Apo-12'-capsorubinal exhibits anti-inflammatory effects and activates nuclear factor erythroid 2-related factor 2 in RAW264.7 macrophages

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Abstract

Apocarotenoids have short carbon chain structures cleaved at a polyene-conjugated double bond. They can be biosynthesized in plants and microorganisms. Animals ingest carotenoids through food and then metabolize them into apocarotenoids. Although several apocarotenoids have been identified in the body, their precise health functions are still poorly understood. This study investigated the anti-inflammatory activities of apo-12'-capsorubinal in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. It was confirmed that apo-12'-capsorubinal was not cytotoxic to the macrophages at the concentrations tested. Apo-12'-capsorubinal treatment led to a marked downregulation of interleukin (IL)-6 protein and *Il6* mRNA levels. This apocarotenoid exhibited more potent inhibitory effects than its parent carotenoids, capsanthin and capsorubin. Furthermore, apo-12'-capsorubinal, but not its parent carotenoids, promoted the nuclear accumulation of nuclear factor erythroid 2-related factor 2 (Nrf2) and upregulated the expression of Nrf2-target genes, such as heme oxygenase 1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO-1), in a dose-dependent manner. Furthermore, a comparison using apo-12'-zeaxanthinal and 7,8-dihydro-8-oxo-apo-12'-zeaxanthinal revealed that the α, β-unsaturated carbonyl group on the polyene linear chain mediated the enhanced nuclear Nrf2 translocation, HO-1 expression, and inhibition of IL-6 production. In contrast, apo-12'-mytiloxanthinal, which harbored a hydroxyl group at C-8 of apo-12'-capsorubinal, did not exhibit any of these activities. These results indicated that the β carbon of the α, β-unsaturated carbonyl group in the linear part of the polyene chain is crucial to the Nrf2-activating and anti-inflammatory effects of apo-12'-capsorubinal. This study will advance our knowledge of the physiological significance of xanthophyll-derived apocarotenoids and their potential use as nutraceuticals and pharmaceuticals.

Keywords

Apocarotenoids; apo-12'-capsorubinal; apo-12'-mytiloxanthinal; anti-inflammatory effect; Nrf2 activation; α, β-unsaturated carbonyl group

Abbreviation

Nrf2, nuclear factor erythroid 2-related factor 2; **HO-1**, heme oxygenase 1; **NQO-1**,

NAD(P)H:quinone oxidoreductase 1; **LPS**, lipopolysaccharide; **IL-6**, interleukin 6

1. Introduction

Carotenoids are yellow- to red-colored fat-soluble pigments produced by plants and microorganisms but not animals. However, animals can ingest various carotenoids present in their diet. Human bodies metabolize carotenoids into various derivatives, including apocarotenoids which have short carbon chains that can be produced enzymatically or non-enzymatically.[1] The most extensively studied apocarotenoids are retinoids, that are, vitamin A derivatives. It is involved in several physiological processes, such as vision,[2] reproduction,[3] and immunity.[4] Recent studies have identified other apocarotenoids in animal tissues such as blood and liver and some foods such as vegetables and fruits and investigated their physiological activities.[5] For instance, β-apo-13-carotenone, one of the non-retinoid apocarotenoids derived from β-carotene and detected in human blood, reportedly exhibits antagonistic effects on some nuclear receptors.[6,7] Apo-12'-capsorubinal, an apocarotenoid related to capsanthin and capsorubin, is detected in not only foods such as some chili peppers[8,9] but also human colostrum.[10] However, the biological activities of apo-12'-capsorubinal and other apocarotenoids with a similar structure have not been reported.

Nuclear factor erythroid 2-related factor 2 (Nrf2) normally forms a complex with the inhibitor protein Kelch-like ECH-associated protein 1 (Keap1), and its transcriptional activity is suppressed.[11,12] Nrf2 activators mediate the release of Nrf2 from its complex, and then Nrf2 translocates to the nucleus and promotes the expressions of heme oxygenase 1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO-1), thus contributing to cellular homeostasis.[13,14] Recent studies have reported the significance of Nrf2 activation in suppressing various chronic inflammatory diseases.[15-18] Several studies are focused on exploring the food-related compounds that activate Nrf2 and could be used to prevent chronic inflammatory diseases.[19,20] To our knowledge, only some lycopene-related apocarotenoids have been shown to exhibit Nrf2-activating effects.[21-23]

Tissue inflammation is crucial for the onset and development of various non-communicable diseases, including diabetes and non-alcoholic fatty liver disease.[24] Macrophages, a type of immune cells, regulate tissue inflammation via the expression of inflammatory cytokines, such as interleukin (IL)-6.[25,26] Dysregulation of inflammatory factor expression leads to the development of non-communicable diseases.[27] Therefore,

food components and their derivatives that regulate macrophage-mediated inflammation are likely to be effective in suppressing and preventing chronic inflammatory diseases. Among the apocarotenoids, paracentrone derived from fucoxanthin and apo-14'-astaxanthinal from astaxanthin reportedly exhibit anti-inflammatory effects in lipopolysaccharide (LPS)-activated RAW264.7 macrophages.[28,29] However, little is known about the anti-inflammatory effects of other xanthophyll-derived apocarotenoids.

This study aimed to investigate the anti-inflammatory and Nrf2-activating effects of apo-12'-capsorubinal in RAW264.7 macrophages. Furthermore, in order to determine a potential structure-activity relationship in apocarotenoids, we compared the activities of apo-12'-capsorubinal; apo-12'-mitiloxanthinal, a hydroxylated derivative of apo-12'-capsorubinal; apo-12'-zeaxanthinal; and 7,8-dihydro-8-oxo-apo-12'-zeaxanthinal, a carbonyl derivative of apo-12'-zeaxanthinal. This study expands our knowledge regarding the physiological significance and potential applications of xanthophyll-derived apocarotenoids.

2. Materials and methods

2.1. Reagents and chemicals. Mouse macrophage-like RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). RPMI 1640, penicillin/streptomycin, and organic solvents were purchased from Fujifilm Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Anti-β-actin (C4) sc-47778 (mouse monoclonal IgG) was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Anti-Lamin B1 (mouse monoclonal), anti-HO-1 (rabbit polyclonal), and anti-Nrf2 (rabbit polyclonal) antibodies were purchased from Proteintech Group, Inc. (Rosemont, IL, USA). As secondary antibodies, goat anti-mouse and anti-rabbit IgG horseradish peroxidase antibodies for western blotting and goat anti-rabbit IgG H&L (Alexa Fluor® 488) for immunocytochemistry were purchased from Abcam (Cambridge, UK). Escherichia coli-derived LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Capsanthin and capsorubin preparation. Capsanthin and capsorubin were fractionated from red paprika extract PapriX-oil HP (Glico Nutrition Co., Ltd., Osaka,

Japan) by silica gel column chromatography eluting with acetone/*n*-hexane (4:6, v/v). Each fraction was purified by high performance liquid chromatography (HPLC) under the following conditions until the purity of each fraction was >99%: Column, Develosil ODS-UG-5 column (250 × 4.6 mm, Nomura Chemical Co., Aichi, Japan); mobile phase, methanol; flow rate, 1.0 mL/min; detection: 450 nm.

2.3. Apocarotenoid preparation. Apo-12'-capsorubinal, apo-12'-mytiloxanthinal, and 7,8-dihydro-8-oxo-apo-12'-zeaxanthinal are synthesized according to a previously reported procedure.[30-33] Zeaxanthin obtained from *Jejuia pallidilutea* strain 11shimoA1[34] was oxidatively cleaved according to previous reports.[29,35] Briefly, 5 mg zeaxanthin and 0.8 mg hexadecyltrimethylammonium bromide were dissolved in 10 volumes of chloroform and then reacted with 10 mL of 2.4 M potassium permanganate solution. After 1 h incubation at room temperature, the chloroform phase was collected and dried on a rotary evaporator. Apo-12'-zeaxanthinal in the oxidation products was isolated by HPLC under the following conditions until the purity was >99%: Column, Develosil C30-UG-5 (250 × 4.6 mm, Nomura Chemical Co., Aichi, Japan); mobile phase, methanol; flow rate, 1.0 mL/min; detection: 450 nm. Apo-12'-zeaxanthinal (*m/z* 367.3 $[M+H]^+$ and 389.3 $[M+Na]^+$) were confirmed using a triple quadrupole mass spectrometer LCMS8040 (Shimadzu, Kyoto, Japan) under the following conditions: Column, Develosil ODS-UG-3 (150 × 2.0 mm, Nomura Chemical Co., Aichi, Japan); mobile phase, methanol; flow rate, 0.2 mL/min; Ionization, electrospray ionization (positive ion mode); nebulizer N_2 gas, 2.0 L/min; drying N_2 gas, 15.0 L/min; de-solvation line temperature, 250 °C; heat block temperature, 400 °C.

2.4. Cell culture. RAW264.7 cells were cultured in RPMI1640 containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. RAW264.7 cells were seeded at 50,000 cells/mL and pre-incubated for 24 h. The cells were further cultured in the presence of carotenoids or apocarotenoids dissolved in dimethyl sulfoxide (DMSO) according to each experiment. Control cells were treated with DMSO only. The DMSO concentration in the media was adjusted to 0.1%, which is not cytotoxic.

2.5. Cell viability. RAW264.7 cells were treated with carotenoids or apocarotenoids for 24 h. Then, WST-1 reagent (10 μL of each well) was added and incubated for an additional 4 h. The absorbance at 450 nm of each well was determined using a microplate reader (Molecular Devices, CA, USA). Media samples containing each carotenoid without cells were used as blanks.

2.6. Gene expression analysis. To evaluate anti-inflammatory effects, RAW264.7 cells were treated with carotenoids or apocarotenoids for 24 h and then were cultured for an additional 6 h in fresh media containing 100 ng/mL LPS in the presence of carotenoids or apocarotenoids. To evaluate the Nrf2 activation effect, carotenoids or apocarotenoids were added for 6 h into the culture media of RAW264.7 cells. Extraction of total RNA from RAW264.7 cells was performed using QIAzol lysis reagent (Qiagen, Hilden, Germany). Reverse transcription of cDNA from the total RNA was performed using ReverTra Ace (TOYOBO, Osaka, Japan) under the manufacturer's instructions. Quantitative PCR analysis was performed using a StepOnePlus real-time PCR system (Applied Biosystems Japan Co., Ltd., Tokyo, Japan). The cDNA was mixed with GeneAce Probe qPCR Mix II (Nippongene, Tokyo, Japan) and PCR reactions were performed under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 30 min and 60 °C for 1 min. mRNA expression was determined using TaqMan gene expression assay (Thermo Fisher Scientific, Frederick, MD, USA): *Il6* (Mm00446190_m1), *Hmox1* (Mm00516005_m1), *Nqo1* (Mm01253561_m1), and *Gapdh* (Mm99999915_g1) which was used as the endogenous control.

2.7. Enzyme-linked immunosorbent assay (ELISA). RAW264.7 cells were treated with carotenoids or apocarotenoids for 24 h and then were cultured for an additional 6 h in fresh media containing 100 ng/mL LPS in the presence or absence of carotenoids. IL-6 level in the collected culture supernatants was determined using a commercially available ELISA kit (Thermo Fisher Scientific, Frederick, MD, USA) according to the manufacturer's protocol.

2.8. Western Blotting. RAW264.7 cells were treated with carotenoids or apocarotenoids for 2 h (for Nrf2 nuclear translocation) or 6 h (for HO-1 expression). Whole cell lysate was obtained by lysing the cells with ice-cold RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate (SDS), 0.1% sodium dodecyl sulfate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor) followed by centrifugation at 12,000 g for 10 min at 4 °C. Nuclear and cytoplasmic proteins were obtained using the LysoPure™ nuclear and cytoplasmic extractor kit (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's protocol. The protein concentration in each extract was determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The extracted proteins (10 μg per lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were reacted with primary antibodies (1:2500) for 1 h at room temperature. Subsequently, secondary antibody (1:20000) was added to the membrane and incubated for 1 h. Proteins were detected using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

2.9. Immunocytochemistry. RAW264.7 cells on culture coverslips (Matsunami Glass Ind.,Ltd., Osaka, Japan) were incubated with apo-12'-capsorubinal for 2 h. The cells were treated with 4% paraformaldehyde for fixation followed by 0.1% Triton X-100 for permeabilization. The cells were reacted with primary antibody (1:100) for 1 h and then with secondary antibody (1:1000) for 1 h at a room temperature. DAPI (1 μg/mL) was used for counterstaining. After removing the secondary antibody, encapsulation was carried out using VECTASHIELD® antifade mounting medium (Vector Laboratories, Inc., Newark, CA, USA) for 1 h. The stained cells were observed using an All-in-one fluorescence microscope BZ-X800 (KEYENCE CORPORATION, Osaka, Japan).

2.10. Statistics. Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using unpaired *t*-tests or one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. Statistical significance was determined at

211 $p < 0.01$ or 0.05.
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214 **3. Results**
215 **3.1. Apo-12'-capsorubinal inhibited IL-6 production from LPS-activated RAW264.7**
216 **macrophages. Fig. 1** shows the molecular structures of the carotenoids and
217 apocarotenoids investigated in this study. It was confirmed in advance that they did not
218 exhibit cytotoxicity (**Fig. 2**). To determine the anti-inflammatory activity of apo-12'-
219 capsorubinal, we evaluated its inhibitory effect on the IL-6 production in activated
220 RAW264.7 macrophages (**Fig. 3**). LPS-induced IL-6 secretion to the culture media was
221 significantly reduced in the cells treated with apo-12'-capsorubinal (**Fig. 3A**).
222 Furthermore, *Il6* mRNA expression was also significantly downregulated by the addition
223 of apo-12'-capsorubinal (**Fig. 3B**). The inhibitory effect of apo-12'-capsorubinal was more
224 potent than the full-length carotenoids capsanthin and capsorubin (**Fig. 3A and B**).
225
226 **3.2. Apo-12'-capsorubinal exhibited Nrf2 activating effect in RAW264.7**
227 **macrophages.** Apo-12'-capsorubinal-treated cells exhibited significantly higher nuclear
228 Nrf2 levels than the untreated cells (**Fig. 4A and B**). Apo-12'-capsorubinal increased
229 nuclear Nrf2 level to more than 3-fold higher than that of untreated cells through
230 promotion of Nrf2 translocation from cytoplasm to nuclear (**Fig. 4A**). In
231 immunocytochemistry, enhanced nuclear localization of Nrf2 was observed in the cells
232 treated with apo-12'-capsorubinal compared to the untreated cells (**Fig. 4B**). Moreover,
233 the apo-12'-capsorubinal-treated cells exhibited higher HO-1 protein levels compared to
234 the untreated cells (**Fig. 5A**). Furthermore, apo-12'-capsorubinal treatment dose-
235 dependently enhanced *Hmox1* mRNA expression, with the 10 μM-treated cells exhibiting
236 10-fold higher mRNA levels than the untreated cells (**Fig. 5B**). The *Hmox1* mRNA
237 expression reached its maximum level at 6 h after 12'-capsorubinal addition and decreased
238 thereafter (**Fig. 5B**). Apo-12'-capsorubinal treatment also upregulated the mRNA
239 expression of another Nrf2 target gene *Nqo1* in a dose-dependent manner (**Fig. 5C**).
240 However, *N*-acetyl-L-cysteine (NAC), which inhibits Nrf2 activation, suppressed apo-
241 12'-capsorubinal-mediated upregulation of HO-1 protein and *Hmox1* mRNA expression
242 (**Fig. 5D**). In contrast, capsanthin and capsorubin did not impact Nrf2 nuclear

accumulation and HO-1 expression (**Fig. 6A and B**). These results indicated that apo-12'-capsorbinal activated Nrf2 primarily owing to the characteristic structure of the apocarotenoid.

3.3. α, β-Unsaturated carbonyl group of apo-12'-capsorubinal contributed to its Nrf2 activating effect. To determine the part of the apo-12'-capsorubinal structure that mediates Nrf2 activation, we constructed apo-12'-zeaxanthinal; 7,8-dihydro-8-oxo-apo-12'-zeaxanthinal, with a carbonyl group introduced at C-8 of apo-12'-zeaxanthinal; and apo-12'-mytiloxanthinal, with a hydroxyl group introduced at C-8 of apo-12'-capsorubinal (**Fig. 1**) and investigated their Nrf2-activating and HO-1-upregulating activities. 7,8-Dihydro-8-oxo-apo-12'-zeaxanthinal, but not apo-12'-zeaxanthinal, promoted nuclear accumulation of Nrf2 and increased both protein and mRNA expression of HO-1 (**Fig. 7A-C**). In addition, apo-12'-mytiloxanthinal failed to promote both nuclear accumulation of Nrf2 and HO-1 expression (**Fig. 7A-C**). These results indicated that the C-8 β carbon of the α, β-unsaturated carbonyl group of apo-12'-capsorubinal was involved in Nrf2 activation and upregulation of HO-1 protein and *Hmox1* mRNA levels.

3.4. α, β-Unsaturated carbonyl group of apo-12'-capsorubinal contributed to its inhibitory effect on IL-6 production. To determine the part of the apo-12'-capsorubinal structure that mediates its anti-inflammatory activity, we assessed the inhibiting activities on IL-6 production of apo-12'-capsorubinal, apo-12'-zeaxanthinal, 7,8-dihydro-8-oxo-apo-12'-zeaxanthinal, and apo-12'-mytiloxanthinal in LPS-stimulated RAW264.7 cells. We observed that compared to apo-12'-zeaxanthinal, 7,8-dihydro-8-oxo-apo-12'-zeaxanthinal showed a stronger inhibiting activity on IL-6 production (**Fig. 8A and B**). Furthermore, apo-12'-mytiloxanthinal did not attenuate either IL-6 protein or *Il6* mRNA levels (**Fig. 8A and B**). These results indicated that the anti-inflammatory action by apo-12'-capsorubinal might be associated with the C-8 β carbon of its α, β-unsaturated carbonyl group.

4. Discussion

Apocarotenoids derived from β-carotene and lycopene are found in vegetables,

fruits, and humans who consume them.[36,37] Previously, we detected fucoxanthin-derived apocarotenoid paracentrone in fucoxanthin-fed mouse tissues.[28] Other studies have detected several apocarotenoids, including apo-12'-zeaxanthinal, in human blood.[38] Apo-12'-capsorubinal, found in foods such as chili peppers,[8,9] has been also detected in human colostrum.[10] Thus, the apocarotenoids found in animal bodies might be ingested via food and synthesized through in vivo metabolism including enzymatic and nonenzymatic process.[1] However, little is known about the biological activities of these apocarotenoids.

To the best of our knowledge, this study was the first report that apo-12'-capsorubinal attenuated LPS-induced expression of IL-6 in RAW264.7 cells. Furthermore, apo-12'-capsorubinal exhibited a stronger activity than its parent carotenoids (**Fig. 3**). Previously, we reported that several apocarotenoids derived from carotenes and xanthophylls, such as β-carotene,[35] astaxanthin,[29] and fucoxanthin,[28] exhibit remarkable anti-inflammatory action against activated RAW264.7 cells. 10,10'-Diapocarotene-dial, a lycopene-derived apocarotenoid, inhibited the transcription of nuclear factor kappa B (NF-κB) and the expressions of the genes encoding inflammatory cytokines.[39] In addition, this apocarotenoid exhibited a more prominent inhibitory activity than its parent lycopene.[39] Since the expression of inflammatory cytokines, including IL-6, is induced by the NF-κB system, the anti-inflammatory mechanism of apo-12'-capsorubinal may involve, at least in part, inhibition of this system. Notably, 10,10'-diapocarotene-dial promoted the transcription of antioxidant response element (ARE), an Nrf2 target.[21] In addition, the treatment of human hepatoma (HepG2) and bronchial epithelial (BEAS-2B) cell lines with apo-8'-lycopenal and apo-10'-lycopenoic acid, respectively, reportedly leads to enhanced Nrf2 accumulation in the nucleus and upregulation of HO-1 and NQO-1.[22,23] In the current study, apo-12'-capsorubinal was found to exert stronger nuclear Nrf2-accumulating and HO-1-inducing activities than capsanthin and capsorubin (**Figs. 4–6**). These findings suggest that the unique structure of apocarotenoids, i.e., the α, β-unsaturated carbonyl group generated by cleavage of the parent carotenoids, may enhance their biological activity.

The α, β-unsaturated carbonyl group is known as a partial structure of active compounds for Nrf2 transcription.[40] Various electrophiles, such as curcumin and 15-deoxy-delta 12,14-prostaglandin J_2 (15d-PGJ_2), mediate their Nrf2 activation effects

depending on this moiety.[41,42] Corroborating these findings, in the current study, the 7,8-dihydro-8-oxo-apo-12'-zeaxanthinal-treated cells exhibited enhanced nuclear Nrf2 accumulation and HO-1 expression, but no such results were observed in apo-12'-zeaxanthinal-treated cells (**Fig. 7**). This finding suggested that the α, β-unsaturated carbonyl group introduced into the linear polyene chain is necessary for Nrf2 activation. Interestingly, the apo-12'-mytiloxanthinal did not exhibit an Nrf2-activating activity despite the presence of the α, β-unsaturated carbonyl group on its polyene linear chain (**Fig. 7**). Previously, Linnewiel et al.[21] compared the activities of some lycopene-related apocarotenoids and reported that the electronic environment of the reactive β-carbon of the α, β-unsaturated carbonyl group can influence Nrf2 activation. In case of apo-12'-capsorubinal, the hydroxylation to the C-8 β carbon of the α, β-unsaturated carbonyl group to make apo-12'-mitiloxanthinal attenuated Nrf2 activation. Furthermore, we observed that the inhibitory effect exerted by 7,8-dihydro-8-oxo-apo-12'-zeaxanthinal was more potent than that of apo-12'-zeaxanthinal, indicating that α, β-unsaturated carbonyl group (C-8) located in the linear polyene chain plays an important role in the anti-inflammatory activity of this apocarotenoid (**Fig. 8**). Our previous study on β-carotene-derived apocarotenoids showed that although both β-apo-8'-carotenal and seco-β-apo-8'-carotenal are C-8' cleavage products, only seco-β-apo-8'-carotenal with intramolecular α, β-unsaturated carbonyl group exhibited anti-inflammatory effects in LPS-activated RAW264.7 cells.[35] In the current study, apo-12'-capsorubinal exhibited potent inhibition of LPS-induced IL-6 production, whereas apo-12'-mytiloxanthinal exhibited no such effect (**Fig. 8**). These results showed that the β carbon of the α, β-unsaturated carbonyl group might also be responsible for the anti-inflammatory action of the apocarotenoids. The anti-inflammatory activity of lycopene-related apocarotenoids has also been shown to be related to the electron density of the β carbon in the α, β-unsaturated carbonyl group.[39] Studies on β-damascone and carotenoid-related aromatic compounds suggested that the α, β-unsaturated carbonyl group enhances both the anti-inflammatory and Nrf2-activating activities of these compounds.[43] Furthermore, electrophiles with α, β-unsaturated carbonyls, such as curcumin, sulforaphane, and 15d-PGJ_2, exhibit anti-inflammatory effects in addition to their Nrf2 activating activities.[44,45] The relationship between Nrf2 activation and anti-inflammatory effect in macrophages

might be attributed to the direct impact of Nrf2 on the transcription of inflammatory factors.[46] In addition, Nrf2 has been shown to exert an indirect impact on inflammatory factors. For instance, Nrf2 mediates the expression of HO-1, which, in turn, inhibits the activities of pro-inflammatory cytokines and mediators.[47,48] However, in the current study, apo-12'-zeaxanthinal was found to inhibit IL-6 secretion but not induce Nrf2 activation (**Figs. 7 and 8**), although its inhibitory effect on IL-6 production is lower than that of 7,8-dihydro-8-oxo-apo-12'-zeaxanthinal. This data means that apocarotenoids can also mediate anti-inflammatory activity through other ways that are independent of Nrf2. The NF-*κ*B system is a representative inflammatory signaling pathway that controls the expression of many inflammatory cytokines. α, β-Unsaturated carbonyl compounds can inhibit NF-*κ*B signaling through several points, including DNA binding activity of p65 subunit and enzymatic activity of I*k*B kinase.[49] Apocarotenoids derived from lycopene,[39] β-carotene,[35] and fucoxanthin[28] inhibited NF-*κ*B signaling. These results suggest that apocarotenoids may exert anti-inflammatory activity through a complex pathway that includes Nrf2 activation. Further studies are warranted to elucidate the relationship between the Nrf2-activating and anti-inflammatory effects of apocarotenoids such as apo-12'-capsorubinal.

In conclusion, apo-12'-capsorubinal markedly suppressed LPS-induced IL-6 production, with more potent inhibitory activity than capsanthin and capsorubin. Furthermore, apo-12'-capsorubinal promoted the nuclear translocation of Nrf2 and upregulated the expressions of its target genes, including *Hmox1* and *Nqo1*. Interestingly, a comparison among apocarotenoids with similar structures revealed that the α, β-unsaturated carbonyl group mediated their Nrf2-activating and anti-inflammatory activities. This study expands our knowledge of the potential benefits of xanthophyll-derived apocarotenoids absorbed from foods and converted via *in vivo* metabolism.

CRediT authorship contribution statement

Naoki Takatani and **Hiroki Miyafusa** contributed equally to this study. **Naoki Takatani:** Conceptualization, investigation, funding acquisition, visualization, and writing - original draft: **Hiroki Miyafusa:** Conceptualization and investigation: **Yumiko Yamano:** Resources and writing - review & editing: **Fumiaki Beppu:** Conceptualization and

writing - review & editing: **Masashi Hosokawa:** Conceptualization, funding acquisition, project administration, supervision, and writing - review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Data availability

Data will be made available on request.

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Figure captions

Fig. 1. Molecular structures of carotenoids and apocarotenoids used in this study.

Fig. 2. Cell viability of RAW264.7 macrophages treated with the carotenoids or apocarotenoids used in this study. The cells were incubated in the media containing 5 μM of carotenoids or apocarotenoid for 24 h. Data are represented as the mean ± SEM (n = 6). Bars with different letters are significantly different ($p < 0.05$).

Fig. 3. Inhibitory effect of apo-12'-capsorubinal on IL-6 expression in LPS-activated RAW264.7 macrophages. The cells were pretreated with 5 μM of carotenoids or apocarotenoid for 24 h and then stimulated with LPS (100 ng/mL) for 6 h in the presence of carotenoids or apocarotenoid. (A) the levels of IL-6 secreted into the culture media and (B) *Il6* mRNA expression. Data are represented as the mean ± SEM (n = 3). Bars with different letters are significantly different ($p < 0.05$).

Fig. 4. Promotion of Nrf2 translocation by apo-12'-capsorubinal in RAW264.7 macrophages. (A) Nuclear and cytoplasmic Nrf2 protein levels and (B) distribution of Nrf2 (green). Nuclei were stained with DAPI (blue). Scale bar indicates 20 μm. The cells were treated with 10 μM of apo-12'-capsorubinal for 2 h. Data are represented as the mean ± SEM (n = 3). $^{\#\#}$, $p < 0.01$.

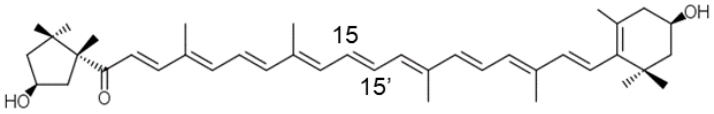
Fig. 5. Upregulation of Nrf2-target protein and gene expression by apo-12'-capsorubinal in RAW264.7 macrophages. (A) HO-1 protein expression in the cells treated with 5 μM of apo-12'-capsorubinal for 6 h. (B) Dose-dependent promotion (6 h) and time course observation (10 μM) of *Hmox1* mRNA expression in apo-12'-capsorubinal-treated cells. Control group was indicated by dotted line in the figure. (C) Dose-dependent promotion (6 h) of *Nqo1* mRNA expression in apo-12'-capsorubinal-treated cells. (D) Cancellation of apo-12'-capsorubinal (5 μM)-induced promotion of HO-1 protein and *Hmox1* mRNA expression by the Nrf2 inhibitor NAC at 1 mM. Data are represented as the mean ± SEM (n = 3). $^{\#\#}$, $p < 0.01$. Bars with different letters are significantly different ($p < 0.05$).

Fig. 6. Apo-12'-capsorubinal specifically activated Nrf2 unlike its parent carotenoids. (A)

Nrf2 nuclear accumulation and (B) HO-1 protein expression levels in the cells treated with apo-12'-capsorubinal, capsanthin, and capsorubin at 5 μM. Data are represented as the mean ± SEM (n = 3). Bars with different letters are significantly different ($p < 0.05$).

Fig. 7. Comparison of Nrf2 activating effects by apocarotenoids with different structures. (A) Nrf2 nuclear accumulation in RAW264.7 cells treated with 5 μM of each apocarotenoid for 2 h. (B) the levels of HO-1 protein and (C) *Hmox1* mRNA expression in the cells treated with 5 μM of each apocarotenoid for 6 h. Data are represented as the mean ± SEM (n = 3). Bars with different letters are significantly different ($p < 0.05$).

Fig. 8. Comparison of inhibitory effects on IL-6 expression by apocarotenoids with different structures. RAW264.7 cells were pretreated with 5 μM of each apocarotenoid for 24 h and stimulated with LPS (100 ng/mL) for 6 h in the presence of apocarotenoids. (A) the levels of IL-6 secreted into the culture media and (B) *Il6* mRNA expression. Data are represented as the mean ± SEM (n = 3). Bars with different letters are significantly different ($p < 0.05$).



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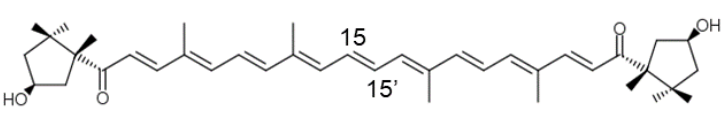

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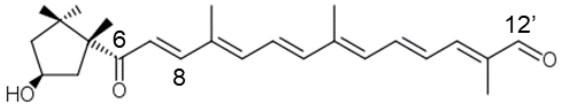
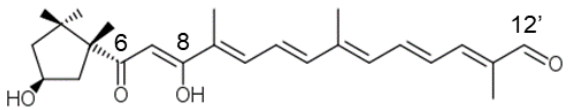


Apo-12'-capsorubinal



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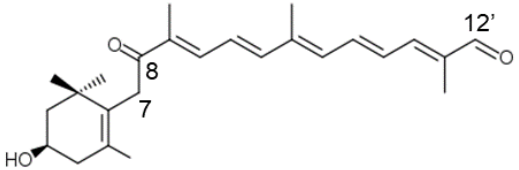

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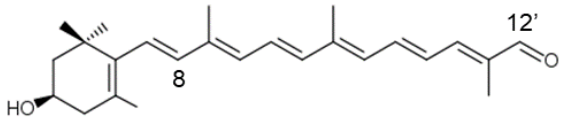


Apo-12'-zeaxanthinal

Fig. 1. Molecular structures of carotenoids and apocarotenoids used in this study.

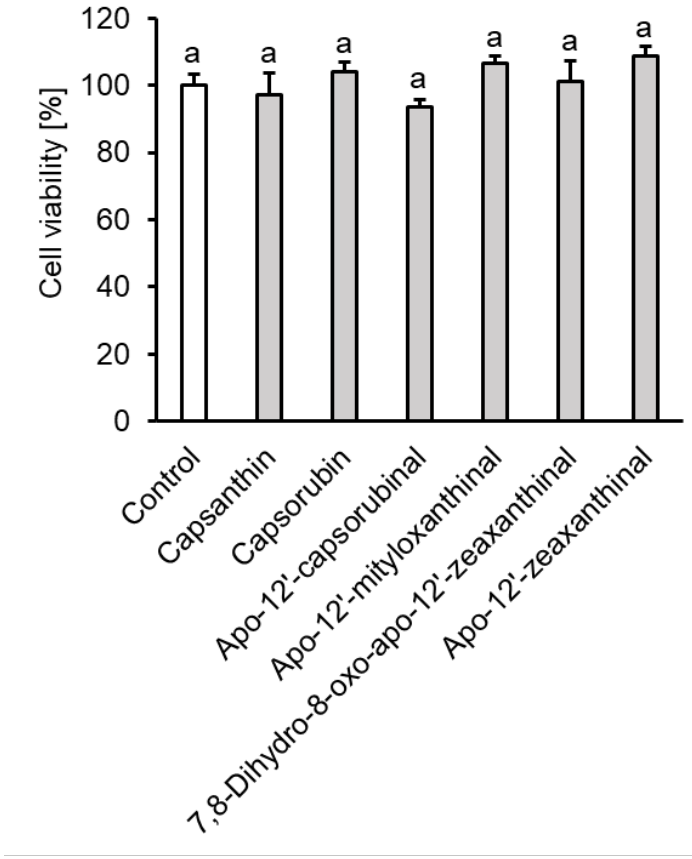
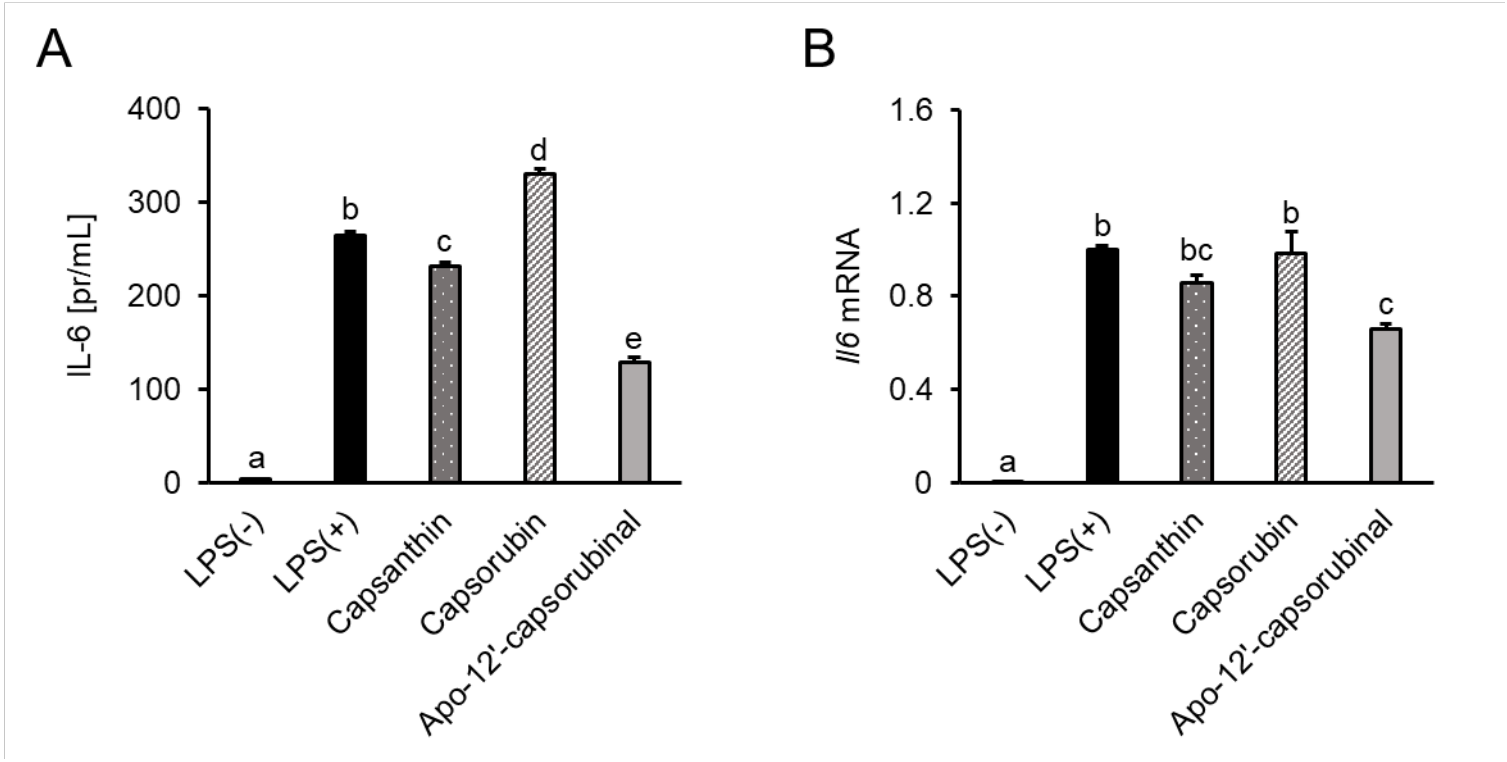


Fig. 2. Cell viability of RAW264.7 macrophages treated with the carotenoids or apocarotenoids used in this study.



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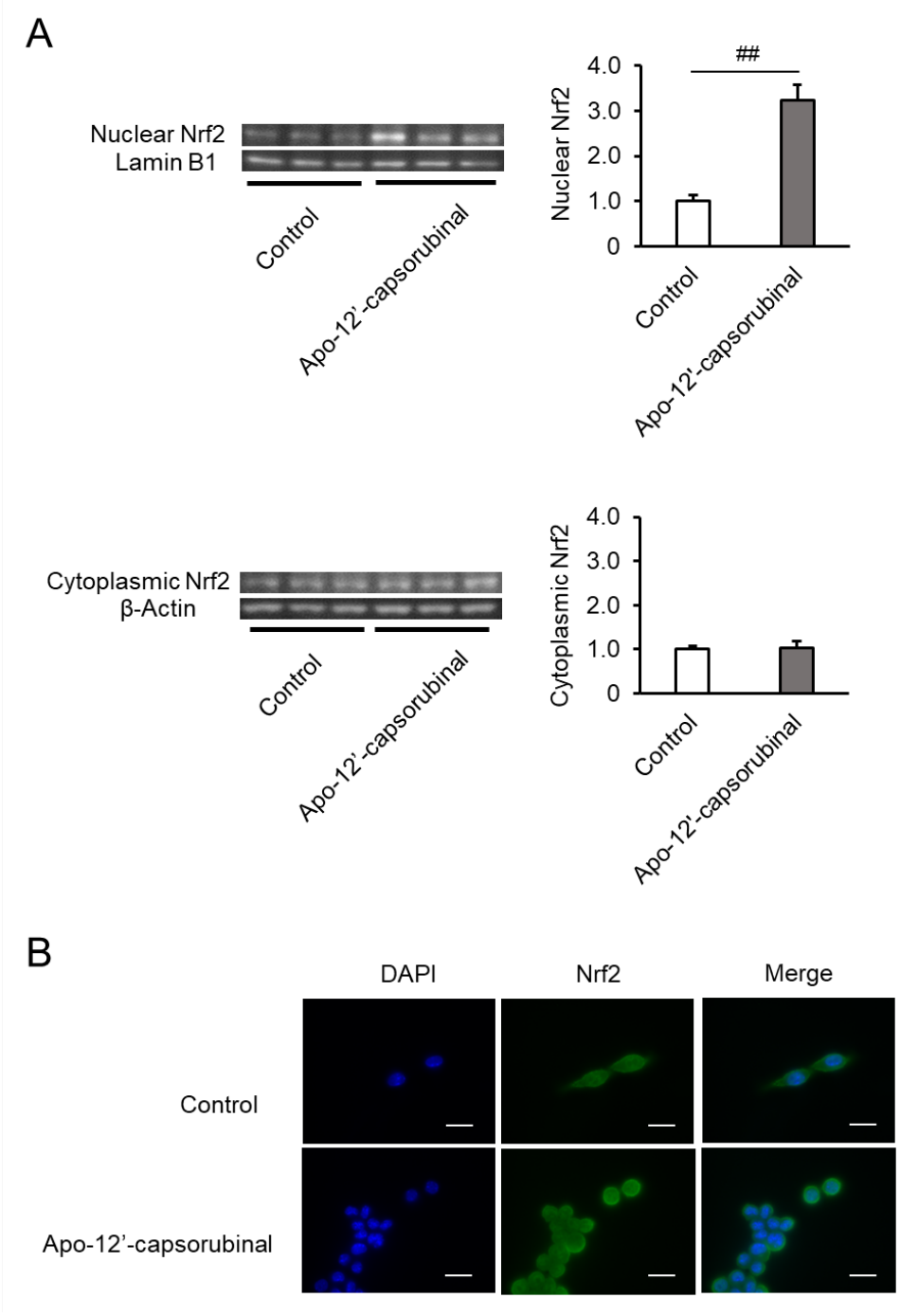

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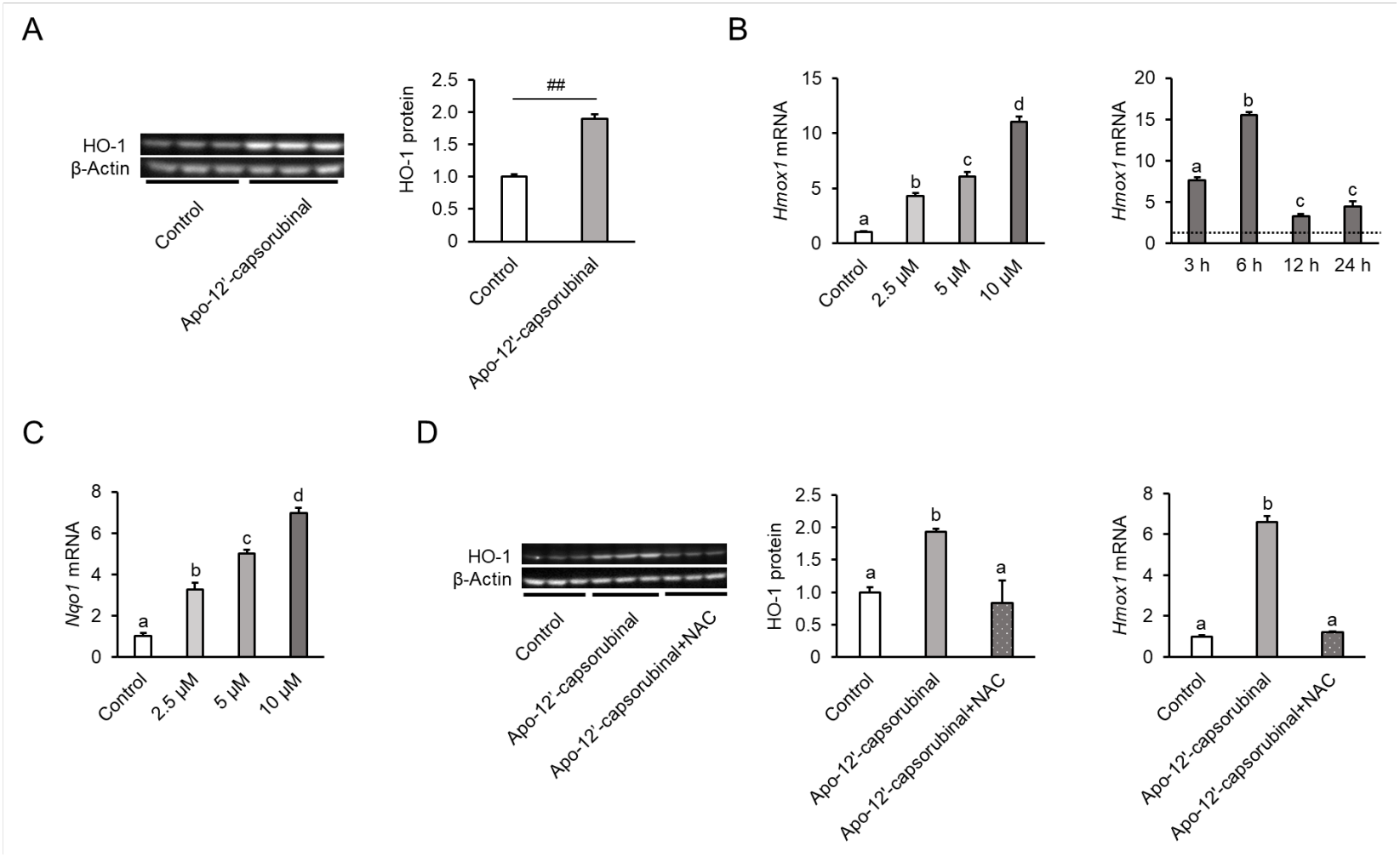

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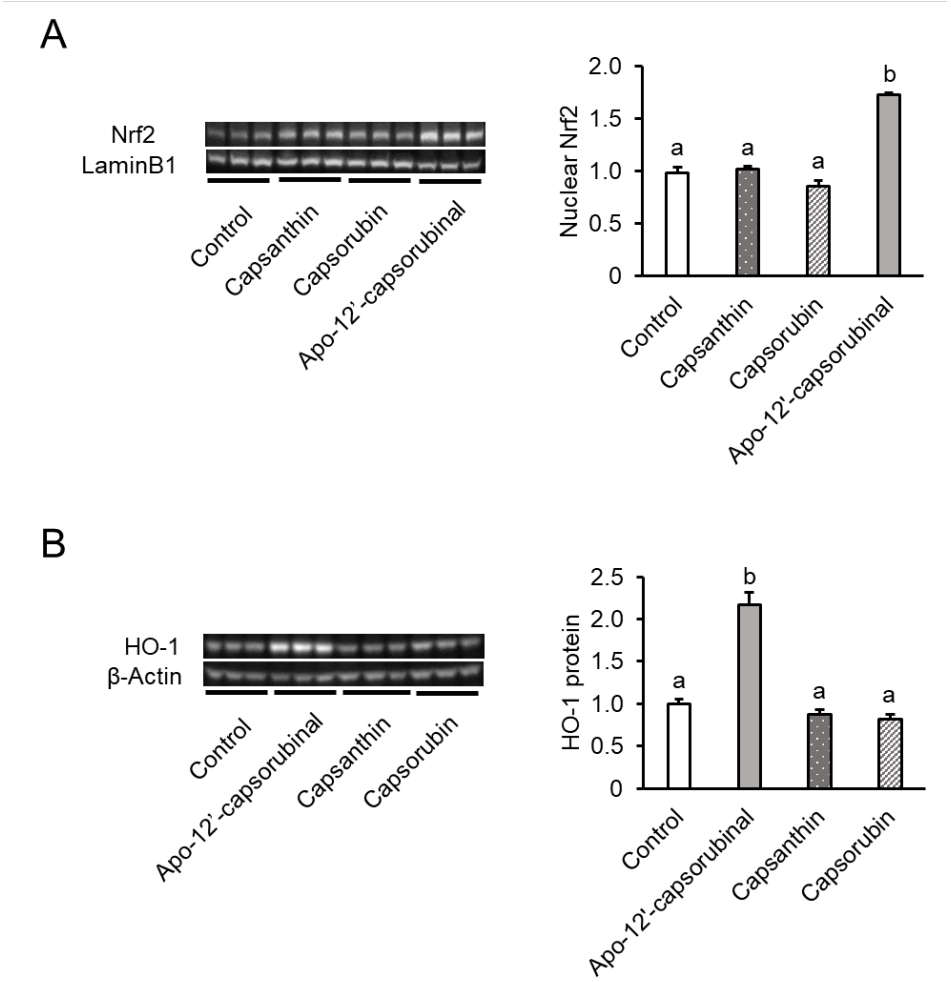

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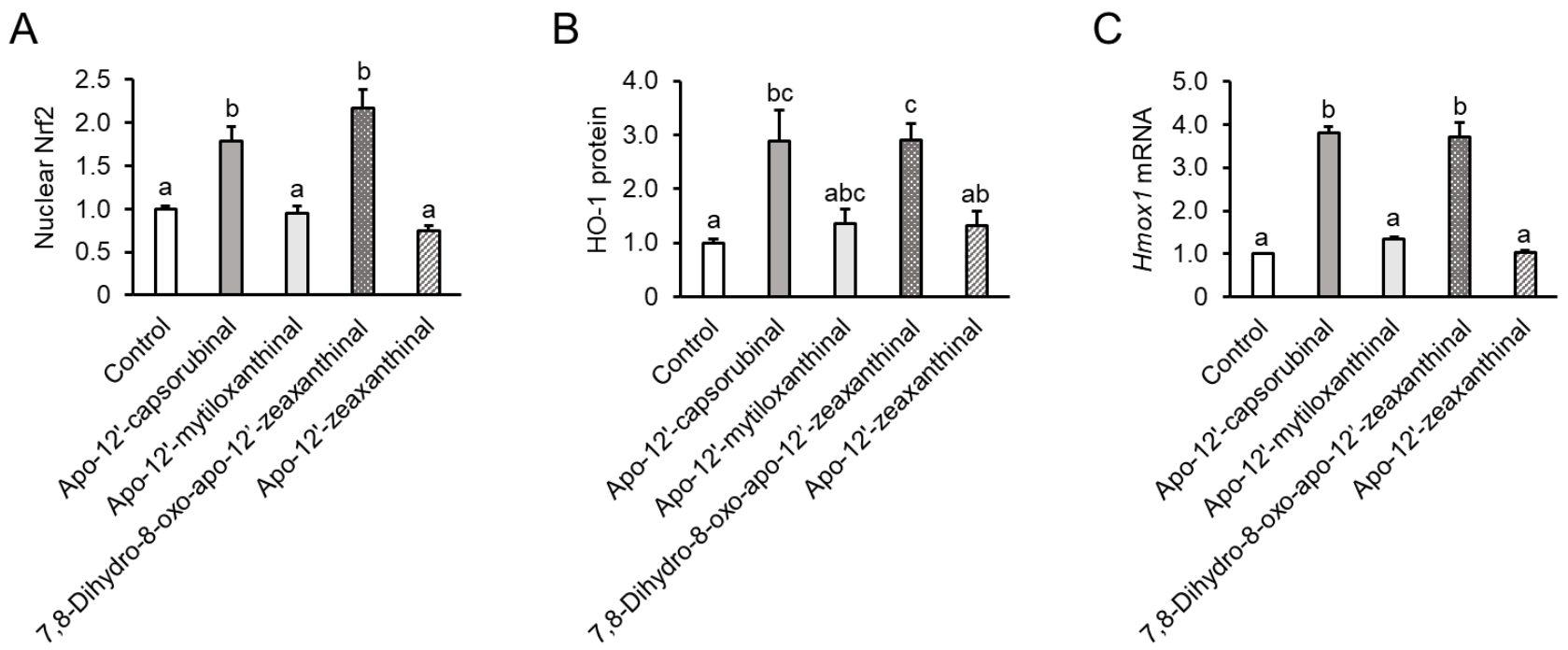

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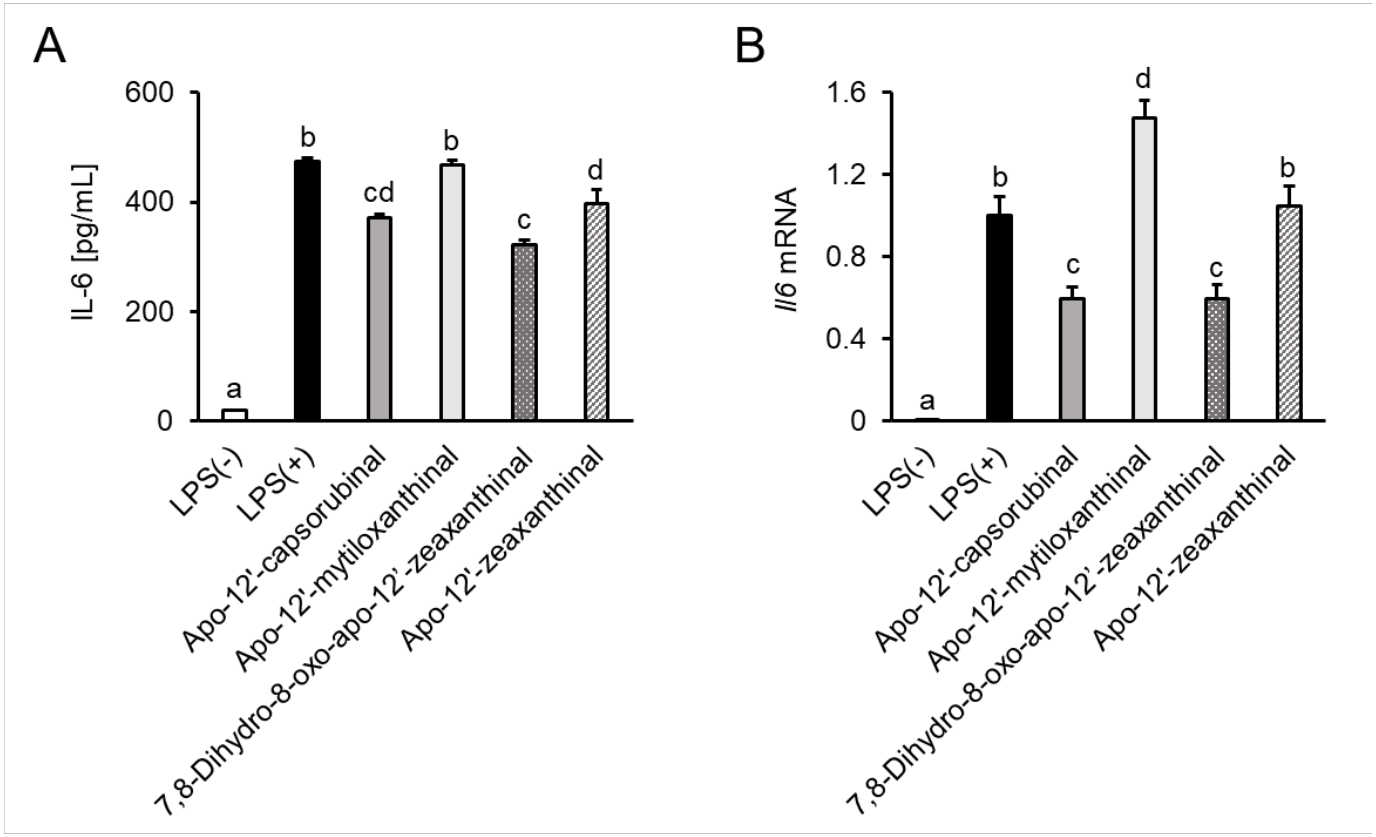

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