# Evaluation of the anti-inflammatory activity of periphytic carotenoids in croton oil-induced zebrafish

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## ABSTRACT

**Introduction and Aim:** Periphyton is the aggregation of micro-floral communities on the surface of submerged objects in the aquatic system. It is an excellent source of nutrients, including functional food carotenoids. The present study was aimed to evaluate the anti-inflammatory activity of carotenoids obtained from sustainable aquatic resource periphyton.

**Materials and Methods:** Periphytons were grown on PVC pipes and subjected to carotenoid extraction. β-carotene and neoxanthin were identified through UPLC analysis. The carotenoid extract was tested for its anti-inflammatory activity in croton oil-induced inflamed zebrafish and was later treated with the carotenoid extract. Inflammatory markers like total protein (TP), myeloperoxidase (MPO), and nitric oxide (NO) in both control and test group animals were measured.

**Results:** Inflammatory markers like TP, MPO, and NO in both control and test group animals were varied significantly ($P<0.01$) between the experimental animal group at subsequent time intervals and treatment with the carotenoid extract.

**Conclusion:** The results are indicative of the anti-inflammatory nature of periphytic carotenoids and their nutritional significance in managing inflammatory conditions in humans. Further studies are required to decipher the specific mechanism of periphytic carotenoid action to understand its therapeutic potential in a broader perspective.

**Keywords:** Periphyton; β-carotene; neoxanthin; zebrafish; anti-inflammatory activity.

## INTRODUCTION

Inflammation is a process of defense mechanism triggered against the infectious agents or during tissue injury with specific morphological and chemical changes in cells and tissues (1, 2). This condition is treatable by nonsteroidal anti-inflammatory drugs (NSAIDs). However, the risk of gastrointestinal and cardiovascular complications associated with the use of NSAIDs to treat the inflammatory disease has paved the way for alternative medicines derived from plant, microbe, and other natural sources. Carotenoids are naturally occurring pigment molecules with immense health benefits such as antioxidants, immune modulation, and are broadly classified as carotene and xanthophylls (3).

Plants, algae, and few microbes can only synthesize carotenoids but not animals. In recent times, microalgae captured considerable interest due to their ability to possess novel carotenoids. It was observed in previous studies that periphytons are an excellent source of microalgae or phytoplankton (4). Periphyton is an aggregation of micro-floral communities on the surface of submerged objects in an aquatic system (5). It consists of many functional molecules like polysaccharides, polyunsaturated fatty acids (PUFA), vitamins and carotenoids, and responsible for the primary productivity in freshwater aquatic systems. The earlier investigations have suggested dietary supplementation of periphyton in a few fish species had a positive effect on improving the growth and immune system (6).

In recent times, functional foods from a natural source are demanding over synthetic drugs due to their potential in combating toxins and other medications (7) and the various health-promoting effects in humans and veterinary animals. The periphyton is ideal for nutraceutical applications due to its sustainable nature and rich nutritional content. The periphyton is limited only to aquaculture practices so far. No studies have previously been attempted to explore the bioactivity of carotenoids from periphyton. Based on these facts, the periphytons were cultured and extracted for carotenoid molecules and tested for their anti-inflammatory property using zebrafish as a model organism.

## MATERIALS AND METHODS

All the chemicals and reagents used in the experiment were procured from Sigma chemical company, USA.

**Periphyton culture and carotenoid extraction**

Periphyton was cultured on 20 PVC pipes in a soil-based, water-filled outdoor cement tank (2×1×1 m) with the fortnightly application of fertilizers like urea, single superphosphate (SSP) and cow dung (8) (Fig. 1). After a month, the periphyton formed on pipes was manually scraped, collected, and subjected to carotenoid extraction using acetone: n-hexane (4:1) biphasic solvent system with slight modification (9). The N-hexane phase containing the carotenoid was treated with 10% KOH to remove chlorophyll and further analyzed it through ultra-performance liquid chromatography (UPLC).

**UPLC experimental condition**

The UPLC analysis was performed according to the previously described method (10). Carotenoid extract of 5µl was injected into the UPLC-H class (Waters) instrument with BEH C-18column (1.76 µm, 50mm×2.1mm) and BEH-C18 (1.76 µm, 2.1×5mm) guard column as a stationary phase. A photodiode array detector (PDA) detector between the wavelengths 260 to 750nm made detection of the carotenoid molecule. Isocratic ratio of (95:5) solvent system A (acetonitrile: methyl alcohol: ethyl acetate; 53:7:40) and solvent system B (methyl alcohol) was used as a mobile phase with a flow rate of 0.2ml/min with a total run time of 8min. β-carotene was used as an internal standard and identified. The identification of neoxanthin was made based on comparing the absorption spectra obtained during the experiment with the published data (11).

**In vivo anti-inflammatory study using Zebrafish (*Danio rario*)**

All the fishes used in this experiment were handled according to the OECD guidelines (12). Zebrafish (*Danio rario*) having a uniform size (2.5±0.2 cm) obtained from a single parent were subjected to acclimatization under laboratory conditions by exposing them to 24hrs light/dark cycle for 10 days in aquaria having water temperature of 25ºC.

Acute toxicity (LC50) of inflammatory agents and test drugs were analyzed according to the OECD method. The fishes (10 numbers) were exposed to the varying concentration of croton oil (1%, 2%, and 3%) and carotenoid extracts (0.5, 1 and 2 mg/ml) for 96 hours. Mortalities were recorded at 24, 48, 72, and 96 hours, and the concentrations, which kill 50 percent of the fish (LC50), were determined.

The fishes were divided into six experimental groups comprising of ten numbers each (n=10). The croton oil (2%) and carotenoid extract (2mg/ml) were prepared in dimethyl sulphoxide (DMSO) and administered to zebrafish through an oral route using micropipette without any regurgitation (13). G1and G2 groups were treated with 5µl of vehicle DMSO and the level of myeloperoxidase (MPO), nitric oxide (NO) in terms of nitrite, and total protein (TP) was measured subsequently at 2nd and 4th hour respectively. G3 and G4 groups were given 5µl of 2% croton oil, and the level of MPO, NO, and TP were measured at 2nd and 4th hour from the initial administration of the inflammatory agent.

G5 and G6 groups were treated with 5µl of 2% croton oil, and after half an hour, 5µl of carotenoid extract (2mg/ml) was administered and the level of MPO, NO and TP were measured at 2nd and 4th hour from the initial administration of the inflammatory agent. The procedures are mentioned in detail below.

**Tissue homogenate preparation**

During the experiment, ten numbers of fish from respective treatments were euthanatized by decapitation and macerated separately with phosphate buffer saline (PBS), centrifuged at 15,000 g under 4ºC for 10 mins. The supernatant obtained was collected and used for further assays.

**Myeloperoxidase, nitric oxide, and total protein estimation**

The myeloperoxidase enzyme assay was performed according to the previously described method with slight modification (14). Instead of serum tissue, homogenate of 10µl was taken in a 96 well microtitre plate, and to this 90µl of PBS and 35µl of 3,3,5,5 tetramethylbenzidine (prepared in phosphate citrate buffer, pH 5.5) were added and mixed well. Finally, the reaction was stopped by the addition of 35µl of 4M $H_2SO_4$, and absorbance was measured at 450nm using ELISA microplate reader (BIO-RAD).

The nitric oxide assay was performed according to the previously described method (15). A tissue homogenate of 100 µl was mixed with 400 µl of Carbonate buffer (500mM sodium bicarbonate, 50mM sodium carbonate. pH 9) and incubated for 1hr under room temperature. Later, to this 100 µl of NaOH (0.35M), 400 µl of $ZnSO_4$ (120mM) was added and allowed to stand for 10 min and centrifuged at 4000 g for 10 min. The supernatant of 150 µl was mixed with 75 µl of sulphanilamide (1%) and 75 µl of N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) (0.1%) in a microtiter plate. After incubation for 10 mins absorbance was read at 540 nm using microtiter plate ELISA reader (Bio-Rad). Nitric oxide in terms of nitrite was calculated based on the standard curve plotted using sodium nitrite.

Total protein concentration was measured using bovine serum albumin (BSA) as standard (16). A Tissue homogenate of 0.5ml was mixed with 5ml of alkaline reagent and incubated for 5 mins. Later, 0.5ml of Folin–Ciacalteu (FC) reagent (1N) was added and incubated again at room temperature for 30 mins. Finally, absorbance was measured at 660nm

using a UV-visible spectrophotometer (Thermo Electron Corporation).

**Statistical analysis**

Comparison among treatments for various parameters was performed by one-way analysis of variance (ANOVA) using VassarStats statistical computation tool followed by Duncan's multiple range test with a significance level at ($P<0.01$).

**RESULTS**

The UPLC analysis was performed to identify the carotenoid molecules present in the periphytic extract. The identification of individual carotenoid molecules was made by comparing the retention time (Rt) of the sample with the authentic standards (Sigma). The carotenoid extract containing β-carotene and neoxanthin were eluted at 2.1min and 0.75min, respectively, and were matched with the standards (Fig.1). The absorption spectrum gives the first level of information about the carotenoid molecule. The conjugated double bond system, which constitutes the light-absorbing chromophore, is responsible for this property. The β-carotene and neoxanthin absorb strongly between 400-500 nm. Hence, in this experiment, the absorption spectrum of β-carotene and neoxanthin in hexane was obtained at 450nm and 436nm, respectively (Fig. 2 and 3).

The animals in group G1 and G2, which received only the vehicle DMSO, showed no significant difference for the activity of inflammatory markers like TP, MPO, and NO at the 2nd and 4th hour, respectively. The increased activity of inflammatory markers after administration of croton oil to animals in the group G3 and G4 at 2nd and 4th hour was suggestive of possible inflammatory reactions. Thus, the animals in the group G3 and G4 showed a significant difference ($P<0.01$) for the activity of inflammatory markers like TP, MPO, and NO. The administration of carotenoid extract over croton oil-induced animals (Group G5 and G6) showed the decreased activity for inflammatory markers and is probably due to the reversing of inflammatory reactions by carotenoid molecules and thus the animals in the group G5 and G6 showed significant difference ($P<0.01$) for inflammatory markers like TP, MPO, and NO at 2nd and 4th hour respectively (table 1 and graph 1).

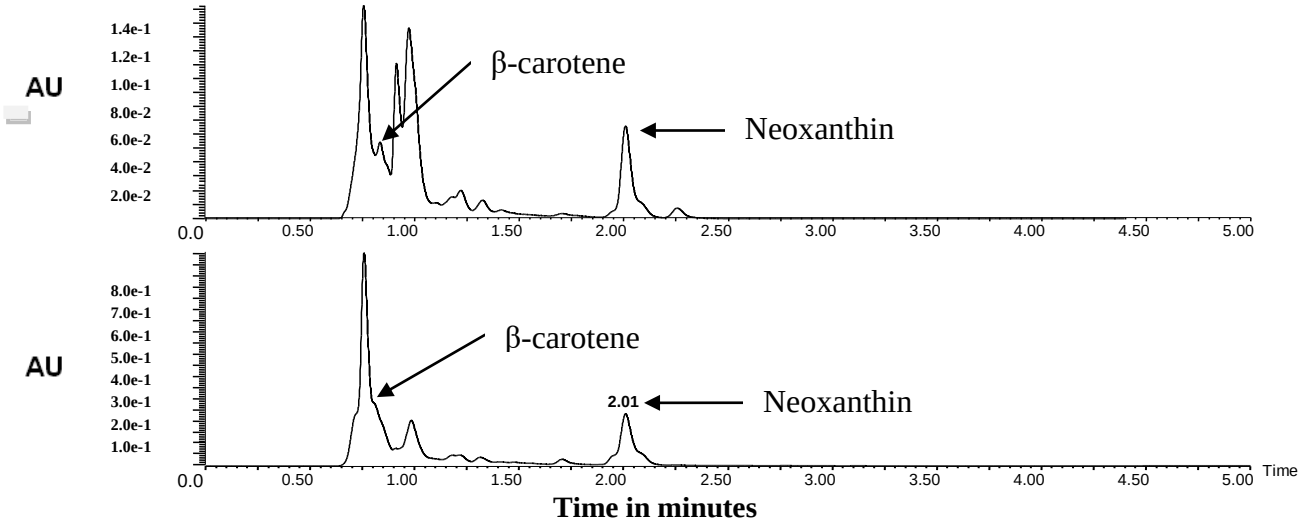


**Fig. 1:** UPLC Chromatogram of periphytic carotenoids (β-carotene and Neoxanthin)

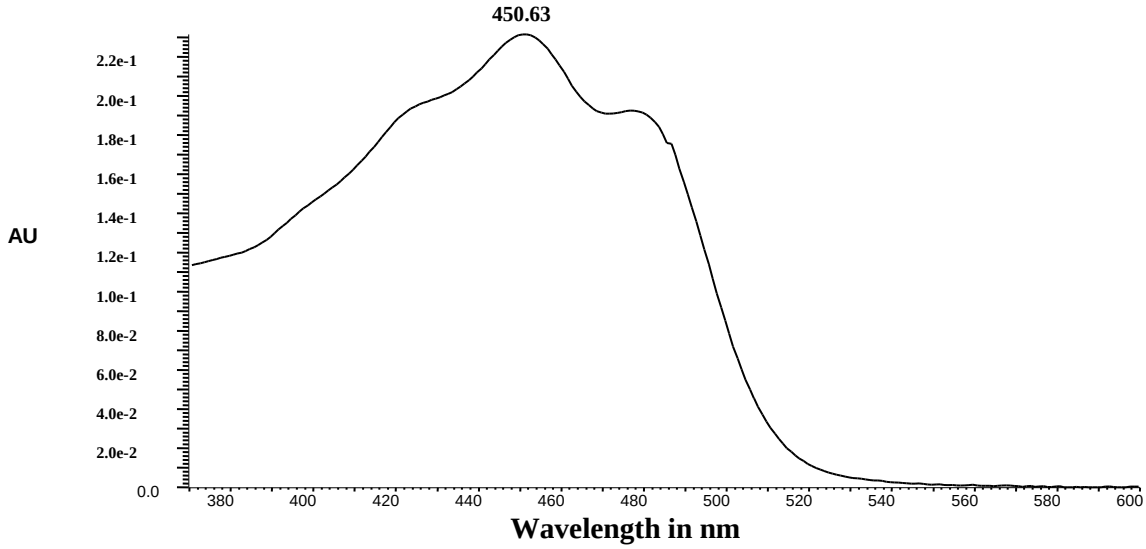


**Fig. 2:** Absorption spectrum of β-carotene at 450nm

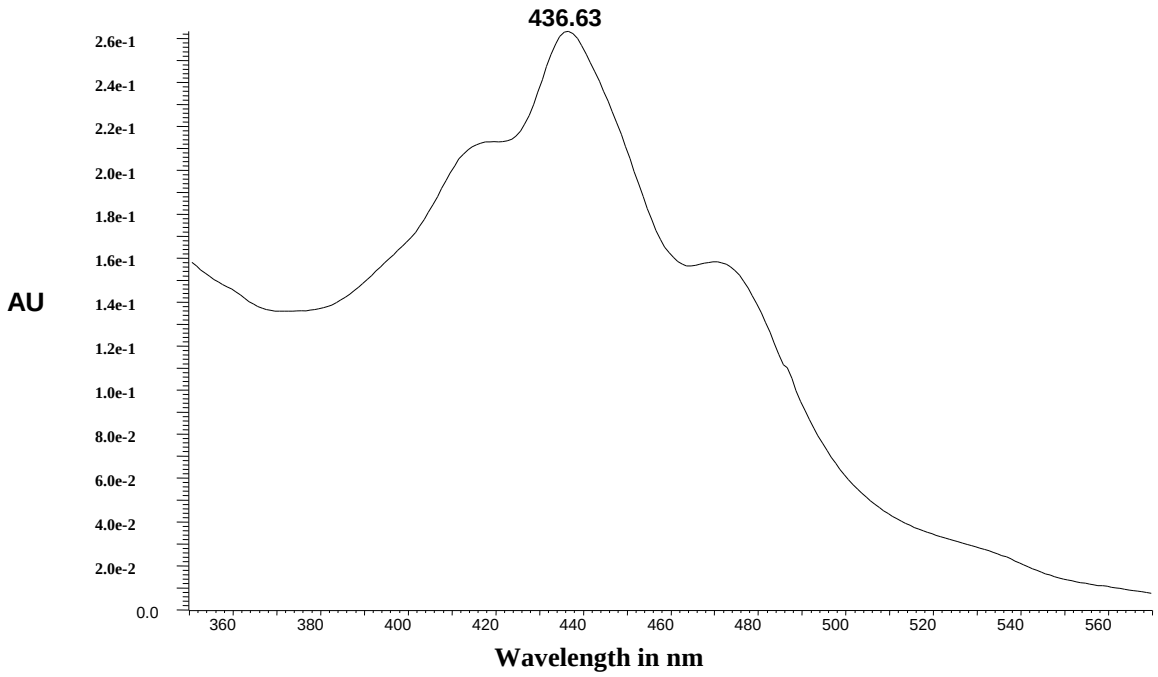


**Fig. 3:** Absorption spectrum of neoxanthin at 436nm.

**Table 1:** The activity of myeloperoxidase, nitric oxide (nitrite), and total protein concentration in experimental groups

| Parameters | G1 | G2 | G3 | G4 | G5 | G6 |
|---|---|---|---|---|---|---|
| **Myeloperoxidase (OD 450)** | 0.465± 0.04[a] | 0.476± 0.02[a] | 0.979± 0.05[b] | 1.102± 0.1[c] | 0.508± 0.0[d] | 0.470± 0.04[e] |
| **Nitric oxide (nitrite, µm/ml)** | 1.600± 0.37[a] | 1.663± 0.3[a] | 6.008± 1.07[b] | 8.879± 1.6[c] | 1.700± 0.4[a] | 1.554± 0.35[a] |
| **Total protein (µg/ml)** | 9.125± 0.67[a] | 9.13± 0.79[a] | 16.907± 3.0[b] | 18.6± 1.68[c] | 9.467± 0.5[d] | 8.955± 1.23[ad] |

**Note**: Results are expressed as Mean ± SD (n=10). Groups (G), G1-Control (2nd hr) G2- Control (4thhr), G3-5µl2% croton oil (2ndhr), G4-5µl2% croton oil (4thhr), G5- 5µl2% crotonoil+5µlCarotenoid extract (2mg/ml, 2nd hr),and G6-5µl2% croton oil+5µlCarotenoid extract (2mg/ml, 4th hr).

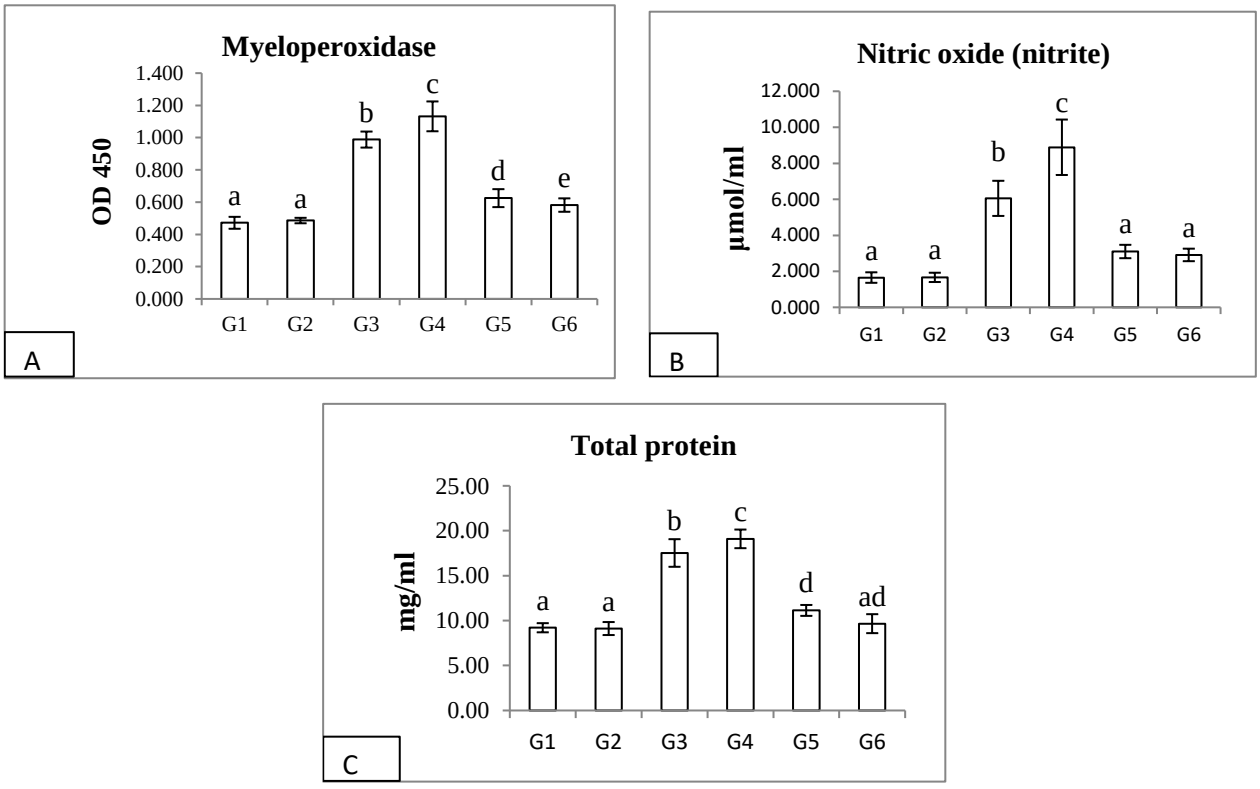


**Fig 4:** The activity of [A] Myeloperoxidase (OD), [B] Nitric oxide (Nitrite), and [C] Total protein concentration between the experimental groups.

## DISCUSSION

### Myeloperoxidase activity

The activity of MPO, NO, and TP between the experimental group is given in table 1. The activity of MPO was increased (G3 and G4) after oral administration of 2% croton oil at a subsequent time interval (Fig. 4A). It led to the inflammation in mucosal epithelial cells of the stomach and intestine. Hence the neutrophil infiltration at the site of inflammation and thereby produced the MPO from its azurophilic granules (17, 18). Interestingly, a low level of activity of MPO was observed after carotenoid treatment in animals of group G5 and G6 (Fig. 4A). In an experiment, peritonitis induced mice showed a lower level of myeloperoxidase activity after treatment with lycopene-rich guava extract compared to non-treated animals. This effect was due to the downregulation of inflammatory mediators and inhibition of gene expression involved in inflammation (19). The topical application of fucoxanthin cream on 12-O-tetradecanoylphorbol-13-acetate (TPA) induced mice lowered the activity of myeloperoxidase enzyme and thus suggested as a natural adjuvant in treating skin inflammation (20). The astaxanthin was found useful in reducing myeloperoxidase enzyme during gout-mediated inflammation (21).

### Nitric oxide activity

The NO activity was increased after administration of 2% croton oil (G3 and G4), and subsequent treatment with carotenoid extract (G5 and G6) reduced the activity of NO at both time intervals (Fig. 4B). Lipopolysaccharide (LPS) stimulated macrophage cells with high nitric oxide was decreased after treatment with mesozeaxanthin and suggested that the effect was due to the downregulation of inflammatory mediator genes by mesozeaxanthin (22). Inhibition of nitric oxide synthase 2 (nos2) was observed in LPS stimulated RAW264.7 cells after treatment with β-carotene and lycopene and concluded that the effect of anti-inflammation of these pigments might be due to their ability to scavenge reactive oxygen (RO) (23). Inhibition of NO production and expression of inducible NO synthase (iNOS) was observed in LPS stimulated RAW264.7 cells over treatment with β-carotene in a dose-dependent manner this anti-inflammatory activity was due to the inhibition of redox-based NF-κB activation (24). In an LPS stimulated HaCaT cells, the expression of iNOS was inhibited by lycopene extracted from a watermelon in a dose-dependent manner, thereby preventing the inflammation (25).

### Total protein concentration

The total protein concentration was increased after administration of 2% croton oil (G3 and G4) and later decreased following treatment with the carotenoid extract (G5 and G6) (Fig. 4C). Endotoxin-induced uveitis (EIU) is a uveal inflammation in small animals and is produced by the administration of bacterial endotoxin. Lycopene treated EIU rats have shown lower total protein concentration in their aqueous humor (26). Similarly, astaxanthin treated EIU rats have also exhibited lower total protein concentration in a dose-dependent manner (27).

## CONCLUSION

Based on these results, it is evident that periphytic carotenoids have immense power to counteract inflammation and may be used to treat inflammatory disorders in humans. Further studies are needed to decipher its specific mechanism of action.

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**CONFLICT OF INTEREST**: None.

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