Chemical Transformation of Astaxanthin from *Haematococcus pluvialis* Improves Its Antioxidative and Anti-inflammatory Activities

Sung Hyun Hwang, Ji Min Kim, Seokjoon Kim, Min Jin Yoon, and Ki Soo Park*

Cite This: ACS Omega 2020, 5, 19120–19130

**1. INTRODUCTION**

Oxidative stress is defined as an imbalance between the production of free radicals and reactive metabolites, known as oxidants or reactive oxygen species (ROS), and their elimination by protective molecules, referred to as antioxidants.1 This imbalance leads to damage to important biomolecules in cells, which may affect the whole organism.2 In addition, oxidative stress has been implicated in the pathogenesis of various disorders, such as chronic diseases, atherosclerosis, neurodegenerative diseases, inflammatory diseases, and aging.3 Therefore, the roles of molecules with antioxidant activity that promote healthy aging and counteract oxidative stress require investigation.4

Carotenoids, which are among the most representative antioxidants, play important roles in controlling the oxidative process. These molecules, possessing long-conjugated double bonds, named as polyene chains, which exert antioxidant activities by quenching singlet oxygen and scavenging radicals.5 Particularly, astaxanthin, nonesterified (Ast-N), monoesterified (Ast-mE), and diesterified (Ast-dE) forms using esterified astaxanthin (Ast-E) in natural extract from *Haematococcus pluvialis* and characterized them by spectrophotometry and high-performance liquid chromatography (HPLC). Additionally, the antioxidant and anti-inflammatory activities of the samples containing three forms of astaxanthin at different ratios were evaluated. The sample containing the maximum level of Ast-mE compared to those of Ast-N and Ast-dE showed the highest antioxidant and anti-inflammatory activities. We also observed the greatest increase in expression of genes related to antioxidant and anti-inflammatory effects in samples containing the highest Ast-mE. These results provide a foundation for in-depth investigation of astaxanthin and other antioxidant molecules, allowing for the development of a practical and cost-effective strategy to improve antioxidant or anti-inflammatory activities of natural extracts that can be used as dietary supplements.

Carotenoids, which are among the most representative antioxidants, play important roles in controlling the oxidative process. These molecules, possessing long-conjugated double bonds, named as polyene chains, which exert antioxidant activities by quenching singlet oxygen and scavenging radicals.5 Particularly, astaxanthin, nonesterified (Ast-N), 3,3′-dihydroxy-β′-β′-carotene-4,4′-dione or esterified (Ast-E), is a red dietary carotenoid present in various microorganisms such as bacteria, yeast, fungi, and microalgae. Astaxanthin has stronger antioxidant activity than various other carotenoids such as lutein, lycopene, α-carotene, and β-carotene.6,7 Moreover, it can protect the skin from injury induced by radiation, chemicals, or toxins; decelerate age-related macular degeneration; and improve immune system function.8 Thus, it has been widely used in various fields, including those regarding foods, dietary supplements, pharmaceuticals, and cosmetics.9,10 The key to these beneficial functions of astaxanthin is its unique molecular structure, which contains hydroxyl and keto moieties on each ionone ring.11

In recent years, researchers have investigated astaxanthin to further improve its antioxidant activities. One study focused on the stereoisomers. Synthetic astaxanthin, which is a racemic mixture of the three isomers (3S,3′S), (3R,3′R), and (3R,3′S), was prepared from petrochemicals, and after the chromatographic separation of each isomer, its antioxidant activities were compared. The accumulation levels of ROS were reduced by 40.12, 30.05, and 22.04% in the presence of the (3S,3′S), (3R,3′R), and (3R,3′S) isomers, respectively.12–14 Furthermore, there is a study that investigated the effect of solubility and/or dispersibility of astaxanthin onto its antioxidant activities. In this work, the authors prepared the disodium disuccinate derivatives of synthetic astaxanthin and found that this derivative exhibited increased scavenging efficiency over equimolar concentrations of Ast-N.15,16 Other researchers investigated the effect of esterification on the antioxidant activities of astaxanthin,
considering the fact that astaxanthin exists in either nonesterified (yeast) or esterified (algae) forms in nature. However, the relative antioxidant capacities of Ast-N, monoesterified (Ast-mE), and diesterified astaxanthin (Ast-dE) remain controversial. Some studies reported stronger antioxidant activity for Ast-N, whereas others indicated that Ast-E (Ast-mE or Ast-dE) is more efficient than Ast-N. Therefore, an in-depth investigation of the antioxidant activities depending on the degree of esterification of astaxanthin is required. In this study, we used Haematococcus pluvialis (H. pluvialis) microalgae, which mainly contains Ast-E with different fatty acids. H. pluvialis is a good source of trans-astaxanthin acyl esters, and it is widely used for astaxanthin products in the industrial perspective because it accumulates the highest levels of astaxanthin in nature. Natural extract in H. pluvialis was obtained by supercritical fluid extraction (sample 1). The Ast-E in the extract was converted to Ast-N through transesterification (sample 2), which was subsequently esterified to Ast-mE and Ast-dE (sample 3). After characterizing the samples containing three forms of astaxanthin at different ratios by spectrophotometry and HPLC, their antioxidant activities as well as anti-inflammatory activities were evaluated. In addition, the expression of genes closely related to antioxidant and anti-inflammatory activities were analyzed to investigate the mechanism of the enhanced bioactivities of astaxanthin.

2. RESULTS AND DISCUSSION

2.1. Chemical Transformation of Esterified Astaxanthin in H. pluvialis. For astaxanthin esters (Ast-E) including Ast-mE and Ast-dE in natural extract from H. pluvialis (sample 1), fatty acids with carbon chains of different length are conjugated to Ast-N through an ester bond. The sample 1 was first converted to Ast-N by removing the fatty acids by transesterification using dehydrated, basic methanol (step 1 in Figure 1). Notably, saponification (hydrolysis) can be used to prepare Ast-N, but a carboxylate anion (R′COO⁻) after the saponification reaction can be produced, which can react to Ast-N in the subsequent esterification reaction, making it difficult to prepare Ast-E with fatty acids of specific lengths (decanoic acid in this work, step 2 in Figure 1). In contrast, the transesterification used in this study generates Ast-N in addition to the fatty acid methyl ester (sample 2) so that the added decanoic acid only reacted with the hydroxyl group of Ast-N in the subsequent Steglich esterification reaction (step 2 in Figure 1). Furthermore, the transesterification that occurs in the absence of water minimizes formation of astacene; one of by-products that can be generated during saponification. As a result, Ast-E with a fatty acid chain of a single length (C10) (sample 3) can be effectively prepared, facilitating the analysis of antioxidant activities of different forms of astaxanthin (Ast-N, Ast-mE, and Ast-dE) with a fixed length of the fatty acid chain.

2.2. Characterization of Samples Containing Three Forms of Astaxanthin. The prepared samples (samples 1−3) containing three forms of astaxanthin (Ast-N, Ast-mE, and Ast-dE, respectively) at different ratios were characterized by HPLC. First, the analytical standard of Ast-N was analyzed by HPLC; as shown in Figure 2A, the retention time was centered at 3.963 min. Similarly, the chromatogram of sample 1 was obtained. As shown in Figure 2B, major peaks for sample 1 appeared at 11.477, 13.627, 16.543, 20.450, and 21.373 min. Our chromatographic profile of sample 1 that contains Ast-E with different fatty acids is almost the same with the one in the previous reports that studied astaxanthin extracted from H. pluvialis. Based on the previous reports, we assigned the position of Ast-mE and Ast-dE in our HPLC results. Because H. pluvialis contains Ast-E bound to fatty acids of different lengths, the first three peaks (between 10 and 18 min) and next peaks (between 20 and 24 min) were predicted to be Ast-mE and Ast-dE, respectively (Figure 2B). We also confirmed that the retention time of astaxanthin-N in sample 2 was centered at 3.943 min after the transesterification reaction (Figure 2C), which agrees well with the results for analytical-grade synthetic Ast-N (Figure 2A). These results clearly demonstrate that fatty acids of different lengths were removed from Ast-E. Finally, the chromatogram of sample 3 after the esterification reaction with decanoic acid was obtained. The results in Figure 2D show that the major peaks for sample 3 were centered at 3.787, 6.863, and 14.973 min. The first peak (3.787 min) corresponds to Ast-N, and the last two correspond to Ast-mE; of the last two, the first matches Ast-mE linked to decanoic acid, and the second matches Ast-mE linked to longer fatty acids. It is known that H. pluvialis contains Ast-E with fatty acids longer than C18. Because these fatty acids are longer than decanoic acids (C10), we assumed that the monoester form with decanoic acid appears first (6.863 min), followed by monoester with longer fatty acids (14.973 min). In addition, the last peak that appeared around 20 min was assigned to diesters with two fatty acids because of the increased molecular weight and hydrophobicity. These results were supported by not only HPLC analysis of pure (synthetic) Ast-N, Ast-mE, and Ast-dE (Figure S1) but also LC−MS analyses of samples 2 and 3 (Figure S2). Notably, Ast-E with fatty acids longer than C18 can be also produced during the esterification reaction (step 2, Figure 1) because H. pluvialis originally has some fatty acids. The relative amounts of all forms of astaxanthin (Ast-N, Ast-mE, and Ast-dE) in the three samples (1, 2, and 3) were determined by measuring the area under the peak (Figure 2). As indicated in Table 1, the content of Ast-mE in sample 3 was higher than that of Ast-dE, suggesting that the composition of sample 3 differs from that of sample 1 in which Ast-dE was more abundant than Ast-mE. Overall, we successfully
synthesized three forms of astaxanthin by chemical transformation.

2.3. In Vitro Antioxidant Activity. The biological activities of the three samples were evaluated. First, in vitro antioxidant

Figure 2. HPLC chromatograms of (A) analytical standard of Ast-N, (B) sample 1, (C) sample 2, and (D) sample 3.
activities were investigated in a DPPH radical scavenging assay, which is regarded as the first approach for evaluating antioxidant activity due its simplicity, speed, and low cost.\(^3^1\) In principle, the hydrogen-donating ability of antioxidants reduces the free radical DPPH (purple) to a stable DPPH (yellow), leading to decreased absorbance at 517 nm.\(^3^2\) As shown in Figure 3, various concentrations of each sample (samples 1–3) were tested to measure their DPPH radical scavenging activities. As an antioxidant, all three samples showed concentration-dependent radical scavenging activities. The radical scavenging activities of samples 1 and 3 were slightly higher than that of sample 2, which was determined by calculating the slope of each linear equation. Importantly, sample 3, containing a higher amount of Ast-mE than that of Ast-dE, showed a slightly higher antioxidant activity compared to that of sample 1, which had a larger amount of Ast-dE than that of Ast-mE. However, the overall difference in vitro antioxidant activities among the three samples was not significant even though sample 3 exhibited the best radical scavenging activity.

### 2.4. Cell Viability.

The cytotoxicities of three samples (1–3) toward B16F10 (murine melanoma), HaCaT (human keratinocyte), and Raw 264.7 (murine macrophage) cells were then assessed in an MTT assay.\(^3^3\) The results in Figure 4 show that samples 1 and 2 were not cytotoxic up to 15 μM toward B16F10, HaCaT, and Raw 264.7 cells, whereas sample 3 exhibited a significant decrease in cell viability below 70% at concentrations higher than 10 μM in Raw 264.7 cells, which are sensitive to environmental conditions (p-value < 0.01).\(^3^4\) Based on these results, we set the maximum concentration to evaluate the cellular activities of the astaxanthin samples. Specifically, we used 5 μM astaxanthin, which is similar to the concentrations used in previous studies.\(^2^6,^3^5\)

### 2.5. Cellular Antioxidant Activity.

We performed the well-established CAA assay, which relies on an intracellular stress-based model.\(^3^6\) Specifically, we first induced oxidative stress in B16F10 and HaCaT cells by exposure to H₂O₂, which can readily penetrate the cell membrane and generate the most reactive form of oxygen, the hydroxyl radical, via the Fenton reaction.\(^7\) Next, DCFH-DA, an oxidation-sensitive indicator, was added to measure ROS production in B16F10 and HaCaT cells. In principle, after entering the cells, DCFH-DA is converted to DCFH by cellular deacetylase enzymes; in the presence of ROS, nonfluorescent DCFH is oxidized to fluorescent DCF. Thus, stronger antioxidant activities lead to lower fluorescence signals produced by preventing DCFH oxidation. We calculated the CAA (%), which is inversely proportional to the fluorescence signal. As shown in Figure 5, among the three samples, sample 2 composed of Ast-N showed the lowest antioxidant capacity in B16F10 and HaCaT cells (Figure 5A,B). Importantly, sample 3 containing more Ast-mE than Ast-dE exhibited higher antioxidant ability than the natural extract from *H. pluvialis* (sample 1), which contains more Ast-dE than Ast-mE. These results demonstrate that sample 3, particularly Ast-mE, is critical for the enhancement of cellular antioxidant activities. Interestingly, unlike the in vitro assay for antioxidant activities (Figure 3), distinct differences in antioxidant activities among the three samples were observed in the cellular antioxidant assay (Figure 5), indicating that the degree of esterification has a greater effect on cellular antioxidant activities than on in vitro antioxidant activities. Based on these results, it is assumed that Ast-mE has superior antioxidant activity due to its easy insertion into the phospholipid bilayer where the polyene chain and terminal ring of the astaxanthin scavenge radicals in cells.

### 2.6. Anti-inflammatory Activity.

It has been reported that antioxidant and anti-inflammatory activities are closely related. In addition, astaxanthin is known as a potent antioxidant that terminates the induction of inflammation in biological systems.\(^5\) To evaluate the anti-inflammatory activities of the samples containing three forms of astaxanthin at different ratios, murine macrophage Raw 264.7 cells were stimulated by LPS to produce and release a wide variety of immunologically active mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. These mediators induce the transcription of nitric oxide synthase, which can generate a large amount of NO and is thought to be involved in cytotoxic effects following inflammation. The resulting level of NO was determined by the Griess reaction, a spectrophotometric assay performed to measure nitrite ion levels.\(^3^8\) Greater production of NO leads to an increased colorimetric signal, indicating less effective anti-inflammatory activities. The results in Figure 6 show the inhibition of NO production (%) by the three astaxanthin samples (1–3). In accordance with the cellular antioxidant activities, the highest anti-inflammatory activity was observed in sample 3 containing more Ast-mE than Ast-dE, supporting the important role of Ast-mE in the antioxidant and anti-inflammatory activities of astaxanthins.

### 2.7. Gene Expression Related to Anti-inflammatory and Antioxidant Activities.

Based on the promising results for the chemical transformation, characteristics, and biological activities of three samples, we investigated the mechanism of these improved activities for sample 3. To better understand the inhibitory effects of each sample on inflammation, the mRNA expression of proinflammatory cytokines was first investigated by qPCR in Raw 264.7 macrophages stimulated with LPS. LPS-activated macrophages produce excessive levels of proinflammatory cytokines including IL-1β, IL-6, and TNF-α, and other inflammatory mediators such as NO to promote the development and progression of inflammation.\(^3^9–^4^1\) As shown in Figure 7A–C, the gene expression levels of proinflammatory cytokines were markedly increased in LPS-stimulated Raw 264.7 macrophages compared to that in resting Raw 264.7 macrophages. In contrast, the different astaxanthin samples (1–3) substantially decreased the mRNA levels of IL-1β, IL-6, and TNF-α. Most importantly, sample 2 was the least effective sample in reducing the levels of IL-1β, IL-6, and TNF-α expression, and sample 3 more effectively reduced the levels of proinflammatory cytokines (p < 0.01) as compared to sample 1. These results indicate that sample 3, which was prepared by chemical transformation, most effectively inhibited the production of proinflammatory cytokines in activated macrophages among the three samples. These results agree with those shown in Figure 6 for the anti-inflammatory effect of astaxanthin.

Next, the expression of genes related to antioxidant activities was analyzed. In addition to its ROS scavenging function, astaxanthin is known to decrease ROS production in LPS-

![ACS Omega](http://pubs.acs.org/journal/acsdof/ACS_Omega)
stimulated macrophages by repressing the expression of NOXs, which are important enzymes that generate ROS in macrophages by donating electrons to oxygen molecules.\(^3,4^{2}\) As shown in Figure 7D, we found that the marked induction of Nox2 expression by LPS was completely abolished by the presence of astaxanthin. Particularly, sample 3 caused the greatest reduction in the expression of Nox2 (\(p < 0.01\)). Furthermore, the expression of nuclear factor E2-related factor 2 (Nrf2), which plays an important role in the endogenous antioxidant defense mechanism and prevents oxidative stress by stimulating the production of antioxidant enzymes, was increased by LPS stimulation; accordingly, the expression of its target gene

Figure 3. DPPH radical scavenging activity of each sample. (A) Sample 1, (B) sample 2, and (C) sample 3. The results are presented as the mean ± S.D. of three independent experiments.
(MnSOD) was also increased (Figure 7E,F). Importantly, sample 3, which was prepared by chemical transformation, was the most effective in reducing the expression of Nrf2 and MnSOD. These results agree with those shown in Figure 5 for the antioxidant effect of astaxanthin. Overall, the highest anti-inflammatory and antioxidant activities were observed in sample 3 containing more Ast-mE than Ast-dE.

3. CONCLUSIONS

In this study, we prepared three samples containing different ratios of Ast-N, Ast-mE, and Ast-dE by chemical transformation. After characterizing the samples, their effects on biological activities including antioxidant and anti-inflammatory activities were evaluated. In addition, we determined how astaxanthin exerts its antioxidant and inflammatory activities through gene expression analysis. Importantly, the chemically synthesized sample 3 containing more Ast-mE than Ast-dE showed the strongest antioxidant and anti-inflammatory activities. These results are encouraging, but the conversion efficiency of astaxanthin was not 100%, particularly in the esterification reaction (step 2, Figure 1), as shown by the HPLC results. Thus, Ast-N remained in sample 3, making it difficult to identify the best structure for the antioxidant activities. Furthermore, the effect of the fatty acid chain length must be evaluated because the biological activities of Ast-E can be changed depending on the different length of fatty acids. However, it should be noted that we can decrease the production cost by using natural extract from *H. pluvialis* instead of the synthetic chromatographically separated one, which enables practical application for its commercialization and mass production. In addition, this is the first study to investigate the effect of esterification of astaxanthin on its biological activities and demonstrate that the sample with a greater portion of Ast-mE through chemical transformation is more effective compared to the one extracted from *H. pluvialis*. This study also suggests that structural conversion via chemical transformation can be applied to Ast-N from other sources to increase antioxidant ability. Therefore, astaxanthin may be applied as a food supplement and/or functional ingredient to reduce oxidative stress. These results provide a foundation for the in-depth investigation of astaxanthin or other antioxidant molecules, allowing for the development of new antioxidant or anti-inflammatory molecules.

4. MATERIALS AND METHODS

4.1. Chemical Transformation of the Natural Extract from *H. pluvialis*. The oleoresin from *H. pluvialis* isolated by supercritical fluid extraction using carbon dioxide (CO2) was purchased from Alphy (Yunnan Alphy Biotech Co., Chuxiong, China) and stored at −20 °C to avoid thermal degradation. The one from *H. pluvialis* contains almost exclusively one isomer (3S,3′S-astaxanthin). It was confirmed that this product contained 10% astaxanthin. The natural extract from *H. pluvialis* (sample 1 dissolved in acetone) was first converted to Ast-N by a transesterification reaction to remove the different fatty acids. Specifically, sodium hydroxide (0.0072 g, Samchun Chemical, Seoul, Korea) was added to 50 mL of methanol (Samchun Chemical) and mixed with anhydrous sodium sulfate (Samchun Chemical) to remove residual moisture. The prepared basic alcohol was filtered through a 0.2-μm syringe filter, which was then incubated with 1 g of the natural extract from *H. pluvialis* in a shaking incubator at 50 °C for 4 h. Next, Ast-N was extracted with petroleum ether (Samchun Chemical). The ether was evaporated, and the resulting Ast-N was dissolved in either dichloromethane (Samchun Chemical) or acetone (HPLC-grade, Samchun Chemical) for the esterification reaction or

![Figure 4. Cytotoxicity in (A) B16F10, (B) HaCaT, and (C) Raw 264.7 cells exposed to different samples. The results are presented as the mean ± S.D. of three independent experiments. *p < 0.05, **p < 0.01 vs control group.](https://dx.doi.org/10.1021/acsomega.0c02479)
HPLC analysis, respectively, and named as sample 2. Finally, Ast-N in sample 2 was transformed to Ast-E by the Steglich esterification method with decanoic acid, a C10 straight-chain-saturated fatty acid (Tokyo Chemical Industry, Tokyo, Japan).29 Specifically, Ast-N in dichloromethane was sequentially mixed with decanoic acid (0.12 mM), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.14 mM; Tokyo Chemical Industry), and 4-dimethylaminopyridine (0.11 mM; Tokyo Chemical Industry), which was then incubated at 20 °C for 2 h. After repeating this process three times, dichloromethane was evaporated. The resulting Ast-E was dissolved in acetone (sample 3) for further analysis. The purity of three samples was estimated to be 10% (sample 1), 11.3 ± 2.7% (sample 2), and 9.1 ± 2.0% (sample 3) by measuring the absorbance at 474 nm (Figure S3). For HPLC analysis, we dissolved each sample in acetone at the concentrations of 0.178 mM (sample 1), 1.18 mM (sample 2), and 0.77 mM (sample 3), respectively.

4.2. High-Performance Liquid Chromatography (HPLC). The astaxanthin samples dissolved in acetone were quantified by measuring the absorbance at 474 nm on a NanoDrop spectrophotometer (Spectramax iD5 multimode microplate reader, Molecular Devices, Sunnyvale, CA, USA) with a calibration curve obtained using synthetic Ast-N (Sigma-Aldrich, St. Louis, MO, USA) (Figure S3). All samples were analyzed by HPLC conducted at 4 °C on a YL9100 system (Younglin, Gyeonggi-do, Korea) with a UV/visible detector (474 nm). The analytical column was a Waters Symmetry C18 column (4.6 × 250 mm, 5 μm, Waters, Milford, MA, USA). The mobile phase consisted of solvent A (methanol:tert-butyl methyl ether:1% (w/v) phosphoric acid, 81:15:4, v/v) and solvent B.
(methanol:tert-butyl methyl ether:1% (w/v) phosphoric acid, 16:80:4, v/v). To selectively separate different forms of astaxanthin, a gradient procedure was employed: 100% of A for 15 min; a linear gradient from 0 to 100% of B for 8 min; 100% of B for 4 min; and 100% of A for 8 min. The flow rate was 1.0 mL/min. tert-Butyl methyl ether, water, and phosphoric acid were purchased from Samchun Chemical (HPLC-grade).

4.3. In Vitro Assay for Antioxidant Activities. To measure the in vitro antioxidant activities, we performed a DPPH radical scavenging assay. Specifically, 20 μL of each sample (samples 1–3) was added to 180 μL of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH; 10 mM; Sigma-Aldrich) dissolved in methanol. After incubation at 37 °C for 15 min, the optical density (OD) was evaluated at 517 nm on a microplate reader. The results indicating DPPH radical scavenging activity (%) were expressed using the following equation: 

\[
\text{DPPH scavenging activity} = \frac{\text{OD}_{control} - \text{OD}_{sample}}{\text{OD}_{control}} \times 100 \%
\]

where \(\text{OD}_{control}\) is the absorbance in the presence of each sample, and \(\text{OD}_{sample}\) is the absorbance in the absence of each sample.

4.4. Cytotoxicity Test. Raw 264.7 (murine macrophage) and B16F10 (murine melanoma) cells were purchased from the Korean Cell Line Bank (Seoul, Korea), and HaCaT (human keratinocyte) cells were purchased from ATCC (Manassas, VA, USA). Raw 264.7, B16F10, and HaCaT cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and 5% CO₂ for 24 h, the cells were washed with 1 × PBS, and then 10 μM DCFH-DA was added to each well, followed by incubation for 1 h at 37 °C. The excess indicator in the medium was removed by washing with 1 × PBS to measure the oxidation reaction that occurs only inside the cells. Next, each sample (samples 1–3) was added to each well along with hydrogen peroxide (H₂O₂, at 1 mM; Sigma-Aldrich), which is known to exhibit the less cytotoxicity on cell growth, the final acetone concentration in the astaxanthin samples was below 5%, which was used as the blank to calculate cell viability. If viability was reduced to less than 70% of the blank, the compound was considered to have cytotoxic potential. The statistical differences were calculated by one-way ANOVA followed by the Dunnett’s test (Minitab, version 18). A two-sided p-value less than 0.05 was considered as statistically significant.

4.5. Cellular Antioxidant Activity Assay (CAA). To measure the production of ROS in B16F10 and HaCaT cells, we used the 2′,7′-dichlorofluorescein 3′,6′-diacetate (2′,7′-DCFH-DA; Sigma-Aldrich), an oxidation-sensitive indicator. In the presence of ROS, DCFH is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF), the absorbance of which can be measured on a microplate reader. All cell lines were first seeded at a density of 0.8–1 × 10⁴ cells/well in 96-well cell-culture plates. After culturing the cells in DMEM supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin at 37 °C and 5% CO₂ for 24 h, the cells were washed with 1 × PBS, and then 10 μM DCFH-DA was added to each well, followed by incubation for 1 h at 37 °C. The excess indicator in the medium was removed by washing with 1 × PBS to measure the oxidation reaction that occurs only inside the cells. Next, each sample (samples 1–3) was added to each well along with hydrogen peroxide (H₂O₂, at 1 mM; Sigma-Aldrich) to induce oxidative stress for 30 min. Finally, the fluorescence intensities were measured on a microplate reader at excitation and emission wavelengths of 485 and 530 nm, respectively. A dose–response curve was established using the blank that had not been treated with H₂O₂. The ROS scavenging activity was expressed as CAA (%) using the following equation:

\[
\text{CAA} = \frac{\text{Is}}{\text{Ic}} \times 100 \%
\]

where Is is the intensity of samples exposed to H₂O₂ in the presence of each sample at different concentrations, and Ic is the intensity of samples exposed to H₂O₂ in the absence of each sample.
of nitric oxide (NO) in Raw 264.7 cells, we determined the NO intensity of the control sample exposed to H₂O₂ in the absence of each sample. The statistical differences were calculated by one-way ANOVA, followed by the Dunnett’s post hoc test.

### 4.6. Anti-inflammatory Assay.
To measure the production of nitric oxide (NO) in Raw 264.7 cells, we determined the NO concentration via the Griess reaction. Raw 264.7 macrophage cells were first seeded at a density of 5 × 10⁴ cell/well into 96-well cell-culture plates. After culturing the cells in DMEM supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin at 37 °C and 5% CO₂ for 24 h, 1 μg/mL lipopolysaccharides (LPS; Sigma-Aldrich) was added to each well except for the control. Each sample (samples 1–3) was added to each well at various concentrations. After incubation for 2 days, 100 μL of the supernatant from each well was collected and mixed with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylene-diamine dihydrochloride in water) in a separate 96-well plate. After incubation at 25 °C for 15 min, the OD at 540 nm was measured on a microplate reader. NO production (%) was calculated using the following equation: 

\[
\text{Absorbance} = (\text{As} / \text{Ac}) \times 100 \%
\]

where As is the absorbance exposed to LPS in the presence of each sample at different concentrations, and Ac is absorbance of the control exposed to LPS in the absence of each sample. For comparison of multiple group data, the statistical differences were calculated by one-way ANOVA, followed by the Dunnett’s post hoc test.

### 4.7. mRNA Analysis.
Raw 264.7 cells were first treated with each sample (samples 1–3) as described above after which their total RNAs were extracted using the NucleoSpin RNA Plus (Macherey-Nagel, Düren, Germany). Next, 150 ng of total RNA was converted to cDNA using an M-MLV cDNA synthesis kit (Enzynomics, Daejeon, Korea), and gene expression was analyzed by quantitative polymerase chain reaction (qPCR). Specifically, qPCR was performed using gene specific primers (500 nM, Table 2) in 1X PreMix (Enzynomics) under the following conditions: 95 °C for 10 min, followed by 35 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s. Each experiment was repeated at least three times, and qPCR data were expressed as the mean ± standard deviation (S.D.). The relative quantification was calculated by the 2^(-ΔΔCt) method and normalized to the respective Rplp0 expression. The statistical differences were calculated by one-way ANOVA followed by the Dunnett’s test (Minitab, version 18).

### Table 2. Gene-Specific Primers for qPCR Experiments

| gene       | forward primer (5’-3’)       | reverse primer (5’-3’)   |
|------------|------------------------------|--------------------------|
| Rplp0      | TGAACATGCTGAAACATCT          | TATAATGCTGCCGTTT         |
| IL-1β      | GTCAACAGAACACATTGACAT        | GCCCATCAGAAGGAGGA        |
| IL-6       | CTGGAAGAGCTCCATCCAGTT        | AAGGAAGGGCTGGTTT         |
| TNF-α      | GGCTGCCGGCAGCTACTG           | AACTTCCCTCTGGATAGATAGCAAT|
| Nos2       | CTACCTAAGATACGAGTGTGA        | CTAACATCACACCTCATATA     |
| Nrf2       | GGCCCAAGCATATCCAGACA         | TGGGGCAACCTGGGAGTAG       |
| MnSOD      | GTGACTTTTGCGGTCCTTTGA        | GCTAACATCTCCAGATT        |

(Figure S3), and viability of Raw 264.7 cells exposed to acetone (Figure S4) (PDF)

### AUTHOR INFORMATION

#### Corresponding Author
Ki Sook Park — Department of Biological Engineering, College of Engineering, Konkuk University, Seoul 05029, Republic of Korea

#### Authors
Sung Hyun Hwang — Department of Biological Engineering, College of Engineering, Konkuk University, Seoul 05029, Republic of Korea
Ji Min Kim — Department of Biological Engineering, College of Engineering, Konkuk University, Seoul 05029, Republic of Korea
Seokjoon Kim — Department of Biological Engineering, College of Engineering, Konkuk University, Seoul 05029, Republic of Korea
Min Jin Yoon — Natural Bio Tree, Seongnam-si, Gyeonggi 13449, Republic of Korea

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c02479

#### Author Contributions
Conceptualization was done by S.H.H., M.J.Y., and K.S.P. Methodology was formulated by S.H.H., J.M.K., S.K., M.J.Y., and K.S.P. Validation was performed by S.H.H. and J.M.K. Resources were provided by M.J.Y. Writing and original draft preparation were performed by S.H.H. Writing and review as well as editing were performed by K.S.P. Project administration was done by K.S.P. All authors have read and agreed to the published version of the manuscript.

#### Funding
This work was supported by “Astaxanthin Chemical Transformation Project” at Natural Bio Tree.

#### Notes
The authors declare no competing financial interest.

### ABBREVIATIONS
Ast-N, nonesterified astaxanthin; Ast-E, esterified astaxanthin; Ast-mE, monosterified astaxanthin or astaxanthin monoester; Ast-dE, diesterified astaxanthin or astaxanthin diester; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMAP, 4-dimethylaminopyridine; CAA, cellular antioxidant assay; DCFH-DA, 2’,7’-dichlorofluorescein 3’6’-diacetate; FBS, fetal bovine serum; IL, interleukin; LPS, lipopolysaccharide; NO, nitric oxide; OD, optical density; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; TNF, tumor necrosis factor; Nrf, nuclear factor E2-related factor
esters in Haematococcus pluvialis A.; Brunet, C.; Sansone, C. On the neuroprotective role of astaxanthin: 2013
Sarada, R.; Ravishankar, G. A. Effective inhibition of skin cancer, 62
commercial applications Astaxanthin: sources, extraction, stability, biological activities and its function of their carotenoid and fatty acid content.
Antioxidant and oxidative stress: A mutual interplay in age-related
independent manners. J. Nutr. Biochem. 2018, 62, 202–209.
(4) Tan, B. L.; Norhaizan, M. E.; Liew, W. P. P.; Sulaiman Rahman, H. Antioxidant and oxidative stress: A mutual interplay in age-related diseases. Front. pharmacol. 2018, 9, 1162.
(5) Ambati, R. R.; Phang, S.-M.; Ravi, S.; Aswathanarayana, R. G. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications—a review. Mar. drugs 2014, 12, 128–152.
(6) Galasso, C.; Orfice, I.; Pellone, P.; Cirino, P.; Miele, R.; Ianora, A.; Brunet, C.; Sansone, C. On the neuroprotective role of astaxanthin: new perspectives? Mar. Drugs 2018, 16, 247.
(7) Naguib, Y. M. A. Antioxidant activities of astaxanthin and related carotenoids. J. Agric. Food Chem. 2000, 48, 1150–1154.
(8) Miao, F.; Lu, D.; Li, Y.; Zeng, M. Characterization of astaxanthin esters in Haematococcus pluvialis by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. Anal. Biochem. 2006, 352, 176–181.
(9) Saw, C. L. L.; Yang, A. Y.; Guo, Y.; Kong, A. N. T. Astaxanthin and omega-3 fatty acids individually and in combination protect against oxidative stress via the Nrf2–ARE pathway. Food Chem. Toxicol. 2013, 62, 869–875.
(10) Rao, A. R.; Sindhuja, H. N.; Dharmesh, S. M.; Sankar, K. U.; Sarada, R.; Ravishankar, G. A. Effective inhibition of skin cancer, tyrosinase, and antioxidative properties by astaxanthin and astaxanthin esters from the green alga Haematococcus pluvialis. J. Agric. Food Chem. 2013, 61, 3842–3851.
(11) Liu, X.; Osawa, T. Cis astaxanthin and especially 9-cis astaxanthin exhibits a higher antioxidant activity in vitro compared to the all-trans isomer. Biochem. Biophys. Res. Comm. 2007, 357, 187–193.
(12) Liu, X.; Luo, Q.; Cao, Y.; Liu, X.; Xiao, H. Antioxidative activity of (3S,3S)− and (3R,3R)−astaxanthin in Caenorhabditis elegans. FASEB J. 2016, 30, 689−681.
(13) Chiou, T. H.; Place, A. R.; Caldwell, R. L.; Marshall, N. J.; Cronin, T. W. A novel function for a carotenoid: astaxanthin used as a polarizer for visual signalling in a mantis shrimp. J. Exp. Biol. 2012, 215, 584–589.
(14) Fassett, R. G.; Coombes, J. S. Astaxanthin: a potential therapeutic agent in cardiovascular disease. Mar. drugs 2011, 9, 447–465.
(15) Hix, L. M.; Lockwood, S. F.; Bertram, J. S. Bioactive carotenoids: potent antioxidants and regulators of gene expression. Redox Rep. 2004, 9, 181–191.
(16) Lockwood, S. F.; Gross, G. J. Disodium diisucinate astaxanthin (CardaxTM): Antioxidant and antiinflammatory cardioprotection. Cardiovasc. Drug Rev. 2005, 23, 199–216.
(17) Aoi, W.; Maoka, T.; Abe, R.; Fujishita, M.; Tominaga, K. Comparison of the effect of non-esterified and esterified astaxanthins on endurance performance in mice. J. Clin. Biochem. Nutr. 2018, 62, 161–166.
(18) Jaime, L.; Rodríguez-Meizoso, I.; Cifuentes, A.; Santoyo, S.; Suarez, S.; Ibáñez, E.; Señorans, F. J. Pressurized liquids as an alternative process to antioxidant carotenoids’ extraction from Haematococcus pluvialis microalgae. LWT—Food Sci. Technol. 2010, 43, 105–112.
(19) Miki, W. Biological functions and activities of animal carotenoids. Pure Appl. Chem. 1991, 63, 141–146.
(20) Cerón, M. C.; García-Malea, M. D. C.; Rivas, J; Acien, F. G.; Fernández, J. M.; Del Río, E.; Guerrero, M. G.; Molina, E. Antioxidant activity of Haematococcus pluvialis cells grown in continuous culture as a function of their carotenoid and fatty acid content. Appl. Microbiol. Biotechnol. 2007, 74, 1112–1119.
activity of extracts from Rhamnus prinoides. In vitro antioxidant, antityrosinase, and cytotoxic activities of astaxanthin from shrimp waste. W. In vitro antioxidant, antityrosinase, and cytotoxic activities of astaxanthin. 329 and Suppressing MAPKs Pathway. Lipopolysaccharide-induced Inflammation and Oxidative Stress in RAW 264.7 Macrophages through Activating Nrf2/HO-1 Signaling and Suppressing MAPKs Pathway. J. Life Sci. 2018, 28, 207–215. Dong, J.; Li, J.; Cui, L.; Wang, Y.; Lin, J.; Qu, Y.; Wang, H. Cortisol modulates inflammatory responses in LPS-stimulated RAW264. 7 cells via the NF-κB and MAPK pathways. BMC vet. Res. 2018, 14, 30. Yang, Y.; Kim, B.; Park, Y. K.; Koo, S. I.; Lee, J. Y. Astaxanthin prevents TGFβ1-induced pro-fibrogenic gene expression by inhibiting Smad3 activation in hepatic stellate cells. Biochim. Biophys. Acta-Gen. Subj. 2015, 1850, 178–185. Gorrini, C.; Harris, I. S.; Mak, T. W. Modulation of oxidative stress as an anticancer strategy. Nat. Rev. Drug Discovery 2013, 12, 931–947. Sun, W.; Lin, H.; Zhai, Y.; Cao, L.; Leng, K.; Xing, L. Separation, Purification, and Identification of (3S, 3′S)-trans-Astaxanthin from Haematococcus pluvialis. Sep. Sci. Technol. 2015, 50, 1377–1383. Dose, J.; Matsugo, S.; Yokokawa, H.; Koshida, Y.; Okazaki, S.; Seidel, U.; Eggersdorfer, M.; Rimbach, G.; Esatbeyoglu, T. Free radical scavenging and cellular antioxidant properties of astaxanthin. Int. J. Mol. Sci. 2016, 17, 103. Chintong, S.; Phatvej, W.; Rerk-Am, U.; Waiprib, Y.; Klaypradit, W. In vitro antioxidant, antityrosinase, and cytotoxic activities of astaxanthin from shrimp waste. Antioxidants 2019, 8, 128. Pillai, M. K.; Santì, L. I.; Magama, S. DPPH radical scavenging activity of extracts from Rhamnus prinoides. J. Med. Plant Res. 2019, 13, 329–334. Jamalzadeh, L.; Ghafoori, H.; Sariri, R.; Rabuti, H.; Nasirzade, J.; Hasani, H.; Aghamaali, M. R. Cytotoxic effects of some common organic solvents on MCF-7, RAW-264.7 and human umbilical vein endothelial cells. 2016. Avicenna J. Med. Biochem. 2016, 4, 1. Nishigaki, I.; Rajendran, P.; Venugopal, R.; Ekambaram, G.; Saktishekaran, D.; Nishigaki, Y. Cytoprotective role of astaxanthin against glycated protein/iron chelate-induced toxicity in human umbilical vein endothelial cells. Phytother. Res. 2010, 24, 54–59. Romano, M. R.; Ferrara, M.; Gatto, C.; Ferrari, B.; Giurgola, L.; Tóthová, J. D. A. Evaluation of Cytotoxicity of Perfluorocarbons for Intraocular Use by Cytotoxicity Test In Vitro in Cell Lines and Human Donor Retina Ex Vivo. Transl. Vis. Sci. Technol. 2019, 8, 24–24. Hamilos, M. I.; Ostojic, M.; Beleslin, B.; Sagic, D.; Mangovski, L.; Stojkovic, S.; Nedeljkovic, M.; Orlic, D.; Milosavljevic, B.; Topić, D.; Karanovic, N.; Wiks, W. Differential effects of drug-eluting stents on local endothelium-dependent coronary vasomotion. J. Am. Coll. Cardiol. 2008, 51, 2123–2129. Hahn, H. J.; Kim, K. B.; An, I. S.; Ahn, K. J.; Han, H. J. Protective effects of rosmarinic acid against hydrogen peroxide-induced cellular senescence and the inflammatory response in normal human dermal fibroblasts. Mol. Med. Rep. 2017, 16, 9763–9769. Vishwakarma, A.; Wany, A.; Pandey, S.; Bulle, M.; Kumari, A.; Kishorekumar, R.; Igamberdiev, A. U.; Mur, L. A. J.; Gupta, K. J. Current approaches to measure nitric oxide in plants. J. Exp. Bot. 2019, 70, 4333–4345. Yoon, S. B.; Lee, Y. J.; Park, S. K.; Kim, H. C.; Bae, H.; Kim, H. M.; Ko, S. G.; Choi, H. Y.; Oh, M. S.; Park, W. Anti-inflammatory effects of Scutellaria baicalensis water extract on LPS-activated RAW 264.7 macrophages. J. Ethnopharmacol. 2009, 125, 286–290.