ORIGINAL ARTICLE

Fucoxanthin Inhibits Cell Proliferation and Stimulates Apoptosis through Downregulation of PI3K/AKT/mTOR Signaling Pathway in Human Ovarian Cancer Cells

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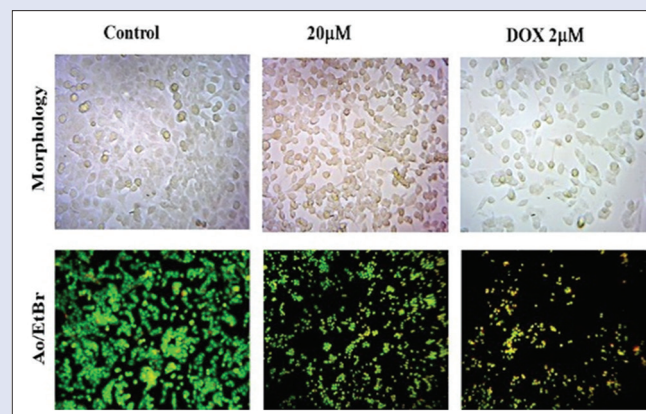
ABSTRACT

Background: Fucoxanthin is rich in seaweed and considered the most efficient anticancer drug because of its powerful antioxidant properties. The aim of this study was to elucidate the role of fucoxanthin on apoptosis via signaling pathway in ovarian cancer cells. First, fucoxanthin exhibited obvious cytotoxicity against A2780 human ovarian cancer cells. **Materials and Methods:** We established the action potential of fucoxanthin on cell proliferation in addition to apoptosis in A2780 cells, which were measured using reactive oxygen species (ROS) generation and the mitochondrial membrane potential (MMP). Apoptotic-related morphological alterations were inspected by acridine orange/EtBr dual staining. In addition, we analyzed caspase family protein expression by enzyme-linked immunosorbent assay analysis. **Results:** Our results established that fucoxanthin stimulates apoptosis as confirmed through reduced cell viability, improved production of ROS, and altered MMP in A2780 cells. Further, the fucoxanthin upregulates the expression of caspase (3, 8, and 9) protein experimentally in A2780 cell. In addition, we uncovered upstream signaling cascade (Akt/mTOR) induced by incubation A2780 cell lines with fucoxanthin that mediated cell apoptosis, migration, and invasion process. **Conclusion:** These findings suggest that fucoxanthin augments apoptosis; reduces cell proliferation, migration, and invasion; and reveals a potential mechanism of fucoxanthin-mediated Akt/mTOR suppression in human ovarian cancer cell line. Hence, fucoxanthin could be reflected as a potential therapeutic agent for ovarian cancer treatment. **Key words:** A2780, apoptosis, fucoxanthin, phosphoinositide 3-kinases/Akt/Mtor, reactive oxygen species

Abbreviations used: ROS: Reactive oxygen species; MMP: Mitochondrial membrane potential; FX: Fucoxanthin; MAPK: Mitogen-activated protein kinase.

SUMMARY

- Ovarian cancer is the major widespread cause of cancer-related mortality among all other gynecologic cancers
- Fucoxanthin potentially represses the cell proliferation particularly cancer cell via Akt/mTOR/S6 kinase signaling pathway in ovarian cancer cells.

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INTRODUCTION

Ovarian cancer is the seventh leading cause of cancer deaths among women worldwide.[1] Epithelial ovarian carcinoma represents approximately 80% of metastatic ovarian cancers. The prognosis for ovarian cancer remains poor overall, with a 40% of patients surviving 5 years.[2] The majority of ovarian cancer patients experience persistent, progressive disease, caused by the attainment of phenotypic resistance toward a variety of conventional chemotherapeutic agents. In spite of enormous advancements in therapeutic options such as surgical therapy and chemotherapy, still it ruins a dispute for the treatment of persistent ovarian cancer.[3] In addition, acquired resistance to chemotherapy is a major limitation in the treatment for ovarian cancer. Hence, it is still essential to recommend an efficient therapeutic drug for ovarian cancer.[4]

The phosphoinositide 3-kinases (PI3K)/AKT/mTOR signaling pathway plays an important role in the regulation of metabolism, cell transcription, proliferation, and survival. Activation of this pathway

has been widely studied in the occurrence and progression of many types of cancers, including colon cancer, breast cancer, ovarian cancer, and endometrial cancer.[5,6] The signaling cascade of PI3K/AKT/mTOR is the most distorted pathway in human cancer lines. It regulates various characteristic features as well as a process, which is most significant for the growth and development of cancer cells.[7] Previous

researchers have reported that ovarian cancer was frequently exerting modifications in one or more molecules of this signaling pathway, such as mutations in the PIK3CA gene, which is a catalytic subunit of Class I PI3K and PIK3R1 gene, which is a regulatory subunit of Class I PI3K and mTOR gene.[8] In addition, the stimulation of oncogenic tyrosine kinase receptors, functional loss of tumor suppression (PTEN), inositol polyphosphate-4-phosphatase, and mutation of the proto-oncogenes (AKT1 and AKT2) have been found in ovarian cancer.[9] In addition to the drug capability of the mechanism of the PI3K/AKT/mTOR signaling cascade, these molecular alterations suggested that targeting the PI3K/AKT/mTOR signaling pathway implies a novel approach for ovarian cancer.[10] Phytochemicals such as flavonoids, isoflavones, terpenes, and carotenoids, which represent a high-quality preference for novel therapeutics as they are predictable as secure and effortlessly acquired, do not exert remarkable lethal effects of long-term therapy and often cytotoxic to cancer cells other than normal cells.[11] Carotenoids are the major groups of phytochemicals which have drawn scientists interest toward examination for therapeutic applications toward cancer progression in modern years.[12] Fucoxanthin (Fx) is an important carotenoid found in brown seaweeds with strong antiproliferative activity and potent induction of apoptosis in a variety of cancer cells. Previous reports have revealed that fucoxanthin has anti-inflammatory, anti-diabetic, anti-obesity, antioxidant, anti-angiogenic, anticancer, and anti-malarial activities.[13,14]

Molecular mechanisms regarding the anti-carcinogenic effects of Fx in various cancer cell lines have been suggested, including blocking of caspase family activation, cell cycle progression, mitochondrial function, PI3K/Akt, mitogen-activated protein kinase, and signal transducers.[15,16] Nevertheless, the therapeutic role of Fx on cancer metastasis remains not clear even though it has abundant biological as well as biochemical properties. The goal of this study was to elucidate the therapeutic effects of Fx against the malignant behavior of epithelial ovarian cancer cells. Besides, we evaluated the Fx-mediated apoptosis mechanism underlying the anti-carcinogenic activity and explored the modification of PI3K/AktmTOR signaling pathways in ovarian cancer cells, thereby offering novel insights into the treatment of this disease.

MATERIALS AND METHODS

Materials

Fx, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), penicillin-streptomycin, fetal bovine serum (heat inactivated), Dulbecco's Modified Eagle Medium (DMEM), glutamine, phosphate-buffered saline, EDTA, JAK1, and STAT 3 were purchased from (Cell Signaling Technology, Beverly, MA, USA). The enzyme-linked immunosorbent assay (ELISA) kit was purchased from Sigma, USA.

Cell culture

The human carcinoma A2780 cell lines were purchased from American Type Culture Collection. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained at 37°C, under a humidified atmosphere of 5% CO_2 plus 95% air incubation.

Evaluation of cytotoxicity by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay

The cytotoxicity effect of Fx was determined by using MTT-based colorimetric assay.[17] A2780 cell lines were seeded on 96-well plates at a density of 5000–6000 cells/well and incubated for 24 h at 37°C. In order to identify the cytotoxic effects of Fx, it was dissolved in DMSO and

A2780 cells and incubated at 37°C for 24 h. After incubation for 24 h, MTT solution (100 μL, 5 mg/mL) was added in each well and the cell culture plates were incubated for 4 h under standard culture condition. The growth medium was then removed, and 100 μL serum-free medium containing MTT was added into the well, thereby formazan crystals were dissolved. The optical density (OD) value was measured using multiwall microplate reader at 570 nm.

Determination of intracellular reactive oxygen species production

Intracellular reactive oxygen species (ROS) was measured using Dichloro-dihydro-fluorescein diacetate (DCFH-DA) (probe), which could pierce into the intracellular matrix; there it is oxidized to fluorescent DCF by a process usually considered to involve ROS.[18] The A2780 ovarian cancer cells were seeded on 6-well plates, incubated with Fx plus using a CO_2 incubator for 24 h. Afterward, the cells were stained and incubated with DCFH-DA for 10 min. At last, the fluorescence intensity was examined using a 485/10 nm excitation filter or a 530/12.5 nm emission filter.

Mitochondrial membrane potential measurement

Mitochondrial membrane potential (MMP) was estimated with Rhodamine-123 (lipophilic, cationic dye). The A2780 cells were cultured and incubated with Fx. Afterward, the treated cells were incubated for 30 min with Rh-123 dye. The alterations in membrane potential were measured by fluorescence microscopy.

Fluorescence microscopic examination of cell death

Dual acridine orange (AO)/ethidium bromide fluorescent staining assay was used to identify the process of apoptosis via morphological changes.[19] The cells were incubated with Fx for 24 h and were instantly examined under an inverted fluorescence microscope to find out cell death.

Caspase activity assays

The caspase activities were measured using a commercial assay kit. In brief, cells were incubated with Fx, and then harvested, washed, and lysed. Cell lysates were incubated with the respective substrate for 1 h. The caspase-3, 9, and 8 activities were assessed through the determination of PNA release at 405 nm OD by ELISA reader.

Western blot

The cells were lysed in radioimmunoprecipitation assay buffer for 30 min, followed by centrifugation at 2000 g for 30 min at 4°C. After centrifugation of the lysate, the supernatant was collected, and total protein concentration was quantified using a NanoDrop spectrophotometer. 50-μg protein samples (containing sample buffer) were denatured at 95°C for 5 min. The denatured proteins were separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (10%) electrophoresis and transferred to a nitrocellulose membrane (0.45 μm). Later, the membrane was blocked with 5% bovine serum albumin for 1 h and incubated overnight at 4°C with definite monoclonal antibodies. After incubation, the membranes were washed three times with blocking buffer and rinsed in phosphate-buffered saline and developed with an enhanced chemiluminescence detection system.

Statistical analysis

All measurements were performed in triplicate, and all values were expressed as the mean ± the standard error. Implication between any two tentative conditions was determined by Student's *t*-test using SPSS

software (version 19) (SPSS Statistics/IBM Corp, Chicago IL, USA). *P* < 0.05 was considered statistically significant.

RESULTS

Cytotoxicity effect of fucoxanthin on A2780 cells

The cytotoxic efficacy of Fx against A2780 cells was detected by MTT assay [Figure 1]. Fx has cytotoxic effects on A2780 cells in a concentration-based mode (5–100 μM), whereas Fx-treated cells reduced cell viability to 80% compared with control cells. Hence, these findings clearly confirmed that Fx treatment potentially encouraged the cell death of A2780 cells.

Effect of fucoxanthin on apoptotic incidence in A2780 cells

Predominantly, the nuclei had been split into less massive fragments, thereby suggesting the formation of apoptotic bodies in Fx-treated cells. Fx (20 μM) treatment result into an increased mass of late apoptotic cells (orange-red colored) in the consequence of damaged cell membrane [Figure 2]. These altered changes were not observed in vehicle cells, which unveiled stained nuclei with intact cell organelles. Furthermore, doxorubicin was used as a standard drug for comparison and confirmation of Fx-mediated apoptosis in A2780 cells. Therefore, our findings suggested that Fx has a significant effect on the nuclear morphological changes, which is closely linked with apoptosis.

Effect of fucoxanthin on intracellular reactive oxygen species generation in A2780 cells

It is well known that ROS production significantly contributed to apoptosis triggering. Similarly, we found that Fx potentially increased the ROS generation in A2780 cells by means of DCFH-DA dye [Figure 3]. In Fx-treated A2780 cells, ROS production was confirmed by prominent fluorescence measurement as compared to control cells. Doxorubicin also effectively enhanced the ROS generation in A2780 cells. Therefore, DCFH-DA staining assay clearly showed that Fx treatment increased the ROS production in A2780 cells.
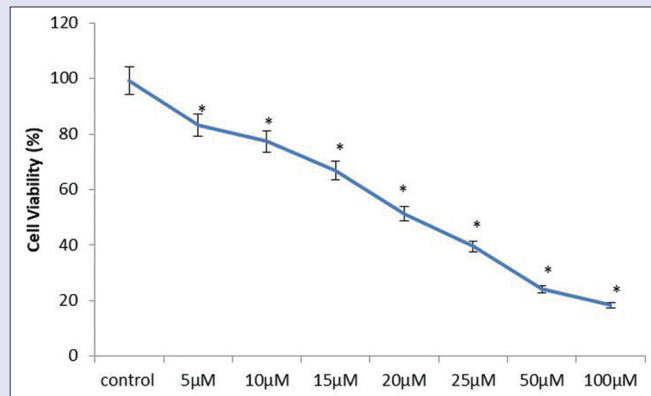
Effect of fucoxanthin on mitochondria membrane potential (ΔψM) damage A2780 cells

MMP (ΔΨm) is a key indicator of the cellular physical condition. It is also an instrumental marker of mitochondrial membrane reliability and ΔΨm reduction, which is an early event in the apoptotic process [Figure 4]. In order to evaluate the effect of Fx on ΔΨm reduction, A2780 cells were stained with Rhodamine 123 dye. Control cells release increased green-colored emission fluorescence, indicating polarized mitochondria. Alternatively, Fx (20 μM)-treated A2780 cells exhibited significantly changed MMP, which is persistently decreased green fluorescence.

Effect of fucoxanthin on caspase-3, -9, and caspase-8 in A2780 cells

Fx treatment markedly increased the activation of caspase-9 as compared with control A2780 cells [Figure 5]. The caspase-3 activation (upregulation) could explain the treatment of Fx through the caspase-9 activation. In addition, Fx treatment significantly enhanced the expression of caspase-8 pattern in A2780 cells.

Effect of fucoxanthin treatment on phosphoinositide 3-kinases, Akt, mTOR, and S6 kinase in A2780 cells

It has been reported that the apoptosis is downregulated by the PI3K/Akt and mTOR/S6 kinase (S6K) signaling pathway that may impact tumor development because various signal transduction mediators may suppress or stimulate carcinogenesis. The PI3K/AKT/mTOR pathway is essential for regulating cell proliferation and survival, which were found to be involved in Fx-mediated cell growth inhibition of A2780 cells. Fx treatment significantly reduced the PI3K phosphorylation and also remarkably decreased pAKT expression in A2780 cancer cells [Figure 6]. Additionally, Fx-treated A2780 cancer cells potentially reduced the mTOR expression. It is clear that ribosomal protein S6K activity is enhanced in several cancer cell lines or tumors. These proteins have also been connected with drug resistance and chemotherapy treatment, with the underlying function in carcinogenesis. In this study, we demonstrated that Fx significantly downregulates S6K expression in A2780 cancer cells.
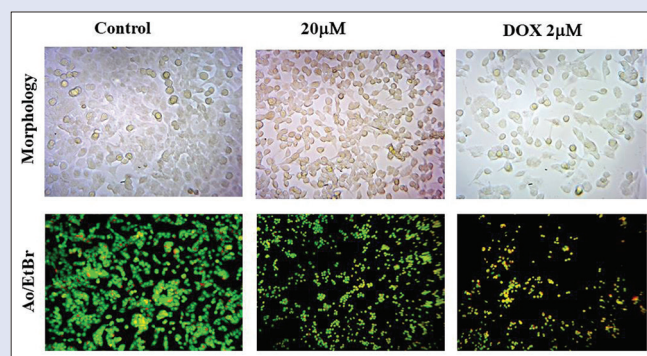

Figure 1: The cytotoxicity effect of fuxoxanthin on A2780 cells determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay. The cells were treated with various concentrations of fucoxanthin (5–100 μM) at 37°C for 24 h. The statistical analysis was carried out using one-way ANOVA. Values are represented as mean ± standard deviation of three experiments. *P* <0.05 was statistically significantly different from the control

Figure 2: Effect of fucoxanthin and doxorubicin on apoptotic incidence by ethidium bromide and acridine orange staining in A2780 cells. Illustrated fluorescence microscopy images show that 20 μM of fucoxanthin- and 2 μM of doxorubicin-treated A2780 cells exhibit enhanced % of apoptotic cells. Green live cells illustrate normal morphology; green early apoptotic cells demonstrate nuclear margination and chromatin condensation. Late orange apoptotic cells confirme fragmented chromatin and apoptotic bodies. The late apoptotic cells illustrate the nucleus of cell demonstrated orange fluorescence by ethidium bromide staining

DISCUSSION

Of the five most common types of gynecologic cancer, ovarian cancer is associated with the highest mortality rate and is the seventh leading cause of cancer death. Half of the people show no signs at the initial
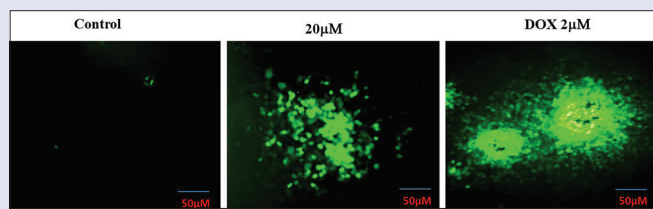

Figure 3: The effect of fucoxanthin on intracellular reactive oxygen species generation was assessed with A2780 cells by using Dichloro-dihydro-fluorescein diacetate (DCFH-DA) staining. A photomicrographic image reveals increased ROS generation in 20 µM of fucoxanthin- and 2 µM of doxorubicin-treated A2780 cells indicates deep Dichloro-dihydro-fluorescein (DCF) fluorescence intensity. The images were examined by floid cell imaging station in 488 nm (ImageJ software, Life Technologies, USA [1.48 version])
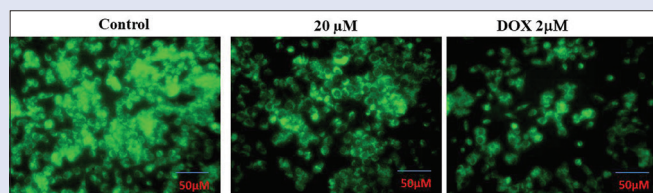

Figure 4: The effect of fucoxanthin on mitochondria membrane potential (ΔψM) damage was estimated with A2780 cells using the Rhodamine 123. 20 µM of fucoxanthin- and 2 µM of doxorubicin-treated A2780 cells decreased fluorescence intensity as indicated in collapsed mitochondria matrix. Then, the images were examined by floid cell imaging station (ImageJ software, Life Technologies, USA [1.48 version])

presentation as such symptoms can take time to develop, therefore most of the patients are diagnosed at the most advanced stage. Consequently, it gives rise to poor treatment response rate to chemotherapies.[20,21] Staging is a key part of deciding the best treatment that determines prognosis and clinical outcome. Stage I shows the overall survival rate of around 84 months. Particularly, Stage IV has high lower survival rate of around 10 months.[22,23] This survival rate is correlated not only with disease stage, but also connected with ovarian cell types. An enormous number of cell types have been recognized in ovarian cancer. However, the majority of the histological subtype is high-grade serious adenocarcinoma, which has the worst survival outcomes.[24,25]

Chemotherapy is a standard approach to reduce the velocity of cancer mortality. Although the recurrence and morbidity of cancer could not be deteriorated by means of the chemotherapeutic process, it is necessary to recognize a positive approach to manage the process of cancer development.[26] Fx can be an effective way to control malignancies by inducing cell cycle arrest and apoptosis. Basically, Fx belongs to a carotenoid family accumulated in the chloroplasts of brown seaweeds.[13] Latest research reports endorse that Fx has abundant pharmacological functions as well as biological activities including antidiabetic, anti-inflammatory, anti-obesity, antioxidant, antitumor, hepatoprotective, cardiovascular, and cerebrovascular protective effects. Most significantly, Fx can be used as both nutritional and therapeutic constituent to prevent or treat chronic diseases.[14,27]

In this study, we studied the effectiveness of Fx by targeting cellular apoptosis against A2780 ovarian cancer cells. Apoptosis, which impairments can be associated in many diseases, including cancer. In cancer cells, apoptosis stimulation is a possible novel approach proposed for cancer therapy.[28,29] Numerous research has examined that flavonoids possess suitable therapeutic properties as apoptotic encouragement, especially in tumor cells including antiviral, antioxidant, anti-inflammatory, and antiproliferative properties.[30,31] Fx also possessed the apoptotic stimulation properties in colon carcinoma of a mouse model.[32] Previous research has shown that Fx significantly suppressed the tumor incidence and cyclinD1 expression in the xenograft mouse model and also it possessed a greater pharmacological activity with the
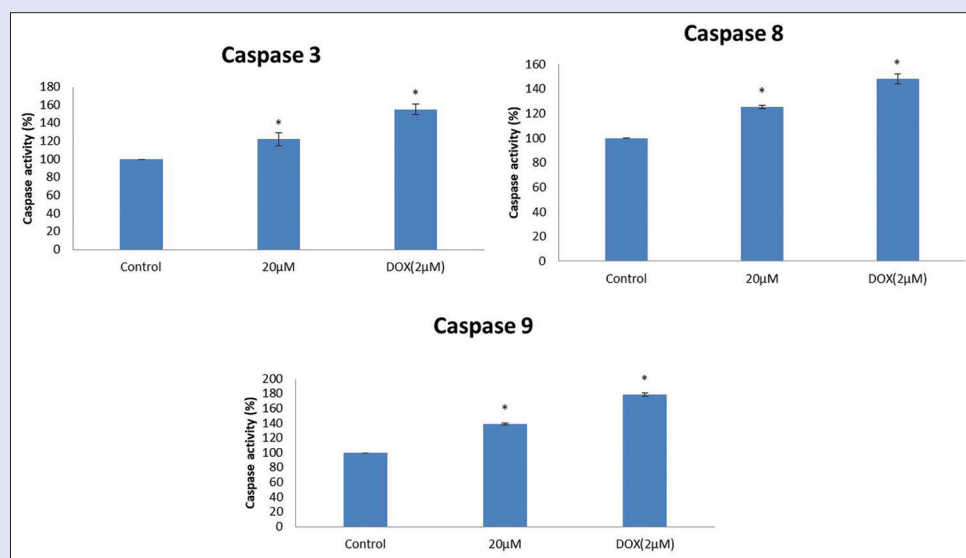

Figure 5: Effect of fucoxanthin treatment on caspase-3, -9, and -8 in A2780 cells. The cells were treated with fucoxanthin for 24 h and harvested. The protein levels were measured through enzyme-linked immunosorbent assay. The details are illustrated in the section of materials and methods section; the figures signify the data of three experiments, each conducted in duplicate. Values are represented as mean ± standard deviation of three experiments. *$P < 0.05$ was statistically significantly different from the control
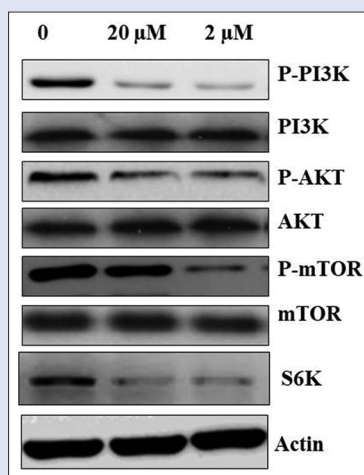
Figure 6: Effect of fucoxanthin treatment on phosphoinositide 3-kinases, AKT, mTOR, and S6 kinase in A2780 cells. The cells were treated with 20 µM of fucoxanthin and 2 µM of doxorubicin for 24 h and collected and total cell lysates were prepared as illustrated in materials and methods section

null side effects in human model as well.[33,34] In this investigation, Fx stimulated structural changes in A2780 cells, which are confirmed by AO/EtBr assessment. Apoptosis is characterized by the apoptotic cells with cell blebbing, shrinkage, fragmentation, and nuclear margination. The combinational therapy of AO/EtBr was used as a reliable evaluation of cellular degeneration. After the staining (AO/EtBr) process, it was illuminated that viable cells were consistently appearing as green, thereby it signifies that the cells with early-stage apoptosis represent greenish yellow color or green yellow-colored fragments, whereas cells with late-stage apoptosis represent orange-colored fragments. It is well documented that the apoptosis is induced either by the depletion of endogenous antioxidants or by the production of ROS. ROS-altering drugs are being predicted as treatment therapy in order to target the destruction of cancer cells.[35,36] In this study, we observed that Fx drastically stimulated ROS production in A2780 cells.

In our study, we explored the curative effect of Fx on cellular apoptotic proteins such as pro-apoptotic marker which includes a family of proteases known as the caspases (cysteinyl aspartate-specific proteases), which are mainly implicated in anticancer drug-induced apoptosis. Our findings define that Fx markedly enhanced the caspase-3, 8, and 9 expression, which indicated the participation of caspases in the apoptosis of A2780 cells. It is well documented that activating both caspase-8 and 9 results in the stimulation of both extrinsic and intrinsic apoptotic pathways.[37] Our results were assessed by ELISA analysis, which cleaved caspase-3, 8, and 9 protein expressions which were largely increased. In addition, the triggering of caspase-8 is closely associated with the extrinsic apoptotic signaling pathway, which is related to the mitochondrial pathway.[38] Also, Fx treatment stimulates the caspase-3 and 9 protein expression similar to standard doxorubicin (an anticancer drug).

In addition, we studied the outcome of Fx on PI3K/Akt/mTOR/S6K signaling cascade in A2780 ovarian cancer cells. The deregulation of the signaling pathways has been implicated as an incident that might rise to tumor formation because various mediators of the signal transduction may possibly suppress carcinogenesis. This signaling pathway is an important regulator of specific cellular processes implicated in cellular proliferation, metabolism, growth, survival, motility, and apoptosis.[39] The activation of the PI3K/AKT pathway promotes tumor cell survival and proliferation of tumor cells in many human cancers. AKT signaling

acts as a downstream target of PI3K in order to standardize cellular processes, whereas mTOR is a significant protein which lies both upstream and downstream of Akt. Akt or mTOR repression eventually inhibited the growth and invasiveness of tumor in cancer cells.[40,41] Our findings recommended that Fx undesirably regulates the PI3K and AKT expression in A2780 ovarian cancer cells and also reduces the mTOR expression in ovarian cancer cells.

Downregulation of Akt/mTOR/S6K pathway has been mainly associated with various cancers. S6K regulates many major cellular and molecular processes associated with cancer and acts through mTOR downstream.[42,43] As a result, this pathway has become an attractive target in cancer therapy. In this regard, Fx destructively regulated the S6K expression in A2780 ovarian cancer cells.

CONCLUSION

Our findings suggested that Fx potentially represses the cell proliferation, largely cancer cell via inhibition of Akt/mTOR/S6K signaling pathway in ovarian cancer cells. Downregulation of S6K resulting in the inhibition of Akt signaling cascade reveals affirmative feedback of Akt pathway regulation via S6K, and it has a remarkable effect in cancer treatment. Fx augments apoptosis; reduces cell proliferation, migration, and invasion; and reveals a potential mechanism of Fx-mediated Akt/mTOR suppression in human ovarian cancer cell line. Therefore, as an active ingredient of marine algae with significant toxicity, Fx may signify a new anticancer drug and will become an emergent therapeutic agent in future.

Conflicts of interest

There are no conflicts of interest.

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