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Original Article

Anti-inflammatory effect of fucoxanthinol as bioavailable marine carotenoid on LPS-stimulated RAW264.7 macrophages through iNOS suppression and nitrogen radical-scavenging

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ABSTRACT

In this study, the anti-inflammatory effect of marine carotenoid fucoxanthinol (FXOH), a deacetylated metabolite of fucoxanthin (FX) was assessed with nitric oxide (NO) reducing capacity on NO production in LPS stimulated RAW 264.7 macrophages. The compounds reduced the induction of nitrite level as an index of NO in the inflammatory cell at low concentration ranges of 6.25-25 μM, and then the inflammatory gene expression, such as iNOS and COX-2, was suppressed in a dose-dependent manner. In addition, the nitrogen-radical-scavenging capacity of the compounds was evaluated in a NO generating system using a Griess assay and the ESR method. These compounds had a potential nitrogen-radical-scavenging capacity; thus the compounds caused the reduction of the nitrite level in cells through suppression of NO production and nitrogen-radical-scavenging activity. Collectively in the results of the assay, the activity of FXOH was higher than that of the FX suggesting that FXOH will have a high bioavailability in the intestinal tract.

Keywords: Fucoxanthinol, Anti-inflammatory effect, Nitric oxide, ESR

INTRODUCTION

A major marine carotenoid, fucoxanthin (FX), was found in edible seaweeds such as *Undaria pinnatifida*, *Hijikia fusiformis* and *Sargassum fulvellum*, and their physiological functions were elucidated, such as anti-carcinogenic effects [1,2], anti-inflammatory effects [3] and radical scavenging activity [4]. A previous study showed that orally administered fucoxanthin (FX) is incorporated into the blood circulation as fucoxanthinol (FXOH), a deacetylated metabolite in the intestinal tract by lipase and esterase from the pancreas or in intestinal cells [5]. Reactive oxygen species, such as the super oxide anion radical (O_2^-), hydroxyl radical (·OH) and peroxyl radicals (ROO·), are widely recognized as being involved in the pathogenesis of various diseases and aging processes. Sachindra *et al*., assessed the antioxidant activities of FX and its two metabolites, FXOH and halocynthiaxanthin which showed antioxidant activities against O_2^- and ·OH [4]. The function of NO has been elucidated in a variety of pharmacological conditions including inflammation, carcinogenesis, atherosclerosis [6,7], but excess NO production or the peroxynitrite radical ($ONOO^-$) produced from reaction with O_2^- caused oxidative damage to the membrane lipid peroxidation, DNA fragmentation and lipoprotein oxidation [8,9]. Preventing of the overproduction of NO may be a way to treat chronic inflammatory diseases [10,11]. In the previous study the anti-inflammatory effect of FX was elucidated in LPS-stimulated inflammatory cells through the suppression of pro-inflammatory mediators, such as NO, PGE2, IL-1β, TNF-α, and IL-6 [12]. The actual anti-inflammatory effect of FX is considered to be due to its metabolite FXOH from the digestive tract into the blood circulation system, but the details are not clarified. Therefore, this study placed an aim to elucidate bioavailability of both compounds through the anti-inflammatory effect on NO production in the inflammatory cell.

MATERIALS AND METHODS

Materials: L-arginine and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), interferon-γ (IFN-γ) and lipopolysaccharide (LPS) were obtained from

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Wako Pure Chemical Co. (Osaka, Japan). 2-(4-Carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) and 4-ethyl-2-hydroxyamino-5-nitro-3-hexenamide (NOR3) were purchased from Dojindo (Kumamoto, Japan). Dulbecco's modified Eagle`s medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (NY, USA).

Cell culture: RAW264.7 cells (mouse macrophages, American Type Culture Collection) were cultured in DMEM medium (including 10 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37 ℃ in a 5 % CO_2 atmosphere.

Cell viability: The MTT assay was used to examine the cell viability due to treatment with a test sample as previously reported [13]. Briefly, after the culture, MTT (0.05 %) was added to each well and incubated for 3 hrs, and then the suspension was carefully removed. The formazan reduced from MTT as an index of survival cells was extracted with DMSO (100 μl) and measured at 570 nm with the reference at 630 nm using a microplate reader (BIO-RAD Model 550, BIO-RAD, USA).

Nitrite production on RAW264.7 macrophages: The cells (5 X 10^5 cells /ml) with or without various concentrations of the compounds (6.25, 12.5 and 25 μM) in the presence of LPS (100 ng/ml), L-arginine (2 mM), and IFN- γ (100 U/ml) were cultured on a 96-well microplate. Cells with or without LPS, IFN-γ and L-arginine were used as the positive control and the control, respectively. After culturing for 16 hrs, the nitrite concentrations in the medium were determined by previously reported procedures [13].

Reverse transcriptase-polymerase chain reaction (RT-PCR): Each compound (6.25, 12.5 and 25 μM) was treated with LPS-stimulated RAW264.7 cells (2.5 X 10^6 cells/ml). The cells were lysed and total RNA from the lysed cells was then isolated according to the manufacturer's instructions using the RNeasy Mini Kit (Invitrogen). The concentration and quality of total RNA were determined using a nanovette micro liter cell (Beckman Coulter DU800, Beckman). cDNA was synthesized from the total RNA using the Super Script Choice system (Invitrogen). Amplification of the cDNA was performed by incubating in LA PCR buffer 0.1 M Tris-HCl buffer (pH8.0, Takara Bio. Inc.,), containing 0.5 M KCl, 0.025 M $MgCl_2$, 250 μM dNTPs and 50 units/ml of Taq DNA polymerase with the iNOS primers: 5`-CCT TGT TCA GCT ACG CCT TC-3`and 5`-CTG AGG GCT CTG TTG AGG TC-3` or the COX2-primers: 5`-GGA GAG ACT ATC ATC AAG ATA GTG ATC and ATG GTC AGT AGA CTT TTA CAG CTC using PCR (Gene Amp PCR System 9700, Applied Biosystems). The following PCR conditions were used; initial denaturation was performed at 94 ℃ for 2 min, followed by 30 thermal cycles at 94 ℃ for 30 s, 60 ℃ for 30 s and at 72 ℃ for 1 min. The PCR product of cDNA (100 ng/μl) was loaded on a DNA chip (Agilent DNA 1000kit, Agilent Technologies) and the electrophoresis was performed with a micro DNA analyzer (Agilent 2100 Bioanalyzer, Agilent Technologies) [14].

NO scavenging activity: The NO scavenging activity of the compounds was provided with an ESR study as previously reported [13]. The reaction mixture of the compound (25 and 100 μM), NOR3 (200 μM) and NO detection reagent, carboxy-PTIO (25 μM) was prepared in PBS and incubated at room temperature for 30 min. The ESR was determined under the described ESR measurement conditions.

ESR measurement: An ESR measurement was performed by ESR spectroscopy (JES-FR30, JEOL) operating in the X-band with a modulation frequency of 100 kHz. The reaction mixture was transferred to a capillary (100 X 1.1 mm I.D., Drumnond Scientific. Co., USA) which was placed in a quartz cell (270 mm long, 5mm I.D., JEOL DATUM Ltd., Japan). The ESR spectra were measured at 9.4 GHz resonant frequency under the following conditions: microwave power, 4 mW; modulation width, 0.1 mT; gain, 320; scan time, 1min; time constant, 0.3 sec. Manganese oxide was used as the internal standard.

Nitrogen radical scavenging activity: NOR3 as an NO donor was used to evaluate the NO or nitrogen-radical-scavenging effect of the compounds [13]. The reaction mixture containing NOR3 (200 μM) with or without the test compounds (6.25, 12.5 and 25 μM) in PBS solution was incubated at room temperature for 60 min, and the nitrite accumulation in the reaction mixture was measured with an NO indicator using the Griess method as previously reported [15]. Briefly, the solution containing the test sample (80 μl), PBS (20 μl), 1 % sulfanilamide containing 5 % phosphoric acid (50 μl) and 0.1 % N-(1-naphthyl) ethylenediamine (50 μl) was incubated in a 96-well plate and the absorbance at 540 nm was measured by a microplate reader (Bio-Rad Model 550, Bio-Rad, USA).

RESULTS AND DISCUSSION

The inflammatory cell system was used to explore the substances for suppressing the overproduction

of NO which can lead to preventing chronic inflammatory diseases [10,11]. Treatment of RAW264.7 macrophages with LPS/IFN-γ has been shown to result in iNOS induction, and excess NO was produced in the cells. The previous studies demonstrated that orally administered FX was rapidly hydrolyzed to FXOH in the gastrointestinal tract and it could be absorbed within several hours after the administration. The enzymes that hydrolyze FX in the gastrointestinal tract might be lipase, cholesterol esterase, carboxylesterase, or others (Fig. 1) [5,16]. In this study, the inhibitory effect of NO production due to both compounds was evaluated in LPS-induced inflammatory RAW264.7 cells. As shown in Fig 2, the nitrite accumulation as an NO indicator in the cells increased due to the LPS treatment. The compounds in low concentration ranges of 6.25, 12.5 and 25 μM were evaluated in the cells. FX showed moderate activity while the FXOH was significantly reduced the nitrite level in a dose-dependent manner (Fig. 2a). The cytotoxicity of these compounds was not in the range of the test concentrations, indicating that the inhibition was due to the effect of the compounds (Fig. 2b). This study was first showed that the inhibitory effect of FXOH was higher than that of FX, which suggested that the metabolite FXOH will be a bioavailable compound in a biological system [17].

In addition, the comparative bioavailability of both compounds was carried out for iNOS suppression and NO scavenging ability. The inhibitory effect on iNOS induction due to FX and FXOH was examined in the LPS-stimulated RAW264.7 cells. The iNOS mRNA gene expression in cells was induced due to the LPS stimulant (Fig. 3 (a)). When the compounds were placed in the cells, the compounds suppressed the iNOS gene expression which was similar to the result in the inhibition of NO production in cells (Fig. 2). The activities of the compounds were higher than those of previously reported polyphenols, such as oroxylin A, quercetin, wogonin and flavonoid glycosides, hyperin and aglimonolide-6-*O*-glucoside [18,20].

The compounds also inhibited gene expression of COX-2 related to inflammation in a dose-dependent manner (Fig. 3 (b)). This result was similar to that previously reported in which FX reduced the levels of iNOS and COX-2 proteins concomitant with reductions in the production of NO [12]. In this study, the potential anti-inflammatory activities of FXOH due to inhibition of iNOS and COX-2 in a macrophage were first evaluated in comparison to that of FX. The suppression of genes due to metabolite FXOH was higher than that of FX, suggesting that FXOH will be a bioavailable compound in the digestive tract into the blood circulation system. It has been shown that LPS exerts its inflammatory effects through the activation of both the MAPK signaling pathway and the classical NF-κB pathway. NF-κB is a mammalian transcription factor for the expression of various pro-inflammatory responses of cytokines along with iNOS, COX-2, TNF-α, IL-1β, and IL-6 [20]. The expression of iNOS and COX-2 genes in murine macrophages has been shown to be dependent on NF-κB activation and both promoters contain the transcription factors for NF-κB [21,22]. A previous article reported that FX inhibited NF-κB activation and the suppression of MAPKs (JNK, ERK and p38) phosphorylation, resulting in reduced levels of pro-inflammatory mediators including NO, PGE2, IL-1β, TNF-α, and IL-6 [12]. FXOH is a potential inhibitor of iNOS and COX-2 induction which will be expected to have a similar inhibitory mechanism through the inhibition of NF-κB activation and MAPKs phosphorylation.

The potential nitrogen-radical-scavenging activity of the compound would contribute to the reduction of the nitrite level in LPS-stimulated cells. These marine carotenoids, such as FX and FXOH are known as antioxidants of the DPPH radical [4]; however the nitrogen-radical-scavenging activity has not yet been clarified. In this study, the NO- or nitrogen-radical-scavenging activity of the carotenoids was examined using NOR3 as an NO donor. The nitrite accumulation from NOR3 in the presence of the compounds was reduced in a dose-dependent manner (1-4 μM) (Fig 4). The reduction of the nitrite level when treated with the compounds was high in comparison with flavonoids, such as aromadendrin, quercitrin and loliolide [13]. To clarify the NO scavenging action of the compounds, an ESR study was performed on the system containing carboxy-PTIO as an NO detection reagent in the presence of NOR3 and the compounds as in a previous report [13]. The carboxy-PTIO radical produced from reaction with carboxy-PTIO and NO did not change in the presence of the compounds (Fig. 5). These results suggested that the compounds would have the potential to inhibit nitrogen radical species except for NO, such as NO_2 or the intermediate radicals, N_2O_3 and N_2O_4, during NO oxidation.

CONCLUSION

This study was clearly elucidated that the marine metabolite carotenoid, FXOH indicated an anti-inflammatory effect involving inhibitory gene expressions, iNOS and COX-2 in LPS-stimulated inflammatory cells. The compounds also showed nitrogen-radical-scavenging activity, suggested that their contribution decreasing the excess NO

production in LPS-stimulated cells. Collectively, in the results of the assay, the higher activities of FXOH than those of FX suggested that the metabolite FXOH will have high bioavailability in the intestinal tract.

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Fig. 1: The chemical structures of marine carotenoids fucoxanthin and its metabolite fucoxanthinol
Fucoxanthinol (FXOH) is produced by deacetylation of fucoxanthin (FX) in the intestinal tract with lipase and esterase.

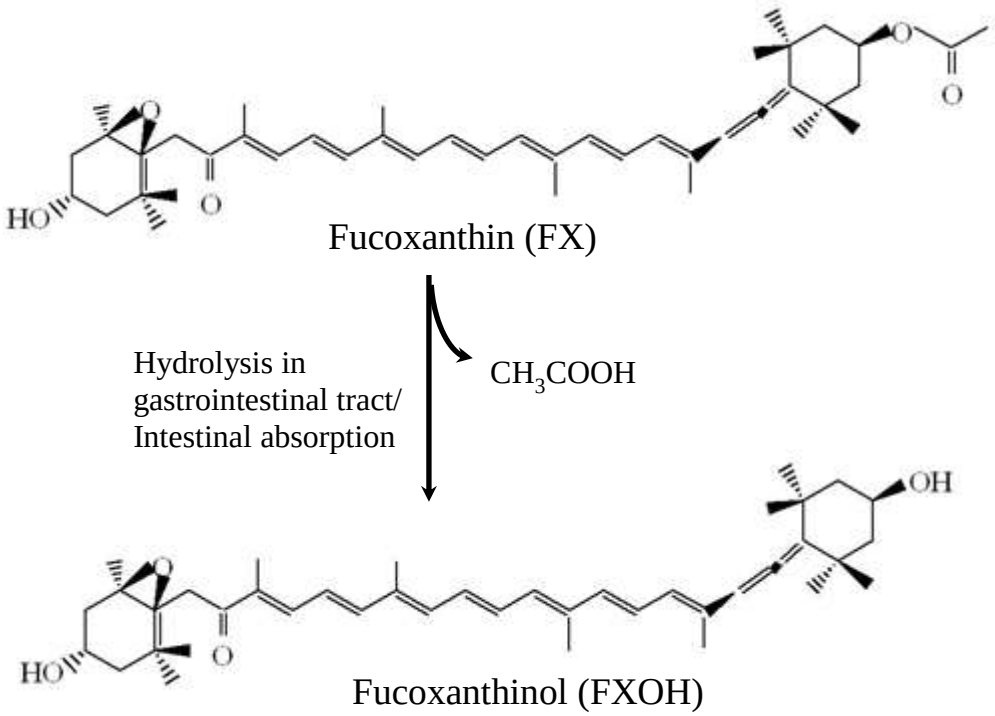
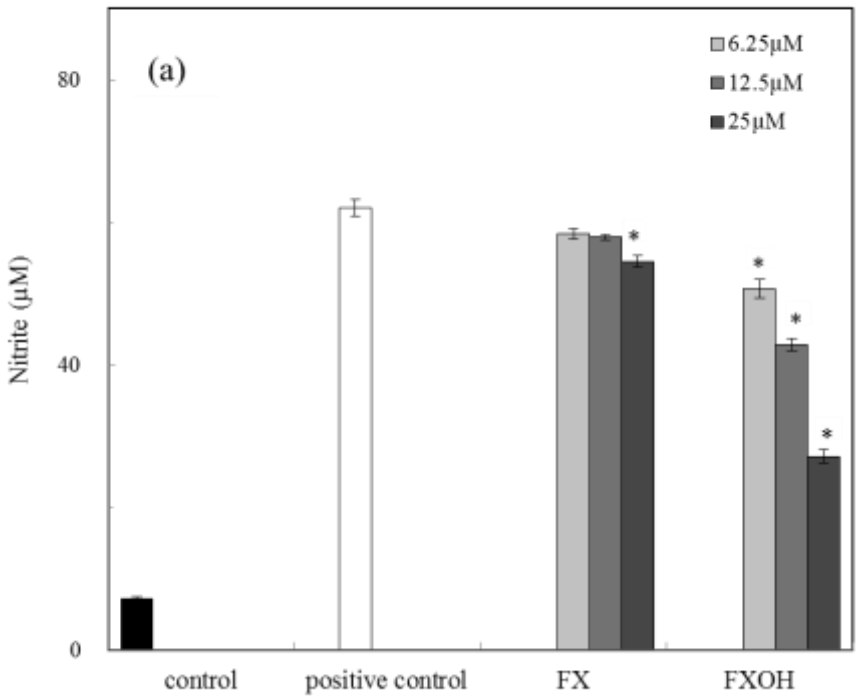


Fig. 2: Inhibition by marine carotenoids of NO production in LPS-stimulated RAW264.7 macrophages
(a) The various concentrations (6.25, 12.5 and 25 μM) of the marine carotenoids, FX and its metabolite FXOH, were evaluated for NO production in LPS-stimulated RAW264.7 macrophages. (b) The cytotoxicity of the test compounds was determined by MTT assay and indicated as survival rate (%) of LPS-treated cells as the positive control. Data were expressed as mean ± SD and the significant difference was analyzed by the student`s *t*-test. **P* < 0.01 indicated as a significant difference from the positive control.



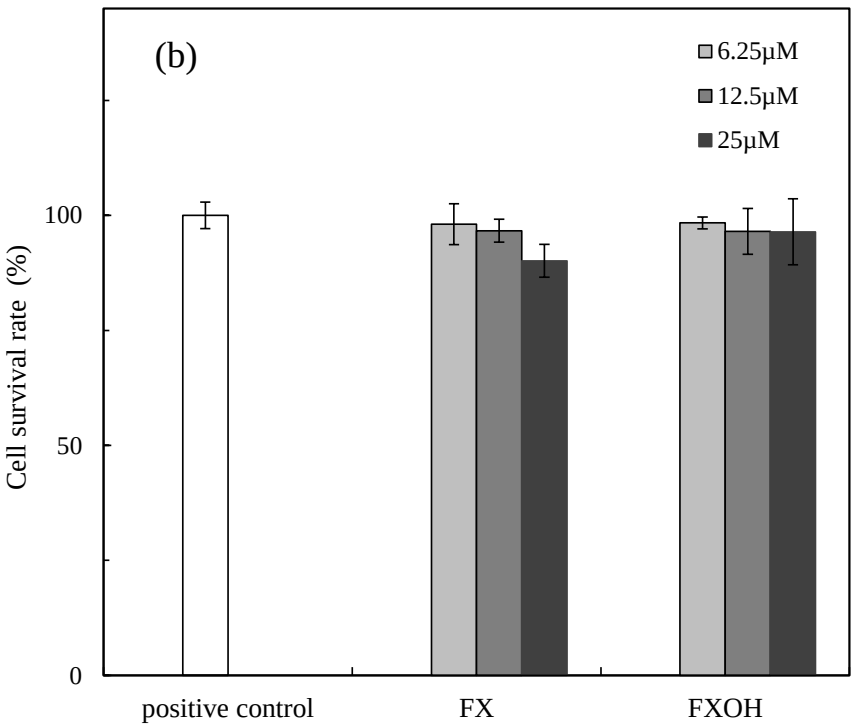


Fig. 3: Inhibitory effect of the marine carotenoids on the gene expression in LPS-stimulated in RAW264.7 macrophages

The inhibitory effect of the iNOS and COX-2 induction due to the marine carotenoids (FX and FXOH) was examined in LPS- stimulated RAW264.7 macrophages. Cells were treated with (positive control) or without (control) LPS/IFNγ. (a) Effect of the marine carotenoids (6.25, 12.5 and 25 μM) on the iNOS *m*RNA expression and (b) suppression of the COX-2 *m*RNA expression. A representative example of two experiments is shown.

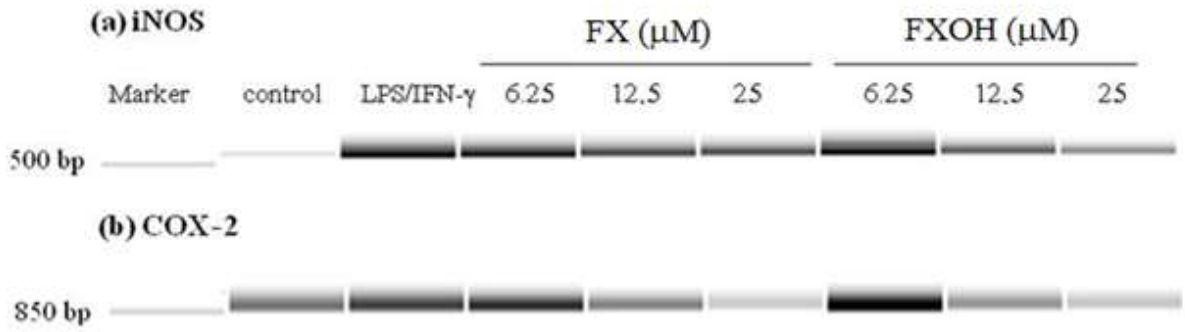


Fig. 4: Nitrogen-radical-scavenging activity of marine carotenoids

The reaction mixture of the test compound (6.25, 12.5 and 25 μM) and NO donor, NOR3 (200 μM) was prepared in PBS and incubated at room temperature for 30 min. (a) control with the test compound and NOR3, (b) NOR3, and with (c) FX, (d) FXOH. Data were expressed as mean ± SD and the significant difference was analyzed by the student`s *t*-test. **P* < 0.01 indicated as a significant difference from the positive control.

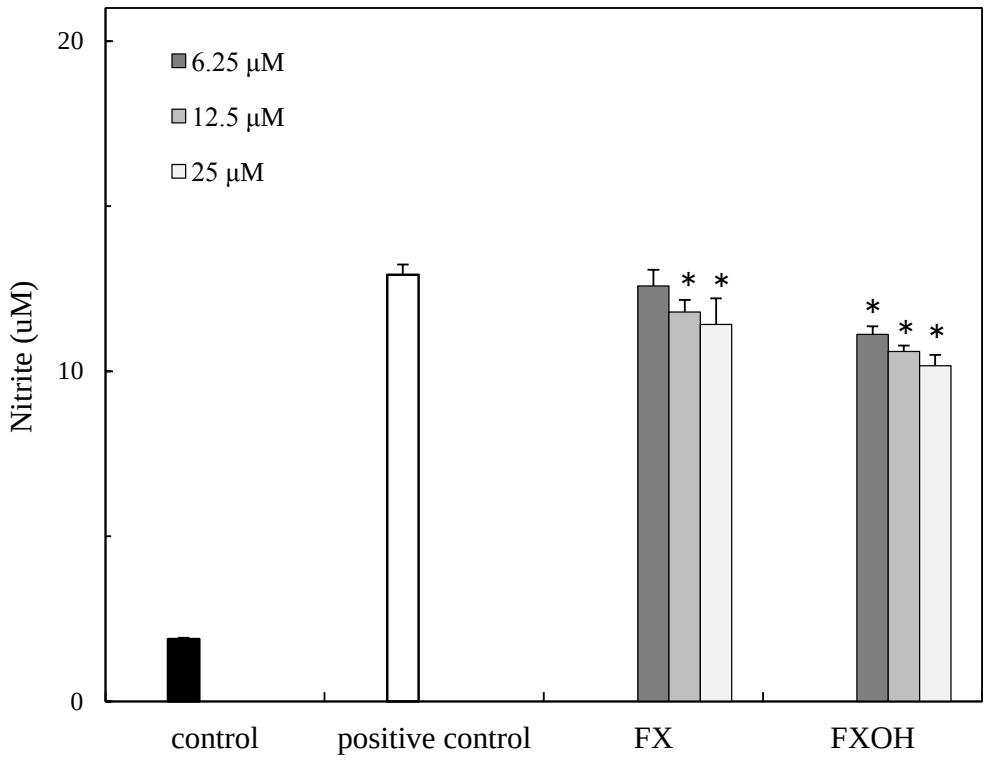
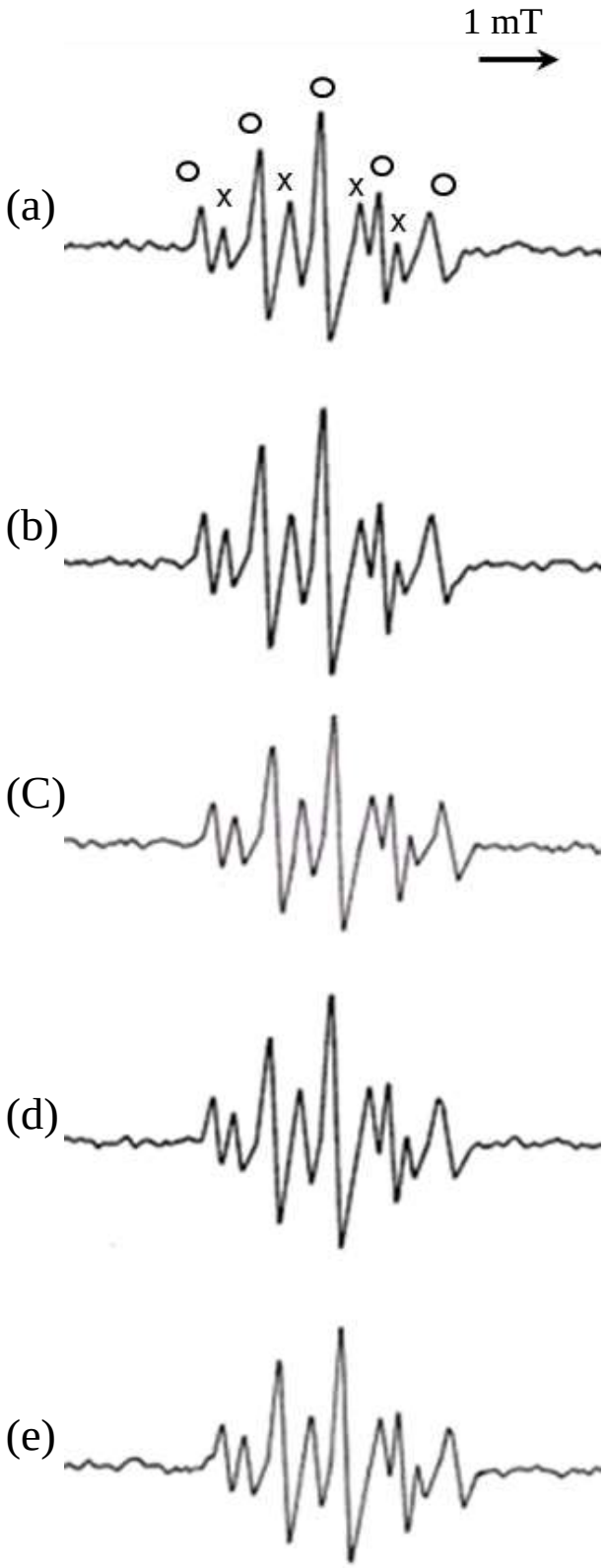


Fig. 5: NO-scavenging activity due to marine carotenoids: The reaction mixture of the test compound (25 and 50 μM), NOR3 (200 μM) and NO detection reagent, carboxy-PTIO (25 μM) was prepared in PBS and incubated at room temperature for 30 min. (a) carboxy-PTIO and NOR3, carboxy-PTIO and NOR3 with FX (b) 25 μM and (c) 50 μM, and FXOH (d) 25 μM and (e) 50 μM. The carboxy-PTIO signal is indicated by the open circle (○) and its detection with the NO-produced carboxy-PTI radical is indicated by the (×) marks.



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