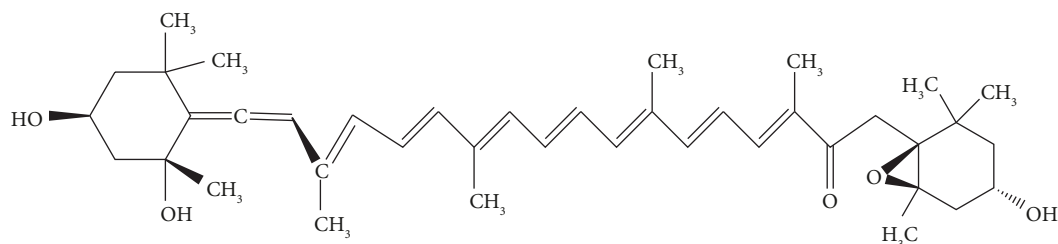
FIGURE 1: Chemical structure of fucoxanthin isolated from hijiki.

2.6. Effect of Fucoxanthin on CAL-27 Cell Mitochondrial Membrane Potential.

CAL-27 cells in the logarithmic growth phase were aseptically cultured by seeding into 6-well culture plates at 5×10^4 cells/mL and then incubated for 24 hours. After 24 hours of incubation with 0, 3, 6, or 9 µg/mL fucoxanthin, the cells were washed twice with PBS and then treated with JC-1 (BD, Tokyo, Japan) for 30 min in the dark at 37°C and 5% CO_2. The results were analyzed using flow cytometry.

2.7. Effect of Fucoxanthin on Glucose Uptake, Adenosine Triphosphate (ATP) Production, Lactate Production, and ROS in CAL-27 Cells.

CAL-27 cells in the logarithmic growth phase were aseptically cultured by seeding into 6-well culture plates at 5×10^4 cells/mL and then incubated for 24 hours. After exposure to fucoxanthin, the cells were harvested, and a small amount of cell suspension was used to determine the cell count using a cell counter. Glucose consumption, ATP production, and lactic acid production (Solaribo, Beijing, China) were assessed by measuring the absorbance at specific wavelengths. Absorbance readings were obtained at 555 nm for glucose, 570 nm for lactic acid, and 340 nm for ATP, adhering to the manufacturer's guidelines and using a microplate reader. The glucose consumption, ATP production, and lactic acid production were calculated from the obtained absorbance values. Measurement of intracellular ROS was performed using 2, 7-dichlorofluorescin-diacetate (Solaribo, Beijing, China). For flow cytometry, cells were collected for analysis.

2.8. Effect of Fucoxanthin on Hexokinase and PK Activity in CAL-27 Cells.

CAL-27 cells in the logarithmic growth phase were aseptically cultured by seeding into 6-well culture plates at 5×10^4 cells/mL and then incubated for 24 hours. After treatment with fucoxanthin, the cells were harvested, and a small amount of cell suspension was used to count the cells using a cell counter. The enzymatic activities of hexokinase and PK (Solaibo, Beijing, China) were evaluated by quantifying the absorbance at 340 nm using a microplate reader, adhering to the manufacturer's guidelines. The hexokinase and PK activities were calculated from the obtained absorbance values.

2.9. Effect of Fucoxanthin on the AKT/mTOR Signaling Pathway and Glycolytic-Related Gene Expression in CAL-27 Cells.

CAL-27 cells in the logarithmic growth phase were aseptically cultured by seeding into 6-well culture plates at 5×10^4 cells/mL and then incubated for 24 hours. After exposure to fucoxanthin, total cellular RNA was extracted with TRIzol. Subsequently, the RNA was solubilized in 25 µL of DEPC water, and the RNA concentration was measured using a NanoDrop spectrophotometer. Next, cDNA was synthesized per the guidelines of the reverse transcription kit (Tokyo, Japan). The reverse transcription system consisted of PrimeScript Buffer RT Enzyme Mix (1.0 µL), RT Primer Mix (1.0 µL), 5× PrimeScript Buffer 2 (for Real-time) (4.0 µL), and RNAse-free dH2O (4.0 µL). The reaction was conducted at 37°C and 85°C for 5 seconds and incubated at 95°C for 30 seconds and then subjected to 40 denaturation cycles at 95°C for 5 seconds and annealing/extension at 60°C for 30 seconds. Table 1 lists the primer amplification sequences used in the reverse transcription quantitative polymerase chain reaction (RT-qPCR).

2.10. Effect of Fucoxanthin on the AKT/mTOR Signaling Pathway and Glycolytic-Related Protein Expression in CAL-27 Cells.

CAL-27 cells in the logarithmic growth phase were aseptically cultured by seeding into 6-well culture plates at 5×10^4 cells/mL and then incubated for 24 hours. Following exposure to fucoxanthin, 150 µL of RIPA lysate supplemented with phosphatase and protease inhibitors was added to each well to extract cellular proteins. After centrifugation and supernatant collection, protein concentrations were assessed using the Bradford method and subsequently normalized. Next, protein samples totaling 30 µg were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, the proteins were transferred onto NC membranes (Millipore Co., Billerica, MA, USA), and the membranes were blocked with 5% skim milk for 2 hours. The membranes were incubated overnight and then exposed to specific antibodies: Cyclin CDK4 (ABclonal Technology, Wuhan, China), Cyclin D1 (ABclonal Technology), p21 (ABclonal Technology), AKT (ABclonal Technology), mTOR (ABclonal Technology), RPS6 (ABclonal Technology), p-AKT (ABclonal Technology), p-mTOR (ABclonal Technology), p-RPS6 (ABclonal Technology), HK (ABclonal Technology), and PKM (ABclonal Technology) diluted at 1 : 2500 and GAPDH (Cell Signaling Technology, Beverly, MA, USA) diluted at 1 : 4000. After three washes with TBST buffer solution for 10 minutes each, the membranes were incubated with suitable secondary antibodies (1 : 4000, ABclonal Technology) at room temperature for 60 minutes. After three

Genes	Sense primers (5′-3′)	Antisense primers (3′–5′)
PK	CTGTGATACGGATCAGAAACCG	TCGTGAGAAATAACAAACCACCT
Hexokinase	CAAA GTGACAGTGGGTGTGG	CTCTGTCACTTCCTGGACCG
RPS6	ATTCAGCGTCTTGTTACTCCAC	GTTCTCTTACTTCCTCCGATTCC
mTOR	GGAGCATTGAGTTGGCTCTC	CTTTGTGAAGCCTTTGTAGA
AKT	CGAGGAGGAGGTGTATCA	ATGGCTTGCACGGAAATGGC
β-Actin	CCTGGCACCCAGCACAAT	GGGCCGGACTCGTCATAC

additional washes with TBST buffer solution, the protein immunoblots were constructed using ECL chemiluminescence reagent. The protein expression levels were analyzed using a Tanon imaging system (Shanghai, China). ImageJ (MD, USA) was used for the image analysis, and the relative gray values representing the target protein expression were normalized to GAPDH, which served as an internal reference.

2.11. Statistical Analysis. Acquired data were analyzed with GraphPad Prism 7.0. Measurement data are presented as means ± standard deviation. The means of the two groups were statistically compared using t-tests. One-way analysis of variance was conducted to compare the means of several groups.

3. Results

3.1. Effect of Fucoxanthin on CAL-27 Cell Viability. Figure 2(a) shows the results of the MTT assays assessing fucoxanthin's cytotoxic effect on CAL-27 cells. CAL-27 cell viability in the groups treated with 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 g/mL fucoxanthin at 6, 12, 18, and 24 hours decreased time- and dose-dependently compared with that of the control group. Fucoxanthin concentrations below 3 μg/mL for 6, 12, and 18 h did not significantly inhibit cell activity ($p > 0.05$). However, 3 μg/mL fucoxanthin for 24 hours significantly inhibited cell viability ($p < 0.05$). After 24 hours, cell proliferation decreased dose-dependently as the fucoxanthin concentration increased (half maximal inhibitory concentration (IC$_{50}$): 6.48 g/mL). Thus, dosages of 3, 6, and 9 μg/mL for 24 hours were selected for fucoxanthin treatment. The results suggest that fucoxanthin is a potential therapeutic drug for tongue cancer, with a higher inhibitory effect on cancer cell proliferation. Various concentrations of fucoxanthin were administered to CAL-27 cells to form cell colonies. CAL-27 cells stained with crystal violet showed dark blue (Figure 2(b)). Each colony was round or oval with clear boundaries. Cell colony numbers decreased as the fucoxanthin concentration increased. CAL-27 cell colony numbers decreased significantly at 9 μg/mL fucoxanthin, indicating that fucoxanthin inhibited CAL-27 cell colony formation dose-dependently.

3.2. Effects of Fucoxanthin on Apoptosis and Mitochondrial Membrane Potential of CAL-27 Cells. The apoptotic rate of CAL-27 cells in the control group was 10.92% (Figure 3(a)). At 3 μg/mL, fucoxanthin increased the apoptosis rate of CAL-27 cells to 14.69%. At 6 μg/mL fucoxanthin, the

CAL-27 cell apoptosis rate increased significantly to 25.20% and continued to increase with the fucoxanthin concentration, reaching 39.90% at 9 μg/mL. In CAL-27 cells, early and late apoptosis rates increased when the fucoxanthin concentration increased. Thus, fucoxanthin effectively promoted CAL-27 cell apoptosis, supporting MTT assay findings on the inhibition of cell proliferation. Flow cytometry was used to determine alterations in MMP in CAL-27 cells treated with various fucoxanthin concentrations. As the fucoxanthin concentration increased, the proportion of red fluorescence in the CAL-27 cells decreased, and the proportion of green fluorescence increased (Figure 3(b)). JC-1 fluorescence results in both the FITC and PE channels in the control cells showed that 98.90% of the CAL-27 cells fluoresced red, and 0.70% of the CAL-27 cells fluoresced green. Increasing the fucoxanthin concentrations to 3, 6, and 9 μg/mL decreased the proportions of CAL-27 showing red fluorescence to 92.50%, 83.70%, and 67.40%, respectively, and increased the proportion of CAL-27 cells showing green fluorescence to 6.89%, 15.50%, and 32.20%, respectively. Thus, fucoxanthin reduced the MMP in CAL-27 cells.

3.3. Fucoxanthin Arrested the Cell Cycle of CAL-27 Cells in G1 Phase. Figure 4(a) presents the flow cytometry results of the intracellular cycle distribution in CAL-27 cells treated with different fucoxanthin concentrations. As the fucoxanthin concentration increased, the number of CAL-27 cells in the G1 phase increased significantly ($p < 0.05$), and the ratio of cells in the S to G2/M phase decreased significantly. At 9 μg/mL fucoxanthin, the number of CAL-27 cells in the G2/M phase was 0, and the S phase cell percentage was 4.49%. These findings indicate that fucoxanthin induced cell cycle arrest at the G1 phase in CAL-27 cells. Subsequently, the expression levels of G1 phase-specific Cyclin CDK4, Cyclin D1, and p21 were detected. Compared with the control group, the expression levels of CDK4 and Cyclin D1 in fucoxanthin group were decreased, and the expression level of p21 was increased (Figure 4(b)).

3.4. Fucoxanthin Inhibited Glycolysis in CAL-27 Cells. Figure 5 illustrates fucoxanthin's regulatory effects on the glycolytic pathway in CAL-27 cells. Glucose uptake, ATP generation, and lactate production measurements revealed that fucoxanthin significantly and dose-dependently inhibited these metabolic parameters in CAL-27 cells compared with those of the control group ($p < 0.05$; Figures 5(a)–5(c)). Compared with the control group, the
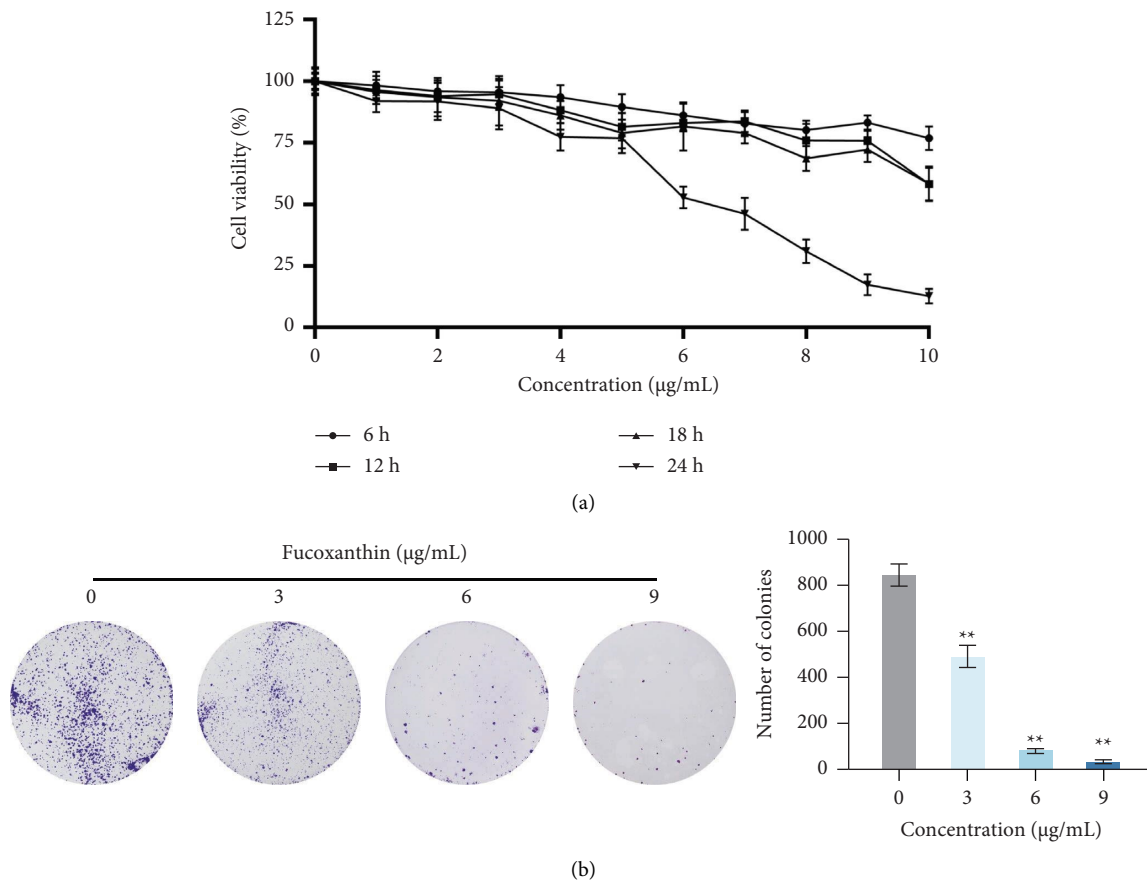
(a)

(b)

Figure 2: Effect of fucoxanthin on CAL-27 cell viability. (a) Inhibitory effect of fucoxanthin on the growth of tongue carcinoma CAL-27 cells compared with control cultures treated with DMEM. Data are presented as means ± standard deviation (SD; $n = 5$). (b) Fucoxanthin's effect on CAL-27 cell colony formation. Colony numbers were counted using ImageJ. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the control group.

content of ROS was significantly increased after intervention with 9 μg/mL fucoxanthin for 24 h (Figure 5(d)). Figures 5(e) and 5(f) show fucoxanthin's effect on glycolysis-related enzyme activities in CAL-27 cells. Hexokinase and PK activity measurements revealed that after 24 hours of fucoxanthin treatment, hexokinase and PK activities were significantly and dose-dependently decreased compared with those of the control group ($p < 0.05$). Thus, fucoxanthin effectively inhibited the activities of glycolysis-related enzymes in CAL-27 cells. These findings provide evidence of fucoxanthin's regulatory effect on the glycolytic pathway in CAL-27 cells.

3.5. Fucoxanthin Inhibited Activation of the AKT/mTOR Signaling Pathway and the Expression of Glycolysis-Related Genes in CAL-27 Cells. Evidence indicates that AKT/mTOR signaling pathways are closely related to the formation and progression of diverse types of cancers. We conducted multiple experiments measuring the expression of potential signaling molecules in response to fucoxanthin stimulation for AKT/mTOR involvement. The mRNA levels of apoptosis-related genes in CAL-27 cells were assessed via RT-qPCR measurements (Figure 6). The antiapoptosis genes

AKT, *mTOR,* and *RPS6* showed no significant changes as the fucoxanthin concentration increased ($p > 0.05$; Figures 6(a)–6(c)). Compared with those of the control group, the expression levels of the glycolysis-related enzyme genes, *hexokinase* and *PKM*, were significantly decreased in the fucoxanthin-treated group ($p < 0.05$; Figures 6(d), 6(e)), indicating that fucoxanthin inhibited these glycolysis-related enzymes' gene expression levels.

3.6. Fucoxanthin Inhibited Activation of the AKT/mTOR Signaling Pathway and the Expression of Glycolysis-Related Proteins in CAL-27 Cells. Relative expression of the proteins AKT, mTOR, and RPS6 did not change significantly as the fucoxanthin concentration increased (Figures 7(a), 7(c), and 7(f)). The antiapoptotic proteins p-AKT, p-mTOR, and p-RPS6 decreased as the fucoxanthin concentration increased. Relative expression of the antiapoptotic protein, p-AKT, decreased significantly with 3 μg/mL fucoxanthin ($p < 0.01$; Figure 7(b)). At 6 μg/mL fucoxanthin, the relative expression levels of p-mTOR and p-RPS6 decreased significantly ($p < 0.01$; Figures 7(d) and 7(g)). These findings demonstrated that fucoxanthin inhibited activation of the AKT/mTOR signaling pathway in CAL-27 cells. At >6 μg/mL
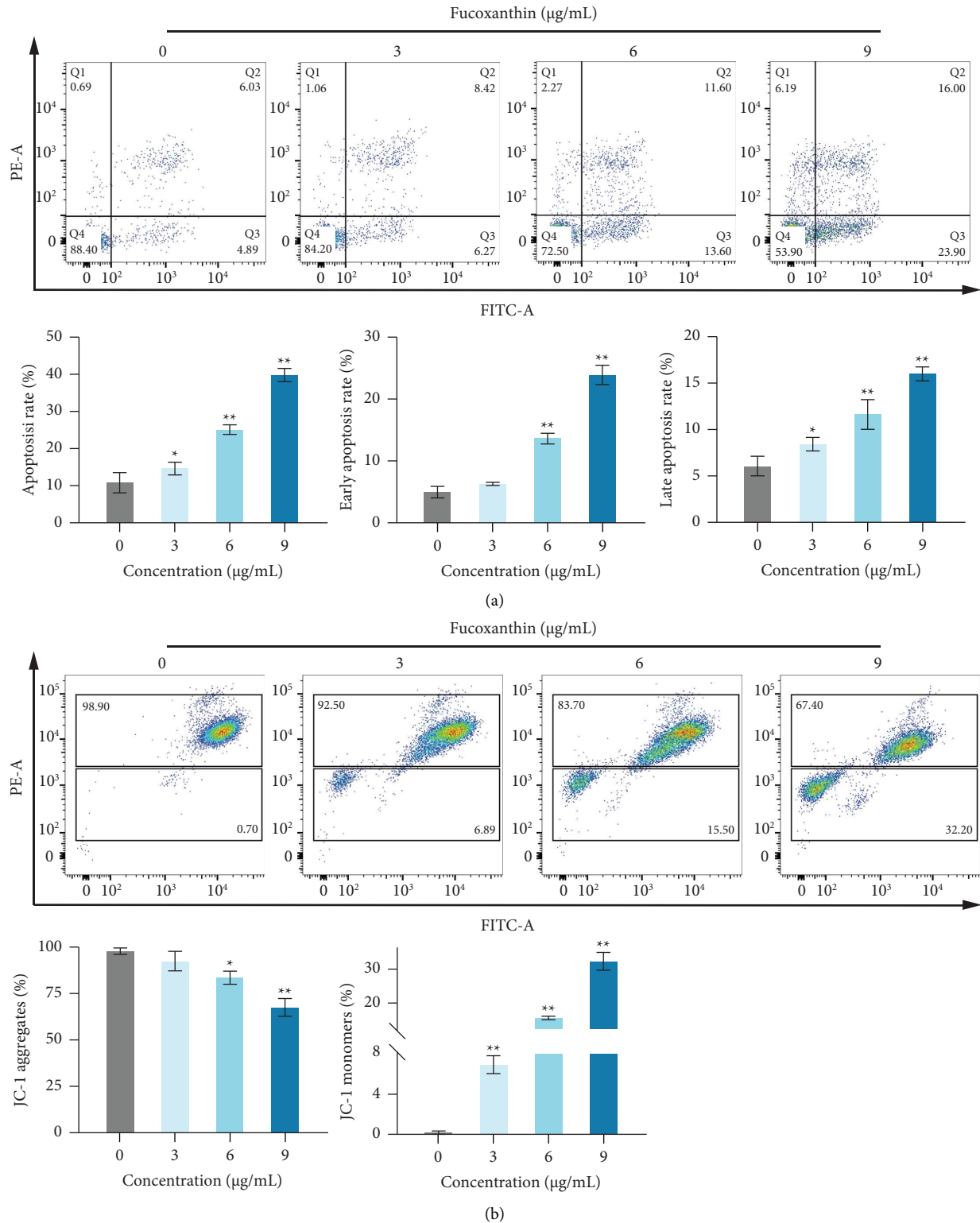
FIGURE 3: Fucoxanthin-triggered apoptosis and mitochondrial membrane potential in CAL-27 cells. Data are presented as means ± SD ($n = 3$). (a) Flow cytometry was used to evaluate apoptosis after 24 h of treatment with 0, 3, 6, and 9 μg/mL fucoxanthin. Flow cytometry analysis of CAL-27 cells divided the cell population into four quadrants based on FITC annexin V and PI staining. Q1 represented dead cells (negative for FITC annexin V and positive for PI); Q2 represented cells in the end stage of apoptosis (positive for both FITC annexin V and PI); Q3 represented cells undergoing apoptosis (positive for FITC annexin V and negative for PI); Q4 represented viable cells that did not undergo apoptosis (negative for both FITC annexin V and PI). (b) Mitochondrial membrane potential of fucoxanthin-treated CAL-27 cells. $^*p < 0.05$ and $^{**}p < 0.01$ compared with the control group.
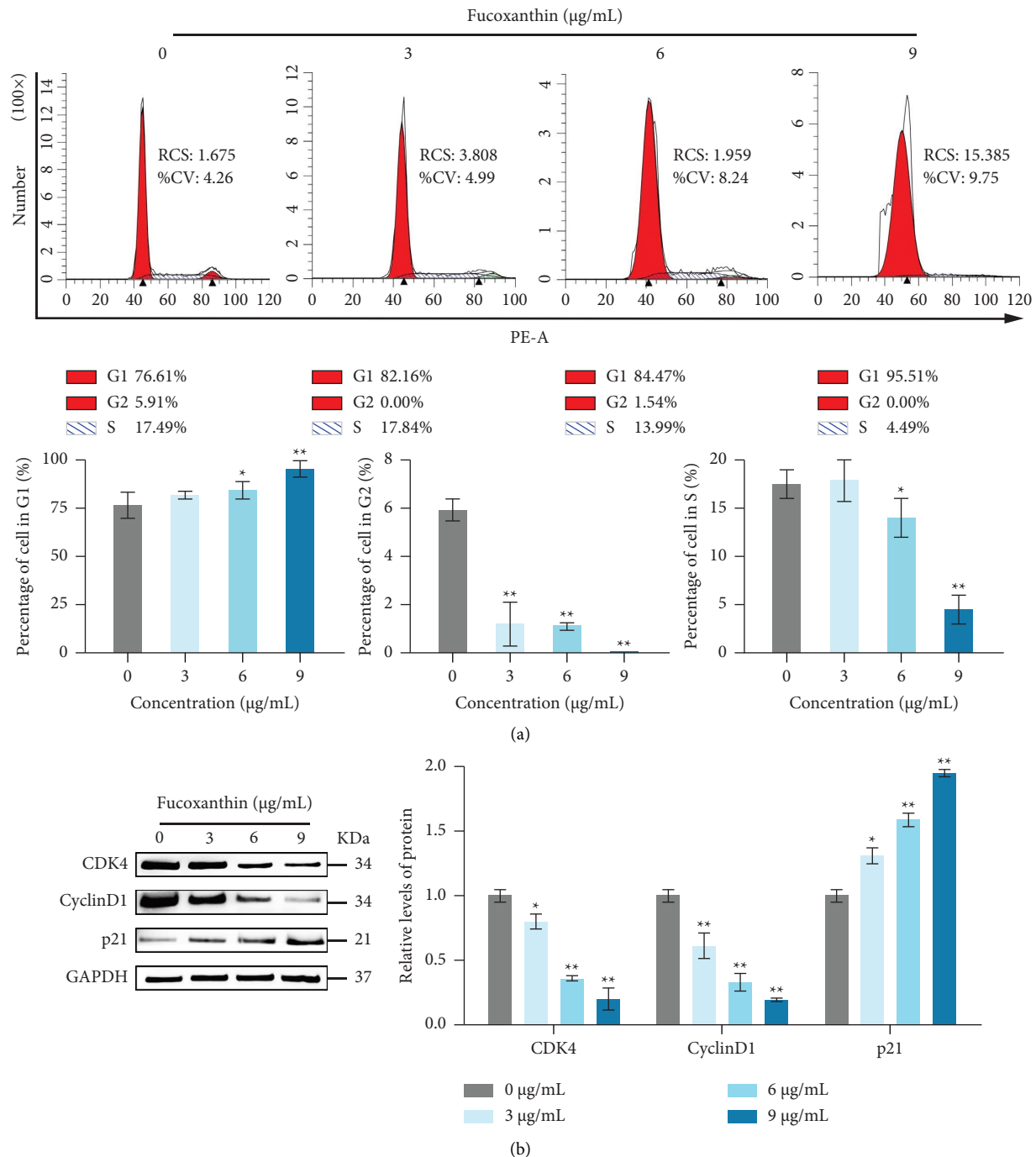
FIGURE 4: Cell cycle distribution of fucoxanthin-treated CAL-27 cells. Data are presented as means ± SD ($n = 3$). (a) Sub-G1 peak (apoptotic peak) after treatment with 0, 3, 6, and 9 μg/mL fucoxanthin for 24 h. The DNA content of cells in the G2 and M phases was twice that of cells in the G0 and G1 phases. Cells in the S phase exhibited a DNA content within the range of the aforementioned extremes. (b) Effect of fucoxanthin on cell cycle-related protein expressions of CAL-27 cells.

fucoxanthin, the relative expression of the glycolysis-related protein hexokinase decreased significantly ($p < 0.05$; Figure 7(h)). Compared with the model group, 24 hours of treatment with different fucoxanthin concentrations significantly reduced PKM protein expression levels (Figure 7(i)). Fucoxanthin at 3 μg/mL significantly decreased the relative expression levels of the glycolysis-related protein, PKM ($p < 0.05$), indicating that fucoxanthin controlled the glycolysis pathway of CAL-27 cells via the AKT/mTOR signaling cascade (Figure 8).

4. Discussion

Because of the lack of adequate therapy, cancer currently ranks as the second most prevalent cause of mortality [18]. Severe adverse effects and chemoresistance are the main
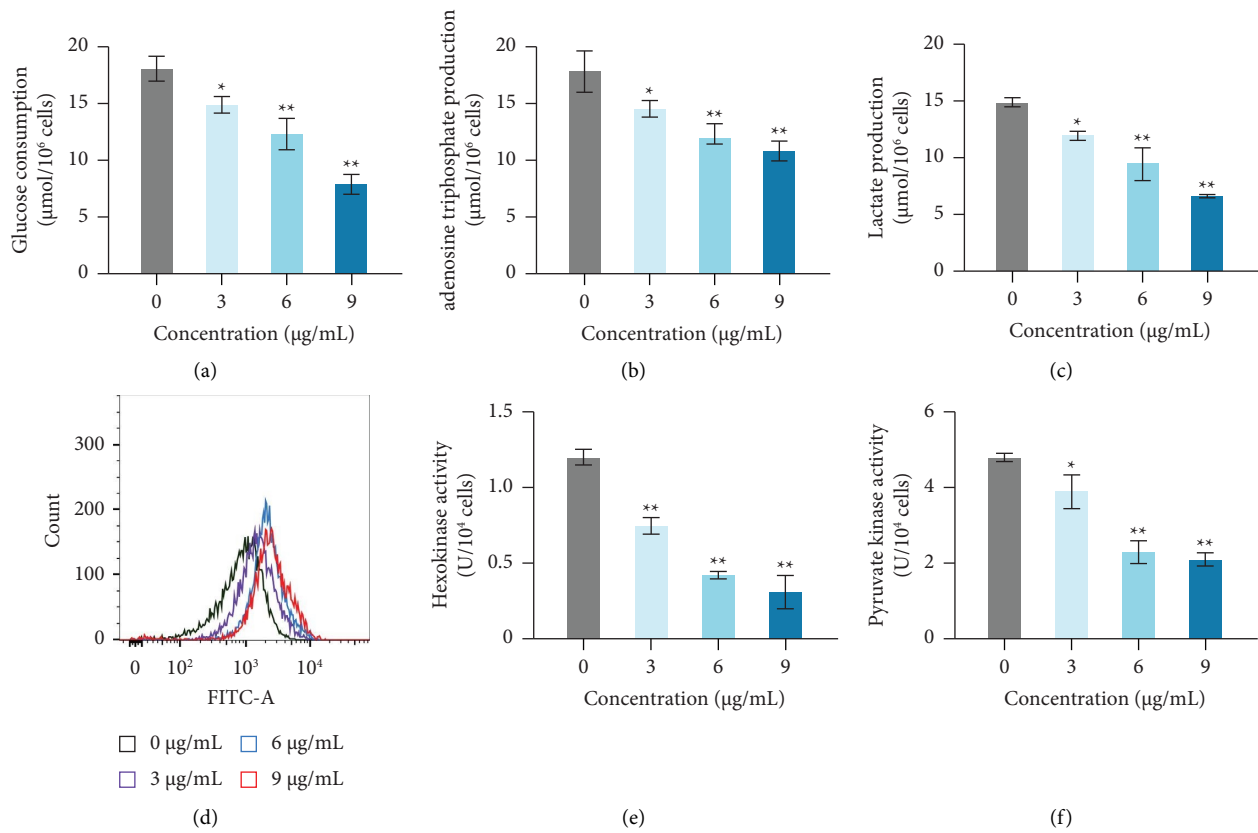
FIGURE 5: Effect of fucoxanthin on glycolytic pathway in CAL-27 cells. Data are presented as means ± SD ($n = 3$). (a) Impact of fucoxanthin on glucose consumption in CAL-27 cells. (b) Influence of fucoxanthin on adenosine triphosphate production in CAL-27 cells. (c) Impact of fucoxanthin on lactate in CAL-27 cells. (d) Influence of fucoxanthin on ROS in CAL-27 cells. (e) Impact of fucoxanthin on hexokinase in CAL-27 cells. (f) Influence of fucoxanthin on PK in CAL-27 cells. $^*p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

FIGURE 6: Influence of fucoxanthin on apoptotic genes in CAL-27 cells. Data are presented as means ± SD ($n = 3$). (a) Effect of fucoxanthin on AKT in CAL-27 cells. (b) Influence of fucoxanthin on mTOR in CAL-27 cells. (c) Effect of fucoxanthin on RPS6 in CAL-27 cells. (d) Influence of fucoxanthin on hexokinase in CAL-27 cells. (e) Effect of fucoxanthin on PKM in CAL-27 cells. $^*p < 0.05$ and $^{**}p < 0.01$ compared with the control group.
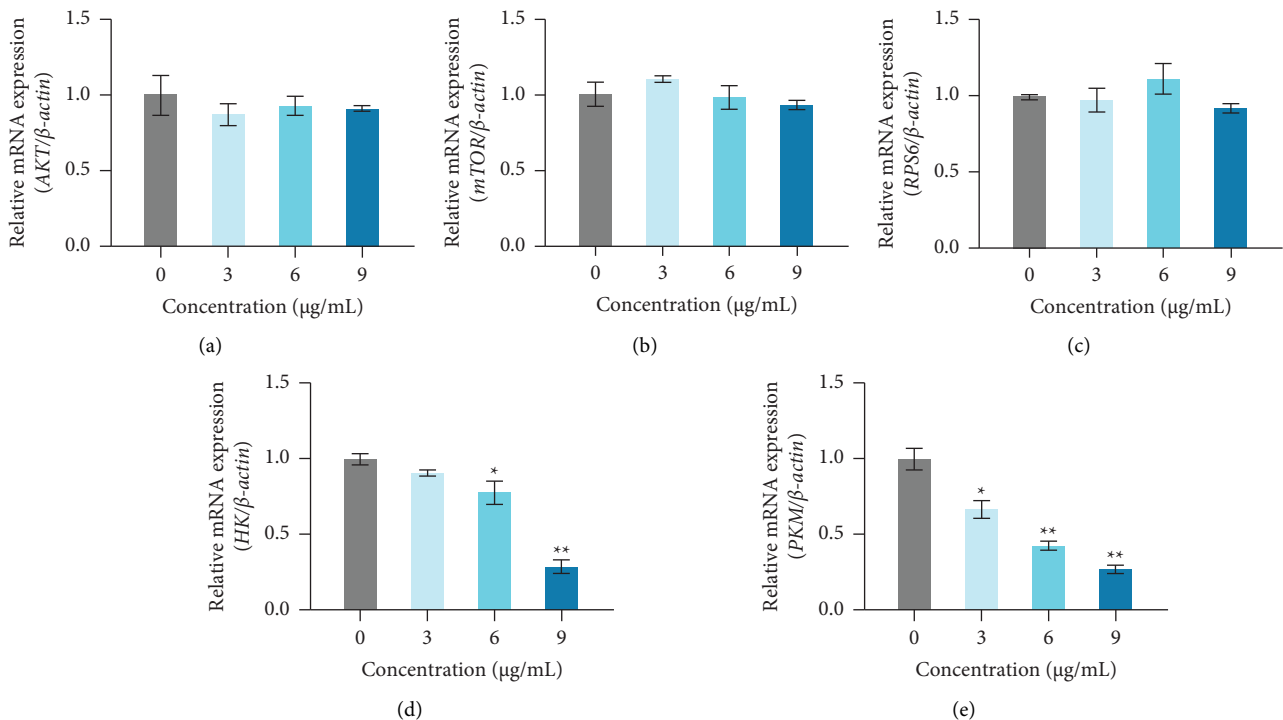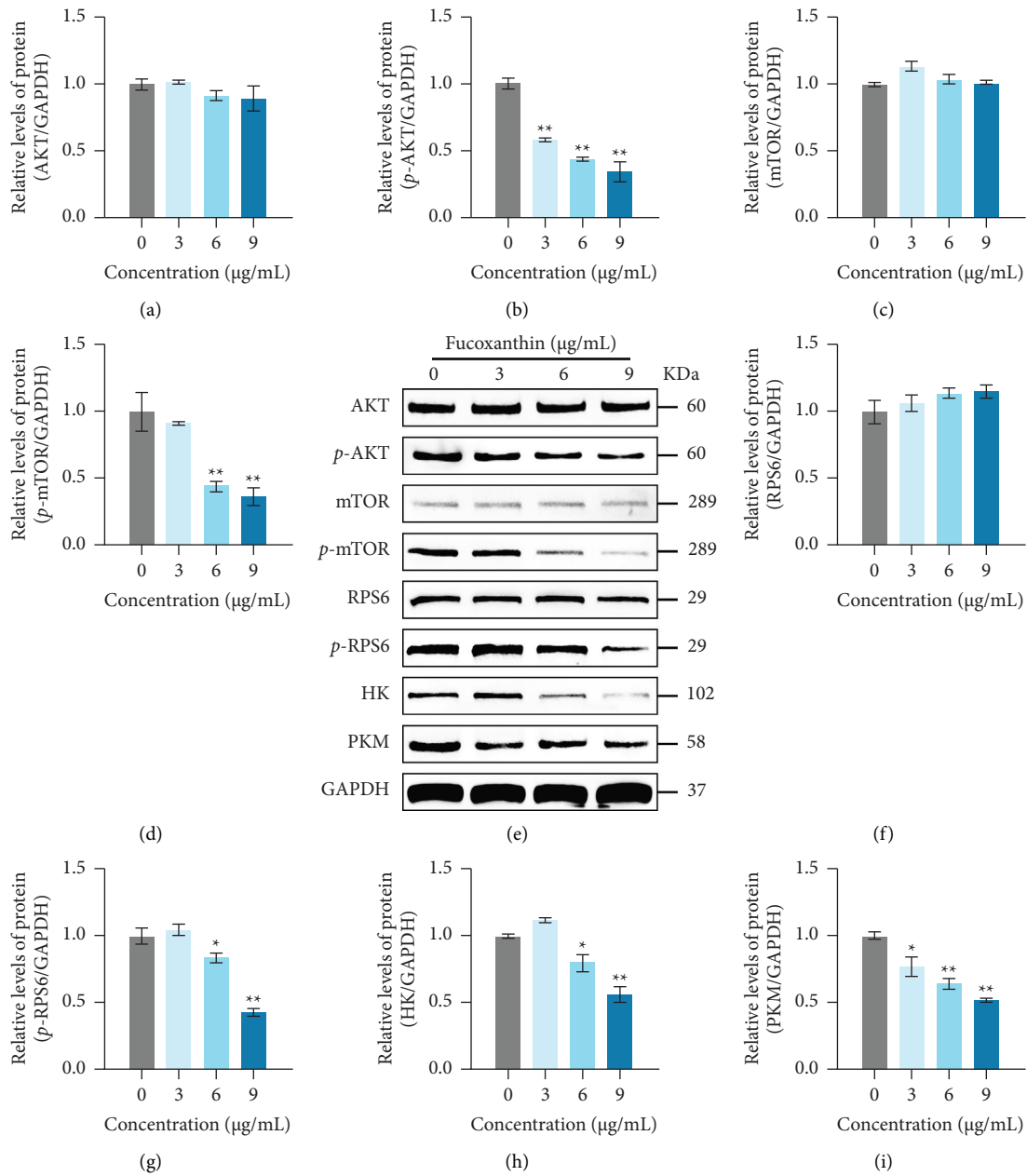
FIGURE 7: Effect of fucoxanthin on AKT/mTOR signaling pathway in CAL-27 cells. Data are presented as means ± SD ($n = 3$). (a) Influence of fucoxanthin on AKT in CAL-27 cells. (b) Effect of fucoxanthin on p-AKT in CAL-27 cells. (c) Effect of fucoxanthin on mTOR in CAL-27 cells. (d) Effect of fucoxanthin on p-mTOR in CAL-27 cells. (e) Influence of fucoxanthin on apoptotic and glycolysis proteins in CAL-27 cells. (f) Influence of fucoxanthin on RPS6 in CAL-27 cells. (g) Impact of fucoxanthin on p-RPS6 in CAL-27 cells. (h) Influence of fucoxanthin on hexokinase protein in CAL-27 cells. (i) Influence of fucoxanthin on PKM in CAL-27 cells.

problems restricting current chemotherapy [19]. Fewer adverse effects and greater pharmacology make natural goods a valuable resource for the pharmaceutical industry [20, 21]. Increasing evidence suggests that fucoxanthin isolated from *S. fusiforme* is a novel anticancer drug that can inhibit tumor development through various pathways. Calabrone et al. discovered that fucoxanthin suppressed prostate cancer cell growth and impeded vascular network formation in endothelial cells. qPCR and membrane antibody microarray showed that fucoxanthin downregulated the expression of various genes, including angiopoietin-2, CXCL5, TGFβ, IL6, STAT3, MMP1, TIMP1, and TIMP2 in prostate and endothelial cells [22]. Wang et al. reported that fucoxanthin significantly inhibits cell adhesion molecule (CAM) expression triggered by inflammatory factors, leading to a reduction in MCF-7 and endothelial cell adhesion [23]. Suppression of the NF-κB signaling pathway inhibited CAM in endothelial cells, as evidenced by decreased phosphorylation levels of IKK-α/β, IκB-α, and NF-κB p65. A previous study revealed that fucoxanthin inhibited
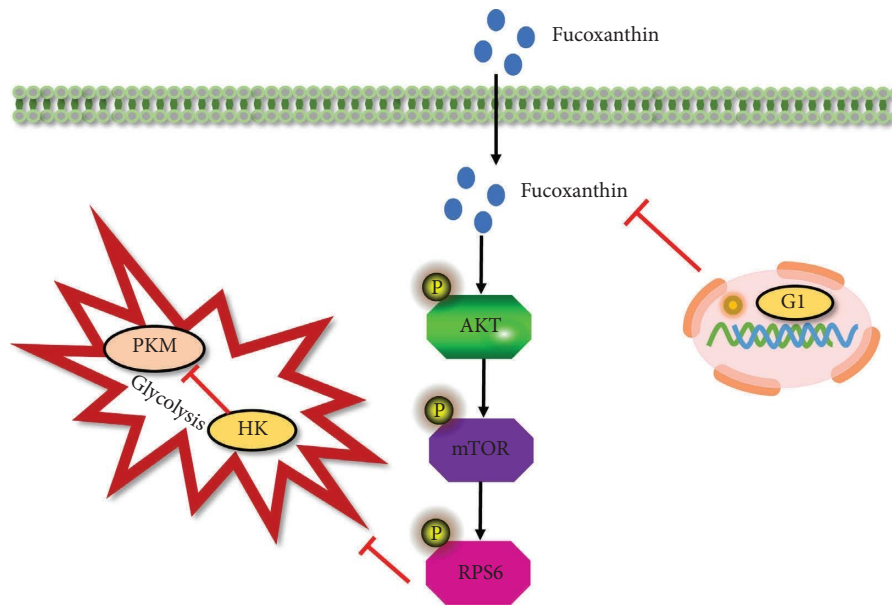
FIGURE 8: Fucoxanthin from *Hizikia fusiforme* inhibited tongue squamous cell carcinoma proliferation via AKT/mTOR-mediated glycolysis.

transendothelial migration of MCF-7 cells [24]. This effect was attributed to the compound's targeting of the epithelial-to-mesenchymal transition, PI3K/AKT, and FAK/paxillin signaling pathways. Additionally, Fang et al. showed that fucoxanthin impedes the proliferation, migration, and invasion of non-small-cell lung cancer (NSCLC) cells in *vitro*. Their study demonstrated arrested cellular division ability at the G0/G1 phase and triggered NSCLC cell apoptosis. The observed outcomes corresponded to the inhibition of PI3K and AKT phosphorylation, resulting in deactivation of the PI3K/AKT signaling pathway. These studies highlight the diverse anticancer properties of fucoxanthin, including inhibition of cell growth, suppression of angiogenesis, reduction of adhesion molecules, and inhibition of migration and invasion.

The current study showed that fucoxanthin time- and dose-dependently inhibited CAL-27 cell activity. Moreover, fucoxanthin effectively inhibited the formation of CAL-27 cell colonies. Flow cytometry results further revealed that fucoxanthin induced CAL-27 cell apoptosis and reduced MMP. These findings demonstrate the significant antiproliferative effects of fucoxanthin on CAL-27 cells. Tumor cells exhibit heightened glucose uptake and utilization of aerobic glycolysis as the primary feature of glucose metabolism. Although aerobic glycolysis is less efficient in net ATP production than in oxidative phosphorylation, it provides energy quickly and can meet the energy demands of rapidly proliferating tumor cells. Lung carcinoma is a highly aggressive cancer and exhibits the foremost etiology of mortality associated with cancer. Metabolomic investigations revealed an accumulation in the biosynthesis of nucleotides, amino acids, glycolysis pathway, tricarboxylic acid cycle, and glutathione metabolism [26]. Inhibiting energy metabolism in cancer cells is a potential approach to cancer treatment, yet it remains a significant challenge to overcome. A previous study revealed that electrostimulation

resulted in mitochondrial dysfunction, thereby inhibiting the electron transport chain and glycolysis pathways. This ultimately led to a severe energy supply crisis, causing cancer cell death [27]. Additionally, neoplastic cells stimulate the glycolytic pathway by elevating glucose uptake. The high glycolysis levels provide energy for unrestricted proliferation of neoplastic cells, and the generated intermediate products promote tumor growth [28].

A primary contributing factor of abnormal activity in the glycolytic pathway in tumor cells is the upregulation of key enzymes involved in the glycolytic pathway [29]. Hexokinase is a crucial rate-limiting factor in the glycolytic pathway. Its primary function is to catalyze the conversion of glucose molecules entering the cell into glucose-6-phosphate. In several cancers, hexokinase is expressed abnormally. PK is the ultimate enzyme restricting the glycolysis rate and converts phosphoenolpyruvate and adenosine diphosphate (ADP) to pyruvate and ATP in the cytoplasm, thereby facilitating tumor cell proliferation. This enzyme has a pivotal function in advancing various tumors. Li et al. discovered that microRNA-let-7b-5p (let-7b-5p) represses the expression of hexokinase-2 through its interaction with the $3'$-untranslated region of hexokinase-2 mRNA. A previous study demonstrated the efficacy of let-7b-5p in restricting breast tumor growth and metastasis by inhibiting hexokinase-2-mediated aerobic glycolysis both in *vitro* and in *vivo* [30]. Andrographolide, a diterpenoid lactone, is a natural anticancer agent because it suppresses cancer cell proliferation. Consequently, andrographolide significantly impeded human lung cancer cell viability and inhibited aerobic glycolysis through reduced lactate generation [31].

The current study showed that fucoxanthin significantly increased the content of ROS and reduced glucose uptake, ATP generation, lactate production, and the enzyme activities of hexokinase and PK. Intervention with different concentrations of fucoxanthin significantly decreased the

expression levels of glycolytic-related proteins, specifically hexokinase and PK, in CAL-27 cells. Flow cytometry results showed that fucoxanthin arrested the CAL-27 cell cycle at the G1 phase. Western blot results showed that the expression levels of Cyclin CDK4, Cyclin D1, and p21 proteins in G1 phase were significantly changed. These results suggested that fucoxanthin can regulate cell glycolysis and effectively impede the rapid proliferation of CAL-27 cells, blocking tumor cell energy supply and inducing cell cycle arrest.

Studies have indicated that the AKT/mTOR signaling pathway is a crucial factor in regulating the glycolytic process [32, 33]. AKT is a critical driving factor of the tumor glycolytic phenotype by upregulating the expression and membrane translocation of glucose transporters and phosphorylating essential glycolytic enzymes, thereby regulating glycolysis [34]. Additionally, AKT can be activated via phosphorylation by phosphatidylinositol-3-kinase (PI3K). Once activated, AKT activates the downstream effector molecule, mTORC, and binds to Raptor, forming mTOR complex 1. RPS6, which facilitates protein synthesis and tumor cell proliferation, can be activated via phosphorylation of p70 ribosomal protein S6 kinase by mTORC1 [35]. Additionally, activation of mTORC1 can modulate the expression levels of glycolytic enzymes by controlling downstream transcription factors [36]. Treatment with triptolide significantly inhibited intrahepatic cholangiocarcinoma (ICC) cell proliferation and glycolysis dose- and time-dependently. Subsequent analysis revealed that the inhibitory effect of triptolide on glycolysis in ICC cells was achieved by targeting the AKT/mTOR signaling pathway. Furthermore, triptolide inhibits tumor cell proliferation and glycolysis in mice with AKT/YapS127A mutations [37]. These results indicate that fucoxanthin can decrease the expression levels of AKT/mTOR pathway-related proteins, including p-AKT, p-mTOR, and p-RPS6, in CAL-27 cells. This suggests that fucoxanthin can modulate the glycolytic pathway in CAL-27 cells via the AKT/mTOR signaling pathway.

5. Conclusions

Fucoxanthin can block cell glycolysis, limit tumor cell energy supply, and inhibit tongue cancer cell proliferation by suppressing the AKT/mTOR signaling pathway, thereby causing cell cycle arrest in the G1 phase. Additional in vivo studies are required to explore the mechanisms underlying fucoxanthin's effects. These findings will establish a scientific foundation for developing fucoxanthin as a therapeutic intervention for tongue cancer.

Data Availability

The data presented in this study are available on request from the corresponding authors.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Xiuqiang Zhang and Haomiao Ding contributed to the conception of the study. Xiuqiang Zhang, Taoyu Liu, Jinchao Ji, Feng Xu, and Lutao Zhang performed the experiments. Xiuqiang Zhang and Haomiao Ding made significant contributions to the analysis and preparation of the manuscript. Xuanan Jin, Xiuqiang Zhang, and Zhenghao Hua performed the data analysis and wrote the manuscript. Bohuai Xu and Haomiao Ding helped perform the analysis with constructive discussion.

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