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Evaluation Of the Potential Cytotoxic, Antimetastatic, and Antioxidant Abilities Of Chrysin and Astaxanthin İn Triple-Negative Breast Cancer Cells

Üçlü Negatif Meme Kanseri Hücrelerinde Chrysin ve astaxanthin'in Potansiyel Sitotoksik, Antimetastatik ve Antioksidan Etkilerinin Değerlendirilmesi

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Öz

Giriş ve Amaç: Üçlü negatif meme kanseri (TNBC), tüm meme kanserleri arasında en kötü genel sağkalım oranına sahiptir. Bu çalışmanın amacı, MDA-MB-231 hücrelerinde chrysin ve astaxanthin'in hücre canlılığı/sitotoksisitesi, metastaz ve oksidatif stres üzerindeki etkilerini araştırmaktır.

Gereç ve Yöntem: Chrysin (5, 10, 15, 20, 25, 40, 50, 75, 90, 100 µg/ml) ve astaxanthin'in (5, 10, 15, 20, 40, 50, 75, 90, 100 µg/ml) TNBC (MDA-MB-231) hücrelerinde hücre canlılığı/sitotoksisitesi üzerindeki etkisi WST-1 ile belirlendi. Chrysin ve astaxanthin'in hücre göçü ve metastaz üzerindeki etkinliği çizik analizi ile belirlendi. Ayrıca chrysin ve astaxanthin'in MDA-MB-231 hücrelerindeki reaktif oksijen türleri (ROS) seviyesi üzerindeki etkisi DCF-DA analizi ile belirlendi.

Bulgular: Astaxanthin, WST-1 verilerimize göre MDA-MB-231 hücrelerinde hücre proliferasyonu üzerine etki göstermedi. Ancak yüksek chrysin dozları MDA-MB-231 hücre hattında hücre canlılığını tüm zaman aralıklarında %70'e kadar düşürdü. Ayrıca chrysin (40 µg/ml) ve astaksantin'e (25 µg/ml) 48 saat maruz kaldıktan sonra MDA-MB-231 hücrelerindeki çizik kapandı. 25 µg/ml dozunda astaxanthin maruziyetinden 24 saat sonra oksidatif strese neden olmadığı ancak 48. saatte yüksek floresans şiddeti gösterdiği tespit edildi. 40 µg/ml krisin uygulamasından sonra ise hem 24. hem de 48. saatte daha fazla floresans yoğunluğu tespit edildi.

Sonuç: Chrysin ve astaxanthin'in MDA-MB-231 hücrelerinde hücre göçü ve hücre içi ROS birikimi üzerine etkileri olabilir ancak hücre çoğalması üzerine etkisi saptanmadı.

Anahtar kelimeler: Üçlü negatif meme kanseri (TNBC); MDA-MB-231; hücre kültürü; chrysin; astaxanthin

Abstract

Aim: Triple-negative breast cancer (TNBC) has the worst overall survival of all breast cancers. The aim of this study was to investigate the effects of chrysin and astaxanthin on cell viability/cytotoxicity, metastasis, and oxidative stress in MDA-MB-231 cells.

Material and Methods: The effects of chrysin (5, 10, 15, 20, 25, 40, 50, 75, 90, 100 µg/ml) and astaxanthin (5, 10, 15, 20, 40, 50, 75, 90, 100 µg/ml) on cell viability/cytotoxicity in TNBC (MDA-MB-231) cells were determined by WST-1. The efficacy of chrysin and astaxanthin on cell migration and metastasis was determined

by scratch assay. In addition, the effect of chrysin and astaxanthin on the level of reactive oxygen species (ROS) in MDA-MB-231 cells was determined by DCF-DA analysis.
Results: Astaxanthin did not suppress cell proliferation in MDA-MB-231 cells according to our WST-1 data. However, cell viability of the MDA-MB-231 cell line at higher chrysin doses decreased to %70 at all-time intervals. After 48 hours of exposure to chrysin (40 µg/ml) and astaxanthin (25 µg/ml), the scratch in the MDA-MB-231 cells was closed. Astaxanthin at a dose of 25 µg/ml was found not to cause oxidative stress at 24 hours after exposure, but a high fluorescence intensity was detected at 48 hours. On the other hand, after the administration of 40 µg/ml chrysin, more fluorescence intensity was detected at both 24 and 48 hours.
Conclusion: Chrysin and astaxanthin may have effects on cell migration and intracellular ROS accumulation in MDA-MB-231 cells but had no effect on cell proliferation.
Key Words: Triple Negative Breast Cancer (TNBC); MDA-MB-231; cell culture; chrysin; astaxanthin

1. Introduction

Breast cancer, the most common type of cancer in women, is classified by the presence and absence of receptors including estrogen, progesterone and human epidermal growth factor 2 (HER2) [1]. The type of breast cancer in which all three receptor proteins are not expressed is considered triple negative breast cancer (TNBC). TNBC is a concept that encompasses fundamentally different diseases with different histological, genomic and immunological profiles [2]. TNBC accounts for 15-20% of all breast cancers and is associated with younger age, a more aggressive clinical course, and a worse prognosis than other histological types [3].

Chrysin, widely used as an herbal medicine in China, is a bioactive flavone with multiple biological properties that can be extracted from plant extracts such as propolis, honey and blue passionflower [4]. Chrysin is a natural compound with antioxidant, anti-inflammatory, anticancer, antihypertensive, antidiabetogenic and anxiolytic properties [5]. In addition, chrysin has potent anti-cancer activity in many types of cancer by inducing cell cycle arrest and intrinsic and extrinsic apoptosis [4]. Although the exact molecular mechanisms of chrysin are not fully understood, it has been reported that it can induce apoptosis in many types of cancer, including breast cancer [6]. It has been reported that it can induce apoptosis by the following mechanisms: facilitating the release of cytochrome C from mitochondria, activating caspase-3 and inhibiting the activity of the X-linked inhibitor of apoptosis protein (XIAP), and reducing protein kinase B (AKT) phosphorylation and triggering the phosphatidylinositol 3-kinase (PI3K) pathway [7]. However, despite the therapeutic effects of chrysin, its bioavailability is poor due to its rapid metabolism, poor absorption, and rapid systemic excretion [8].

Astaxanthin is an orange-red pigmented molecule, a member of the carotenoid family, with high antioxidant properties. The people generally get astaxanthin, which is biosynthesized by microalgae, bacteria, and fungi, from seafood such as salmon, shrimp, lobster, crab, trout, and fish roe. [9]. Astaxanthin, a xanthophyll carotenoid, can exist both inside and on the surface of the phospholipid membrane. Therefore, astaxanthin may exert its effects against reactive oxygen species (ROS) both at the surface and inside of phospholipid membranes [10]. Astaxanthin has anti-oxidant, anti-inflammatory, anti-cancer, anti-lipid peroxidation, immunomodulatory, UV protection and anti-aging properties, and its antioxidant activity is greater than that of coenzyme Q10 or vitamin E [11]. Astaxanthin has shown anti-proliferative activity in many cancer lines, including breast cancer [12]. Astaxanthin induces intrinsic apoptosis through inhibition of PI3K, AKT and mitogen-activated protein kinase (MAPK) signalling [13]. In addition, astaxanthin reduces the expression of anti-apoptotic proteins such as Bcl-2, resulting in the induction of caspases and consequently the apoptosis of cancer cells [14].

MDA-MB-231 cells are highly resistant to chemotherapy and radiotherapy. Therefore, new therapeutic agents need to be developed. Chrysin and astaxanthin have been reported to induce apoptosis through similar pathways. However, the effects of these two antioxidants on oxidative stress-mediated cell death in MDA-MB-231 cells have not been investigated. The aim of this study is to investigate the effects of chrysin and astaxanthin on cell viability/cytotoxicity, apoptosis, metastasis, and oxidative stress in chemo- and radioresistant breast cancer cells (MDA-MB-231).

2. Material and Methods

2.1. Cell culture

The ER/PR- Her2/neu MDA-MB-231 breast cancer cell line was used to determine the effects of chrysin and astaxanthin on cell viability, migration, and reactive oxygen species ROS of TNBC cells. The MDA-MB-231 cell line was obtained from the ATCC (Rockville, MD, USA). MDA-MB-231 cells were grown in medium containing DMEM-F12 (Dulbecco's modified Eagle medium F12), 10% FBS (Fetal Bovine Serum) (v/v), 1% penicillin/streptomycin (v/v) under 5% CO2 and 37°C conditions. Experiments were started when MDA-MB-231 cells reached confluence [15].

2.2. Determination of the effectiveness of chrysin and astaxanthin on cell viability

The effects of chrysin and astaxanthin on cell proliferation in MDA-MB-231 cell lines were determined using the WST-1 method. For the WST-1 experiment, MDA-MB-231 cells were seeded in 96-well plates at $1x10^4$ cells/well in 100 µl. The MDA-MB-231 cell line was then allowed to proliferate and differentiate for 24 hours [16]. After microscopic observation of the cells under appropriate conditions, chrysin (5, 10, 15, 20, 25, 40, 50, 75, 90, 100 µg/ml) [17, 18] and astaxanthin (5, 10, 15, 20, 40, 50, 75, 90, 100 µg/ml) [19-21] were applied to MDA-MB-231 cells at different and specific doses. Cell proliferation was analyzed using the WST-1 cell proliferation reagent kit (Roche). Briefly, 10 µl of WST-1 compound was added to each well and after incubation for 3 hours at 37°C, readings were taken at 450 nm (reference wavelength 620 nm) on the multiscan spectrophotometer at 24, 48 and 72 hours. The absorbance of control cells, to which no substance was applied, was determined as 100% and the percentages of the applied doses were calculated by comparison with the control. The experiment was performed in four replicates [16].

2.3. Determination of cell migration rates using the in vitro scratch assay method

The cell scratch test was used to evaluate the effects of chrysin and astaxanthin on the migration ability of MDA-MB-231 cells. Cells were plated in 6-well plates at $5x10^5$ cells/well and allowed to cover the Petri dish surface in a monolayer (24 hours). A linear scratch was then made into the monolayer cells using a sterile 10 µl pipette tip. After scratching, the tip was washed with fresh medium to remove any residue. Chrysin and astaxanthin were applied before the medium was added on the cells, 5 ml of scratch assay medium containing 3% FBS was added to the cells. In order to make accurate measurements, reference lines were created by drawing a straight line with a very thin pencil close to the edges of the scratch. In the first stage, the cells were photographed with a microscope immediately after the drawing process and this photograph was taken as the 0. hour (starting) photograph. Similarly, cells were photographed after 24 and 48 hours to make the necessary calculations. The remaining distance between the drawn part was calculated using Image J software [22].

2.4. Determination of reactive oxygen species level in cells

2′,7′-Dichlorofluorescin diacetate (DCF-DA) is an oxidation-sensitive dye. It is a non-fluorescent compound, but when taken up by cells it is converted to the fluorescent form in the cell cytosol. Chrysin and astaxanthin were applied to MDA-MB-231 and control cells in 12-well plates with round coverslips while the level of intracellular reactive oxygen species (ROS) was determined. The plates were then washed once with PBS. A 0.5 ml of DCF-DA solution prepared in serum-free medium was added to the cells to a final concentration of 10 µM (from stock solutions prepared in 10 mM DMSO and stored in aliquots at 20°C). The cells were kept in the dark at 37°C for 30 minutes, wrapped in aluminium foil. They were quickly and gently washed 3 times with PBS and examined under a fluorescence microscope after fixation with paraformaldehyde. Cells incubated with 250 µM H_2O_2 were used as a positive control [23, 24].

2.5. Statistical analysis

The statistical analyses of the study were performed using GraphPad Prism 6 program. The comparison of cell viability of MDA-MB-231 cell lines with control cells was performed by one-way ANOVA test followed by Dunnet post-hoc test. The statistically significant values were defined as $p<0.05$. All analysis were performed independently in at least triplicate.

3. Results

3.1. Effects of chrysin and astaxanthin on the viability of MDA-MB-231 cells

The half-maximal inhibitory concentration (IC_{50}) values of astaxanthin and chrysin are shown in Table 1.

Table 1. Half-maximal inhibitory concentration (IC_{50}) values of astaxanthin and chrysin.

	24 h	48 h	72 h
Astaxanthin	Unstable	Non determined	366.9
Chrysin	111.6	Unstable	138.0

The cytotoxic effects (24, 48 and 72 hours) of doses of chrysin (5, 10, 15, 20, 25, 40, 50, 75, 90, 100 µg/ml) and astaxanthin (5, 10, 15, 20, 40, 50, 75, 90, 100 µg/ml) were investigated on the TNBC cell line (MDA-MB-231). MDA-MB-231 cells were exposed to chrysin at different doses ranges for 24, 48 and 72 hours. According to our WST-1 data, especially lower doses of chrysin (10 and 15 µg/ml) increased cell viability statistically significantly at 24- and 72-hours exposure ($p<0.0001$). However, cell viability of the MDA-MB-231 cell line at higher chrysin doses decreased to %70 at all-time intervals (Fig. 1).

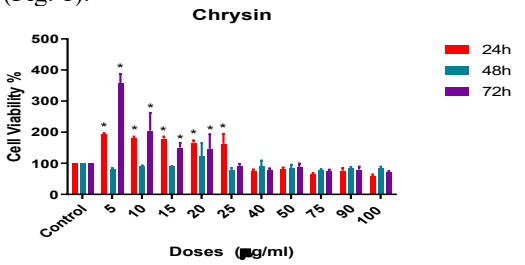


Fig. 1. Effects of chrysin on cell proliferation in MDA-MB-231 cells.

Astaxanthin was added to MDA-MB-231 cells at various doses and treated for 24, 48 and 72 hours. After treatment, WST-1 cell proliferation agent was used for cell viability/cytotoxicity experiment according to the manufacturer's protocol. According

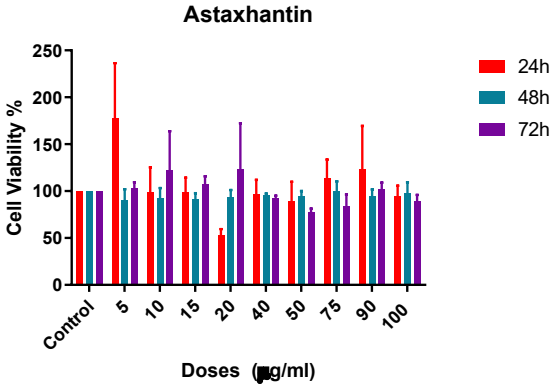


Fig. 2. Effects of astaxanthin on cell proliferation in MDA-MB-231 cells.

to our WST-1 data of astaxanthin, there was no statistically significant result ($p>0.05$). The effect of astaxanthin was found to be unstable and not precise at all time intervals (Fig. 2).

In the WST-1 cell proliferation assay, a dose of 40 µg/ml for chrysin and 25 µg/ml for astaxanthin were determined to be the most effective doses, and therefore these doses were used in the cell migration assay and reactive oxygen species analyses.

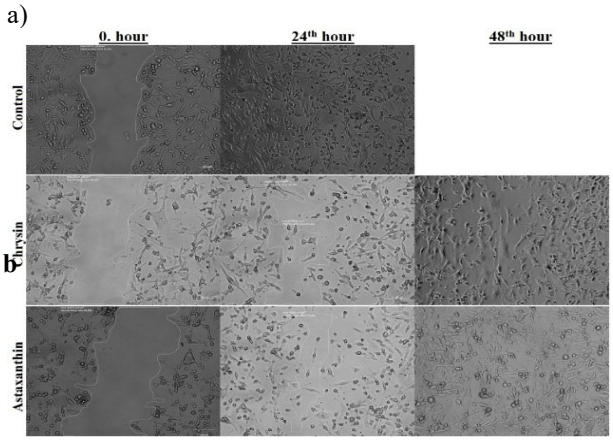
3.2. Effect of chrysin and astaxanthin on migration of MDA-MB-231 cells

In the scratch assay, MDA-MB-231 cells were examined at 0, 24 and 48 hours after being treated with chrysin and astaxanthin, and their images were recorded. After performing in vitro scratch assay method on MDA-MB-231 cells, we have observed that the scratch is healed up at 24th hour. After exposure of the MDA-MB-231 cell line to chrysin (40 µg/ml) for 48 hours, the scratch closed. Interestingly after 25 µg/ml dose of astaxanthin treatment, we have found that scratch is covered at 48th hour (Fig. 3).

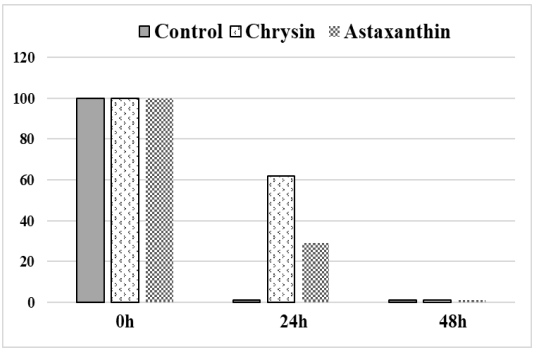
3.3. Effect of chrysin and astaxanthin on the level of reactive oxygen species in MDA-MB-231 cells

ROS is a key regulator of many cellular activities, including cellular metabolism, cell cycle and programmed cell death. Fluorescent DCF-DA was used to determine the effect of chrysin and astaxanthin on intracellular ROS accumulation in the MDA-MB-231 cell line. After 24 and 48 hours of treatment with chrysin and astaxanthin, cells were imaged using an inverted microscope with a fluorescent attachment (Fig. 4). According to our DCF-DA cellular ROS assay experiment, minimum florescence light was observed at 24th and 48th hours in control group. It was found that astaxanthin did not induce oxidative stress at 24 hours after exposure at a dose of 25 µg/ml; however, a much higher fluorescence intensity was detected at 48 hours. On the other hand, after application of 40 µg/ml chrysin, more fluorescence intensity was detected at both 24 and 48 hours ($p:0.0028$).

a)



b



b)

Fig. 3. Effects of chrysin and astaxanthin on cell migration in MDA-MB-231 cells.

(a. Images were obtained by scanning with a fluorescence microscope at a 10× magnification objective. b. The closure areas of the cells in the scratch test assay are given as a percentage compared to the control.)

4. Discussion

Breast cancer is a very common and extremely heterogeneous disease with many different subtypes [25]. Treatment options for TNBC, an aggressive form of breast cancer, are very limited. Therefore, it is very important to develop new drugs to understand the molecular basis of the disease [26].

Chrysin may suppress most cancer-related pathways and inhibit cancer by inducing apoptosis and also attenuating autophagy-induced cell death [27]. Chrysin effectively inhibits cell proliferation while inducing apoptosis in breast cancer cell lines, and it may downregulate cyclin D1, causing cell cycle arrest in G1 [28]. Chrysin has been studied in many different breast cancer cell lines. The combination of

a

chrysin and pyrotinib treatment increased autophagy in BT-474 and SKBR-3 cells [29]. Chrysin T47D decreased proliferation in breast cancer cells [30] and showed cytotoxic effects [31, 32]. In MCF-7 cells, it showed an antiproliferative effect by inducing apoptosis [33]. Chrysin impaired genomic stability in MCF-7 and BT474 cells, prevented cell survival and increased the sensitivity of MCF-7 cells to chemotherapeutic drugs. It also disrupted DNA

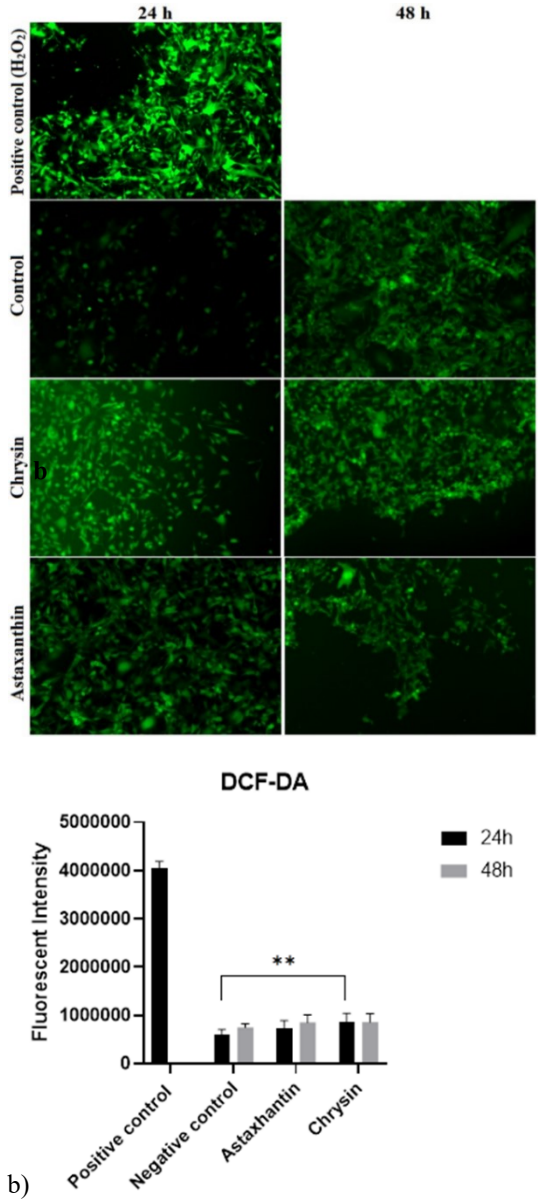


Fig. 4. Effects of chrysin and astaxanthin on the level of reactive oxygen species in MDA-MB-231 cells.
(a. Fluorescence microscopic image of cells. b. Measurement of the fluorescence intensity that indicates the increase in ROS in cells treated with chrysin and astaxanthin. Groups with the asterisk symbol (**) on the graph indicate that they are statistically. (p:0.0028))

double-strand break repair and caused accumulation of DNA damage [34]. The combination of curcumin and chrysin in nanocapsule form induced apoptosis in MDA-MB-231 cells and showed cytotoxic activity [35]. Chrysin amino acid derivatives decreased Bcl-2, increased Bax and induced apoptosis in MDA-MB-231 and MCF-7 cells [36]. It was reported that Chrysin nanoparticles inhibited cell proliferation via PI3K/JNK, induced apoptosis via p53 and acted as metastasis inhibitor by suppressing matrix metalloproteinases (MMPs) in MDA-MB-231 derived xenograft model [37]. Chrysin was shown to inhibit the growth of MDA-MB-231 cells and induce cytoplasmic lipid accumulation in cells but did not cause apoptosis and cell death [38]. Chrysin was shown to downregulate MMP-10 and suppress MDA-MB-231 cell migration and invasion. Furthermore, chrysin was shown to induce E-cadherin expression and decrease vimentin, snail and slug expression in TNBC cells. In conclusion, chrysin was reported to play a role in antimetastatic activity by inhibiting the Akt pathway and regulating MMP-10 expression [39]. It was reported that chrysin and 1,2,3,4,6-penta-O-galloyl-β-D-glucose (5GG) combined treatment synergistically induced apoptosis, arrested cell cycle and inhibited cell proliferation and colony formation in MDA-MB-231 cells [40]. It has been shown that the administration of chrysin in combination with radiotherapy to MDA-MB-231 cells increased Bax and p53 gene levels, decreased Bcl-2 expression and increased apoptosis. It was also reported that increased apoptosis correlated with decreased expression of cyclin D1 and p-STAT3. [41]. MDA-MB-231 cells are highly resistant to chemotherapy and radiotherapy. Chrysin applied in our study had no effect on cell proliferation, but chrysin induced oxidative stress. Chrysin can induce oxidative stress in MDA-MB-231 cells and induce MDA-MB-231 cells to undergo apoptosis as reported in the literature. In addition, according to our results, we found that chrysin suppressed metastasis in MDA-MB-231 cells. However, there is only one study that investigated the effect of chrysin on metastasis in MDA-MB-231 cells, and in that study, it was reported that it suppressed metastasis through MMP-10 [39]. In this sense, our study results may provide preliminary findings for newly designed studies.
Astaxanthin has been reported to induce apoptosis in breast cancer cell lines, with negligible effects on MCF-10A [42, 43]. Astaxanthin induces cancer cell apoptosis by decreasing Bcl-2 expression [14]. Co-administration of astaxanthin and carbendazim was shown to have anti-proliferative effects in MCF-7 cells, arresting the cell cycle at the G2/M phase and alleviating ROS production associated with carbendazim treatment [44]. Astaxanthin suppressed

proliferation of T47D and BT20 cells [45], blocked cell cycle progression of SKBR3 cells at G0/G1, suppressed proliferation and induced apoptosis [13]. Astaxanthin was demonstrated to have no effect on cell viability in MCF-7, SKBR3 and MDA-MB-231 cell lines [46]. Astaxanthin-mediated nanoparticles were reported to have strong cytotoxic effects in MDA-MB-231 cells [47]. Astaxanthin decreased cell viability and Bcl-2 expression and induced apoptosis and DNA damage in MDA-MB-231 and T47D cells. Astaxanthin also caused more cell death in T47D cells compared to MDA-MB-231 cells. In addition, the cytotoxic effect of astaxanthin on MCF10A cells, its effects on apoptosis and DNA damage are insignificant compared to MDA-MB-231 and T47D [14]. Astaxanthin blocked proliferation, reduced cell number and cell migration in MCF-7 and MDA-MB-231 cells [43]. In our study, astaxanthin applied had no effect on cell proliferation, but we found that astaxanthin could induce oxidative stress. Astaxanthin can suppress cell proliferation and induce oxidative stress in MDA-MB-231 cells. According to our results, we also determined that astaxanthin suppressed metastasis in MDA-MB-231 cells. The number of studies investigating the effect of astaxanthin on TNBC cells, a type of resistant breast cancer, is very limited, so our study may provide data for new studies.

5. Conclusion

Chrysin and astaxanthin need to be investigated as new treatment agents in resistant TNBC cells due to their apoptosis induction in various types of cancer. Therefore, in our study, the effects of chrysin and astaxanthin on cell viability/cytotoxicity, apoptosis, metastasis, and oxidative stress were investigated. MDA-MB-231 cells are known to be highly resistant to chemotherapy and radiotherapy. Chrysin and astaxanthin did not have any significant effects on the proliferation of MDA-MB-231 cells, but it was found that chrysin and astaxanthin could induce ROS accumulation. Chrysin and astaxanthin were also found to suppress metastasis. According to our results, it can be said that chrysin and astaxanthin may stimulate cell death by inducing ROS accumulation in TNBC cells, which are resistant breast cancer cells, and may play a role in suppressing metastasis.

A limitation of this study is that chrysin and astaxanthin were not tested in healthy breast cell lines. However, this is a preliminary study and chrysin and astaxanthin will be studied in several breast cancer cell lines and healthy breast cell lines to compare the results. In addition, the fact that annexin-V and caspase protein activity were not investigated to determine the source of the cytotoxic effect of chrysin and astaxanthin on tumour cells is another limitation. In particular, it is necessary to investigate the type of cell death caused by chrysin and astaxanthin by determining the PI3K and AKT pathways, caspase 3 protein level, and apoptosis/necrosis.

6. Acknowledgement

Ethics Committee Approval

Since a commercial cell line was used in the study, there is no need for an ethics committee.

Conflict of Interest

The authors declared no conflict of interest.

Financial Disclosure

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