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Araştırma Makalesi – Research Article

Therapeutic Effect of Astaxanthin on 5-Fluorouracil-Induced Ovarian Damage in Rats

Astaksantin'in Sıçanlarda 5-Florourasil ile İndüklenen Over Hasarı Üzerindeki Terapötik Etkisi

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ABSTRACT

Although astaxanthin (ASX) is one of the most studied antioxidant molecules, its curative effect against ovarian damage caused by 5-fluorouracil (5-FU) has not been demonstrated to date. It was therefore aimed to investigate whether ASX is therapeutic against 5-FU-induced ovotoxicity in this study. Rats were first exposed to 5-FU (100 mg/kg) and then treated ASX (250 µg/kg) for three days. Oxidative stress (OS), inflammation and apoptosis markers were determined using spectrophotometric methods. Ovarian tissues were also evaluated histologically. The levels of OS, inflammation and apoptosis biomarkers increased by 5-FU administration ($p<0.05$). Treatment with ASX significantly alleviated these markers ($p<0.05$). These findings reveal that ASX may exert an ovoprotective effect by reducing pro-inflammatory mediators and enhancing antioxidant status in ovarian tissue.

Keywords- 5-fluorouracil, Astaxanthin, Inflammation, Ovarian damage, Oxidative stress

ÖZ

Astaksantin (ASX) üzerinde en çok çalışılan antioksidan moleküllerden biri olmasına rağmen, 5-florourasil (5-FU)'in neden olduğu yumurtalık hasarına karşı iyileştirici etkisi bugüne kadar gösterilmemiştir. Bu nedenle bu çalışmada ASX'in 5-FU ile indüklenen ovotoksisiteye karşı terapötik olup olmadığının araştırılması amaçlandı. Sıçanlar önce 5-FU'ya (100 mg/kg) maruz bırakıldı ve ardından üç gün boyunca ASX (250 µg/kg) tedavisi

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gördü. Oksidatif stres (OS), inflamasyon ve apoptoz belirteçleri spektrofotometrik yöntemlerle belirlendi. Ayrıca yumurtalık dokuları histolojik olarak da değerlendirildi. OS, inflamasyon ve apoptoz biyobelirteçlerinin seviyeleri 5-FU uygulamasıyla arttı ($p<0.05$). ASX ile tedavi bu belirteçleri önemli ölçüde iyileştirdi ($p<0.05$). Bu bulgular, ASX'in yumurtalık dokusunda pro-inflamatuar mediatörleri azaltarak ve antioksidan durumu güçlendirerek yumurtalık koruyucu bir etki gösterebileceğini ortaya koymaktadır.

Anahtar Kelimeler- 5-florourasil, Astaksantin, İnflamasyon, Oksidatif stres, Yumurtalık dokusu hasarı

I. INTRODUCTION

The incidence of cancer is increasing all over the world, and chemotherapeutic drugs are used alone or in combination in the treatment of cancer [1, 2]. Since chemotherapeutics have a low therapeutic index, they affect not only target malignant cells but also healthy cells [3]. 5-fluorouracil (5-FU) is classified within the antimetabolite chemotherapeutics and is frequently used against colorectal, head and neck, breast, esophageal, pancreas, cervical, stomach, renal and bladder cancers for many years [4]. It exhibits anticancer activity mainly by inhibiting DNA synthesis [5]. In addition to leukopenia, hemolytic anemia and thrombocytopenia, 5-FU administration may cause some side effects, such as stomatitis, mucositis, diarrhea and various organ toxicity, including ovotoxicity [4, 6, 7]. Increasing evidence has revealed that tissue toxicity induced by 5-FU is associated with oxidative stress (OS) and inflammation due to increased reactive oxygen species (ROS) production and decreased glutathione (GSH) levels [5]. Redox balance is a vital feature of the organism and OS is caused by an imbalance of the redox system in the body [1]. ROS are produced in the organism depending on redox reactions of endogenous and exogenous sources. While ROS within physiological limits are essential for cell signaling, ROS within the pathological margin cause damage to cellular macromolecules, including lipids, nucleic acids and proteins [8]. Excessive ROS is tried to be neutralized by enzymatic molecules, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and non-enzymatic molecules, such as GSH [9]. Failure to balance the increase in ROS by antioxidant systems causes OS [3]. It causes inflammation by increasing the levels of inflammatory markers, such as tumor necrosis factor-alpha (TNF-α) and myeloperoxidase (MPO). The cooperation of OS and inflammation activates apoptosis, causing permanent tissue damage and tissue death [1]. Since there is no approved treatment protocol or a specific antidote to be used against the toxic effects of chemotherapeutic use, different toxicity prevention strategies are currently being studied meticulously [10, 11]. In this context, the therapeutic effects of molecules with antioxidant and anti-inflammatory properties against chemotherapeutic-induced tissue damage are frequently investigated [1, 3, 5].

Carotenoids, a group of antioxidants, contain over 700 different compounds produced by phytoplankton, algae, plants, fungi and bacteria [3]. Astaxanthin (ASX) is a red-orange xanthophyll carotenoid that can be produced in algae, plants and some animals, including crab, salmon and flamingo [12]. ASX is considered a powerful antioxidant due to its structure consisting of a conjugated double bond and ketone groups [13]. The physiological actions of ASX are antioxidant, anti-inflammatory and anticancer activities [8]. Although ASX has been shown to reduce the toxicity of various chemotherapeutic agents in liver, brain and kidney tissues [14-16], there is no research on its effect on 5-FU-induced ovotoxicity. This study was conducted to investigate the therapeutic effect of ASX, which is known to have antioxidant and anti-inflammatory activities, against 5-FU induced ovarian injury model, and also the mechanism related to effect was investigated for the first time.

II. MATERIAL AND METHOD

A. Experimental Design

All experiments were performed in accordance with the approval of Local Animal Ethics Committee of Karadeniz Technical University (Protocol number: 2021/29). Only rats whose estrus stage was confirmed by the examination of vaginal smear samples taken from the animals were used [5]. ASX and 5-FU were purchased from Sigma-Aldrich (MO, USA) and dissolved with 5% dimethyl sulfoxide (DMSO) [17, 18] and physiological saline [5, 19], respectively. All the treatments were given via intraperitoneally. The rats were randomly divided into 4 groups (A-D) with six subjects in each group. Group A was given physiological saline on day 1 and 5% DMSO for the following three days. Group B was given 5-FU (100 mg/kg) on day 1, followed by 5%DMSO for 3 days. Group C were treated with 5-FU on day 1, followed by ASX (250 µg/kg/day) for 3 days. Group D was given physiological saline on day 1, followed by ASX (250 µg/kg/day) for 3 days. The doses of ASX [20, 21] and 5-FU [5, 19] were determined according to previous related studies. All rats were sacrificed by cervical dislocation on day 5 [5, 22] and ovarian tissues were removed. Half of the tissues were harvested at -80°C for biochemical examinations, while the other parts were preserved in histological evaluation.

B. Biochemical Analysis

Ovarian tissues (approximately 25 mg) were homogenized in 2 mL of phosphate-buffered saline using a homogenizer (IKA, T25 Ultra-Turrax, Staufen, Germany). The protein levels of the supernatants were determined using a commercial colorimetric kit (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL) according to the manufacturer's recommendations. Amount of malondialdehyde (MDA) were determined using previously described method [23]. Briefly, 250 µL of supernatant was mixed with 1.5 mL of phosphoric acid and 0.5 mL of thiobarbituric acid and incubated in a boiling water bath for 1 h. Then, the tubes were centrifuged at 1800xg for 10 min and the absorbances of the supernatants were read at 532 nm using a microplate reader spectrophotometer (Molecular Devices, CA, USA). 1,1,3,3-tetramethoxypropane was used as a standard and tissue MDA levels were expressed as nmol/mg protein [24].

Total oxidant status (TOS) and total antioxidant status (TAS) levels were determined using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey). Oxidative stress index (OSI) was calculated with the following formula [25]:

$$\text{OSI (arbitrary unit)} = (\text{TOS/TAS}) \times 100 \quad (1)$$

The levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), SOD, CAT, TNF-α, MPO and cleaved caspase-3 were evaluated by ready-to-use enzyme-linked immunosorbent assay (ELISA) kits (Finetest, Wuhan, China). The absorbances were measured at 450 nm using a microplate reader spectrophotometer (Molecular Devices, CA, USA). Then, the concentration-absorbance graph was generated for the standards and the levels of the investigated parameter in the samples were determined according to this graph by using the absorbance values of the samples.

C. Histological Analysis

Tissue samples were fixed in 10% formaldehyde for two days and then placed in paraffin blocks after routine tissue follow-up. Sections of 5 µm were stained with hematoxylin&eosin and examined under a light microscope (Olympus BX50, Tokyo, Japan). The prepared slides were evaluated using previously described scale [26, 27].

D. Statistical Analysis

Statistical analysis was performed using statistical package for the social sciences (SPSS) version 23.0 (Chicago, IL, USA) statistical software. The conformity of the data to the normal distribution was evaluated with Kolmogorov-Smirnov test. Since the data did not show a normal distribution, they were expressed as median and 75^{th} and 25^{th} percentile (IQR). Kruskal Wallis and followed by Mann-Whitney U tests were used for statistical analysis between groups. $p<0.05$ is considered statistically significant.

III. RESULTS

All biochemical findings were shown in Table 1. Results indicated that 5-FU administration elevated MDA, TOS, OSI and 8-OHdG levels compared with Group A (all p=0.004). Treatments with ASX significantly decreased these levels (all p=0.004).

The TAS, SOD and CAT levels in Group B were significantly lower than Group A (all p=0.004). Hovewer, ASX alleviated these levels significantly (p=0.025, p=0.004 and p=0.004, respectively).

There were significant elevations of ovarian TNF-α, MPO and cleaved caspase-3 levels in 5-FU-treated group as opposed to Group A (all p=0.004). Administration of ASX significantly decreased these levels compared to Group B (all p=0.004). Interesingly no significant differences were between control and ASX (*per se* group) groups in terms of biochemical markers (p>0.05).

Table 1. Levels of biochemical parameters in rat ovarian tissues among the groups

	Control	5-FU	5-FU+ASX	ASX
TOS (μM H_2O_2 equivalent/L)	11.9 (11.3-12.1)	28.3 (18.6-40.6)[a]	10.7 (10.5-10.9)[b]	11.6 (9.65-13.5)
TAS (mM trolox equivalent/L)	0.58 (0.55-0.70)	0.31 (0.28-0.37)[a]	0.59 (0.51-0.66)[b]	0.58 (0.52-0.64)
OSI (arbitrary unit)	2.24 (2.14-2.31)	9.26 (6.63-11.0)[a]	1.85 (1.62-2.15)[b]	2.10 (1.57-2.33)
MDA (nmol/mg protein)	25.0 (23.9-26.1)	81.4 (78.7-89.8)[a]	18.9 (14.0-25.2)[b]	22.0 (13.7-26.1)
8-OHdG (ng/mg protein)	2.22 (1.83-2.94)	9.85 (9.19-11.2)[a]	3.04 (1.58-3.75)[b]	2.38 (1.94-3.40)
SOD (ng/mg protein)	3.97 (3.71-4.28)	1.25 (0.97-1.33)[a]	3.37 (2.78-5.30)[b]	4.09 (3.04-5.39)
CAT (mIU/mg protein)	841.2 (734.5-1007.0)	293.2 (185.7-369.8)[a]	838.4 (693.6-944.0)[b]	857.0 (825.8-1074.8)
MPO (ng/mg protein)	5.24 (4.73-6.62)	10.2 (8.11-10.9)[a]	4.04 (3.36-5.80)[b]	5.36 (3.44-7.13)
TNF-α (pg/mg protein)	152.9 (129.1-170.3)	425.7 (354.0-518.0)[a]	189.9 (184.0-220.0)[b]	167.3 (130.0-206.2)
Caspase-3 (ng/mg protein)	0.99 (0.86-1.37)	8.43 (8.01-8.84)[a]	1.09 (0.70-1.69)[b]	0.88 (0.62-1.67)

Data were expressed as medians with a 25th and 75th percentile interquartile range (IQR). P-values according to Kruskal-Wallis and the Mann-Whitney U test. [a]p<0.05 compared with control group, [b]p<0.05 compared with 5-FU group. 5-FU: 5-fluorouracil, ASX: astaxanthin, TOS: total oxidant status, TAS: total antioxidant status, OSI: oxidative stress index, MDA: malondialdehyde, 8-OHdG: 8-hydroxy-2'-deoxyguanosine, SOD: superoxide dismutase, CAT: catalase, MPO: myeloperoxidase, TNF-α: tumor necrosis faktor-alpha.

All histological findings were presented in Table 2 and Figure 1. Administration of 5-FU significantly increased vascular congestion, edema, follicular degeneration and leukocyte infiltration levels in the ovarian tissue (p=0.006, p=0.002, p=0.019 and p=0.046, respectively) (Table 2 and Figure 1). ASX treatment alleviated these parameters significantly compared to Group B (p=0.006, p=0.002, p=0.019 and p=0.046, respectively).

Table 2. Histopathological findings of experimental groups

	Control	5-FU	5-FU+ASX	ASX
Vascular congestion	0.5 (0-1)	2 (1.75-3)[a]	0.5 (0-1)[b]	0.5 (0-1)
Edema	0 (0-1)	2 (2-2.25)[a]	1 (0-1)[b]	0 (0-1)
Hemorrhage	0 (0-0)	0 (0-0.25)	0 (0-0)	0 (0-0)
Follicular degeneration	0 (0-0)	1 (0-1)[a]	0 (0-0)[b]	0 (0-0)
Leukocyte infiltration	0 (0-0)	0.5 (0-1)[a]	0 (0-0)[b]	0 (0-0)

Data were expressed as medians with a 25th and 75th percentile interquartile range (IQR). P-values according to Kruskal-Wallis and the Mann-Whitney U test. [a]p<0.05 compared with control group, [b]p<0.05 compared with 5-FU group. 5-FU: 5-Fluorouracil, ASX: astaxanthin.

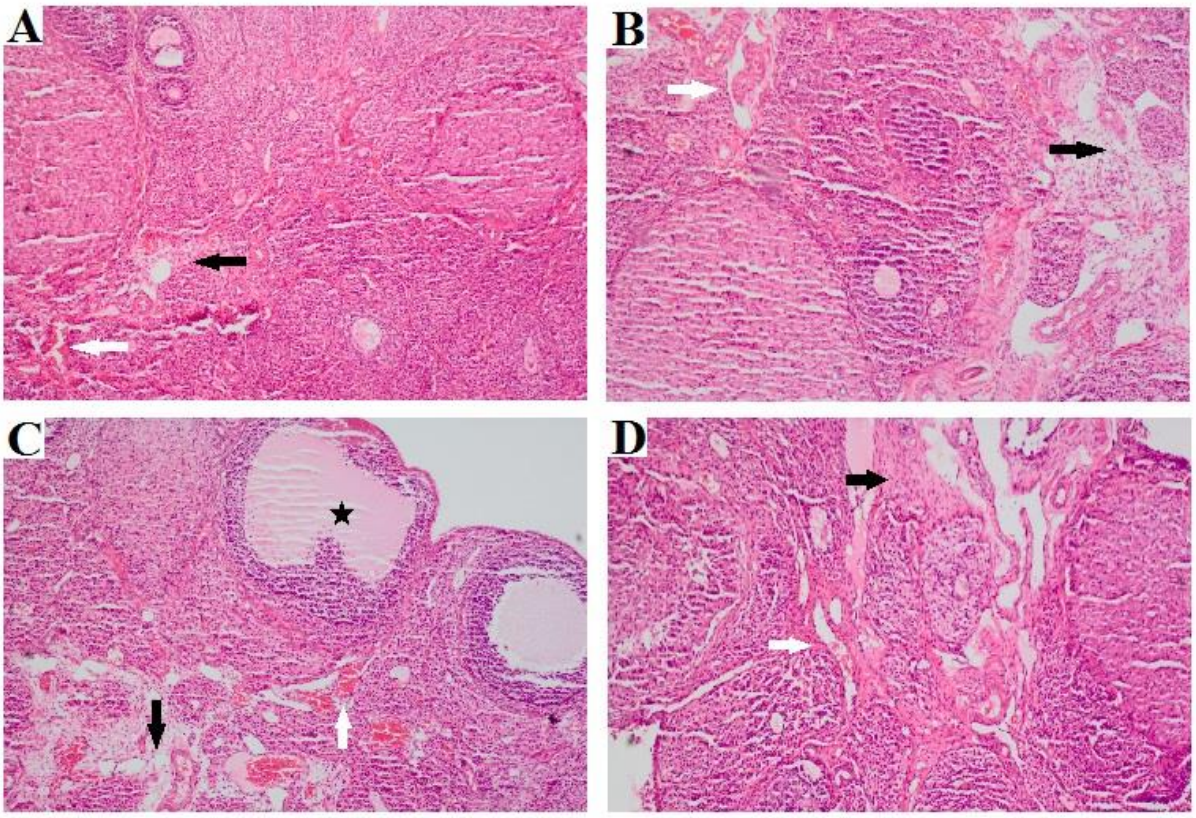

Figure 1. Histopathological examination of rat ovarian tissues-stained H&E (×100). (A) Control group: Normal ovarian tissue structure, with mild vascular congestion (white arrow) and edema (black arrow) in the interstitial area. (B) ASX group: Ovarian tissue containing mild vascular congestion (white arrow) and edema (black arrow) in the interstitial space. (C) 5-FU group: In ovarian tissues, there were severe vascular congestion (white arrow), moderate edema (black arrow) and degenerative follicle structure containing leukocyte infiltration (black star). (D) 5-FU+ASX group: An ovarian tissue structure very similar to the control group, with mild vascular congestion (white arrow) and edema (black arrow) in the interstitial area.

IV. DISCUSSION

The ovarian follicle reserve created in fetal life is not renewed throughout the female's life. Follicle excretion occurs with the onset of ovulation cycles, especially with puberty. For this reason, the female continues her life with the gradual decrease in the number of oocytes in her ovaries. Events that accelerate this physiological decline in an uncontrolled manner, such as pesticide/radiation exposure and chemotherapeutic use, cause a decrease in fertility rates in women [28]. Elimination of chemotherapy-induced reproductive toxicity is therefore crucial for fertility [29]. Ovarian tissue is one of the tissues most affected by chemotherapeutics. Utilization of chemotherapeutics can cause ovarian damage in the acute period, and early menopause in the chronic period [30, 31]. The results revealed that 5-FU administration increased vascular congestion, edema, follicular degeneration, and leukocyte infiltration findings in rat ovarian tissue. These findings demonstrate that a single dose of 5-FU (100 mg/kg) has been successfully established with an experimental ovarian injury model. Moreover, this was consistent with the results of previous studies showing that 5-FU causes ovarian damage in experimental models [5, 7, 32]. Histological data showed that 5-FU followed by 3 days of ASX treatment significantly improved parameters indicative of 5-FU-induced ovarian damage. This situation was evaluated as a sign that ASX has an ovoprotective effect. Although many beneficial biological properties of ASX have been demonstrated in experimental models [8, 33], investigation of its ovoprotective effect has become popular in recent years [28]. Our histological findings were consistent with the results of previous studies demonstrating the ovarian protective effect of ASX [11, 34, 35].

Increased ROS, inflammation and apoptosis, and suppression of antioxidants are main mechanisms of 5-FU-induced tissue damage [36]. If the oxidant/antioxidant balance is disrupted, it causes OS [37, 38]. While ROS, which can occur endogenously in the organism, are important for cell signaling, high levels of ROS damage cellular functions [39-41]. It is thought that OS is an important harmful factor affecting the development of follicles, oocytes and embryos, and increased OS can cause subfertility and infertility [34, 35]. ROS attacks initiate lipid peroxidation (LPO) in membranes and this chain process continues with the formation of unstable and reactive products. MDA is considered the most important indicator of LPO level [14]. DNA is one of the most important targets of ROS and shows increased 8-OHdG DNA damage. 8-OHdG levels in ovarian tissues

were therefore measured to determine the level of free radical-mediated DNA damage in this study [27]. TOS and TAS measurements are useful, fast and simple methods to evaluate the complex oxidative mechanism of a pathology [16, 22]. SOD and CAT are two of the most important enzymes protecting the cell against ROS attacks [15, 25]. Our results showed that a single dose of 5-FU administration significantly increased the levels of MDA, TOS, OSI and 8-OHdG in rat ovarian tissue and decreased the levels of TAS, SOD and CAT, which reflect the capacity of the antioxidant system. These data indicated that increased OS and decreased antioxidant system capacity were involved in the pathogenesis of ovarian tissue damage caused by 5-FU, as demonstrated in histological findings. These findings were consistent with the results of studies showing that 5-FU cause tissue damage by increasing OS, DNA damage and suppressing the capacity of antioxidant system [5, 37, 42, 43]. ASX (250 µg/kg) was used to attenuate the oxidative damage of 5-FU on ovarian tissue in this study. Our results showed that the parameters representing OS (MDA, TOS, OSI and 8-OHdG) decreased and the parameters representing antioxidant capacity (TAS, SOD and CAT) increased in the ovarian tissue of 5-FU+ASX group rats. ASX has stronger antioxidant properties than conventional antioxidant molecules, such as vitamin C, vitamin E, and β-carotene. For example, its antioxidant capacity is 100-500 times that of vitamin E and 15 times that of carotenoids [34]. It directly scavenges ROS by donating electrons and neutralizing the free radical to a non-reactive form. In addition, its structural properties create the potential of ASX to break free radical chain reactions by donating electrons, thus preventing LPO. It may interact synergistically with other antioxidants, such as vitamin C due to transmembrane alignment in the lipid bilayer resulting from the amphipathic character of ASX [41]. Since ASX is in an amphipathic structure, it can reduce DNA damage by crossing membranes [34]. Cell membranes are vulnerable to ROS attacks due to their polyunsaturated fatty acids content. Due to the chemical structure of ASX, its polar groups overlap with the polar regions of the cell membrane, while the non-polar central region of the molecule is clamped to the non-polar interior region of the membrane. Thus, ASX protects cell membranes against ROS attacks [44]. It has been proven that ASX supports intracellular antioxidant defense mechanisms and decreases LPO levels by inactivating ROS in various studies [35, 41]. Previous studies have shown that ASX can bind to major and minor grooves of DNA and protect DNA against OS from Fenton reaction [45]. It is thought that ASX's ROS scavenging activity and its potential to bind to DNA play a central role in the elimination of OS and DNA damage exacerbated by 5-FU. Consistently, it was previously reported that ASX could exert tissue protective activity by modulating OS, DNA damage and the capacity of antioxidant system in various experimental models [1, 11, 12, 14-16].

Inflammation has been suggested as a second mechanism of 5-FU-induced toxicity [29]. OS and inflammation are closely related cellular processes that stimulate one another. Indeed, ROS-activated inflammatory signaling further exacerbates ROS production, creating a vicious circle. Thus, OS and inflammation are two major risk factors for numerous human diseases and thus are critical therapeutic targets [41]. TNF-α and MPO are considered two of the main inflammatory biomarkers [46]. The elevated levels of these markers in 5-FU-treated rats indicates that 5-FU toxicity was mediated by inflammation. This finding was consistent with the results of previous studies showing that 5-FU cause tissue damage by increasing inflammation [5, 7, 32]. ASX application to 5-FU administered rats restored these changes with its previously demonstrated anti-inflammatory activity. It has been reported that the anti-inflammatory activity of ASX is due to its ability to inhibit Janus kinase/signal transducer and activator of transcription (JAK/STAT3), p38 mitogen-activated protein kinase (p38 MAP kinase) and extracellular-signal-regulated kinase (ERK1/2) signaling pathways and to reduce OS and thus inflammation by activating the nuclear factor erythroid 2–related factor 2 (Nrf2) pathway [46, 47]. In addition to these molecular pathways, it is most widely reported that ASX can block one of the most important inflammatory pathways, the nuclear factor kappa B (NF-κB)-dependent signaling pathway, and inhibit gene expression of downstream inflammatory mediators, including TNF-α and IL-6 [46]. In this study, it is thought that ASX's reduction in inflammation induced by 5-FU in ovarian tissue is due to its potential to modulate ROS scavenging activity and pro-inflammatory transcription factors. Consistent with these results, it has been previously described in different experimental models that ASX prevent tissue damage by suppressing the inflammation levels [11, 15, 35].

Apoptosis is proceeded by the activation of caspases, which are cysteine aspartyl specific proteases. Increased OS and inflammation levels can trigger apoptosis through caspase-3 activation [27]. Therefore, it has been reported that compounds with anti-apoptotic properties may be useful agents in the prevention of chemotherapeutic-induced toxicity [22]. The elevated caspase-3 levels in 5-FU-treated rats were a sign that 5-FU toxicity is mediated by apoptosis. This was consistent with previous study results highlighting the role of apoptosis in 5-FU-induced tissue toxicity [6, 43, 48]. ASX application to 5-FU administered rats restored these changes with its previously demonstrated antioxidant and anti-inflammatory activity. It is emphasized that the anti-apoptotic property of ASX is not only a reflection of its antioxidant and anti-inflammatory properties, but also modulates the expression of some key proteins involved in apoptosis signaling. ASX can exhibit anti-apoptotic effects by inducing anti-apoptotic B-cell lymphoma 2 (Bcl-2) expression and inhibiting pro-apoptotic Bcl-2-associated X protein (Bax) expression. Consistent with our results, the tissue protective effect of ASX by

reducing apoptosis in various experimental models has been previously emphasized in various studies [11, 15, 34].

V. CONCLUSION

This study showed that ASX attenuated ovarian damage induced by 5-FU in rats for the first time. It can be posited that ASX may possess therapeutic potential against 5-FU-induced reproductive system disorders. However, the use of ASX against 5-FU-induced ovotoxicity needs to be supported by more extensive *in vivo* and clinical studies.

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