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DOI:10.34172/PS.2022.44

Please cite this article as: Valinezhad Sani F, Kamalian S, Abdi H, Ghofrani G, Boustan A, Mosaffa F. Fucoxanthin inhibits the proliferation of ABCC2-over expressing cisplatin-resistance ovarian cancer cells via inducing apoptosis. Pharm Sci. 2022. doi:10.34172/PS.2022.44

Received Date: 17 Jun 2022

Accepted Date: 11 Nov 2022

This is a PDF file of an article which was accepted for publication in Pharmaceutical Sciences. It is assigned to an issue after technical editing, formatting for publication and author proofing

Fucoxanthin inhibits the proliferation of ABCC2-over expressing cisplatin-resistance ovarian cancer cells via inducing apoptosis

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Abstract

Background: The development of multidrug resistance (MDR) is a major barrier to achieving effective chemotherapy in cancer. Studies have shown that epithelial ovarian cancer initially responds to platinum-based therapy, however, the recurrent type is often resistant to treatment and is associated with high mortality. Fucoxanthin, a natural component found in marine algae, possesses various pharmacologic properties. This study evaluated the cytotoxicity and resistance reversal activity of fucoxanthin on multidrug resistance-associated protein 2 (MRP2)-overexpressing, cisplatin-resistant ovarian cancer cells (A2780RCIS) and their parental cells (A2780).

Methods: Cell viability was evaluated in the presence of different concentrations of fucoxanthin or cisplatin or fucoxanthin/cisplatin combination using the MTT assay. Propidium iodide staining and sub-G1 analysis were used to evaluate fucoxanthin potential for cell cycle modification and apoptosis induction in cancer cell lines.

Results: The results showed that fucoxanthin was able to cause similar toxicity in both cell lines via apoptosis induction. Co-treatment of cells with cisplatin (3.125 to 100 μ M) and nontoxic concentrations of fucoxanthin (1 and 2.5 μ M) did not reverse resistance to cisplatin in A2780RCIS cells.

Conclusion: Although fucoxanthin was not able to modify cisplatin-resistance in ovarian cancer cells, it was equally effective in inducing apoptosis and death in both A2780 and A2780RCIS cells, indicating it is not an MRP2 substrate.

Keywords: cisplatin, cytotoxicity, fucoxanthin, multidrug resistance (MDR), ovarian cancer.

Introduction

Among gynecologic cancers, ovarian carcinoma causes the highest mortality. Because of the lack of effective screening methods and explicit symptoms associated with early-stage disease, most patients with ovarian cancer are diagnosed at late stages. These patients are treated by surgical excision of the cancer followed by platinum/taxane combination chemotherapy. Although, this first line treatment regime is effective, in up to 75% of ovarian cancer patients, recurrence occurs which is followed by acquired drug resistance.¹ Therefore, the development of new therapies, drug-resistance modulators or chemosensitizers with low toxicity and minimum undesirable side effects has gained clinical interest in recent years.²

Fucoxanthin, is an orange-colored pigment from marine algae that contributes 10% of the total carotenoids in nature.³ It has been used for centuries as a traditional supplement and fucoxanthin-rich brown seaweed is used as food in East Asian countries diets. Recent reports have shown pharmacological potentials of fucoxanthin including anticancer, anti-inflammatory, antioxidant, neuroprotective, anti-hyper-lipidemia and anti-obesity activities.⁴ Different potential mechanisms have been reported for the anti-carcinogenic effects of fucoxanthin such as DNA fragmentation by activation of endogenous nucleases,⁵ suppression of cyclin D expression and proteosomal degradation and activation of the caspase pathway.⁶ Liu et al. have reported that the anti-cancer properties of fucoxanthin

are related to increased intracellular calcium levels through enhanced expression of connexin genes and gap junction intracellular communication.⁷ Recently, it has been reported that the combination of fucoxanthin with other chemotherapeutic drugs was beneficial in breast and lung cancer therapies.^{8,9} In the present study for the first time we examined the likely benefits of fucoxanthin application in ovarian cancer therapy and overcoming drug resistance in this cancer using cell line models. We evaluated the toxicity and cisplatin-resistance reversal activity of fucoxanthin in A2780RCIS cells already proved to be resistance against cisplatin via overexpressing MRP2 (ABCC2), a well-known transmembrane ABC efflux transporter which pumps cisplatin out of the cells.^{10, 11} We also compared the effects of fucoxanthin in the cisplatin resistant cells with cisplatin-sensitive ovarian cancer cells, A2780.

Materials and Methods

Cell lines and reagents

Drug-sensitive and resistant human ovarian cancer cell lines, A2780 and A2780RCIS were kindly provided by Dr Hermann Lage, Institute of Pathology, Charite Hospital, Berlin, Germany. Both cell lines were cultured in RPMI-1640 medium supplemented with 10% of fetal bovine serum, 1% penicillin (100 units/ml) and streptomycin (100 mg/ml) at 37^{0C} in a humidified atmosphere with 5% (v/v) CO₂. Fucoxanthin and cisplatin were purchased from Sigma (USA) and Ebewe pharma (Austria) respectively. 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) was provided from Alfa Aesar (USA). Propidium iodide (PI) was obtained from Sigma (USA). All other chemicals and reagents obtained from commercial sources were of analytical or cell culture grade.

Cell cytotoxicity assay

The cytotoxic effect of fucoxanthin was measured by MTT assay. A2780 and A2780RCIS cells were seeded in 96-well dishes (5.0×10^3 cells/well). After 24 h incubation, the cells were exposed to different concentrations of fucoxanthin (0, 2.5, 10, 20, 60, 80 μ M) for 48 and 72 h. After treatment the cells were incubated with 10 μ l MTT (5 mg/ml) for 4 h, then the medium was discarded and formazan crystals were solubilized with DMSO (100 μ l/well). The spectrophotometric absorbance was measured at 570 and 630 nm (reference wavelength) using a microplate reader, and the percentage of viable cells was calculated by the ratio of OD test/OD control.

Evaluation of cisplatin-resistance reversal activity

To assess the cisplatin-resistance reversal effect of fucoxanthin, A2780RCIS cells were seeded into 96-well plates, then treated with different concentrations of cisplatin (0, 3.125, 6.25, 12.5, 25, 50 and 100 μ M) alone or in the presence of non-toxic concentrations of fucoxanthin (1 or 2.5 μ M) for 48 and 72 h, and the cell viability was determined via MTT assay as previously described.

Sub-G1 and cell cycle distribution analysis

To explore the mechanism of cell death caused by fucoxanthin, propidium iodide staining method was applied. Ovarian cancer cell lines at a density of 3.5×10^5 cells/ml were cultured in 6-well plates overnight, then exposed to fucoxanthin (20 μ M) for 48 h. Then cells were harvested and centrifuged at $400 \times g$ for 5 min. The pellets were resuspended in 400 μ l PI staining solution (50 μ g/ml) containing sodium citrate (0.1 %), Triton X-100 (0.1%) and RNase A (0.1 mg/ml) and then incubated for 30 min in the dark. Following incubation, the cells were centrifuged and washed twice with ice-cold phosphate buffered saline (PBS) and resuspended in ice cold PBS. Finally, the cells were analyzed by flow cytometry using FL2 channel (BD FACS Calibur, USA). The percentage of cells in different phases of cell cycle and sub-G1 (as a hallmark of DNA fragmentation and apoptosis induction) was calculated with Flowjo7.6.1 software.

Microscopic examination of the cell morphologies

A2780 and A2780RCIS were seeded at a density of 3.5×10^5 cells per well of 6-well plates. Following an overnight incubation, the cells were treated with fucoxanthin (20 μ M) or cisplatin (3.9 μ M) for 48 h and microscopic images were captured from different treatments by a CCD camera connected to an inverted phase-contrast microscope (INVERSO-TC100, Medline Scientific Limited, United Kingdom) for visualization of the appearance changes of the cells following the treatments.

Statistical analysis

All results were expressed as mean \pm standard deviation (SD) from three independent experiments. In order to compare the data from different groups, Student's t test or one-way ANOVA with post-hoc Dunnett's test were applied using prism version 8 software. *P* value < 0.05 was considered significant.

Results

The effect of fucoxanthin on the cell viability of A2780 and A2780RCIS cancer cell lines

To evaluate the fucoxanthin effects on the viability of A2780 and A2780RCIS cancer cells, the cells were incubated with different concentrations of fucoxanthin for 48 and 72 h, and then MTT assay was performed. As shown in Fig. 1, fucoxanthin resulted in a concentration-dependent inhibitory effect on both cisplatin sensitive and resistant ovarian cancer cells. There was no significant difference between IC₅₀ of fucoxanthin at 72 h incubation times in each cell line and fucoxanthin toxicity toward cisplatin-resistant A2780RCIS cells was nearly equal to its lethal effects on A2780 cisplatin-sensitive cells (Table 1). Fucoxanthin at concentration of 2.5 μ M had no significant effect on the proliferation of A2780 and A2780RCIS cells. Therefore, 1 and 2.5 μ M concentrations of fucoxanthin were selected for cisplatin-resistance reversal experiment.

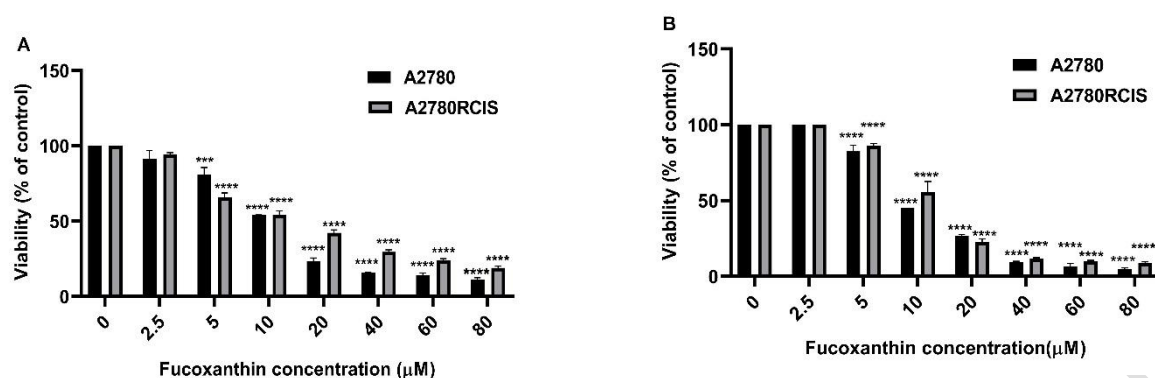


Fig. 1. The effects of fucoxanthin on the viability of A2780 and A2780RCIS cells. The cells were treated with different concentration of fucoxanthin for 48 (A) and 72 h (B), then cell viability percentage was measured using MTT assay. Each experiment was carried out at least in triplicate and the results were presented as mean \pm S.D. *** $P < 0.001$, **** $P < 0.0001$ compared to untreated control cells (one-way ANOVA followed by post-hoc Dunnett's test).

The effect of fucoxanthin on cisplatin-resistance phenotype in A2780RCIS cells

In order to assess the cisplatin-resistance reversal effect of fucoxanthin, first the sensitivity to cisplatin was compared between two cell lines. As shown in Fig. 2, cisplatin significantly decreased the proliferation rate of A2780 and A2780RCIS cells in a time and dose-dependent manner. Cisplatin IC50 values obtained for two cell lines confirmed that it was much more toxic against parental A2780 cells in comparison with the resistant cell line A2780RCIS following both 48 and 72 h incubation periods (Table 2). Furthermore, the cytotoxicity of different concentrations of cisplatin in presence of non-toxic concentration of fucoxanthin (1 or 2.5 μ M) was assessed in both cell lines following 48 and 72 - cotreatments. Our results showed that non-toxic concentration of fucoxanthin couldn't change the sensitivity of both cell lines toward cisplatin (Fig. 3).

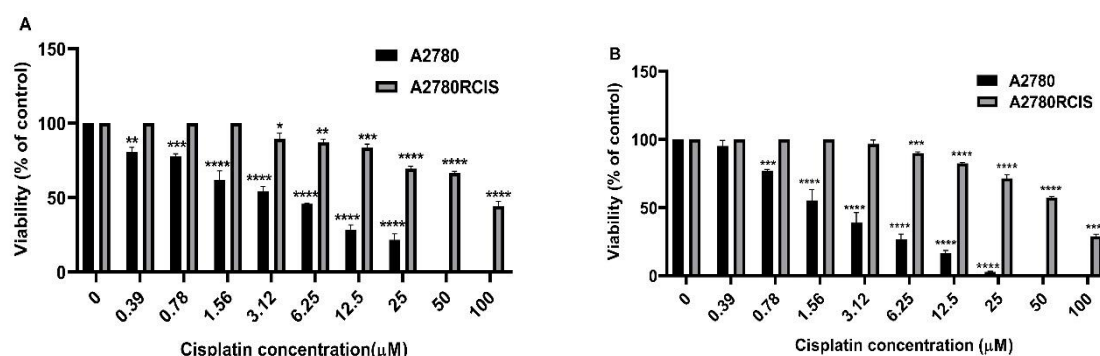


Fig. 2. The effects of cisplatin on the viability of A2780 and A2780RCIS cell lines. The cells were treated with different concentrations of cisplatin for 48 (A) and 72 h (B), then the sensitivity of ovarian cancer cell lines to cisplatin was measured by MTT assay. Each experiment was performed three times

and the results were shown as mean \pm SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ compared to control groups (one-way ANOVA followed by post-hoc Dunnett's test).

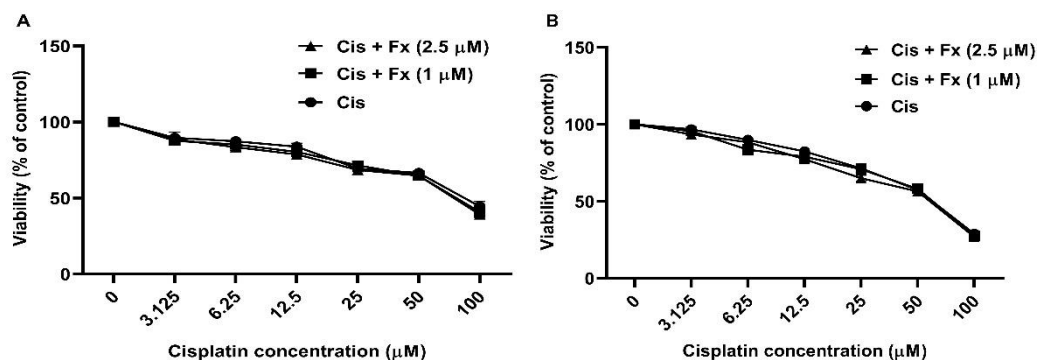


Fig. 3. The effects of fucoxanthin on the toxicity of cisplatin in A2780RCIS cells. The cells were treated for 48 (A) and 72 h (B) with different concentrations of cisplatin in absence or presence of fucoxanthin (1 and 2.5 μ M). Each experiment was performed three times and the results were shown as mean \pm SD. There were no significant differences between viability of cisplatin treated and cisplatin + fucoxanthin cotreated cells at all tested concentrations of cisplatin (one-way ANOVA followed by post-hoc Dunnett's test). Cis (Cisplatin), Fx (Fucoxanthin).

The apoptosis-inducing effect of fucoxanthin on A2780 and A2780RCIS cancer cell lines

The apoptosis-inducing effect of fucoxanthin was evaluated using PI staining of DNA content and flow cytometry. Internucleosomal degradation of DNA, resulted from the activation of endogenous endonuclease, is one feature that happens during apoptosis and cells with the fragmented DNA appear in the sub-G1 area of PI fluorescent histograms. While cisplatin treatment (3.9 μ M) resulted in about 20% significant increase in the apoptotic (sub-G1) cell count in A2780 cells (4B and 5A) compared to untreated ones (4A and 5A), at the same concentration it was not able to cause any significant change in apoptotic cell percentage in A2780RCIS cells (4E and 5B) compared to control cells (4D and 5B) which was in conformity with the MTT viability assay results. In contrast, when A2780 and A2780RCIS cells were treated with 20 μ M fucoxanthin for 48 h, the majority of cells (about 68 % and 97 %, respectively) of both cell lines became apoptotic and had fragmented DNA (4C, 4F, 5A and 5B).

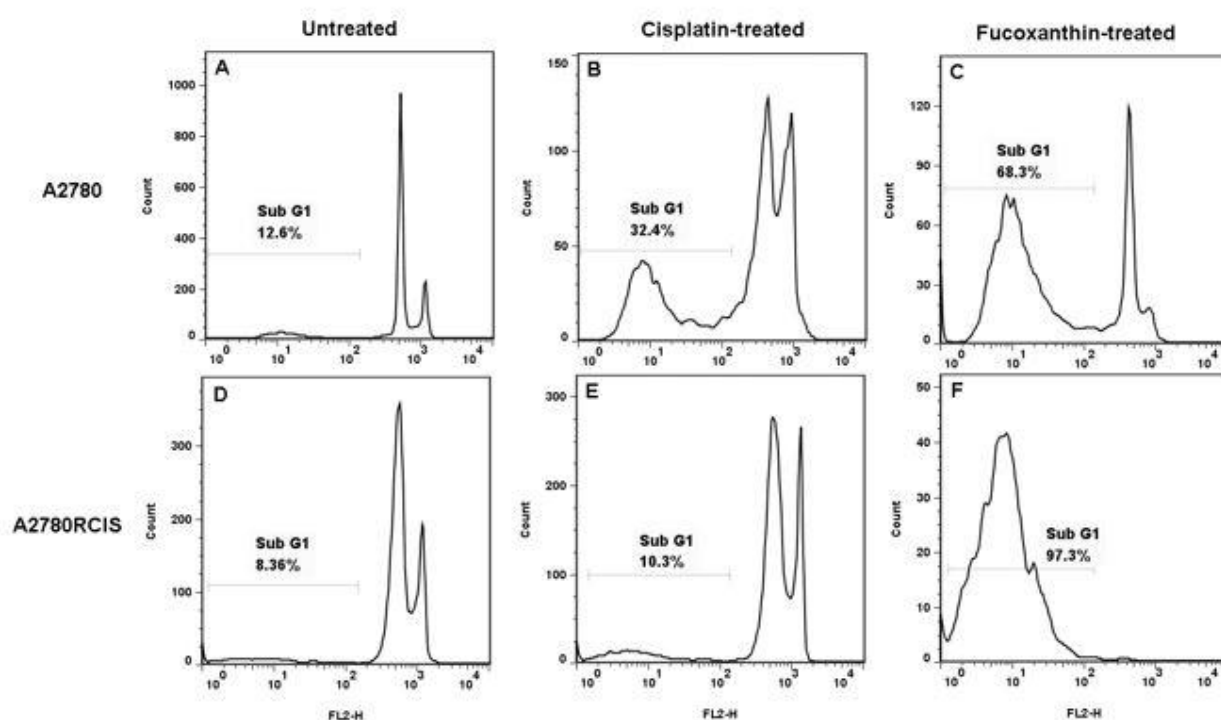


Fig. 4. Evaluation of apoptosis induction using PI staining method and flow cytometry. The A2780 (A) and A2780RCIS (B) cells were treated with Cisplatin (3.9 μ M) or fucoxanthin (20 μ M) for 48 h. The sub-G1 peak, as an indicative of apoptotic cells, was analyzed and compared to untreated cells using flow cytometry. The histograms are representative of three independent experiments.

The Effects of fucoxanthin on Cell Cycle Progression

The effects of fucoxanthin and cisplatin on cell cycle distribution of A2780 and A2780RCIS cells were assessed by PI staining and flow cytometry. As shown in Fig. 5, 48 h-treatment with cisplatin (3.9 μ M) caused a significant G2 arrest in A2780 cell (5A), but not in resistant A2780RCIS cells (5B) compared to the controls. Following 48 h treatment with fucoxanthin (20 μ M) no significant changes were observed in the distribution of A2780 cells in the cell cycle phases compared with untreated ones (5A) and almost all of A2780RCIS cells had fragmented DNA (5B).

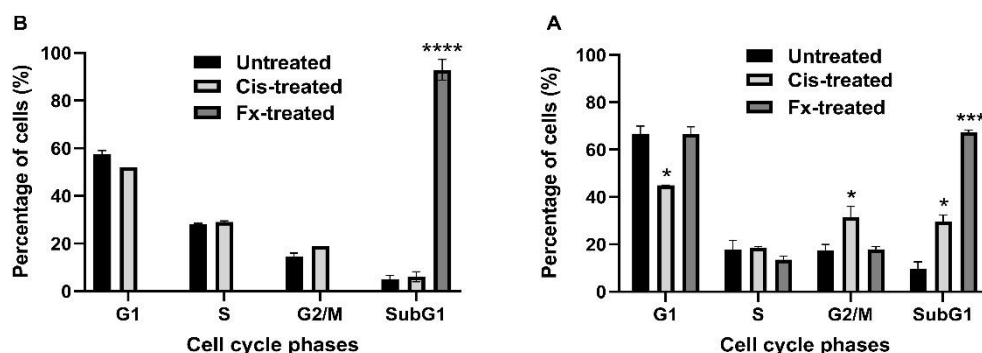


Fig. 5. Cell cycle distributions of fucoxanthin or cisplatin-treated ovarian cancer cells measured with the flow cytometry analysis. A2780 (A) and A2780RCIS (B) cells were either untreated or treated with fucoxanthin (20 μ M) or cisplatin (3.9 μ M) for 48 h, then stained with PI, and analyzed by flow cytometry. The proportion of cells at each phase of cell cycle was presented as percentage of cells in the total cell cycle (cells in G1+S+G2/M). The proportion of sub-G 1 cells was also presented as percentage of the entire cell experiments. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ compared to untreated control cells (one-way ANOVA followed by post-hoc Dunnett's test).

The effects of fucoxanthin treatment on morphology of the cells

The exposure of A2780 and A2780RCIS cells to 20 μ M fucoxanthin for 48 h clearly induced the morphological changes indicative of apoptotic cell death in both cell lines including rounding up, reduction of cell size, nuclear condensation and fragmentation and formation of apoptotic bodies (Fig. 6). The same morphological changes were observed in A2780 parental cells exposed to cisplatin (3.9 μ M) but not in their cisplatin resistant derivatives, A2780RCIS cells.

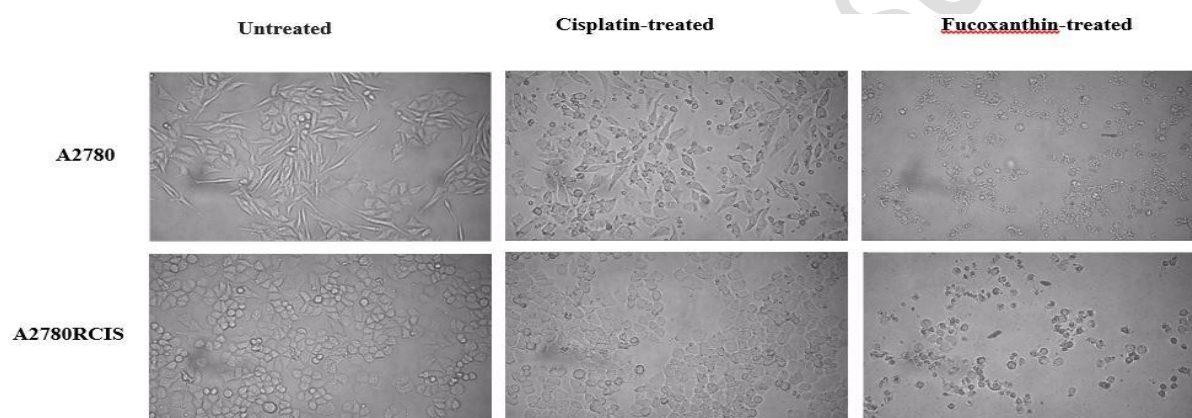


Fig. 6. The effect of fucoxanthin on A2780 and A2780RCIS cell proliferation. The cells treated with fucoxanthin (20 μ M) or cisplatin (3.9 μ M) for 48 h and the images were captured by an inverted phase-contrast microscope ($\times 200$).

Discussion

Ovarian cancer is one of the most common gynecologic cancers with increasing mortality rates in most parts of the world.¹² Although, cisplatin has been widely used as a first line treatment for ovarian cancers, unfortunately, the phenomenon of resistance to it is one of the main obstacles which leads to the ineffectiveness of platinum-based chemotherapy treatments. Efforts to find effective chemosensitizers or new therapies against drug-resistance ovarian cancer cells, especially natural compounds with less side effects for the human body can lead to the development of more successful therapies in the future.

Fucoxanthin is a well-known natural dietary carotenoid commonly found in seaweeds and diatoms. There are many pharmacological evidence about its health-promoting effects including anti-cancer¹³ and anti-inflammatory properties,¹⁴ its protective effects against doxorubicin-induced cardiotoxicity,¹⁵

diabetic nephropathy,¹⁶ alcoholic liver injury¹⁷ and other metabolic disease. The multi-potent health effects of fucoxanthin are attributed to its unique chemical structure including an allenic bond, an acetyl group, a conjugated carbonyl group, and 5,6-monoepoxide.⁴ The long traditional consumption of fucoxanthin supports its safety. Furthermore, it has been shown that fucoxanthin did not cause any toxicity or adverse effects in different study models and can be considered as a safe pharmaceutical component.¹⁸

In the present study we evaluated the effect of fucoxanthin on the survival of cisplatin sensitive and resistant ovarian cancer cells as well as its effect on reducing the resistance to cisplatin in MRP2 overexpressing, cisplatin-resistant ovarian cancer cells. Following the treatment of the cells by cisplatin, the results obtained by MTT viability assay, PI staining of DNA content and microscopic examination all validated the resistance of A2780RCIS cells to cisplatin compared to its parental A2780 cells. The results of cytotoxicity assay also indicated that fucoxanthin, when used alone for the treatment of the cells, has a concentration-dependent cytotoxicity in both cell line. Unlike cisplatin, fucoxanthin showed almost similar toxicity in both sensitive and resistant cells, which reveals that this compound is not a substrate for the MRP2 pump and therefore, has the potential to induce death in cisplatin-resistant ovarian cancer cells. Other researchers previously reported that P-gp (ABCB1)-overexpressing breast, liver and ovarian cancer cell lines, MCF-7/ADR, Hep-G2/ADR and SKOV-3/ADR, which were resistant to doxorubicin showed the same degree of resistance toward fucoxanthin compared to their parental cells, MCF-7, Hep-G2 and SKOV-3 cells. They concluded that fucoxanthin is a substrate of P-gp transporter which leads to its efflux by the pump.¹⁹ We also found that fucoxanthin at nontoxic concentration of 1 and 2.5 μ M couldn't increase the sensitivity of A2780RCIS cells to cisplatin at 48 or 72 h. Combination therapy which implied that these concentrations of fucoxanthin is not an inhibitor or modulator of MRP2 activity. In the study conducted on MCF-7/ADR, Hep-G2/ADR and SKOV-3/ADR cells, fucoxanthin at nontoxic concentration enhanced doxorubicin cytotoxicity in these cells. In addition, it increased the accumulation of the P-gp substrates, doxorubicin and rhodamine123, in the resistant cells which was suggestive that it is an inhibitor or modulator of the function of P-gp.¹⁹ In the next step, to investigate the mechanism of fucoxanthin cytotoxicity, ovarian cancer cells treated with 20 μ M fucoxanthin were evaluated by PI staining followed by flow cytometry and microscopic examination. The results showed that unlike cisplatin that was able to induce G2/M arrest in sensitive ovarian cancer cells, fucoxanthin induced apoptosis in these cells without affecting cell proportions in different phases of the cell cycle. The key cytotoxicity mechanism of cisplatin is the formation of DNA intra- and interstrand cross-links that causes bulky DNA damage. As an effort to repair the cisplatin-induced DNA damage a checkpoint response prevents the cells from cell-cycle progression and they arrest in G2M phase and finally die via apoptosis.²⁰ As fucoxanthin did not cause any cell cycle arrest in the ovarian cancer cells, it probably activates an apoptotic pathway other than the pathway activated by DNA damage responses in these cells. In previous studies on the other cancer types and cell lines conducted with the aim of cell death characterization induced by fucoxanthin, apoptosis was frequently

demonstrated to be responsible mechanism for cell toxicity, however, various pathways were reported to be involved in induction of apoptosis.^{5,6} Fucoxanthin is a well-known antioxidant that also shown to be pro-oxidant. Reactive oxygen species (ROS) are well recognized as mediators of DNA damage. In hepatic BNL CL.2 cells, 6-h fucoxanthin (5 μ M) treatment augmented reactive oxygen species (ROS) levels in the cells, which returned to the control level at 12 h of incubation.²¹ In another study on human promyelocytic leukemia cells, HL-60, 24 h-fucoxanthin (10 μ M) treatment did not affect the ROS level at all. The authors suggested that ROS was not involved in the main pathway of apoptosis induced by fucoxanthin in these cells. This finding was in correlation with our results as we also noticed no detectable alteration in the cell cycle distribution (naturally accompanied by DNA damage response) in the 48 h-fucoxanthin-treated ovarian cancer cells. As fucoxanthin caused the loss of mitochondrial membrane potential at an early stage of treatment, they also concluded that fucoxanthin leads to apoptosis via the mitochondria in a manner different from oxidative stress. Fucoxanthin also induced cleavages of procaspase-3 and rabbit anti-poly (ADP-ribose) polymerase (PARP) which demonstrates the apoptosis induced by fucoxanthin was caspase-3 dependent.²² There are also other studies which reported the main role of caspase-3 in fucoxanthin-induced apoptosis in the human cervical cancer cells (Hela),²³ human bladder cancer cells (EJ-1 and T24),²⁴ osteosarcoma cells (Saos-2)²⁵ and endometrial cancer cells (HEC-1A).²⁶

Conclusion: Although, fucoxanthin was not able to modify cisplatin resistance in A2780RCIS ovarian cancer cells, it was highly effective in inducing apoptosis and death in both cisplatin sensitive and MRP2 overexpressing resistant ovarian cancer cells which demonstrates it is not an MRP2 substrate. To determine whether any clinical benefits exist for administration of fucoxanthin in treating cisplatin-resistant ovarian cancers additional detailed investigations including animal studies in xenograft cancer models are required.

Acknowledgment

The authors gratefully acknowledge of the Vice-Chancellor of Research in Mashhad University of Medical Sciences, Mashhad, Iran (Grant number 980871) for approval and financial support of this research.

Author contribution Statement

FVS conceived of the study, designed the study and drafted the manuscript. SK carried out the tests and performed the statistical analysis. HA, SG and AB coordinated the studies. FM designed the study, and helped to revising the manuscript. All authors read and approved the final manuscript.

Disclosure Statement

The authors report no conflict of interest.

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Table 1

Fucoxanthin IC50s in A2780 and A2780RCIS ovarian cancer cell lines.

line Cell	Fucoxanthin IC50 (μM) - 48 h	Fucoxanthin IC50 (μM) - 72 h
A2780	11.00 ± 0.53	10.94 ± 0.25
A2780RCIS	13.13 ± 1.06*	11.57 ± 0.50

The cells were treated with different concentrations of fucoxanthin for 48 and 72 h, then the sensitivity of ovarian cancer cell lines to cisplatin was measured by MTT assay and the IC50 values of fucoxanthin were determined. Each experiment was performed three times and the results were shown as mean ± SD. * $P < 0.05$ compared to sensitive cells incubated with fucoxanthin for 48 h (unpaired Students' t test).

Table 2

Cisplatin cytotoxicity in A2780 and A2780RCIS ovarian cancer cell lines.

Cell line	Cisplatin IC50 (μM) - 48 h	Cisplatin IC50 (μM) - 72 h
A2780	3.97 ± 0.56	2.51 ± 0.43 [#]
A2780RCIS	88.93 ± 4.90****	63.76 ± 2.07**** ^{##}

Cells were treated with different concentrations of cisplatin for 48 and 72 h, then the sensitivity of ovarian cancer cell lines to cisplatin was measured by MTT assay. The IC50 values of cisplatin were determined. Each experiment was performed three times and the results were shown as mean ± SD. **** $P < 0.0001$ compared to A2780 cells incubated with cisplatin for 48 h or 72 h, [#] $P < 0.05$ and ^{##} $P < 0.01$ compared to the same cell line treated with cisplatin for 48 h (unpaired Students' t test).