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Astaxanthin treatment ameliorates ER stress in polycystic ovary syndrome patients: a randomized clinical trial

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Astaxanthin (ASX), as a natural carotenoid compound, exists in various types of seafood and microorganisms. It has several possible beneficial therapeutic effects for patients with polycystic ovary syndrome (PCOS). Patients with PCOS also suffer from endoplasmic reticulum (ER) stress. In the present work, it was hypothesized that ER stress could be improved by ASX in PCOS patients. Granulosa cells (GCs) were obtained from 58 PCOS patients. The patients were classified into ASX treatment (receiving 12 mg/day for 60 days) and placebo groups. The expression levels of ER stress pathway genes and proteins were explored using Western blotting and quantitative polymerase chain reaction. To assess oxidative stress markers, follicular fluid (FF) was gained from all patients. The Student's *t* test was used to perform statistical analysis. After the intervention, ASX led to a considerable reduction in the expression levels of 78-kDa glucose-regulated protein (GRP78), CCAAT/enhancer-binding protein homologous protein (CHOP), and X-box-binding protein 1 compared to the placebo group, though the reduction in the messenger RNA (mRNA) expression level of activating transcription factor 6 was not statistically significant. However, ASX significantly increased the ATF4 expression level. GRP78 and CHOP protein levels represented a considerable decrease in the treatment group after the intervention. In addition, a statistically significant increase was found in the FF level of total antioxidant capacity in the treatment group. Based on clinical outcomes, no significant differences were found between the groups in terms of the oocyte number, fertilization rate, and fertility rate, but the ASX group had higher rates of high-quality oocytes, high-quality embryo, and oocyte maturity compared to the placebo group. Our findings demonstrated that ER stress in the GCs of PCOS patients could be modulated by ASX by changing the expression of genes and proteins included in the unfolding protein response.

Trial registration **This study was retrospectively registered on the Iranian Registry of Clinical Trials website (www.irct.ir; IRCT-ID: IRCT20201029049183N, 2020-11-27).**

Abbreviations

ASX	Astaxanthin
PCOS	Polycystic ovary syndrome
ER	Endoplasmic reticulum
GCs	Granulosa cells
FF	Follicular fluid
GRP78	Glucose regulated protein78
CHOP	C/EBP homologous protein
ATF4	Activating transcription factor4
ATF6	Activating transcription factor 4
XBP1	X-box binding protein 1

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GAPDH	Glyceraldehyde-3-Phosphate dehydrogenase
ROS	Reactive oxygen species
NRF2	Nuclear factor E2-related factor 2
OS	Oxidative stress
UPR	Unfolding protein response
RCT	Randomized clinical trial
ICSI	Intracytoplasmic sperm injection
IBD	Inflammatory bowel disease
8-OHdG	8-Hydroxy-2′-deoxyguanosine
BMI	Body mass index
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
Tes	Testosterone
AMH	Anti-Müllerian hormone
PRL	Prolactin
TAC	Total antioxidant capacity
SOD	Superoxide dismutase
MDA	Malondialdehyde
PVS	Perivitelline space
ZP	Zona pellucid
ART	Assisted reproductive technology

Polycystic Ovary Syndrome (PCOS) is one of the most prevalent endocrine and metabolic dysfunctionsin women of reproductive age. It is the most common cause of anovulatory infertility, affecting 6–10% of these women[1]. PCOS is a heterogeneous disease that includes both endocrine and metabolic disorders. It has various symptoms, such as insulin resistance, hyperandrogenism, gonadotropin disorder, and anovulation dysfunction. The pathogenesis of PCOS is unknown; however, it is believed that this disease is linked to oxidative stress (OS), mitochondrial dysfunction, chronic low-grade inflammation, and metabolic disorders that impair normal ovarian function[2–5]. The endoplasmic reticulum (ER) is a critical organelle in all eukaryote cells that regulates the quality of secreted proteins[6,7]. Calcium homeostasis, protein folding, cell differentiation, lipid metabolism, and protein translocation are all controlled by ER homeostasis[8,9]. Nonetheless, the ER function capacity may exceed its normal limits under certain conditions, such as nutrient deprivation, acid–base instability, hypoxia, and reactive oxygen species (ROS) accumulation. These factors cause fluctuations that interfere with ER stability. Additionally, the accumulation of misfolded or unfolded proteins in ER causes ER stress induction[10–14]. ER stress is a critical local factor that interacts with OS and inflammation. The unfolding protein response (UPR) is activated in cells due to ER stress. UPR is a highly conserved process during evolution[15]. GRP78, a 78-kDa glucose-regulated protein, is a central factor in initiating UPR[16]. It activates 3ER transmembrane molecules, including RNA-dependent protein kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6), to initiate downstream UPR processes[16]. The apoptosis pathway is triggered by prolonged or severe ER stress[17].

Cell death caused by ER stress is mediated by CCAAT/enhancer-binding protein homologous protein (CHOP)[18]. The primary function of IRE1 is to activate the UPR-related gene via X-box-binding protein 1 (XBP1)[19]. Furthermore, the principal roles of PERK are to reduce protein translation via eIF2 phosphorylation and to control transcription via ATF4 phosphorylation[20]. ATF6 activates the nucleus' transcription factor, after moving from ER into the Golgi[21]. All three major UPR pathways have been found to regulate the pro-inflammatory transcriptional program via transcription factors, such as activator protein-1 and nuclear factor-κB(NF-κB)[22]. UPR and ER are essential in granulosa cells' physiological and pathological events (GCs)[23].

UPR activation and ER stress play critical roles in the pathogenesis and progression of human diseases, particularly genetic disorders, autoimmune diseases, metabolic dysfunction, cancer, and neurodegenerative diseases[24,25]. Recent studies indicate that ER stress occurs in ovarian cells, influencing follicle formation, oocyte maturation, and ovulation[26–31]. Also ER stress has been linked to ovarian disease in a few studies. Excess androgen in GCs can activate the ER stress pathway, leading to apoptosis via death receptor 5[32]. It is still difficult to develop an effective treatment for PCOS patients. In addition to different therapies for PCOS, lifestyle and diet changes have been suggested in this regard[33]. Diet and dietary factors play a major role in disease management. It has been shown that antioxidant interventions can improve PCOS complications, such as hormonal imbalances and metabolic disorders[34]. As a natural carotenoid compound, astaxanthin (ASX) is present in various kinds of seafood and microorganisms. Pluvialis-extracted ASX has been found to be safe and well-tolerated in daily dosages of 2–12 mg by the US Food and Drug Administration (FDA)[35,36]. Natural ASX has not been reported to be hazardous at any dose or for any duration of time in human investigations[37]. Various beneficial biological effects and activities are exhibited by ASX, including immunomodulatory and anti-inflammatory activity, protection against UV damage, cardioprotective effects, alleviation of metabolic syndrome, prevention of neuronal damage, anti-diabetic activity, inhibition of cell membrane peroxidation, and anti-aging and anti-cancer activities[38,39]. Additionally, previous studies have revealed the suppression of ER stress by ASX[40,41]. Thus, the present work aimed to determine the impacts of ASX supplementation on ER stress markers and OS in the GCs of infertile PCOS patients.

Methods

Trial design. Infertile PCOS patients aged 18–40 years were enrolled in this randomized clinical trial (RCT). They all met the Rotterdam criteria[42] and were advised to undergo intracytoplasmic sperm injection (ICSI). PCOS was the only endocrinopathy in all of them and oligomenorrhea with oligo-ovulation due to the higher prevalence rate was the main reason for the inclusion of these patients.

Patients were excluded from the trial if they fulfilled any of the following criteria:

Severe endometriosis (stages 3 and 4 in terms of the revised AFS-rAFS classifying the endometriosis), FSH > 10 mg/mL, hyperprolactinemia, Cushing's disease, ovarian tumors, thyroid disease, severe male factor infertility (particularly non-obstructive azoospermia), drug history affecting ovarian function in the 3 months prior to the study (steroids and oral contraceptive pills [OCPs]), female infertility factors other than cervical and tubal factors, any autoimmune disease, systemic disorders like metabolic syndrome, severe obesity and malnutrition (body mass index [BMI] over 35), hyperlipidemia, diabetes, and cardiovascular disease.

The participants were recruited from patients who were candidates for the ICSI protocol at Omid Clinic, Tehran, Iran, between November 2020 and September 2021. Although male factor indication for ICSI utilization appears to be constant, non-male factor indications remain controversial[43]. Some have advocated for the universal application of ICSI to all oocytes, regardless of the cause of infertility[44,45]. Moreover, several studieshave shown that conventional insemination of defective oocytes does not result in fertilization, whereas the use of ICSI increases fertilization and improve clinical outcome[43,46]. On the other hand, previous studies have shown that oocyte quality is low in patients with PCOS[47,48]. In our center, it has also been seen that PCOS patients with more dysmorphic oocytes have a lower fertilization rate after in vitro fertilization (IVF) compared to ICSI; accordingly, we employed ICSI, even though there was no male factor. The Ethics Committee of Tehran University of Medical Sciences approved the project (code:IR.TUMS.REC.1399.340) in accordance with relevant guidelines/regulations. The study was registered on the Iranian Registry of Clinical Trials (IRCT) website (IRCT-ID: IRCT20201029049183N1). Only a part of the results of the clinical trial registered with IRCT is presented in this paper. Before the intervention, each participant signed the informed consent form.

Intervention. ASX 12 mg/day (2 × 6 mg capsules; Astareal, Tokyo, Japan) was orally given to the participants in the treatment (ASX) group for 60 days. In the placebo group, the patients received 2 capsules containing edible paraffin every day for 8 weeks with the same appearance as ASX. The capsules were simultaneously produced by a similar company. According to a former study, the ASX dose was selected as 12 mg orally per day[37]. In addition to following their normal daily routine, patients had to notify the researchers of any changes to their activity level or diet. To monitor participants' dietary intake or activity levels, a 3-day food diary was collected during the study from all participants (1 weekend day and 2 weekdays). The Nutritionist IV program was used to estimate dietary intake. Further, patients completed a validated form of the 7-item International Physical Activity Questionnaire (IPAQ) at the start and end of the trial. To ensure compliance during the trial, all participants received daily reminders to take their supplements and were also asked to return the empty supplement bottles.

Randomization. First, 58 women were included in the work. The participants were randomly assigned to control (placebo) and intervention (ASX) groups through blocked randomization. The block size was 4 and contained letters A and B (representing the intervention and control groups). Sequentially numbered sealed opaque envelopes were used to conceal the contents. To avoid bias, participants were kept apart from other researchers during randomization. Figure 1 illustrates the study map. In this study, the statistician, patients, and investigator were unaware of the grouping, and the decipherer was not part of the research team. The ASX and placebo capsules were randomly distributed to the trial participants in identical bottles containing 60 capsules. It should be noted that the medicinal content of each bottle was labeled in the form of a code by someone other than the research team, and the research team was unaware of its interpretation.

Ovarian stimulation protocol. An antagonistic regimen was administered to all patients (a flexible antagonist regime) for controlled ovarian stimulation. Prior to the ovarian stimulation cycle, all patients received OCPs (Ovocept LD, Abureihan, Iran) for 21 days. Briefly, 150–300 IU/day of recombinant follicle-stimulating hormone (Gonal-F, Merck Serono SA, Switzerland) was prescribed from the third day of the menstrual cycle. The optimum dosage was set considering the estradiol concentration and ovarian response. After monitoring the ovaries, when at least 2 follicles with the size of 14–15 mm were present, 0.25 mg/day cetrorelix acetate, Cetrotide (Merck Serono SA, Switzerland) was administered as the gonadotropin-releasing hormone antagonist. Cetrotide consumption was discontinued after reaching a diameter of 18 mm ≥ 2 for follicles; however, 10,000 IU human chorionic gonadotropin (hCG) was administered (Ovitrelle, Merck Serono SA, Switzerland), and oocytes were retrieved after 36 h through transvaginal ultrasound-guided aspiration[49].

Sample preparation. Samples were prepared according to a previous study[49]. Follicles were aspirated without blood contamination and flushing on the oocyte retrieval day. All FFs were collected from each patient and centrifuged at 3000 *g* for 15 min. Furthermore, 5 mL of the supernatant was aliquoted and kept at − 80 °C for OS marker measurement. GCs were isolated by transferring the FF-derived pellet over 5 mL of Ficoll-Hypaque density gradient centrifugation (Lymphodex, Inno-Train, Germany). After centrifugation at 400 *g* for 20 min, the cells from the interface were gathered and rinsed at 600 *g* for 5 min. Moreover, 1 mL of phosphate-buffered saline (PBS; Sigma, Germany) with 1% bovine serum albumin (BSA; Sigma, Germany) was used for cell resuspension. Then, the cells were passed through a 40-µm cell strainer (BD Biosciences, CA, USA), and GCs were collected accordingly. Next, the cells were processed for the extraction of RNA and protein.

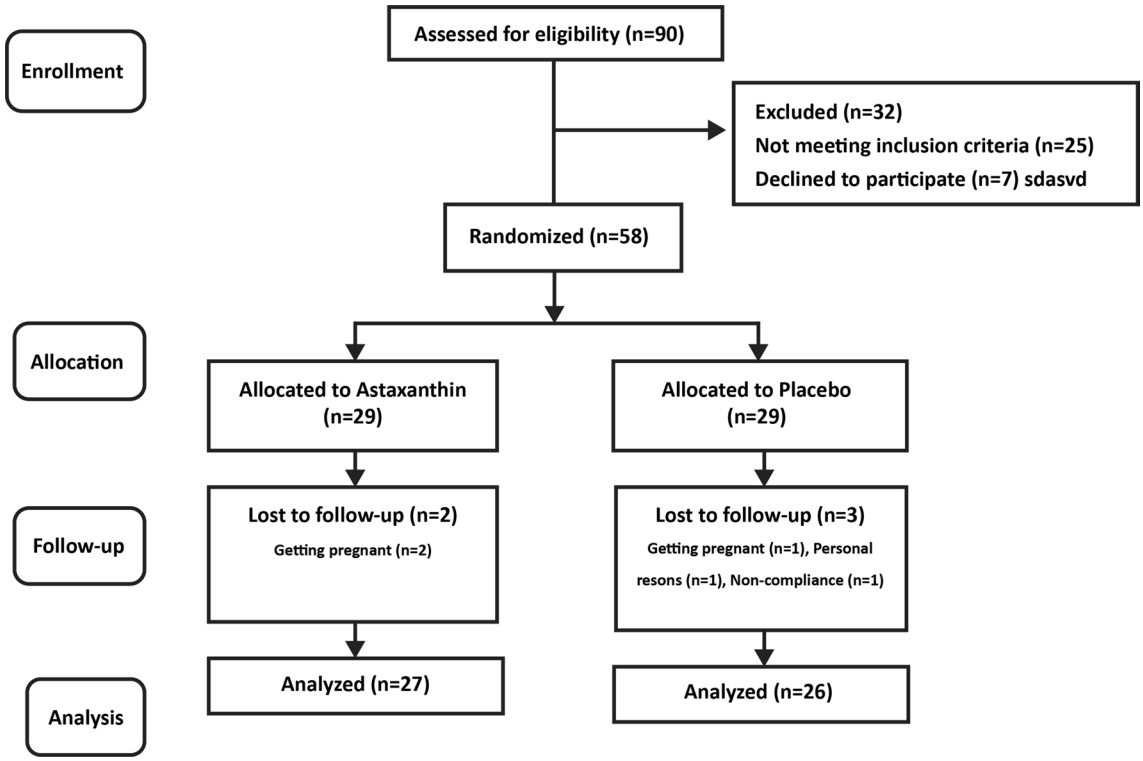

Figure 1. Summary of patient flow through the study.

Follicular fluid analysis. Follicular fluid (FF) was assessed for superoxide dismutase (SOD), total antioxidant capacity (TAC), and malondialdehyde (MDA) using colorimetric assay kits (Navand Salamat, Tehran, Iran). All OS parameters were detected in duplicate.

RNA extraction and real-time polymerase chain reaction. Total RNA was manually extracted from GCs through a solution of RNX-PLUS (SinaClon, Tehran, Iran) based on the manufacturer's protocol. The RNA concentration and purity were calculated via a spectrophotometer (Biochrom WPA Biowave, Cambridge, UK). Then, a complementary DNA (cDNA) synthesis kit (SinaClon, Tehran, Iran) was used to perform cDNA synthesis based on the manufacturer's protocol. A RealQ Plus 2 × Master Mix Green (Amplicon, Denmark) was applied to define the gene expression levels. Additionally, polymerase chain reactions (PCRs) were conducted with specific primers for XBP1, ATF4, ATF6, GRP78, CHOP, and the glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene. All the reactions were performed twice by a Light Cycler 96 System (Roche, Germany). The messenger RNA (mRNA) expression of genes corresponding to the calibrator sample was calculated using the Livak method ($2^{-\Delta\Delta Ct}$; Livak and Schmittgen, 2001). In the real-time PCR, 25 samples in each group were used for analysis. Table 1 presents the specific primer sequences.

Gene	Primer
GRP78	F: CTGTCCAGGCTGGTGTGCTCT R: CTTGGTAGGCACCACTGTGTTC
CHOP	F: GGTATGAGGACCTGCAAGAGGT R: CTTGTGACCTCTGCTGGTTCTG
ATF4	F: TTCTCCAGCGACAAGGCTAAGG R: CTCCAACATCCAATCTGTCCCG
ATF6	F: CAGACAGTACCAACGCTTATGCC R: GCAGAACTCCAGGTGCTTGAAG
XBP1	F: CTGCCAGAGATCGAAAGAAGGC R: CTCCTGGTTCTCAACTACAAGGC
GAPDH	F: CGC CAG CCG AGC CAC ATC R: CGC CCA ATA CGA CCA AAT CCG

Table 1. Specific primers used for real-time quantitative PCR. *GRP78* Glucose regulated protein78, *CHOP* CCAAT/enhancer-binding protein homologous protein, *ATF4* activating transcription factor4, *ATF6* activating transcription factor 4, *XBP1* X-box binding protein 1, *GAPDH* glyceraldehyde-3-Phosphate dehydrogenase

Western blot analysis. As described earlier[50], RIPA buffer was employed to lyse cells to extract total protein. After centrifugation at 14,000 rpm and 4 °C for 20 min, a BCA Protein Quantification kit was used to measure the protein concentration based on the manufacturer's instructions. An equal volume of 2X Laemmli sample buffer was added to the cell lysates. Next, the lysates (20 µg) were exposed to SDS-PAGE, followed by boiling for 5 min and transferring them to a 0.2-µm immune-Blot polyvinylidene difluoride membrane (Cat No: 162–017,777; Bio-Rad Laboratories, CA, USA). Then, the membranes were blocked with 5% BSA (Cat No: A-7888; Sigma Aldrich, MO, USA) for 1 h in 0.1% Tween 20. Subsequently, the membranes were incubated with Anti-GRP78 (Cat No: ab21685, Abcam), anti-chop (Cat No: ab194533, Abcam), and anti-beta actin-loading control antibodies (Cat No: ab8227; Abcam) at room temperature for 1 h. After washing the membranes with TBST, incubation was performed with a secondary antibody, goat anti-rabbit IgG H&L (HRP; Cat No: ab6721; Abcam). Next, incubation with enhanced chemiluminescence was conducted for the membranes for 1–2 min. The densitometry of protein bands was calculated using Gel Analyzer version 2010a (NIH, USA). Thirty-three samples in the Western blot, 17 in the treatment group, and 16 in the placebo group were used for analysis.

Clinical data. Data on harvested oocytes, including the number of mature oocytes, the quality of the oocytes, and the percentage of high-quality oocytes, were obtained on the day of the puncture. It was determined that the high-quality oocyte rate was equal to the number of good-grade oocytes divided by the total number of retrieved oocytes × 100 for each participant. According to previous data[51,52], 6 factors were used to assess the quality of individual oocytes, including oocyte morphology, range of possible oocyte sizes, ooplasm features, the structure of the perivitelline space (PVS), zona pellucida (ZP), and the morphology of the polar body. Oocyte morphology can be "worst" (characterized by an overall dark color and/or ovoid shape),"average" (characterized by a somewhat darker overall color and/or ovoid shape), or "best" (characterized by a normal color and shape). The range of possible oocyte sizes encompassed worst (abnormally tiny or huge, below 120 µ or larger than 160 µ), average (do not differ from best by more than 10 µ), and best (greater than 130 µ and less than 150 µ). The ooplasm feature was another intended factor (worst: extremely granular and/or vacuolated and/or demonstrating numerous inclusions; average: moderately granular and/or showing a small number of inclusions; best: showing no granularity or inclusions). The other factors were the structure of the PVS (worst: excessively large PVS, lack of PVS, or highly granular PVS; average: moderately enlarged/small/less granular PVS; best: best size PVS and the absence of granules in PVS) and ZP (worst: extremely thin or thick < 10 µ or > 20 µ; average:do not deviate from best by more than 2 µ; best: best zona > 2 µ and < 18 µ). The last factor was the morphology of the polar body, which can be the worst or multiple PBs (granular and/or either small or large PBs), average (fair but not excellent), and best (normal size and shape). The total oocyte score (TOS) was calculated by adding up the values assigned to each of the parameters; in addition, values of − 1, 0, and 1 represented the worst, average, and best, respectively. Oocytes may have a + 6 TOS and − 6 TOS at their highest and lowest levels, respectively. Oocytes were checked 16–18 h after ICSI to determine fertilization rates. Data on embryo development was gathered 2–3 days after ICSI, including number, quality, and high-quality embryo rate. In this study, the high-quality embryo rate was calculated as the number of high-quality embryos divided by the number of successful fertilizations × 100[53] for each participant. The embryo quality is determined by its cell number (blastomeres), its fragmentation, and the presence of multiple nuclei, pits, and vacuoles[54]. In accordance with the ASEBIR (Association for the Study of Reproductive Biology) criteria, grade A and B cleavage embryos were classified as high quality based on these factors. The rates of chemical pregnancy (as determined by the hCG test) and clinical pregnancy (as determined by ultrasound to observe the gestational sac) were computed during the study.

Statistical analysis. The data were presented as means ± SDs. The Kolmogorov–Smirnov test was used to confirm the normal distribution of data. Statistical analyses were performed by the paired Student's *t*test or independent sample *t*test and Fisher's exact test using SPSS version 22 (SPSS Inc., Chicago, Ill, USA). *P* values less than 0.05 were considered statistically significant. To compare gene expression as the primary outcome in this trial and due to a paucity of comparative research in this field, the standardized effect size was used to determine the sample size. Considering the large standardized Cohen's effect size (d = 0.8), a type I error of 5% (a = 0.05), and a type II error of 20% (b = 0.2; power = 80%), the sample size was obtained 26 in each group. Considering the 10% loss to follow up, the sample size was calculated to be 29 subjects in each group.

Ethics approval and consent to participate. The Ethics Committee of Tehran University of Medical Sciences approved the project (Ethics committee reference number: IR.TUMS.REC.1399.340) and all research was performed in accordance with relevant guidelines/regulations. Written consent was obtained from all participants.

Results

Generally, during the intervention stage, 5 subjects had to withdraw from the study, including 3 subjects in the placebo group (2 cases for personal reasons and 1 case due to pregnancy) and 2 cases in the ASX group (due to pregnancy). Finally, 53 patients participated in this study, including 27 and 26 cases in the intervention and placebo groups, respectively (Fig. 1). No adverse symptoms or effects with the ASX supplementation were reported by the participants during the trial, and they had good compliance with the intervention. No significant differences were also found in the duration of infertility, mean age, BMI, and hormonal profile between the treatment and control groups at the beginning of the study (Table 2). According to gene expression results, the expression levels of CHOP ($P < 0.0001$; Fig. 2a), GRP78 ($P < 0.05$; Fig. 2b), and XBP1 ($P < 0.001$; Fig. 2c) were significantly decreased in the treatment group than in the placebo group. Although in the treatment group, the mRNA expression level of ATF6 ($P = 0.073$; Fig. 2d) was reduced, this reduction was not significant statistically.

Variables	Mean ± SD Placebo (n = 26)	Mean ± SD Intervention (n = 27)	*P*-value
Age (years)	30.84 ± 4.84	30.36 ± 5.16	0.745
BMI (kg/m^2)	26.24 ± 1.59	26.12 ± 1.56	0.802
Infertility duration (year)	3.386 ± 1.93	4.24 ± 2.02	0.147
Mean menstruation duration (day)	6.81 ± 0.9	6.48 ± 1.29	0.338
Mean menstrual cycle duration (day)	42.36 ± 10.05	44.12 ± 14.4	0.634
Baseline FSH (μIU/ml)	3.92 ± 1.11	4.19 ± 1.15	0.416
Baseline LH (μIU/ml)	9.01 ± 3.54	8.85 ± 3.13	0.874
Baseline Tes (ng/ml)	1.18 ± 0.56	1.24 ± 0.55	0.713
Baseline AMH (ng/ml)	9.21 ± 1.96	8.06 ± 3.01	0.132
Baseline PRL (ng/ml)	12.36 ± 1.8	13.14 ± 2.27	0.203

Table 2. Baseline parameters in individual group. Significance ($p < 0.05$) was assessed by t-test. *BMI* body mass index, *FSH* follicle-stimulating hormone, *LH luteinizing hormone, Tes* testosterone, *AMH* anti-Müllerian hormone, *PRL* prolactin.

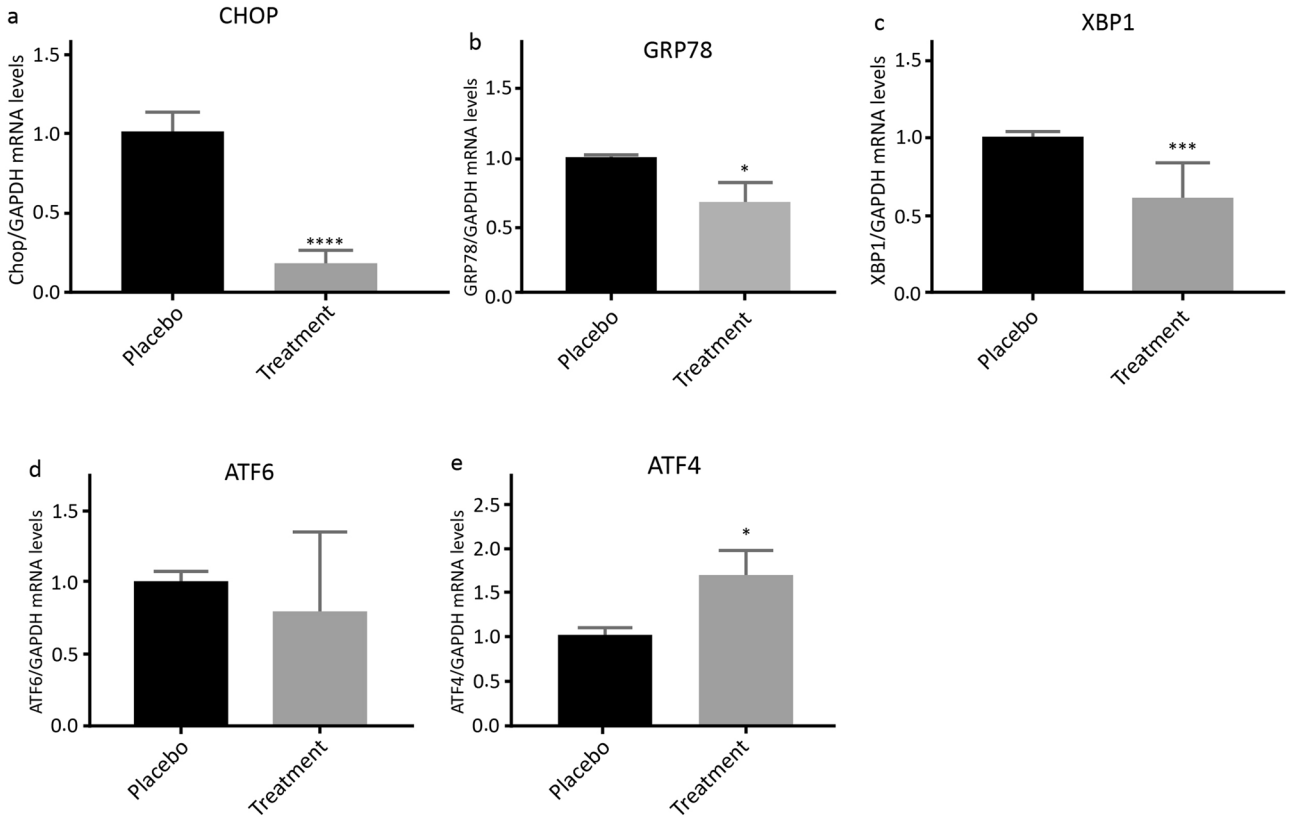

Figure 2. The fold changes levels of CHOP (**a**), GRP78 (**b**), XBP1 (**c**), ATF6 (**d**), and ATF4 (**e**) in GCs of placebo and treatment groups. Statistical significance ($p < 0.05$) was assessed by t-test. The results showed that fold changes levels of ATF4 was significantly increased in the intervention group ($P < 0.05$). After intervention, it was found that in the ASX group, the fold changes levels of CHOP, GRP78, and XBP1 were significantly decreased compared to the control group, while the reduction level of ATF6 was not significant between two groups ($P > 0.05$). *P*: placebo; T: treatment. Placebo: n = 25, Treatment: n = 25. Differences between groups; $^{*}p < 0.05$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

However, the ATF4 expression level ($P < 0.05$; Fig. 2e) significantly increased compared to the placebo group. Moreover, the expression levels of CHOP and GRP78 protein significantly decreased after the intervention in the treatment group than in the control group ($P < 0.0001$; Fig. 3a and $P < 0.001$; Fig. 3b, respectively). Based on the results of the FF analysis (Table 3), a statistically significant increase was found in the TAC level in the treatment group ($P < 0.05$). Nonetheless, there was no considerable difference in the FF levels of MDA and SOD between the treatment and control groups ($P > 0.05$). According to Table 4, no statistically significant difference was detected in the number of retrieved oocytes, the number of embryos, and fertilization rates ($P > 0.05$), though

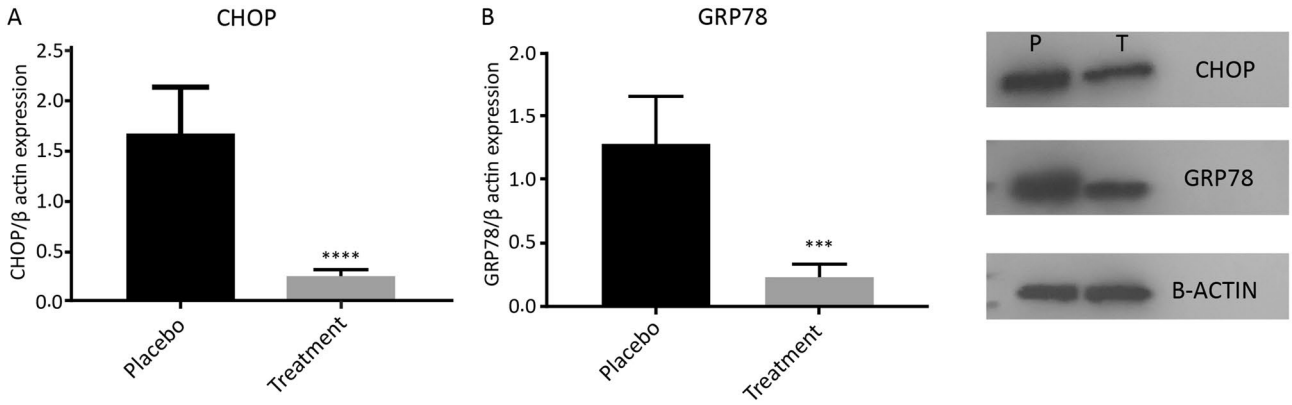

Figure 3. The protein expression levels of CHOP and GRP78 in the GCs of placebo and treatment groups. Western blot analyzed of the protein expression of Grp78 and Chop normalized to β-actin. Following intervention, the protein expression of Grp78 and Chop significantly reduced in treatment group compared to control group. Statistical significance ($p < 0.05$) was assessed by t-test. *P*: placebo; T: treatment. Placebo: n = 16, Treatment: n = 17. Differences between groups; ***$p < 0.001$ ****$p < 0.0001$.

Variables	Mean ± SD Placebo (n = 26)	Mean ± SD Intervention (n = 27)	*P*-value
SOD (U/ml)	173.4 ± 27.32	177.5 ± 19.85	0.55
TAC (mmol Fe2 + /l)	0.28 ± 0.06	0.32 ± 0.04	0.02*
MDA (μm/l)	2.55 ± 0.82	2.18 ± 0.89	0.14

Table 3. Comparison of OS markers in FF of placebo and treatment groups. Significance ($p < 0.05$) was assessed by t-test. Differences between groups. *TAC* total antioxidant capacity, *SOD* superoxide dismutase, *MDA* malondialdehyde. *$p < 0.05$.

Variables	Mean ± SD Placebo (n = 26)	Mean ± SD Intervention (n = 27)	*P*-value
Number of retrieved oocytes	22 ± 4.88	21.26 ± 4.34	0.56
Rate of MII (mature) oocyte	68.81 ± 10.34	76.39 ± 8.07	0.004**
Rate of high quality oocyte	59.19 ± 10.45	67.9 ± 6.38	0.006**
Rate of fertilization	77.86 ± 10.13	81.24 ± 7.39	0.17
Number of embryos	11.04 ± 2.61	12.07 ± 2.49	0.14
Rate of high quality embryo	63.38 ± 13.68	70.24 ± 10.44	0.04*

Table 4. Comparison of clinical outcomes of placebo and treatment groups. Statistical significance ($p < 0.05$) was assessed by t-test. Differences between groups. *$p < 0.05$, **$p < 0.01$.

the proportion of MII and high-quality oocytes rates were considerably higher in the study group than in the control group. Additionally, the embryo quality improved following ASX therapy. The results indicated that the rate of high-quality embryos was significantly higher in the study group than in the control group ($P < 0.05$). It was also found that the ASX group had a 44.44% chemical pregnancy success rate compared to the placebo group (34.61% success rate; (9/26, Fisher's exact test; $P = 0.577$; Fig. 4). Additionally, the clinical pregnancy rate was 30.76% (8/26) in the placebo group and 37.03% (10/27) in the ASX group (Fisher's exact test; $P = 0.773$; Fig. 4).

Discussion

Based on the findings of the present clinical trial on the effects of ASX on infertile patients with PCOS, the FF levels of MDA were reduced by ASX, while SOD and TAC levels represented an increase. Moreover, a 60-day course of ASX in the present work resulted in increased ATF4 mRNA expression levels. However, the expression levels of GRP78, CHOP, XBP1, and ATF6 mRNA were reduced compared to the placebo group. Our findings revealed that the protein levels of GRP78 and CHOP were also reduced after the pharmacological intervention. There is strong evidence that OS can result in ER stress[55]. ER stress was prevented by the administration of antioxidants, confirming the potential effects of OS on inducing ethanol-elicited ER stress[40,56,57]. In turn; OS is also induced by the accumulation of unfolded proteins in ER by various mechanisms. Mitochondrial oxidative phosphorylation is stimulated by the activated BiP, a key signal molecule in the ERstress pathway, to produce ROS as a by-product. Furthermore, the enzymes of the NADPH oxidase (NOX) family increase ROS production,

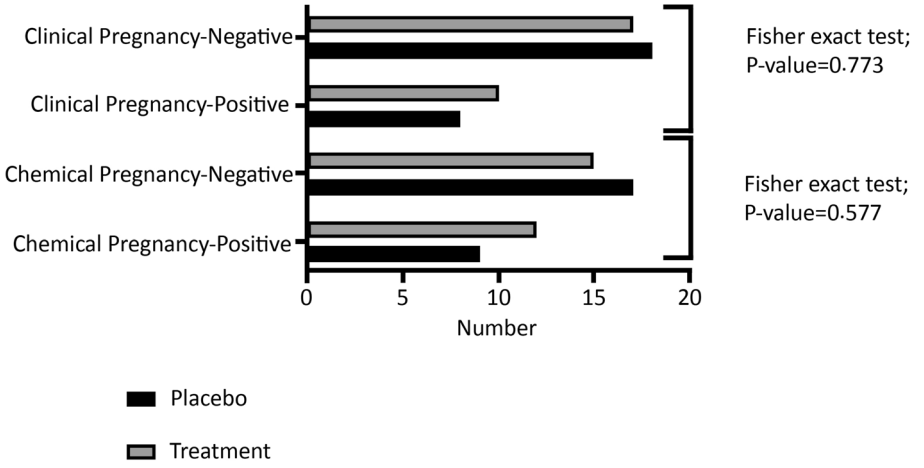

Figure 4. Comparison of clinical and chemical pregnancy rate between study groups. The chemical pregnancy rate was 44.44% (12/27) in the ASX group and 34.61% (9/26) in the placebo group (Fisher's exact test; 1-sided $P=0.327$, 2-sided $P=0.577$). Moreover, the clinical pregnancy rate was 37.03% (10/27) in the ASX group and 30.76% (8/26) in the placebo group (Fisher's exact test; 1-sided $P=0.424$, 2-sided $P=0.773$). Placebo: n = 26, Treatment: n = 27.

particularly through Nox2 and Nox4 isoforms under ER stress[58–60]. The role of OS and ER stress in PCOS pathogenesis is highlighted by recent publications[23,61]. To determine whether ASX is effective in treating PCOS, the expression levels of mRNA and protein in the ER stress pathway were measured in our study. Our study focused on ER stress levels in the GCs of infertile PCOS patients who received ASX or not. Former investigations revealed an incremented expression of UPR genes such asATF4, ATF6, CHOP, and XBP-1 in GCs in PCOS patients[23]. According to our findings, the mRNA levels of GRP78, spliced XBP1, and CHOP were significantly decreased compared to the placebo group; however, the mRNA level of ATF4 was significantly increased in the ASX group. Various studies demonstrated that the activation of UPR and ER stress is associated with chronic diseases, including inflammation, diabetes, obesity, inflammatory bowel disease, neuromuscular inflammation disease, respiratory inflammation disease, PCOS, and arthritis[16]. Based on the results of studies on type 2 diabetes and insulin resistance, hyperglycemia has a close relationship with ER stress[61]. It was found that androgens can increase the GRP78 expression level[62]. Previous studies have confirmed the activation of ER stress by hyperandrogenism in PCOS[32]. ER stress includes some molecular cascades comprising some enzymes and transcription factors to restore homeostasis[16]. Based on a previous paper, GRP78 and UPR activator proteins (PERK, IRE1, and ATF6) increased in PCOS patients compared to healthy subjects[23]. Our results indicated that GRP78 mRNA and protein levels were significantly reduced by pharmacological intervention with ASX. A rise in the GRP78 expression in human diseases is an indicator of elevated protein misfolding conditions in ER, as well as a marker, to measure ER stress[16]. Treatment with ASX led to a reduction in the GRP78 expression level, indicating that ER stress conditions were reduced by interventions in PCOS patients, at least partially. The findings also showed a significant reduction in XBP1 mRNA expression after treatment with ASX in PCOS patients. Therefore, it is to be expected that ASX reduces XBP1 and thus decreases UPR target genes. It was concluded that the XBP1 expression level was reduced, while the ATF4 expression demonstrated a significant increase. According to former research, the expression of Nuclear factor E2-related factor 2(NRF2) is promoted by ATF4 by inducing genes included in antioxidant functions[63]. Our work revealed that ASX might have a role in NRF2 promotion by increasing ATF4. As a result of GRP78 activation, the ATF6 branch of the UPR travels to the Golgi, where it is processed by S1P and S2P proteases. Entering the activated cytosolic domain of ATF6 into the nucleus induces the expression of genes that augment the degradation and translation attenuation of misfolded proteins[64]. Hence, ATF6 appears to provide cell protection against ER stress. Nevertheless, some reports suggest that ATF6 may also have a pro-apoptotic function[65]. Moreover, CHOP and XBP1 are transactivated by ATF6[66]. Our results suggest that ASX is associated with a decrease in UTR target genes by decreasing the expression of AFT6, though reduced AFT6 expression was statistically insignificant in our work. Under severe or chronic ER stress, apoptosis is started via CHOP, as well as other mechanisms[16]. A CHOP activity was found in the ovary physiological circumstances and PCOS[66]. In the current work, ASX reduced the CHOP mRNA and protein expression levels after the intervention. By the reduced expression levels of GRP78 and CHOP, the survival branches of UPR are induced by ASX in GCs in PCOS patients. Further, it was reported that by increasing ATF4, the PERK and CHOP expression levels increased as well[67,68]. Consistent with our results, in the study of Bhuvaneswari et al., the liver tissue was protected by ASX against higher fructose and fat diet-induced damage by reducing the levels of PERK and ATF6[59]. However, Shen et al. concluded that the brain damage was attenuated by ASX in an experimental Parkinson's disease model by reducing CHOP and GRP78 levels[69]. Wang et al. demonstrated that ethanol-induced cardiomyopathy was inhibited by ASX in mice by reducing the levels of ATF6, PERK, GRP78, ATF4, and CHOP[41]. Demir et al. demonstrated that the testicular tissue was protected by ASX against torsion/detorsion-induced injury through the antioxidant activity and suppression of endoplasmic stress. The levels of ATF6, GRP78, and CHOP markers

were significantly reduced due to ASX treatment[70]. Numerous studies have shown that the activation of UPR and ER stress is implicated in chronic diseases, including inflammation, diabetes, obesity, inflammatory bowel disease, arthritis, neuromuscular inflammation disease, respiratory inflammation disease, and PCOS[16]. Investigations on type 2 diabetes and insulin resistance revealed a close relation between hyperglycemia and ER stress[61]. It was also found that androgens can increase the GRP78 expression level[62]. Higher levels of testosterone are produced in women with PCOS, along with other androgen hormones. By increasing these hormones in women with PCOS, multiple complications appear, including weight gain, acne, infertility, excessive growth of hair in the body or face, and absent or irregular menstrual periods[61]. A close relation was evidenced between signaling pathways for OS, inflammation, and ER stress[71,72]. It can be deduced that ER stress can be partially modulated by ASX antioxidant effects in PCOS patients. Although several studies have identified OS in patients with PCOS, the experimental data regarding OS markers are inconsistent[73–76]. These contradictory results could be due to small sample sizes and using different methods. It appears that antioxidant therapy might be beneficial to women with PCOS based on the OS status of these women. Our work confirmed that the FF level of TAC was increased by ASX supplementation for 60 days, while there was no considerable difference in the FF levels of MDA and SOD between the treatment and control groups. According to various animal trials, ASX can reduce OS by decreasing 8-hydroxy-2′-deoxyguanosine (8-OHdG)[77] and MDA[78], though it can increase antioxidant enzymes[78,79]. A limited number of clinical trials have also been performed in this regard. The results of a systematic review and meta-analysis on the antioxidant effect of ASX in humans showed that ASX might be operative in the reduction of OS as ASX may decrease the total quantity of the specific lipid peroxidation effectively (ISP and MDA) and improve plasma antioxidant capability (TAC) while increasing a definite antioxidant enzyme (SOD). However, the antioxidant effects of ASX on humans are unclear[80]. Reproductive potential is lowered in women with PCOS regardless of ovulatory state[81] due to changes in oocytes[82], embryo, and endometrial competence[83], along with infertility-related co-morbidities, as well as a higher chance of pregnancy complications[84]. Virtually, every factor that is associated with PCOS also affects reproductive potential independent of PCOS. Meiotic abnormalities and poor oocyte quality are more common in women with PCOS due to the elevated OS that drives the excessive generation of ROS[81].

Ultimately, this study discovered that administering ASX to PCOS patients before assisted reproductive technology (ART) cycles enhanced the proportion of MII and high-quality oocytes, as well as the rate of high-quality embryos. It seems that in our study, ASX might have been associated with the high-quality rate of oocyte and embryo by decreasing OS. Compared to the control group, the fertilization rate of the ASX group was not statistically greater ($P = 0.17$). In the present study, ASX had no significant effect on the fertility rate compared to the placebo. There was an increase in chemical pregnancy rates from 34.61% in the placebo group to 44.44% in the ASX group. There was also a rise in the rate of clinical pregnancy from 30.76% in the placebo group to 37.03% in the ASX group. Although the ASX group had a higher rate of chemical and clinical pregnancies, these changes were not statistically significant. The small sample size, ASX dosage, exposure length, and other factors may have contributed to the lack of statistical significance in our investigation.

One strength of our study is the coverage of all PCOS phenotypes, providing a considerably larger potential to derive generalizable results. Moreover, managing the nutritional intake and physical activity are beneficial aspects of our research. Nonetheless, this study had some limitations as well. Given that our sample size was relatively small, it may be difficult to detect small changes in response to ASX treatment. In addition, our study's follow-up period was short; non-significant improvements in ART outcomes may be meaningful with longer follow-ups. The study was also limited by the lack of objective measurement of patient compliance; more precisely, the serum or plasma levels of ASX could not be measured in this study. However, these results may shed fresh light on the potential involvement of ASX in modulating several conditions in PCOS patients. Future studies are needed with larger sample sizes and various doses and durations to corroborate our findings. It is recommended that ASX concentrations in serum or plasma be measured as well.

It is concluded that the molecular pathways of ER stress can be modified by ASX as a natural supplement through antioxidant activity. Therefore, it can influence the expression of genes and proteins involved in the UPR in the GCs. Furthermore, OS markers may be modulated by ASX in the FF of patients. In addition, ASX may improve some of the ART outcomes for PCOS patients. Thus, ASX administration may be beneficial for PCOS patients. Eventually, the results of our study indicated that ER stress could be used as a potential therapeutic target in PCOS.

Data availability

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

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Author contributions

F.A. and M.J.: designed and performed the experiments, provided the samples, statistical analysis, and prepared the manuscript; A.A.: assisted with study design, performed the study protocol and revised the manuscript; M.S.: assisted in performing the study protocol and revised the manuscript. S.L.: Data collection and data analysis. The final version was approved by all authors for submission.

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Competing interests
The authors declare no competing interests.

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Review

The Role of Astaxanthin as a Nutraceutical in Health and Age-Related Conditions

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Abstract: The current review provides an up-to-date analysis of scientific data on astaxanthin (ASX) sources and experimental studies on its health benefits as a potent antioxidant in the aging process. ASX is a liposoluble carotenoid nutrient and reddish-orange pigment, naturally synthesized by numerous microalgae, yeasts, and bacteria as secondary metabolites. Provides a reddish hue to redfish and shellfish flesh that feed on ASX-producing microorganisms. The microalga *Haematococcus pluvialis* is the most important source for its industrial bioproduction. Due to its strong antioxidant properties, numerous investigations reported that natural ASX is a more significant antioxidant agent than other antioxidants, such as vitamin C, vitamin E, and β-carotene. Furthermore, several data show that ASX possesses important nutraceutical applications and health benefits, especially in healthy aging processes. However, further studies are needed for a deeper understanding of the potential mechanisms through which ASX could lead to its effective role in the healthy aging process, such as supporting brain health and skin homeostasis. This review highlights the current investigations on the effective role of ASX in oxidative stress, aging mechanisms, skin physiology, and central nervous system functioning, and shows the potential clinical implications related to its consumption.

Keywords: astaxanthin; health benefit; human aging; reactive oxygen species; antioxidant supplementation; neuroprotection; skin protection; carotenoid

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1. Introduction

Natural compounds can display therapeutic effects against various chronic conditions, from inflammation to cancer [1,2]. Nutraceuticals are food components that have both nutritional and medicinal properties, and have been used since 1980 as an essential part of

complete wellness and health [3,4]. Nutraceuticals can be extracted from plants, fungi, bacteria, or animal products, then concentrated and administered in a suitable pharmaceutical dosage form to prevent or treat some human pathological conditions [2].

Astaxanthin (ASX) is a liposoluble carotenoid and a reddish-orange pigment. ASX plays a role in circulating lipoproteins and cell membranes, and has significant antioxidant and anti-inflammatory activity [5,6]. It can be naturally synthesized by numerous microalgae, yeasts, and bacteria as a secondary metabolite. It supplies a reddish hue to the to redfish, sea bream and salmon flesh and crustaceans (crabs and shrimps) that feed on ASX-producing microbes [7,8]. The microalga *Haematococcus pluvialis* is considered the most important source of its industrial biological production [7,9,10]. However, commercial production of ASX has traditionally been done by chemical synthesis [11].

ASX is also used commercially for feed production. As salmon cannot naturally synthesize ASX, the salmon grown on farms fail to develop the characteristic flesh color of their wild counterparts. ASX is used together with canthaxanthin in feed to dye salmon flesh, as well as for trout, and shrimp [12]. Likewise, ASX gives the characteristic color to egg yolk and broiler chicken carcass.

Research carried out on ASX shows that it helps reduce the negative effects of aging by neutralizing reactive oxygen species (ROS) and reactive nitrogen species (RNS) within the body's cells which lead to overloading of systems. defense and the consequent oxidative damage [13,14]. Recently, ASX was found to have a several times greater effect than that of β-carotene on singlet oxygen quenching, and an antioxidant function of up to 100 times more significant than vitamin E against lipid peroxidation [15]. ASX is an antioxidant and anticancer agent that prevents cardiovascular diseases, diabetes, and neurodegenerative disorders, and stimulates the immune system [14,16–21]. The anti-aging role of ASX has been attributed to its antioxidant and anti-inflammatory properties, preventing age-related muscle deterioration and improving energy generation in the mitochondria [22,23]. ASX can help eliminate free radicals produced during exercise and aerobic metabolism in muscles [24]. It can also help boost immunity, resist fatigue, and delay aging [25].

The global consumption of many sources of ASX in food and nutraceuticals continues to increase. ASX products are used for pharmaceutical applications in dosage forms, such as tablets, capsules, soft gels, creams, oils, biomass, and granulated powders [26]. Due to its beneficial properties, ASX can also be used as a food colorant and an antioxidant to improve foods' nutritional value and sensory quality. The European Commission considers ASX a food dye signed as E161 [27]. In 2019, the average daily intake (ADI) was set at 0.2 mg/day by the Additives and Products or Substances used in Animal Feed (FEEDAP) committee of the European Food Safety Authority (EFSA) [28].

The potential pharmacological effects of ASX, which include anti-inflammatory and antioxidant activities, as well as neuro and skin-protective effects, have brought it to public attention. Previous studies have demonstrated that, due to its better biosafety and high bioavailability, ASX has strong therapeutic potential against many diseases, such as in modulating neuroinflammation [29], ocular diseases [30], cardiovascular, aging, neurodegenerative, respiratory, and liver disease [31]. In this study, we summarize the therapeutic impact of ASX in controlling oxidative stress related to the aging process, skin conditions, and the effects on the central nervous system.

2. Structure and Sources of Astaxanthin

ASX belongs to a family of naturally occurring organic pigments called carotenoids. It is a keto-carotenoid classified as a xanthophyll with the chemical IUPAC's name 3,3′-dihydroxy-β, β-carotene-4,4′-dione. In nature, ASX molecules exist as stereoisomers, geometric isomers, and in free and esterified forms, among which the most abundant are (3S, 3′S) and (3R 3′R) stereoisomers. The empirical formula of ASX is $C_{40}H_{52}O_4$. The structures of two natural enantiomers of ASX are shown in Figure 1.

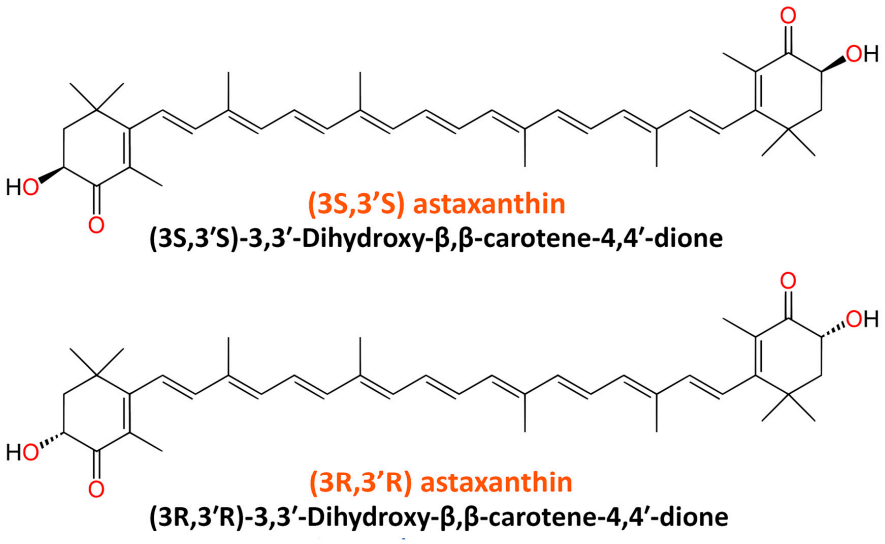

Figure 1. Chemical structure of two natural enantiomers of astaxanthin.

ASX is produced by several marine and freshwater microorganisms, including microalgae, fungi, lichens, and bacteria (Table 1). It can also be obtained from redfish and crustaceans that feed on ASX-producing microorganisms [32].

Table 1. Diversity of the natural sources of ASX.

Group of Organisms	Representative	References
	Haematococcus pluvialis	[9,10]
Plantae (microalgae)	*Chlorella zofingiensis* *Chlorococcum* *Chromochloris zofingiensis* *Chlamydomonas reinhardtii* *Diatoms*	[33–35]
Fungus (yeasts)	*Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) *Yarrowia lipolytica* § *Saccharomyces cerevisiae* §	[36–38]
Lichene	*Clodia aggregata,* *Concamerella fistulata,* *Usnea amaliae,* *Usnea densirostra*	[39]
Bacteria	*Corynebacterium glutamicum* § *Cyanobacteria* (*Synechococcus sp.*) *Agrobacterium aurantiacum* *Paracoccus carotinifaciens* *Escherichia coli* §	[38,40,41]
Animalia	Redfish Crustaceans (*Euphausia superba, Pandalus borealis, Calanus finmarchicus, etc.*) Wild salmon (*Oncorhynchus* species)	[4,7,26]

§ Genetically modified organism (GMO).

Microalgae and yeasts are very important commercial sources of natural ASX. Microalgae are already gaining popularity as a source of food, which makes their implementation in

the food system easier [42]. The microalgae *Haematococcus pluvialis* synthesizes the highest amount of ASX in nature, which makes it an optimal choice for the commercial production of ASX. *Haematococcus pluvialis* may contain up to 3.8% of ASX on a dry weight basis. A naturally cultivated form of ASX is produced from the microalgae *Haematococcus pluvialis*. ASX is also produced by algal species, such as *Neochloris wimmeri, Enteromorpha intestinalis, Chlorococcum, Chromochloris zofingiensis*, etc. [35]. Hence, using algal-based ASX could help overcome its chemical production.

Aerobic Gram-negative bacterium *Paracoccus carotinifaciens sp. nov.* and basidiomycetous yeast *Xanthophyllomyces dendrorhous (syn. Phaffia rhodozyma*) also produce high-value carotenoid and are used for ASX production at the industrial scale. High-titer production of ASX by the semi-industrial fermentation of *Xanthophyllomyces dendrorhous* could be easily scaled-up to an industrial application for producing this xanthophyll [43]. Yeast *Phaffia rhodozyma* was first isolated from the exudates of trees in mountainous regions of Japan and Alaska during the early 1970s [44]. The yeast was recognized as very special because due to its ability to synthesize ASX. Later, *Phaffia rhodozyma* was approved by European Commission for use as a pigment source in feeding stuff for salmon and trout [45]. Several species of Lichens are also producing ASX, although not in industrially exploitable quantities [46].

The content of ASX found in salmon were recorded in the range of 26–38 mg/kg in the flesh of wild salmon (*Oncorhynchus* species) and 6–8 mg/kg in the flesh of farmed Atlantic salmon, which is a reason for consumers' preferences for wild salmon over farmed salmon. Big trout may contain 6 to 25 mg/kg of ASX in the flesh [47]. Recent findings are associated with the possible use of crustacean by-products as a source of natural ASX [10]. Shrimp, crabs, krill, crayfish, and copepods can also serve as a source of ASX [48].

ASX is also produced through chemical synthesis and genetic engineering. In the second half of the 20th century, ASX was successfully synthesized from isophorone, *cis*-3-methyl-2-penten-4-yn-1-ol, and a symmetrical C_{10}-dialdehyde. Although the production by chemical synthesis is a more cost-effective way to obtain ASX, this process does not promise to give a pure chemical compound, but a combination of different isoforms, some of which cannot be found in nature; thus, synthetic or chemical ASX molecules have different activities than their natural counterparts [12,49–51]. The biological effect of natural ASX is much stronger than its synthetic analog, which may be due to the presence of all three isomers in synthetic ASX: two enantiomers (3′R 3R and 3′S 3S) and one mesoform (3R 3′S), while natural ASX only has 3S, 3′S or 3R, 3′R (Figure 1) [52]. Around 95% of natural ASX is mono- or di-esterified with fatty acid molecules, while synthetic ASX is free.

Studies demonstrate that natural ASX is 6000 times more powerful than vitamin C, 770 times more active than coenzyme Q10 (CoQ_{10}), 100 times more potent than vitamin E, and five times more powerful than β-carotene in trapping energy from singlet oxygen, one of the most common ROS found in the human body [53–55]. Interestingly, ASX neutralizes ROS by either donating or accepting electrons without being damaged in this process [56]. Its distinct chemical characteristics make it unique when compared to other antioxidants.Even though their structures are similar, ASX contains 13 conjugated double bonds, while β-carotene only has 11. Oxo groups are located in the fourth and fourth prime locations in the cyclohexene structure. The length of the electron-rich, conjugated double bonds determines the antioxidant potential of carotenoids. When compared to β-carotene and vitamin E, ASX has greater efficacy due to an extension of the conjugated double bond system [57]. The molecule of ASX is relatively polar due to the presence of hydroxyl groups in positions 3 and 3′. While nonpolar carotenoids, including β-carotene and lycopene, are present between the lipid bilayer of membranes and may cause the phospholipid molecules' intermolecular packing to become disrupted [58,59], ASX with polar end groups that extend toward the head group areas of the lipid bilayer can cross the membrane. The structure of the lipids that make up the membrane remains preserved [5,58,59]. As a consequence, ASX halts free radical chain reactions and scavenges lipid peroxyl radicals to function as a chain-breaking antioxidant. ASX crossing the cell lipid bilayer membrane, allows its

terminal rings to efficiently scavenge ROS on the membrane surface while its polyene chain traps ROS inside the membrane [5]. Consequently, ASX acts as a scavenger of free radicals in the inner membrane layer and simultaneously could control oxidation on the membrane surface.

ASX also acts as a powerful antioxidant without having any pro-oxidative properties [60]. As a result, ASX effectively neutralizes destructive ROS while being gentle on the body's cells. Thus, its unique structure and flexibility to neutralize free-radicals from both hydrophilic and hydrophobic boundaries of a cell membrane make it a stronger antioxidant compared to all other antioxidants, such as β-carotene, vitamin E, etc. ASX counteracts potentially harmful free radicals/ROS by trapping energy (quenching) and through the transfer of electrons or hydrogen abstraction (scavenging) [56].

The world market of ASX exceeds USD 400 million per year, and the share of natural ASX on it is about one percent, while its market value is 2–7 times higher than that of the synthetic analog [61]. According to experts, ASX production worldwide will reach 190 tons by 2024. Synthetic ASX is mainly used for aquaculture [61]. Therefore, the urgent task is to increase the production of natural ASX, especially due to the high market demand.

An alternative to chemical synthesis is the genetic modification of microorganisms and an increase in the amount of ASX they produce. Several methods are being developed for this purpose. Thus, using the genetically modified organisms (GMO) *Yarrowia lipolytica*, *Escherichia coli*, and *Saccharomyces cerevisiae*, an enhancement in the synthesis of ASX to 8–10 mg/g DCW occurred [38,62,63]. However, the commercial application of these genetically modified microorganisms and the use of their products in food or feed will need to undergo special regulations to ensure their safety. Studies whose purpose was to establish ways to increase the synthesis of ASX by microorganisms without changing their genetic code, as well as effective methods for extraction and delivery systems, have also been conducted [64]. It was also found that the synthesis of ASX is promoted by a decrease in the content of nitrates and phosphates in the medium and an increase in the amount of ferrous iron and sodium chloride, as well as an increase in light exposure [25,65]. The biotechnological productions of ASX and other carotenoids by yeasts or *Escherichia coli* have been described by researchers [38]. Astaxanthin productivity in engineered *Chlamydomonas reinhardtii* has been evaluated by Perozeni et al. [34]. Their encouraging results show that the host used could be competitive with the current *Haematococcus pluvialis* which is the main cultured organism for the industrial production of astaxanthin.

ASX is used in fish feed. However, the high temperature of the extrusion technique, used for processing fish feed, affects its stability and antioxidant capacity [66]. The retention of ASX is usually around 92% if the temperature during the extrusion process is at 90 °C, but it decreases to 85% at 100 °C.

3. Astaxanthin in the Food Industry

Most of the ASX used in fish feed production comes from synthetic sources. This synthetic ASX indirectly becomes part of human food. Food producers use ASX for its antioxidative property and the coloring it gives to foods. Hence, using ASX in food production could be a potential health management plan. Different products can be used to ensure that enough ASX becomes a part of everyday intake, for example, dairy products, fruit drinks, soy products, and protein shakes. The naturally produced ASX should be preferred to achieve this goal because the naturally produced ASX has a higher bioavailability, and lower cost.

On the other hand, chemically produced ASX uses petrochemical compounds resulting in damage to the environment [66]. However, several limitations make the use of natural in industry ASX difficult. For example, natural ASX is usually unstable and degrades under temperature and long-term storage. In addition, due to its highly conjugated and unsaturated structure ASX can be damaged during the production process and technical steps, i.e., light, heat, storage conditions, etc. Another problem with ASX is its low solubility

in water, leading to a lower availability during intake. Therefore, it also forms an emulsion over the water similarly to other fatty acid compounds.

The chemical characteristics of ASX and other carotenoids, as well as several dietary and non-dietary factors, all affect how well they are absorbed [67]. Researchers have looked at how several animal species, such as mice, rats, dogs, and humans, absorb ASX from various sources. A double-blind experimental study reported that 28 physically fit men received 250 g of wild or farm-raised salmon every day for 4 weeks (5 mg ASX per day). Plasma ASX concentrations plateaued at 39 nmol/L after 6 days of administration with wild salmon (3S, 3′S isomer), and at 52 nmol/L after the administration of farmed salmon (3R, 3′S). It is interesting to note that after ingesting salmon from an aquaculture farm, the plasma levels of ASX were considerably higher on days 3, 6, 10, and 14, but not on day 28. These findings point to a similarity between the ASX isomer pattern in human plasma and that of the consumed salmon. Furthermore, it appears that even when ASX is taken from several sources, maximal levels can be attained during the first week of consumption when ASX intake is persistent [68]. Carotenoids are lipid-soluble compounds, and dietary lipids have a beneficial impact on ASX absorption. When administered in an oil-based formulation, ASX seems to be absorbed at a greater rate. Eight male adult participants were given a single dosage of 40 mg of ASX in three distinct lipid-based formulations (n = 8 for each group) in open parallel research. These three lipid-based preparations improved the bioavailability of ASX, although the preparation with the greatest proportion of the hydrophilic synthetic surfactant showed the greatest bioavailability. Consequently, such findings imply that ASX should be taken along with dietary fats to enhance bioavailability [11,69]. Thorough investigations should aim to reproduce these findings in dosages equal to those indicated by the different organizations, including the EFSA and FDA (US Food and Drug Administration), given the limited number of people involved in these bioavailability trials.

Several methodologies have been proposed to enhance the bioavailability of ASX. One of the strategies relies on encapsulation, which raises another question concerning what kind of compound should be used for encapsulation. Polymeric compounds are generally preferred in this regard. Still, one has to be sure that the selected polymeric compound does not alter the chemical nature of ASX and is also biodegradable once consumed. Proteins are also known for their emulsification properties. Milk proteins could act as a potential emulsifier due to their natural way of production and known health benefits. Sodium caseinate is an example of a protein successfully used to increase the stability of an ASX nano-dispersion. Such dispersions are usually prepared with the help of the emulsification evaporation technique. Researchers managed to obtain an optimum dispersion for ASX by carrying out three passes through a homogenizer using a pressure of 30 MPa at 25 °C [12].

Acute and sub-chronic toxicity of the ASX-rich biomass of *Haematococcus pluvialis* has been studied in Wistar rats. It was found that the oral LD_{50} was more than 12 g/kg body weight and showed no adverse effects in either male or female rats. Based on their findings, the researchers concluded that the recommended doses of ASX as a dietary supplement should be 2–6 mg/day or 0.07–0.1 mg/kg/day for an average individual weighing 60 kg [70]. ASX obtained from *Haematococcus pluvialis* has been recommended in a 24 mg/day dose for no more than 30 days in Europe, Japan, and the USA [71].

Considering the safety issues, the allowed levels of ASX in food supplements were up to 8 mg/day, and the acceptable daily intake for adults ranged from 0.034 to 0.2 mg ASX/kg body weight [28].

The consumption of ASX is beneficial not only for humans but also for animals. For example, the administration of 0.25 mg/kg body weight per day helped increase the milk yield and improve the health status of buffaloes [72]. Moreover, ASX also helped manage heat stress and inflation in egg-laying and broiler hens [73]. ASX also helped in combating heat stress in Karan and Sahiwal heifers [74].

Limited evidence in the literature devoted to showing improvements in ASX bioavailability reveals that this goal has not garnered significant attention. Novel delivery strategies,

including various types of formulations, such as nanoparticles, topical application cream, and defined phospholipid complexes offer significant promise and are worthy of further exploration in attempts to enhance the bioavailability of this interesting beneficial molecule.

4. The Role of Astaxanthin in Managing Oxidative Stress

The imbalance of oxidation or the antioxidant mechanism in body cells facilitates the development of too many ROS and free radicals, resulting in oxidative stress. An essential mediator in the pathogenic development of illnesses is the increase in oxidative factors. These may interact with proteins, lipids, and DNA to cause protein inactivity, lipid oxidation, and damage to DNA in a chain reaction, which results in a wide range of disease conditions [75,76]. Cancer, cardiovascular disorders, autoimmune disease, ischemic disease, atherosclerosis, diabetes mellitus, and hypertension are the most common diseases caused by oxidative stress [77].

The role of ASX in the suppression of oxidative stress is significant. The antioxidant protection system becomes weaker with age. ROS associated with aging can be produced endogenously or exogenously, however, the mitochondrial ROS has the most prominent contribution to the aging process because mitochondrial dysfunction caused by oxidative stress is regarded as a key contributor to aging [78]. The body becomes more sensitive to oxidative stress and is prone to many diseases caused by the lack of antioxidant protection [79]. Several health concerns affecting seniors are mediated by oxidative stress and imbalances between pro-oxidants, such as ROS, and antioxidants, including the oxidation of blood lipids (cholesterol and triglyceride), increasing the risk of heart disease, pain, and stiffness in joints, cognitive decline, including mental awareness, information handling, and memory [13]. Research continues to validate the most effective ways to help reduce oxidative stress [2,80].

Therefore, ASX, being the most prominent antioxidant agent, is considered a source with active antioxidant properties and a distinctive nutritional supplement that can fight against oxidative stress and related damages to maintain health [56]. Dietary supplementation with ASX at any age can help combat oxidative stress and promote better health and well-being throughout life.

Findings demonstrate that natural ASX is an extremely potent scavenger of ROS and a valuable ingredient for healthy aging formulations. It reduced oxidative stress in subjects and improved the serum lipid profile by normalizing serum triglycerides and increasing the levels of beneficial HDL-cholesterol [16]. The intake of ASX has been shown to prevent mitochondrial oxidative stress and improve the overall integrity of the mitochondrial membrane. This apparently leads to an increased energy generation capacity and improved cell energy status [16,17]. In addition, topical preparations containing ASX have been used in anti-aging formulations. A recent study has utilized lipo gel and hydrogel that contained ASX and other algal extracts for topical application [16].

After conducting 12 randomized clinical trials, including 380 participants, Ma and colleagues found that ASX could reduce the levels of biomarkers of oxidative stress and inflammation. Its intake decreased the concentration of blood malondialdehyde, improved the superoxide dismutase activity, and reduced serum isoprostane concentration in overweight patients [81].

A recent in vitro study has reported that the supplementation of 20 μM ASX on MCF-7 cells showed considerable pro-oxidant activity with a 53.3% increase in ROS in comparison to a 17.3% increase in the control. Findings of this study also mentioned that this effect improved (68.1% increase in ROS) the synergistic treatment of cells with a mixture of ASX, β-carotene, and lutein [82]. These results indicate that ASX, despite its well-known antioxidant properties that protect cells against oxidative damage, may potentially cause oxidative stress in cancer cells [83,84].

5. The Role of Astaxanthin in the Aging Process

Aging in humans is a dynamic and progressive phenomenon that is accompanied by numerous health challenges, varying from individual to individual due to several factors, including genetics, lifestyle choices, environmental factors, and life events [79]. The body's antioxidant and repair processes become less effective with age. Premature aging is also closely linked to oxidative stress. Aging is typically accompanied by reduced cellular energy production and increased free radical production. This leads to the overloading of defense systems and oxidative damage. From a biological point of view, aging involves accumulating oxidative damage in cells and tissues. Maintaining a healthy lifestyle, along with a balanced and nutritious diet, is linked to healthy aging and prolonged periods of better health [2]. As a result, there is a growing need for healthy items that are appropriate for the elderly population. Effective antioxidants could help in promoting healthy aging [85].

ASX could be regarded as a highly promising candidate geroprotector [23]. It efficiently protects the mitochondrial double membrane system to improve its efficiency in energy production [86].

Younger people are naturally better protected from free radicals and others toxins through the balanced activity of the mitochondria, efficient antioxidant and DNA repair systems, and active protein degradation machinery. ROS, otherwise known as pro-oxidants, are formed as by-products of a normal metabolism in our bodies when food is converted into energy. The mitochondrial respiratory chain is also one of the major sources of cellular ROS generation. Immune cells fighting bacterial infections also release ROS. High levels of ROS can initiate harmful alterations in key biomolecules, such as lipids, proteins, and DNA [13,87].

A recent study has investigated the anti-aging effects of ASX in the accelerated aging model [18]. The research used a combination of D-galactose (galactopyranose having D-form) and jet lag to induce aging in the mice model, and 0.01% ASX was administered in one of the groups to study the anti-aging effects. The results indicate that six weeks of ASX supplementation significantly prevent liver deterioration by stimulating D-galactose metabolism. Moreover, the antioxidant status and muscle functions improved in ASX-supplemented mice compared to the control group [18].

At the molecular level, ASX modulates several crucial cell-signaling pathways, such as JAK-STAT, NF-kB, and PPARγ pathways. In brief, ASX is a promising nutraceutical supplement for treating various health issues, such as hair loss, where inflammation and oxidative stress play a critical role in the onset and subsequent progression of the health issue. ASX helped improve cognitive function in healthy, aged individuals. A human trial (n = 44) that evaluated ASX supplementation with a 12 mg daily dose for 12 weeks suggested that ASX may help protect against age-related cognitive decline [88]. A randomized clinical trial was carried out involving 32 healthy human participants between 60–70 years of age with confirmed signs of oxidative stress to evaluate the effect of ASX on aging. This study demonstrated that the supplementation of a lysosomal formulation of dark chocolate, having 7 mg of co-crystalized ASX with enhanced bioavailability, displayed fascinating effects on the improvement in oxidative status in aging human participants. It suggested the potential benefits of a combination of ASX with dark chocolate [89].

5.1. The Role of Astaxanthin in Skin Aging

ASX supports normal healthy skin by improving skin elasticity and moisture and reducing wrinkle formation (Table 2). ASX has been shown to have anti-inflammatory, immune-modulating, and DNA repair properties, which can effectively maintain skin health [90].

Table 2. Summary of some pre-clinical and clinical studies evaluating potential role of ASX in the management of aging, including skin and brain aging.

Routes of Administration	Concentrations	Experimental Model	Goals/ Health Benefits	Reference
Oral	0.25 mg/kg BW/day	Buffaloes	Increase of milk production and improvement of overall health	[72]
Oral	10, 20, 40, and 80 mg/kg	Broiler hens	Management of heat stress and inflammation	[73]
Oral	0.25 mg/kg BW/day	Heifers	Prevention of heat stress	[54]
Oral	0.01% ASX	Mice	Improvement of the oxidative status and muscle function	[18]
Topical	20 J/cm^2	Mice	Prevention of photoaging caused by UV irradiation	[91,92]
Oral	25 mg/kg	Rats	Protection from oxidative damage caused by cerebral ischemia-reperfusion injury	[93]
Transcutaneous intrathecal (i.t.) injection	10 µL of 0.2 mM	Rats	Protection against spinal cord injury-induced neuronal loss, demyelination, and functional deficit	[94]
Oral	5 mg per/day	Human	Study of the bioavailability of ASX	[68]
Oral	40 mg	Human	Study of the bioavailability of ASX	[69]
Oral	12 mg/day	Human	Prevention age-related cognitive decline	[88]
Oral	7 mg/day	Human	Improvement of the oxidative status	[89]
Oral	6 mg or 12 mg	Human	Prevention of age-related skin damage and improvement of skin conditions	[95]
Oral	4 mg/day	Human	A strong antioxidant effect and facial skin rejuvenation	[46]
Oral	4 mg	Human	Reduction the skin damage caused by exposure to UV rays	[96]
Oral	6 or 12 mg/day	Human	Prevention of age-related dementia	[97,98]

It was observed that the ASX supplementation of 6 or 12 mg prevented the secretion of inflammatory cytokine from keratinocytes and reduced the secretion of matrix metalloproteinase-1 by dermal fibroblast, thus preventing skin damage and helping in the maintenance of healthy skin in participants [95]. Another study has demonstrated that ASX reduced the transepidermal water loss attributed to ultraviolet exposure by decreasing the expression of aquaporin 3 and other proteins, thus reducing skin damage [91].

ASX supplementation (4 mg/day) for four weeks has been shown to rejuvenate the skin by reducing lipid oxidation and corneocyte desquamation in subjects above 40. The promising effects of ASX were mainly attributed to its antioxidant properties [99].

Human studies showed that 6 mg/day of ASX for six to eight weeks might reduce wrinkles, water loss, and age spots. ASX also improved elasticity, moisture content, and skin texture, and the effects seem to be enhanced when combined with the application of ASX topically [11]. In a double-blind trial in Japanese subjects, 4 mg of ASX supplementation, reduced the skin damage caused by exposure to UV rays [96]. ASX supplementation significantly reduced skin damage and helped maintain skin moisture compared to the placebo group [11]. Skin changes include a loss of elasticity and the proper function of oil glands, thinning skin layers, and the accumulation of pigments. These and other factors cause wrinkles, age spots, and dry/loose/sagging skin.

Chung et al. detected experimentally that ASX significantly inhibited the ultraviolet-induced cytotoxicity and cell death of epidermal keratinocytes [100]. The clinical studies support the benefits of ASX supplementation (3–6 mg/d) on photoaged skin [101]. The administration of ASX reduced UV-induced wrinkle formation and increased collagen fibers in the skin [92].

5.2. The Role of Astaxanthin in the Brain Aging

Brain aging is associated with decline in cognitive function and motility. As ASX is capable of crossing the blood-brain barrier and its intake could have a healing effect on brain aging [102]. Recent research has validated ASX's ability to protect the central nervous system. Much of this research has been centered on the neuroprotective benefits of ASX (Table 2). The neuroprotective effects of ASX were rewieved by Fakhri et al. from a clinical perspective [103].

In this area, two human clinical trials were carried out in Japan [97,98]. The first study took ten elderly subjects with age-related forgetfulness and administered 12 mg of ASX each day for 12 weeks [98]. The researchers found efficacy for age-related decline in cognitive and psychomotor function. The second study was randomized, double-blind, and placebo-controlled: a study on human volunteers. After 12 weeks at either 6 mg or 12 mg of daily ASX, subjects were found to have decreased phospholipid hydroperoxides levels (which accumulate in people with dementia) and improved erythrocyte antioxidant status. The researchers concluded that ASX supplementation might contribute to the prevention of dementia in humans as they age [97]. Human brain cells were subjected to an oxidative stress-induced neuronal cell damage system at Nagoya University in Japan. Significant protection was found in cells pre-treated with ASX [104].

Additionally, pre-treatment with ASX inhibited the generation of ROS. The authors concluded that the neuroprotective effect of ASX depends upon its antioxidant potential and mitochondria protection; therefore, it is strongly suggested that treatment with ASX may be effective for oxidative stress-associated neurodegeneration and a potential candidate for natural brain food. ASX can protect against damage from ischemia, the condition where there is a deficient supply of blood to the brain due to an obstruction of the arteries, which results in stroke, brain cell death, and impaired brain function [105]. The researchers attributed ASX's benefits to its intense antioxidant activity. Another study found that pretreatment with ASX for five hours, and again one hour before ischemia, protected against brain damage [93]. ASX was found to be a potent agent against neurodegenerative disorders. ASX reduced brain cell death. Lastly, ASX displayed an ability to improve the proliferation of neural stem cells. The flurry of activity in 2009 and 2010 was not the first research on ASX benefits for the brain; a series of tests on rodents before this at the International Research Center for Traditional Medicine in Japan showed ASX's potential as a supplement for the brain [106]. In the first experiment, blood pressure was reduced by the introduction of ASX to hypertensive rats. Blood pressure is a causative factor for many eye and brain diseases. The researchers went on to examine the effects of ASX on stroke-prone rats. After five weeks of continuous supplementation, the stroke incidence was delayed in the treated group. Next, it was established that the possible mechanism for these in vitro findings is nitric oxide suppression [107,108].

The same study demonstrated a neuroprotective effect on ischemic mice. In this case, ischemia was induced by blocking the carotid artery [8]. In humans, this condition can be caused by plaque buildup, which can block blood flow through the carotid artery in the neck, the primary source of blood to the brain. This plaque buildup can lead to many types of dementia [94].

ASX can alleviate the adverse effects of homocysteine accumulation, glutamate excitotoxicity, and oxidative stress on neuronal cells [109]. ASX reduces neuronal deficits and protects the rat brain from oxidative damage due to ischemia-reperfusion injury [93].

6. Other Pharmacological Activities of Astaxanthin

ASX is a powerful anti-inflammatory and antioxidant molecule due to its role in maintaining the integrity of mitochondrial membranes [110]. The antioxidant properties of ASX have been actively studied. ASX was useful for improving the chronic inflammation process caused by lipopolysaccharide *Escherichia coli* O55, which affects the mucous membrane of the oral cavity [111].

Studies show that ASX helps balance the immune system and suppress overactive immune responses that can create inflammation [112]. ASX supports cardiovascular health by improving blood lipid profiles in healthy seniors. As a bioactive compound, ASX has beneficial health effects for humans in preventing degenerative syndromes, such as cancer and cardiovascular disease [113]. A high dose ($\geq$20 mg/day) of ASX showed a significant antioxidant effect after a 3-week intervention [114].

The antioxidant activity of ASX is many times higher than other antioxidants, such as β-carotene or α-tocopherol [115]. This property explains its therapeutic effects on certain metabolic disorders. ASX has been demonstrated to improve glucose metabolism in diabetics [116]. The 8-week administration of ASX supplementation improved the glucose level and reduced visceral body fat mass [117]. The randomized clinical trial was performed to investigate the potential effects of ASX supplementation on lipid peroxidation, insulin sensitivity, and anthropometric indices in participants with type 2 diabetes mellitus. The results showed that ASX can improve lipid metabolism in humans [107].

ASX has a protective effect against cholesterol and triglyceride oxidation. ASX also helps boost mitochondrial energy delivery, which allows the heart muscle to contract more powerfully and efficiently [56,118]. Research indicates that most diseases associated with the brain result from oxidation and/or inflammation. Antioxidants that can cross the blood-brain barrier are essential for people to protect the brain and central nervous system as they age. The human body may lose the ability to produce high levels of the antioxidants that are normally synthesized internally, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. Additionally, the human body is now subjected to unprecedented levels of oxidation caused by environmental factors, such as pollution, widespread toxic metals, contaminants, processed food, and high levels of stress [119]. All of these lead to an assault on vital organs during aging, particularly in the brains and eyes [120,121]. ASX helped support eye health and protected the eyes by reducing oxidative damage and improving blood flow in capillaries. Studies of individuals with age-related macular degeneration have demonstrated significant improvements in retinal health when given ASX and other carotenoids [122]. Liposome-encapsulated ASX demonstrated a hepatoprotective effect in lipopolysaccharide-induced acute hepatotoxicity [123].

ASX is safe in human clinical trials, and its intake has been shown to reduce cellular DNA damage and pro-inflammatory milieu [16].

The involvement of oxidative stress in antimicrobial therapy has remained an important issue over the years [124]. Significant antibacterial activity of crude ASX extract obtained from *Haematococcus pluvialis* was found against *Escherichia coli*, *Salmonella typhi*, *Vibrio cholera,* and *Staphylococcus aureus* by Rather and coworkers [125]. Results of this study showed that 10 µL of ASX extracted from *Haematococcus pluvialis* has the highest antibacterial potential (10.2 $\pm$ 0.20 mm) against *Escherichia coli*, while having the least antibacterial potential (6.1 $\pm$ 0.0 mm) against *Vibrio cholera* [125].

A global pandemic on novel coronavirus COVID-19 leads to severe morbidity and mortality worldwide. SARS-CoV-2 causes elevated levels of inflammatory factors, including interleukin-6 and tumor necrosis factor-alpha [112]. Subjects with comorbidities showed an increased risk of acute disease prognosis and of developing severe symptoms [126]. Scientists and therapists are searching for effective antiviral, anti-inflammatory, and antioxidative agents that would be useful in preventing the progression of COVID-19 [127,128]. ASX demonstrates great potential in reducing complications of COVID-19, considering its antioxidant, anti-inflammatory, autophagy-modulatory, and anti-apoptosis activities [112,129].

7. Concluding Remarks

ASX is a xanthophyll reddish-orange carotenoid that shows significant biomedical applications. It is synthesized naturally by different living organisms, such as microalgae, fungi, lichens, and bacteria; it can also be produced biotechnologically. Besides, the reddish flesh of some animals (salmon, shrimps, lobsters, crayfish, etc.) is due to feeding on the ASX-producing organisms. Oxidative stress is a key contributor to several diseases,

including aging and age-related disease. The significant antioxidant, anti-inflammatory, neuroprotective, skin-protective, immunomodulator, antimicrobial, and anticancer activity, as well as the ability to improve lipid metabolism, make ASX a promising compound for the prevention or even treatment of different health conditions (Figure 2). An additional important role of ASX has been reported, i.e., suppressing the development of lifestyle-related diseases, such as diabetes. Strong evidence shows that ASX holds great promise for those wishing to prevent cognitive diseases and maintain general brain health. The implications of the studies cited above are extremely exciting, as the proportion of the elderly and the number of patients with cognitive decline increase in the population. Researchers validated the significant benefits of ASX supplementation for healthy aging. Consequently, the demand and research for natural ASX for human health are increasing extensively worldwide. This review highlighted important ASX-associated clinical trials and explored many clues for research on the nutritional aspects of healthy ASX to learn much more about its value for healthy aging and for the management of age-related disorders.

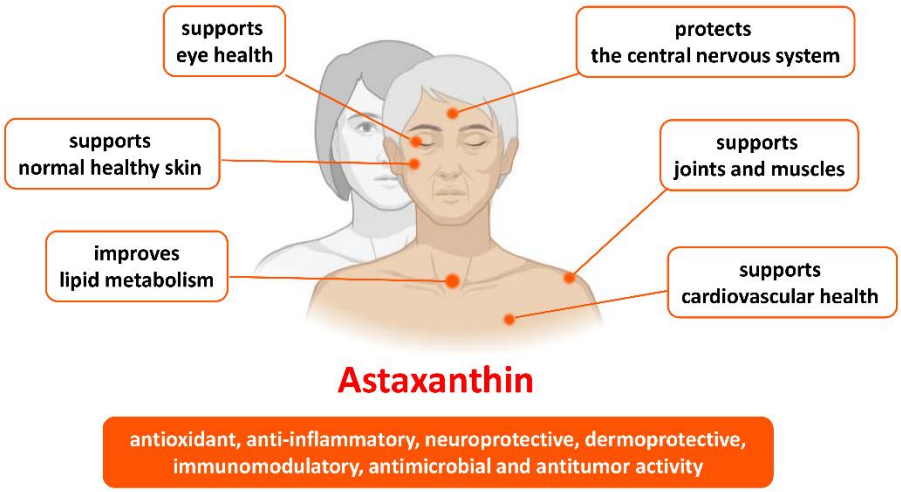

Figure 2. Benefits of astaxanthin in health and age-related conditions.

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ORIGINAL ARTICLE

WILEY

Randomized clinical trial of astaxanthin supplement on serum inflammatory markers and ER stress-apoptosis gene expression in PBMCs of women with PCOS

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Abstract

Polycystic ovarian syndrome (PCOS) is related to pro-apoptotic and pro-inflammatory conditions generated by Endoplasmic reticulum (ER) stress. This study aimed to determine the effect of Astaxanthin (ASX), as carotenoid with potent antioxidant and anti-inflammatory properties, on serum inflammatory markers, apoptotic factors and ER stress-apoptotic genes in peripheral blood mononuclear cells (PBMCs) of women with PCOS. This randomized, double-blind clinical trial included 56 PCOS patients aged 18–40. For 8 weeks, subjects were randomly assigned to one of two groups: either 12 mg ASX ($n=28$) or placebo ($n=28$). Real-time PCR was used to quantify gene expression associated with ER stress-apoptosis in PCOS women's PBMCs. The levels of TNF-α, IL18, IL6 and CRP were determined by obtaining blood samples from all patients before and after the intervention using Enzyme-linked immunosorbent assay (ELISA). Also, the levels of active caspase-3 and caspase-8 were detected in the PBMC by ELISA kit. Furthermore, we evaluated the efficacy of ASX on disease symptoms. Following the 8-week intervention, ASX supplementation was able to reduce the expression of GRP78 ($p=0.051$), CHOP ($p=0.008$), XBP1 ($p=0.002$), ATF4 (0.038), ATF6 (0.157) and DR5 (0.016) when compared to the placebo. However, this decrease was not statistically significant for ATF6 ($p=0.067$) and marginally significant for GRP78 ($p=0.051$). The levels of TNF-α ($p=0.009$), IL-18 ($p=0.003$), IL-6 ($p=0.013$) and active caspase-3 ($p=0.012$) were also statistically significant lower in the therapy group. However, there was no significant difference in CRP ($p=0.177$) and caspase-8 ($p=0.491$) levels between the treatment and control groups. In our study, ASX had no significant positive effect on BMI, hirsutism, hair loss and regularity of the menstrual cycle. It appears that ASX may benefit PCOS by changing the ER stress-apoptotic pathway and reducing serum inflammatory markers; however, additional research is required to determine this compound's potential relevance.

KEYWORDS
apoptosis, astaxanthin, ER stress, inflammatory markers, PBMC, polycystic ovary syndrome

1 | INTRODUCTION

Among women of childbearing age, PCOS is the most common endocrine condition and the major cause of infertility.[1] There is a wide variation in the prevalence of this syndrome across countries.[2] It may be affected by factors such as racial diversity and physical composition.[3] According to the Rotterdam agreement, more than 15% of women have PCOS.[4,5] There are numerous characteristics associated with PCOS, such as hyperandrogenism (HA), metabolic disturbances, irregular menstruation, anovulation, hirsutism and infertility.[6] There is no clear explanation for the aetiology of PCOS, but genomic and environmental factors are believed to contribute to this condition.[7,8] According to recent research, chronic low-grade inflammation has a significant role in the development of PCOS.[9] In fact, as a pro-inflammatory condition, PCOS is associated with cardiovascular disease and type 2 diabetes.[10] In numerous investigations comparing women with this syndrome to controls, tumour necrosis factor (TNF-α), interleukin-6 (IL-6) and interleukin-18 (IL-18), as well as high-sensitivity C-reactive protein (hs-CRP), were shown to be significantly elevated.[11,12] It was observed that there is a positive correlation between TNF-α and IR. Additionally, IR may increase the level of IL-6, a known risk factor for cardiovascular disease in females. There is also a correlation between increased levels of IL-1α, IL-1β, IL-2, IL-8, IL-9, IL-15, IL-17, IL-23 and interferon-γ (IFN-γ) with PCOS. Researchers hypothesize that hyperandrogenism in PCOS may have stimulated the local macrophages, thereby contributing to the pro-inflammatory milieu.[13–16] In addition, numerous studies have demonstrated the involvement of apoptotic dysregulation in the development of PCOS. Caspases play a critical role in the process of apoptosis. There is evidence suggesting that caspases 1, 3, 7, 8 and 9 are involved in the development of PCOS.[17–19] Studies have also looked at the connection between PCOS and Endoplasmic reticulum (ER) stress. ER stress has been found to be higher in PCOS/obese PCOS patients as well as mouse models for PCOS compared to non-PCOS controls in previous investigations.[20–22] ER stress develops as a pathologic condition when unfolded or misfolded proteins accumulate in tissues with excessive protein production. The induction of ER stress can occur under physiological and pathological circumstances such as glucose deprivation, inflammation and oxidative stress (OS), as well as high free fatty acid levels, abnormal Ca^{2+} regulation and hypoxia.[23] Under ER stress, unfolded protein response (UPR) occurs to protect the cells.[23] UPR is regulated by the 78-kDa glucose-regulated protein (GRP78) and the three transmembrane proteins it contains: protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6).[24] If ER stress cannot be alleviated, the cell undergoes apoptosis.[25] It's still not clear how ER stress triggers apoptosis, and the mechanism may vary depending on the stimulus and cell type. Cell apoptosis under ER stress is clearly linked to overexpression of the UPR transcription factor C/EBP homologous protein (CHOP).[25] In apoptosis brought on by ER stress, the CHOP-targeted death receptor 5 (DR5) plays an important role by boosting the generation of the autocrine death ligands signal, which in turn causes more cell death.[26–28] A recent study has demonstrated that human and mouse cumulus cells undergo apoptosis after testosterone-induced ER stress, via induction of DR5.[29] A number of studies have suggested that DRs and caspase-8 may contribute to the promotion of apoptosis subsequent to ER stress.[26,27,30,31] It has been shown that, caspase-8 has the ability to initiate the activation of caspase-3 and caspase-7.[32] ER stress, on the other hand, exerts a close relationship with inflammation and OS. It has been demonstrated that all three main UPR pathways regulate the transcriptional program that promotes inflammation through transcription factors like NF-κB (Nuclear factor-κB) and activator protein-1 (AP-1).[33]

PCOS is often associated with inflammation, obesity, insulin resistance (IR) and HA, which are all common symptoms and potential causes. These factors have a strong correlation with OS. In addition, excessive androgen levels can lead to OS, IR and inflammation. The interaction of these factors contributes to the development and worsening of ovulation disorders in PCOS, particularly in individuals who are overweight or obese.[34,35] Recently, there has been increasing emphasis on the use of antioxidants as an adjuvant treatment for PCOS. It has been reported that ASX, as a xanthophyll carotenoid found in several microorganisms, has been reported to be a potent antioxidant with no negative effects. It has been found that ASX has anti-inflammatory, antiapoptotic, antioxidative, anticancer, neuroprotective and immunomodulatory effects, among others.[36] According to considerable in vivo and in vitro research, ASX appears to have anti-inflammatory properties in mammals, raising the prospect that it could be a useful treatment for disorders related to inflammation.[37] Moreover, prior investigations have shown that ASX suppresses ER stress.[38–40] Meanwhile, recent research from our lab suggested that ASX may be able to influence ER stress and OS in the granulosa cells of PCOS patients.[41] As OS induces ER stress and plays a pivotal role in the pathogenesis of PCOS, and also considering the interactions between ER stress, inflammation, OS and apoptosis, in this study, we examined the effect of ASX on serum inflammatory markers, active caspase levels and gene expression related to ER stress- apoptosis in PBMC of women with PCOS. Furthermore, we evaluated the efficacy of ASX on disease symptoms.

2 | METHODS

2.1 | Trial design and participants

Between October 2021 and May 2022, 56 PCOS patients aged 18–40, were enrolled in the current randomized, double-blind trial at the Omid Clinic in Tehran, Iran, which is registered at http://www.irct.ir: IRCT20201029049183N2. Prior to any intervention, all participants signed informed consent forms (ethics committee reference number: IR.TUMS.MEDICINE.REC.1400.1051). Pregnant women and women with metabolic disorders, including thyroid disease, Cushing's syndrome, hyperprolactinemia, diabetes mellitus, congenital adrenal hyperplasia and impaired glucose tolerance, as well as current or particular past diet or physical activity programs

(within the last 3 months) were not included in the study. In addition, all of the patients in our research had no medical history of inflammatory or autoimmune diseases. For 8 weeks, participants were randomized into two groups and given either a placebo ($n = 28$) or 12 mg capsules in terms of colour, shape, size, packaging and other attributes. Astareal Company (Tokyo, Japan) provided ASX capsules and placebos. Checking the capsule containers and sending daily text messages reminding participants to take their supplements and placebos were used to gauge compliance. The compliance was ensured through the use of 24-h food diary records completed during the study. Additionally, a modified version of the Nutritionist IV program, which is based on Iranian foods, was utilized to estimate the dietary intake of patients. In addition, we utilized the Persian version of the 7-item International Physical Activity Questionnaire (IPAQ) to monitor and regulate physical activity levels. The scoring system provided by the IPAQ was used to classify physical activity levels into three categories: inactive, minimally active and health-enhancing physically active. Regarding clinical signs, a 21–35-day menstrual cycle was considered regular. The study also used the Ferriman-Gallwey criteria to determine the hirsutism score, as well as the Ludwig visual score for hair loss.

2.2 | Assessment of outcomes

Inflammatory markers were considered as the primary outcomes, and biomarkers of apoptosis, gene expression, clinical features of hyperandrogenism (hirsutism and hair loss), and irregular menstruation cycle were considered as the secondary outcomes.

2.3 | Randomization and blinding

The study began with the inclusion of 56 women. We used blocked randomization to assign participants to either a control (placebo) or intervention (ASX) group at random. Participants and researchers were kept in the dark about randomization and allocation to the study. An overview of the study map can be seen in Figure 1.

2.4 | Isolation of lymphocyte cells

Density gradient centrifugation with Ficoll–Hypaque (Lymphodex, Inno-Train, Germany) was used to isolate lymphocytes from the blood. It is best to describe the procedure as follows: fresh blood is combined with an equal volume of Phosphate Buffer Saline (PBS), and then carefully layered over Ficoll in a 2:1 ratio. The sample was subjected to 1100 g of centrifugal force for 25 min at 20°C. As a final step, the middle layer was slowly transferred to a fresh falcon and twice washed with PBS at 300 g for 10 min.

2.5 | RNA extraction and real-time PCR

The extraction of RNA was done manually. The RNX-plus Solution was utilized in the process of RNA isolation (Cinnacolon, Tehran, Iran). Until cDNA could be made, it was held at −20°C. A spectrophotometer was used to determine the quantity of total RNA extracted from each sample (Biochrom WPA Biowave, Cambridge, UK). Then, we synthesized complementary DNA, using a cDNA synthesis kit (Sinnaclon, Tehran, Iran) according to the manufacturer's instructions. Quantitative RT-PCR was used to evaluate the expression of XBP1, ATF4, ATF6, GRP78, CHOP, DR5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene using Lightcycler 96 System (Roche, Germany) and Real Q Plus 2x Master Mix Green (Amplicon, Denmark). mRNA expression calculations were calculated using the Livak technique ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). Table 1 displays the primer sequences.

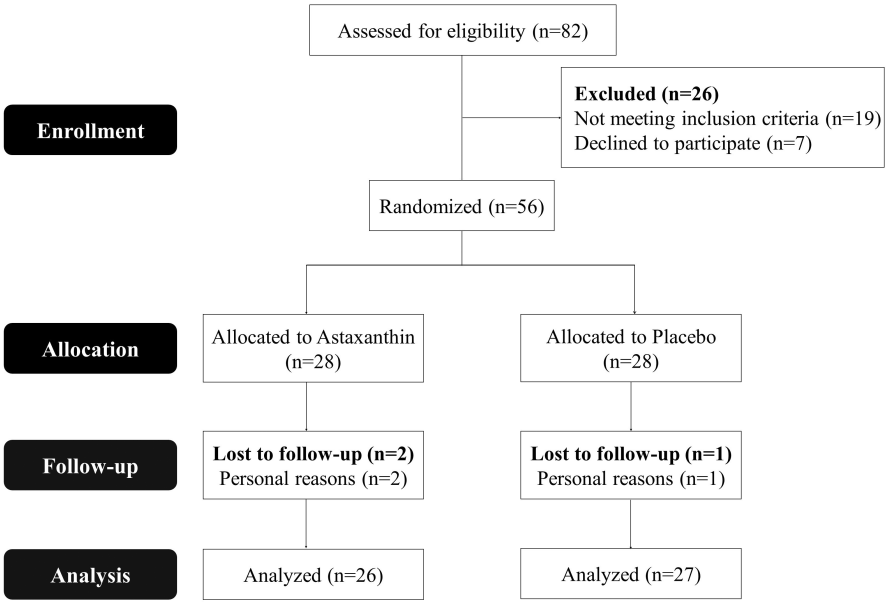

FIGURE 1 Flow diagram of patients' recruitment.

TABLE 1 Forward and reverse primers used for real-time quantitative PCR.

Gene	Primer
GRP78	F: CTGTCCAGGCTGGTGTGCTCT R: CTTGGTAGGCACCACTGTGTTC
CHOP	F: GGTATGAGGACCTGCAAGAGGT R: CTTGTGACCTCTGCTGGTTCTG
ATF4	F: TTCTCCAGCGACAAGGCTAAGG R: CTCCAACATCCAATCTGTCCCG
ATF6	F: CAGACAGTACCAACGCTTATGCC R: GCAGAACTCCAGGTGCTTGAAG
XBP1	F: CTGCCAGAGATCGAAAGAAGGC R: CTCCTGGTTCTCAACTACAAGGC
DR5	F: AGCACTCACTGGAATGACCTCC R: GTGCCTTCTTCGCACTGACACA
GAPDH	F: CGC CAG CCG AGC CAC ATC R: CGC CCA ATA CGA CCA AAT CCG

Abbreviations: ATF4, activating transcription factor4; ATF6, activating transcription factor 4; CHOP CCAAT, enhancer-binding protein homologous protein; DR5, Death receptor 5; GAPDH, glyceraldehyde-3-Phosphate dehydrogenase; GRP78, glucose regulated protein78; XBP1, X-box binding protein 1.

2.6 | Enzyme-linked immunosorbent assay

At the beginning and end of the intervention, 10 mL of venous blood samples were collected to determine serum levels of TNF-α, IL-18, IL-6, CRP, caspase-3 and caspase-8. Abcam enzyme-linked immunosorbent assay (ELISA) kit utilized according to the manufacturer's instructions for TNF-α, IL-18 and IL-6 (abcam ab181421, ab215539 and ab178013, USA, respectively). In addition, CRP was evaluated using the particle enhanced turbid metric approach in this study (Pishtaz-Teb Diagnostics, Tehran, Iran). Also, we utilized Invitrogen Thermo Fisher Scientific kits (Invitrogen, Camarillo, CA, USA), for detection the levels of caspase-3 (KHO1091) and caspase-8 (BMS2024) in the PBMC, in compliance with the manufacturer's protocols. The results were presented per μg of protein found in PBMC lysates.

2.7 | Statistical analysis

The Kolmogorov–Smirnov test was utilized to confirm the parameters' normality, and data were reported as means± SD (standard deviations). In accordance with the proposed formula for parallel clinical trials (Kelsey et al.), and serum CRP levels as a key variable, we calculated a sample size of 25 participants per each group[42] based on type I error of 5% ($\alpha=0.05$), type II error of 20% ($\beta=0.20$; power = 80%). Given the anticipated 5% attrition rate, the sample size for each group was determined to be 28 individuals. Statistical analysis was performed using SPSS version 22 with a significance level of $p<0.05$. In order to compare the baseline characteristics of the two groups, we utilized a chi-square test for categorical data and an independent sample *t*-test for continuous data. Also, variables change between the two intervention groups were compared by independent sample *t*-tests. For intra-group changes, Mcnemar test used for categorical variable. In order to avoid potential bias, analysis of covariance (ANCOVA) was used to adjust for the effects of baseline values (dependent variable) such as BMI and age. The correlations between the serum levels of the cytokines with the signs of diseases and baseline variables were investigated using Pearson's correlation coefficient.

3 | RESULTS

In this randomized clinical trial (RCT), 56 participants were first split into two groups of 28. Ultimately, 53 participants completed the trial: 26 in the ASX group and 27 in the placebo group; all dropped out in both groups was for personal reasons (1 and 2 patients in ASX and placebo groups, respectively) (Figure 1). The compliance rate in our study was high, with more than 90% of capsules consumed in both groups during the study. Throughout the trial, no adverse effects were noted in association with the ASX supplement in PCOS women. There were no significant differences between the ASX group and placebo in terms of age, BMI, basal hormones (LH, FSH and Testosterone), disease duration, smoking and alcohol consumption ($p>0.05$) (Table 2); this also applies to intake of nutrients (Table 3). About physical activity, all of the patients in our study engaged in minimally active category. In terms of inflammatory cytokines, IL-18, IL-6, TNF-α and CRP, there were no statistically significant differences between the two groups at the baseline ($p>0.05$) (Table 4). Table 4 indicated that, ASX treatment was related to a significant drop in IL-18 ($p=0.003$), IL-6 ($p=0.013$) and TNF-α ($p=0.009$) versus to placebo. In addition, there was no discernible effect of ASX supplementation on CRP level ($p=0.177$), and BMI ($p=0.571$) (Tables 2 and 3). In the treatment group, CHOP ($p=0.0008$; Figure 2A), ATF4 ($p=0.038$; Figure 2B), XBP1 ($p=0.002$; Figure 2C) and DR5 ($p=0.016$; Figure 2D) gene expression levels were considerably lower than the placebo group. Furthermore, the treatment group had lower expression levels of ATF6 ($p=0.067$; Figure 2E) and GRP78 ($p=0.051$; Figure 2F) compared to placebo group, but these differences were not statistically significant for ATF6 and only marginally significant for GRP78. Additionally, there was a reduction in the levels of active caspase-3 ($p=0.012$) and caspase-8 ($p=0.491$) in the treatment group; however, this reduction was statistically significant only in caspase-3 (Figure 3).

In Table 5, it is evident that there were no significant differences between the two groups at the baseline in terms of normal menstrual cycle, hirsutism and hair loss. The study found that there was a slight improvement in menstrual cycle irregularity in both the ASX and placebo groups after intervention. However, this improvement was not statistically significant ($p=0.386$). The results indicated that, normal menstrual cycle increased after ASX therapy in the treatment group compared to the baseline values ($p=0.031$). In addition, no significant changes were observed in hirsutism ($p=0.398$) and hair loss ($p=0.784$) in both groups.

Finally, as shown in Table 6, serum levels of IL-18, IL-6, TNF-α and CRP were analysed for correlations. Correlation analysis showed

TABLE 2 Baseline characteristics of study participants.

Variables	Mean ± SD placebo ($n = 27$)	Mean ± SD intervention ($n = 26$)	p-Values
Age (year)	31.19 ± 4.57	30.42 ± 4.69	0.552[a]
Smoking (yes)	3 (11.11)	0	0.235[b]
Alcohol consumption (yes)	1 (3.7)	3 (11.53)	0.350[b]
Disease duration (year)	4.38 ± 1.9	5.11 ± 1.77	0.157[a]
FSH (μIU/mL)	5.48 ± 1.67	4.98 ± 1.16	0.214[a]
LH (μIU/mL)	9.35 ± 3.21	10.59 ± 2.45	0.121[a]
T (ng/mL)	0.43 ± 0.32	0.47 ± 0.31	0.639[a]
BMI (Kg/m^2)			
Baseline	26.55 ± 1.89	26.08 ± 1.89	0.369[a]
End	26.51 ± 1.76	25.89 ± 2.20–0.19	0.265
Mean changes	−0.04 ± 1.21	±0.48	0.571

Note: Statistically significant ($p < 0.05$), percent change in brackets.

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone.

[a]Based on independent *t*-test.

[b]Based on Chi-square.

TABLE 3 Dietary intake of study participants throughout the study.

Variables	Mean ± SD placebo ($n = 27$)	Mean ± SD intervention ($n = 26$)	p-Values
Energy(kcal/day)			
Baseline	2264 ± 213.7	2319 ± 207.9	0.349
End	2298 ± 178.1	2337 ± 142.3	0.387
Mean changes	34.01 ± 249.6	17.97 ± 231.0	0.809
Carbohydrates (g/day)			
Baseline	300.3 ± 39.0	316.7 ± 69.00	0.289
End	293.2 ± 24.72	292.9 ± 20.26	0.224
Mean changes	20.73 ± 49.44	26.91 ± 83.05	0.742
Protein (g/day)			
Baseline	81.66 ± 11.09	80.35 ± 13.68	0.701
End	82.55 ± 10.78	84.53 ± 9.21	0.476
Mean changes	0.884 ± 13.31	4.184 ± 14.98	0.400
Fat (g/day)			
Baseline	79.47 ± 10.02	77.66 ± 12.54	0.564
End	78.14 ± 12.61	75.94 ± 11.09	0.502
Mean changes	−1.32 ± 9.89	−1.72 ± 11.82	0.894
SFA (g/day)			
Baseline	15.28 ± 2.97	12.13 ± 2.60	0.218
End	14.22 ± 2.55	15.14 ± 2.85	0.221
Mean changes	−1.05 ± 2.39	−1.14 ± 2.33	0.891
PUFA (g/day)			
Baseline	22.17 ± 3.11	21.18 ± 2.98	0.244
End	20.64 ± 3.302	19.71 ± 3.16	0.301
Mean changes	−1.53 ± 1.57	−1.47 ± 1.97	0.902
MUFA (g/day)			
Baseline	25.08 ± 2.52	24.32 ± 3.01	0.323
End	24.79 ± 2.63	24.71 ± 2.65	0.984
Mean changes	−0.28 ± 1.22	0.45 ± 2.15	0.124

Note: p (based on independent *t*-test), statistically significant ($p < 0.05$).

Abbreviations: MUFA, mono unsaturated fatty acid; PUFA, poly unsaturated fatty acid; SFA, saturated fatty acid.

TABLE 4 Comparison of serum inflammatory markers between the two groups.

Variables	Mean ± SD placebo ($n = 27$)	Mean ± SD intervention ($n = 26$)	p-Values[a]	p-Values[b]
IL18 (pg/mL)				
Baseline	276.9 ± 43.46	287.0 ± 78.22	0.562	0.038[c]
End	285.9 ± 51.01	259.5 ± 63.30	0.099	
Mean changes	9.05 ± 22.07	−27.52 ± 43.92	0.003[c]	
IL6 (pg/mL)				
Baseline	13.25 ± 3.20	14.19 ± 2.90	0.266	0.146
End	12.93 ± 3.00	12.82 ± 2.61	0.885	
Mean changes	−0.31 ± 1.43	−1.37 ± 1.55	0.013[c]	
TNF-α (pg/mL)				
Baseline	18.81 ± 1.78	19.25 ± 2.57	0.478	0.002[c]
End	18.25 ± 1.60	17.35 ± 1.29	0.029[c]	
Mean changes	−0.56 ± 1.25	−1.89 ± 2.21	0.009[c]	
CRP (mg/L)				
Baseline	2.42 ± 1.08	2.78 ± 1.35	0.290	0.319
End	2.25 ± 0.83	2.39 ± 1.07	0.603	
Mean changes	−0.17 ± 0.62	−0.39 ± 0.54	0.177	

Abbreviations: CRP, C-reactive protein; IL18, interleukin-18; IL6, interleukin-6; TNF-α, tumour necrosis factor.

[a]Based on independent *t*-test.

[b]Based on linear mixed effects model (the included variables were: basic value of dependent variable, treatment type, Age and BMI).

[c]Statistically significant.

that serum CRP correlated positively with hirsutism ($r_s = 0.343$; $p = 0.012$) and hair loss ($r_s = 0.245$; $p = 0.047$). However, no significant correlation was shown between other variables.

4 | DISCUSSION

This RCT examines the effects of ASX consumption on the ER stress-apoptosis pathway and serum inflammatory markers in PCOS women. The result indicated that, in PCOS women, 8 weeks of oral treatment of 12 mg ASX decreased serum levels of TNF-α, IL-18, IL-6 and CRP. In the treatment group mRNA expression levels of CHOP, ATF4, XBP1 and DR5 decreased compared to the placebo group; however, this reduction was not statistically significant in ATF6, and it also had a borderline effect on GRP78. Also, ASX had no significant positive effect on BMI, hirsutism, hair loss and regularity of the menstrual cycle in women with PCOS. A persistent low-grade inflammation condition has been reported in patients with PCOS, including elevated leukocyte numbers and dysfunction of pro-inflammatory cytokines.[43] Cardiovascular disease and Type 2 diabetes mellitus (T2DM) have been associated with this inflammatory condition.[44] There is a correlation between chronic inflammatory processes and elevated levels of inflammatory cytokines, such as IL-1β, TNF-α and IL-6.[45,46] In addition, it has been reported that, PCOS has been linked to greater levels of hsCRP, TNF-α, IL-6 and IL-18 compared in healthy controls.[11,12,47] High levels of IL-18 in PCOS patients were found to be correlated with insulin sensitivity and obesity.[45] It has been observed that IL-18 induces TNF-α, resulting in the production of IL-6.[48] This suggests that higher IL-6 and TNF-α levels in PCOS may be associated with IR, HA and obesity.[49–51] IL-6 and TNFα stimulate the liver to produce CRP.CRP is commonly used to measure chronic low-grade inflammation in PCOS studies. A growing body of research suggests that CRP is a biomarker of intravascular inflammation and a key indicator of cardiovascular disease.[52] Elevated levels of CRP may potentially elucidate the heightened susceptibility of some patients with PCOS to the beginning of cardiovascular disease (CVD) at an earlier age.[53] ASX is a potent antioxidant that effectively inhibits the initiation of inflammation in various biological systems.[54] According to previous research, ASX has been shown to reduce inflammatory cytokine expression and modify inflammatory signalling pathways.[55] It has been shown that ASX reduces TNF-α, IL1, IL6 and IL18 and modulates NF-kB and JAK/STAT.[56–59] Also, according to a recent review, ASX may be a promising treatment option for chronic inflammatory illnesses and may also protect against skin and gastrointestinal ailments and vascular stiffness by controlling inflammation.[55] Similar to findings from prior studies, our investigation found that the levels of TNF-α, IL-18 and IL-6 reduced dramatically in the therapy group, it was while the reduction in CRP levels was comparable. Wei Xia et al. discovered in a systematic review and meta-analysis that taking ASX was associated with a decrease in CRP levels. This association was noticed when ASX was administered for at least 12 weeks or at high doses above 12 mg/day.[60] It appears that if ASX had been supplied at a higher dose and for a longer duration in our trial, the CRP reduction would have been significant.

Excessive ER stress has been implicated in the aetiology of PCOS and is thought to contribute to cell death. Studies have

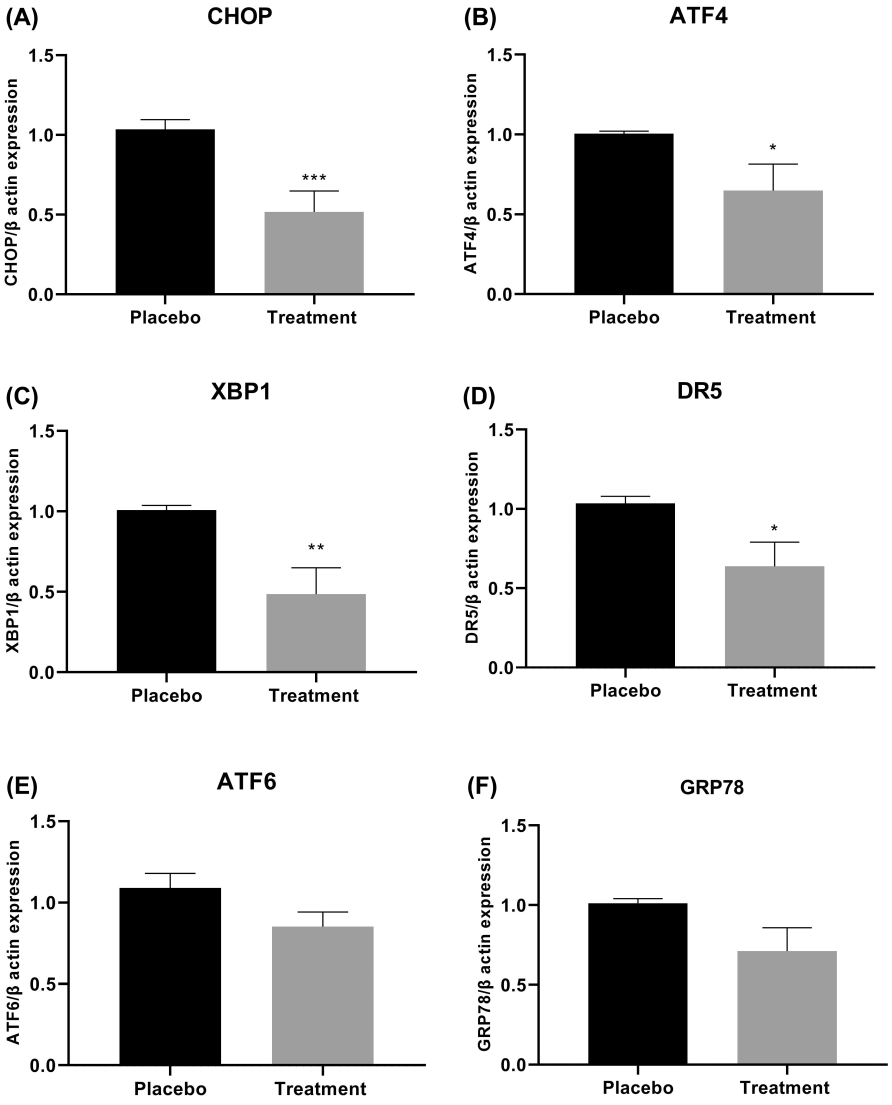

FIGURE 2 The fold changes levels of CHOP (A), ATF4 (B), XBP1 (C), DR5 (D), ATF6 (E) and GRP78 (F) in pbmcs of placebo and treatment groups. Statistical significance ($p < 0.05$) was assessed by *t*-test. Differences between groups; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

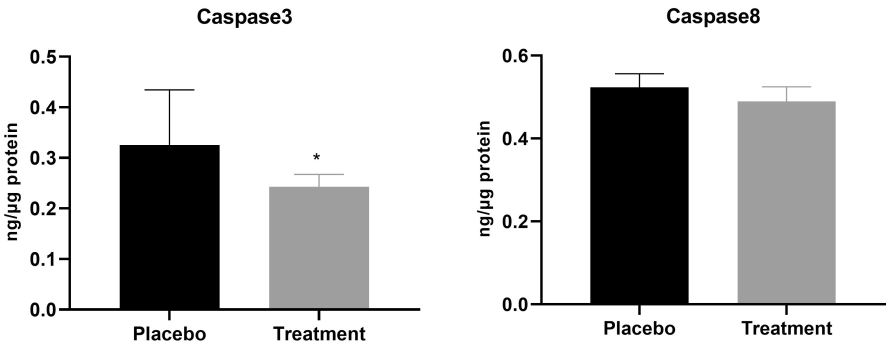

FIGURE 3 Protein concentrations of active caspase-3 and caspase-8 measured using ELISA in PBMCs of placebo and treatment groups. Statistical significance ($p < 0.05$) was assessed by *t*-test. Differences between groups; $^{*}p < 0.05$.

shown that ER stress inhibitors can ameliorate many PCOS symptoms, which may be a novel entry point for treating PCOS.[61] The ER protein homeostasis is controlled by the UPR mechanism. While in normal settings, the UPR is not engaged, it can be activated in the presence of certain stressors.[62] GRP78 is an essential component in maintaining the homeostasis of the ER.[39] During pathological conditions, GRP78 activates the UPR pathway by releasing three ER stress sensors. Due to this, the degree of ER stress is closely correlated with the expression of GRP78.[24] ATF4 serves as another indicator of ER stress; it has been shown that ATF4 induces CHOP expression under ER stress. CHOP increases levels of pro-apoptotic molecules such as DR5 in response to extensive or persisted ER stress.[62] ATF6, as another branch of UPR, stimulates protein folding and degradation pathways associated with the ER in a manner analogous to IRE1-XBP1.[63] ER stress, on the other hand, exerts a close relationship with inflammation and OS. It has been demonstrated that all three main UPR pathways regulate the transcriptional program that promotes inflammation

TABLE 5 Clinical symptoms of PCOS at baseline and after 2 months of treatment with ASX and placebo.

Variables	Placebo ($n=27$) Number (%)	Intervention ($n=26$) Number (%)	p-Values[b]
Normal menstrual cycle (yes)			
Before intervention	6 (22.22)	4 (15.38)	0.727
After intervention	7 (25.92)	10 (38.46)	0.386
Intra-group comparison (p[a])	1.00	0.031*	
Hirsutism (yes)			
Before intervention	8 (29.62)	11 (42.30)	0.398
After intervention	8 (29.62)	11 (42.30)	0.398
Intra-group comparison (p[a])	1.00	1.00	
Hair loss (yes)			
Before intervention	10 (37.03)	14 (53.84)	0.274
After intervention	11 (40.74)	12 (46.15)	0.784
Intra-group comparison (p[a])	1.00	0.500	

Note: Statistically significant * ($p<0.05$), percent change in brackets.

[a]Based on Mcnemar test.

[b]Based on Chi-square.

TABLE 6 Correlations between pro-inflammatory cytokines and Baseline parameters.

	IL-6		IL-18		TNF-α		CRP	
	r_s	p	r_s	p	r_s	p	r_s	p
Age (year)	−0.222	0.110	−0.091	0.515	0.185	0.186	0.002	0.989
BMI (kg/m^2)	−0.114	0.110	−0.061	0.662	0.148	0.289	−0.028	0.840
FSH (μIU/mL)	0.140	0.318	0.124	0.377	−0.074	0.598	−0.199	0.152
LH (μIU/mL)	−0.122	0.384	−0.026	0.854	0.002	0.989	0.079	0.574
T (ng/mL)	0.115	0.414	−0.123	0.379	−0.074	0.600	−0.110	0.431
Disease duration (year)	0.161	0.251	0.080	0.569	−0.041	0.773	0.102	0.468
Normal menstruation cycle	0.164	0.242	−0.022	0.877	0.069	0.626	−0.043	0.762
Hirsutism	0.176	0.207	−0.175	0.210	−0.076	0.588	0.343	0.012*
Hair loss	0.220	0.113	0.106	0.449	0.018	0.900	0.245	0.047*
Smoking	0.146	0.296	0.077	0.582	−0.002	0.990	0.109	0.437
Alcohol consumption	−0.035	0.801	0.044	0.756	0.022	0.876	0.080	0.570

Note: p (based on Pearson correlation coefficient), statistically significant * ($p<0.05$).

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone.

through transcription factors like NF-KB and AP-1.[33] In the present study, we determined the effect of ASX on ER stress-apoptosis in the PBMCs of PCOS patients; the results demonstrated the expression levels of CHOP, GRP78, XBP1, ATF4, ATF6 and DR5 mRNA decreased compared to the placebo group. Similar to our findings, Bhuvaneswari et al. found that ASX decreases the levels of ATF6 and PERK, which preserve liver tissue from the harmful effects of a high fat and fructose diet[64]; on the other hand, it has been shown that ASX attenuated brain injury by reducing levels of GRP78 and CHOP.[40] Recent research by Wang et al. demonstrated that administration of ASX prevents mice from developing ethanol-induced cardiomyopathy by lowering their levels of PERK, ATF6, ATF4, GRP78 and CHOP.[39] Similarly, in another study, Selim Demir et al. found that in the testicular TID model, high levels of ER stress cause testicular tissue damage and that ASX prevents this damage by decreasing the level of ER stress.[65] A number of studies have suggested that DRs and caspase-8 may contribute to the promotion of apoptosis subsequent to ER stress.[26,27,30,31] It has been shown that, caspase-8 has the ability to initiate the activation of caspase-3 and caspase-7.[32] A growing body of evidence supports a strong correlation between the therapeutic effects of ASX and its anti-apoptotic properties.[66] In our prior investigation, we demonstrated that the intake of ASX by infertile women with PCOS resulted in improved levels of apoptotic factors in both serum and follicular fluid, as well as modulation in gene and protein expression related to the apoptosis pathway in granulosa

cells.[67] In the present study, in line with recent studies, we found that, the levels of caspase-8 and caspase-3 reduced in the therapy group, however this reduction was comparable in caspase-8. Based on our data, it can be inferred that PCOS patients exhibit decreased expression levels of ER stress-apoptosis pathway genes when treated with ASX. This suggests that ASX may activate the surviving branches of the UPR in PBMCs. However, given the strong association between ER stress, oxidative stress, apoptosis and inflammation, it may be argued that regulating the ER stress pathway can help to balance apoptotic pathway and improve inflammatory conditions in PCOS patients.

Multiple studies have examined various therapy approaches that have shown promise in reducing symptoms associated with PCOS, including infertility, menstrual cycle regularity, BMI, IR and HA.[68–70] To the best of our knowledge, this is the first study that investigates the effect of ASX supplement as an adjuvant therapy on PCOS symptoms. The results of our study indicated that, ASX improve menstrual cycle irregularity (38.46% vs. 25.92% of patients in ASX and placebo groups, respectively), but the change was not statistically significant ($p=0.386$). Also, there were no significant changes in hirsutism ($p=0.398$) and hair loss ($p=0.784$) in both groups. There may be several factors that contribute to the variations between our study and other studies, including the type and amount of supplement, participant characteristics (such as age, genetics and body measurements), pharmacokinetic factors, the duration of the participants' follow-up, and the number of participants. From our perspective, the small sample size is a significant limitation of the present study. Perhaps using a larger sample size in the study would have resulted in statistically significant clinical changes.

The results of this investigation suggest that a positive correlation exists between CRP levels and clinical manifestations of HA (hirsutism and hair loss). However, no significant correlation was shown between other variables. A research study by Nehir et al. demonstrated a positive correlation between FAI and CRP, TNF-α and α-1 glycoprotein.[71] Additionally, prior research has shown a link between inflammatory indicators and HA in PCOS.[72,73] At present, there is no clarity regarding whether inflammation induces HA by stimulating theca cell androgen synthesis, or whether HA itself initiates the inflammatory response. Some research on PCOS patients suggests that androgens can stimulate inflammatory cells and initiate the inflammatory process. On the other hand, some experts believe that chronic inflammation is the primary factor behind androgen production and ovarian dysfunction.[74]

One remarkable component of our study is the inclusion of all PCOS phenotypes, which considerably increases the possibility of generating data that can be generalized to a larger population. However, it is important to acknowledge that the present study does possess several limitations. Initially, there was a lack of objective measures, such as the quantification of ASX levels in serum or plasma, which made it difficult to evaluate patient compliance The present analysis yielded findings that indicate the potential influence of ASX on the modulation of gene expression related to the ER stress pathway. Nevertheless, it is imperative to assess post-translation alterations and various other aspects. Ultimately, it is conceivable that the comparatively brief duration of the intervention could have played a role in the development of insignificant results. Hence, it is imperative to undertake further study over an extended duration and with larger sample sizes in order to validate our findings.

In conclusion, our findings show that 8 weeks ASX administration in PCOS patients can modulate ER stress-apoptosis in PBMCs by changing the expression of genes implicated in the UPR process and apoptosis. ASX, on the other hand, reduced serum levels of pro-inflammatory markers, such as IL-18, TNF-α, IL-6 and CRP in PCOS patients. More extensive research is required to determine the potential role of ASX.

AUTHOR CONTRIBUTIONS

Masoome Jabarpour: Data curation (equal); formal analysis (lead); investigation (lead); methodology (equal); project administration (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Fardin Amidi:** Conceptualization (lead); funding acquisition (equal); methodology (lead); project administration (equal); resources (equal); software (lead); supervision (lead); validation (equal); writing – review and editing (equal). **Ashraf Aleyasin:** Conceptualization (supporting); project administration (equal); supervision (supporting); visualization (supporting); writing – review and editing (equal). **Maryam Shabani Nashtaei:** Writing – review and editing (equal). **Mojtaba Saedi Marghmaleki:** Writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

CONSENT

Not applicable.

TRIAL REGISTRATION

This study was retrospectively registered on the Iranian website (www.irct.ir) for clinical trial registration (http://www.irct.ir: IRCT20201029049183N2, 12/04/2022).

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Effects of Astaxanthin Supplementation on Skin Health: A Systematic Review of Clinical Studies

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Abstract

Astaxanthin (AST), a naturally-occurring keto-carotenoid found in several species of bacteria and microalgae, has demonstrated diverse biological activities in vitro and in vivo. There is growing commercial interest in the application of astaxanthin in nutraceuticals and cosmeceuticals, due to its purported photoprotective, DNA repair, antioxidant, and anti-inflammatory benefits. This systematic review therefore aimed to summarize current clinical evidence on the effects of astaxanthin supplementation on skin health. Using the following combinations of broad Major Exploded Subject Headings (MesH) terms or text words [astaxanthin OR AST OR ASX OR carotenoid OR xanthophyll] AND [skin OR derm*], a comprehensive search of PubMed, EMBASE, Medline, Clinicaltrials.gov, and Google Scholar databases found a total of eleven clinical studies. There were six randomized, placebo-controlled, double-blind trials, while the rest were prospective, open-label studies. In many of the randomized, controlled trials reviewed, AST supplementation improved skin texture, appearance (wrinkles), and moisture content at the end of the study period. AST also appeared to protect against UV-induced skin damage. No serious adverse events were reported in any of the studies. However, most available studies had a relatively small sample size and were conducted on healthy Japanese females. Many of the studies were also funded by commercial entities, with potential conflicts of interests. This was difficult to account for in our analyses. Overall, there is some clinical data to support the benefits of astaxanthin supplementation (in the range of 3 to 6 mg/d) on skin health, especially for photoaged skin.

Keywords: anti-ageing; astaxanthin; carotenoid; clinical trials; skin; systematic review; xanthophyll.