# Carotenoids identified from slipper-shaped oyster *Magallana bilineata* powder residue exhibit potent biological activities

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## Abstract

**Carotenoids are a diverse class of biologically active compounds that contribute significantly to human health, serving vital functions in nutrition and overall well-being. *Magallana bilineata*, a commercially important oyster species, yields a shelf-stable powder residue possessing bioactivities with unknown specific compounds. Carotenoids are key marine bioactive compounds, but their presence in oysters remains underexplored. The present study aimed to identify the bioactive compounds from oyster powder residue through mass spectrometry for optimum utilization and value creation as a biomedical resource. The study employed solvent extraction of oyster powder residue, followed by fractionation using octadecylsilyl (ODS) column chromatography, liquid chromatography-mass spectrometry (LC-MS)-guided profiling, and ultra-high-performance liquid chromatography-ultra-high-performance liquid chromatography-elevated energy mass spectrometry-elevated energy mass spectrometry (UHPLC-MS$^E$) analysis to identify carotenoids, with bioactivity assays conducted to assess the cytotoxic, antimicrobial, antioxidant, and anti-inflammatory properties. The study obtained two HPLC fractions and enabled the identification of carotenoid compounds based on retention times and UHPLC-MS$^E$, with elemental compositions inferred from the observed mass-to-charge ratios. The bioactivities of the two HPLC fractions, identified as zeaxanthin in fraction 1 and a zeaxanthin/lutein isomeric mixture in fraction 2, were assessed. The zeaxanthin/lutein isomeric mixture exhibited higher effectiveness in MCF-7 cancer cell inhibition ($IC_{50}$ = 93.29 ± 0.07 μg/mL) than cisplatin, but both HPLC fractions showed strong antibacterial activity against *Klebsiella pneumoniae* and *Escherichia coli*. Moreover, notable antioxidant activity was observed in both fractions for 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity assays, while zeaxanthin demonstrated anti-inflammatory activity (43.68 ± 0.11%) comparable to aspirin (43.49 ± 0.17%). These findings suggest that the observed biological activities of the HPLC fractions may be the consequences of the adaptive response and filter-feeding behaviors of oysters, which result in the accumulation of bioactive carotenoids. This study offers a promising perspective on applying mass spectrometry techniques for advanced compound extraction and identification, and on utilizing oyster powder residue as a sustainable approach to waste valorization and as a functional ingredient for biomedical applications.**

*Keywords:* Anti-inflammatory, Antioxidant, Carotenoids, *Magallana bilineata*, Mass spectrometry

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## 1. Introduction

Carotenoids are a diverse group of bioactive compounds with important health benefits, playing crucial roles in human nutrition and well-being [1]. Their structure is defined by a 40-carbon backbone comprising eight isoprene units arranged in either cyclic or linear end structures, resulting in various *cis* and *trans* isomeric configurations [2]. These compounds are categorized into two groups: xanthophylls, which contain oxygen, and carotenes, which are purely hydrocarbon compounds without oxygen [3]. Carotenoids have garnered increasing scientific interest due to their various biological properties, including antioxidant, anticarcinogenic, neuroprotective, reparative, antiproliferative, and anti-inflammatory activities [3,4]. In addition to their pharmacological potential, they are also widely utilized in nutraceuticals, cosmeceuticals, and photoprotective formulations targeting conditions linked to oxidative stress [5]. These functional properties are attributed to their unique structural feature, a polyene chain consisting of 8–13 conjugated double bonds, that serves as a chromophore for light absorption and enables efficient neutralization of reactive oxygen species [5]. Furthermore, structural modifications such as glycosylation, esterification, cyclic or acyclic arrangements, and geometric isomerism significantly influence their biological efficacy [5,6].

Oysters are widely valued for their nutritional content, offering high-quality protein, essential vitamins and minerals, omega-3 fatty acids, and biologically active polysaccharides [7]. They serve not only as a staple protein source but also support the livelihood of many coastal communities. Through their filter-feeding mechanisms, oysters ingest phytoplankton, microalgae, and organic matter, enabling them to accumulate a variety of bioactive compounds. This natural feeding strategy positions oysters as a promising source of functional biomolecules with potential health and pharmaceutical applications [8]. Various bioactive compounds in oysters, including peptides, polysaccharides, sterols, and other small molecules, have been recognized for their medicinal potential [9]. Several studies have identified specific oyster species as promising sources of pharmaceutical agents due to the bioactivities of their protein hydrolysates [10]. Extracts from oyster species *Crassostrea madrasensis* and *Magallana bilineata* have been shown to possess strong antioxidant and anti-inflammatory properties, attributed to their peptides, polysaccharides, and low molecular weight compounds [11–13]. Notable antioxidant activity has been detected in the processing residue of *C. iredalei*, highlighting the potential of oyster by-products in bioactive compound recovery [14]. Additionally, polysaccharides from Pacific oyster *C. gigas* have exhibited anti-tumor activity by promoting splenocyte proliferation, enhancing IL-2 secretion, and inhibiting HepG2 cell growth [15], along with antioxidant, antihypertensive, and hypoglycemic effects [16]. Recently, semi-purified proteins extracted from the hemolymph of the rock oyster *Saccostrea glomerata* demonstrated antimicrobial activity, particularly in suppressing pneumococcal infections [17].

The slipper-shaped oyster *Magallana bilineata* (formerly *Crassostrea iredalei*), identifiable by its distinctive purple-black adductor muscle scar, holds considerable commercial value as this species is endemic to the Philippines [18]. Our group has developed a technology to increase the market value of *M. bilineata* by processing it into an oyster extract powder, a shelf-stable culinary product [19]. To maximize waste utilization, we also recovered the oyster powder residue, a by-product from oyster powder processing that contains a significant amount of important amino acids and antioxidant activity [14]. Moreover, our previous work involved liquid chromatography mass spectrometry (LC-MS)-guided metabolite profiling of solvent fractions from oyster powder residue, suggesting the presence of several bioactive groups such as alkaloids, terpenoids, peptides, and cytotoxic macrolides [20]. However, the specific bioactive compounds responsible for the observed bioactivities remain unidentified.

The chemical complexity of marine-derived extracts necessitates the use of advanced analytical techniques for effective separation, detection, and identification of active constituents. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become a powerful tool in marine bioprospecting, enabling the high-sensitivity detection and structural elucidation of compounds within complex biological matrices [21]. This technique offers enhanced specificity by separating isobaric and interfering substances, improving the accuracy of compound identification [22]. LC-MS/MS has been successfully applied in the dereplication of bioactive compounds from marine sponge and clam [23,24] and the detection of toxins in oyster tissues [25,26]. Moreover, it has also proven effective for peptide characterization in hydrolyzed marine proteins [27]. However, the application of LC-MS/MS to identify bioactive constituents in marine invertebrates, especially

oysters, has not been extensively documented. Thus, the present study aims to identify and characterize bioactive compounds from *M. bilineata* powder residue using LC-MS/MS-based analytical techniques. The proposed method involves fractionating the active compounds from the *M. bilineata* using octadecylsilyl (ODS) column chromatography, followed by reversed-phase HPLC, LC-MS-guided isolation, and profiling through liquid chromatography coupled with tandem elevated mass spectrometry ($MS^E$). Herein the identification and bioactivities of the carotenoids from oyster powder residue were demonstrated, providing a sustainable approach to waste valorization and functional ingredient development.

## 2. Materials and methods

### 2.1. Materials

Analytical-grade solvents, including acetonitrile, ethanol, ethyl acetate, methanol, and trifluoroacetic acid, were procured from Fujifilm Wako Chemicals (Japan). Formic acid was obtained from Honeywell-Fluka™ (Seelze, Germany). LC-MS calibrants, leucine-enkephalin (3 mg) and sodium iodide (2 μg/μL in a 1:1 mixture of 2-propanol and water), were sourced from Waters Corporation (Milford, USA). Cisplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and Folin-Ciocalteu were purchased from Sigma–Aldrich (Massachusetts, USA). Media used for antibacterial testing, including nutrient broth, nutrient agar, and Mueller-Hinton agar, were obtained from Merck (Darmstadt, Germany). The MCF-7 human breast cancer cell line (ECACC 86012803) was acquired from DS Pharma Biomedical (Japan) for cytotoxicity evaluation.

### 2.2. Sample collection and oyster powder residue processing

Fresh slipper-shaped oyster *M. bilineata* were collected from a seafood market in Dumangas, Iloilo, Philippines (10.8484° N, 122.6955° E) in June 2023. The University of the Philippines Visayas Museum of Natural Sciences confirmed the taxonomic identification of the sample and was catalogued under accession number UPVMI 03853. The preparation of the oyster powder residue followed the method described by Peralta et al. [14], with modifications. Oysters (100 kg) were thoroughly washed with water to remove external debris and immersed in hot water to facilitate shell opening and tissue extraction. A portion of the oyster meat (500 g) was boiled in water at a 1:0.5 (*w/v*) ratio for 5 min and homogenized. The resulting homogenate was filtered through a fine mesh to extract the liquid fraction, and the remaining solid residue was manually chopped into smaller pieces. The residue was dried in a custom-built cabinet dryer at 60–80 °C for 8 h under continuous agitation. The dried material was ground into a fine powder by a mechanical grinder, and stored in polyethylene bags at 4 °C until further analysis.

### 2.3. Solvent extraction

A 10-g portion of the oyster residue powder was extracted three times with methanol at a 1:1 (*v/v*) ratio. The dried methanol extracts (8.7 g) were subsequently extracted with water and ethyl acetate (1:1 *v/v*). The resulting dried water fraction (7.0 g) was subjected to column chromatography using octadecylsilyl (ODS). Elution was performed stepwise with 300 mL of methanol–water mixtures, increasing methanol content from 20% to 100% in 20% increments, yielding five fractions. The 80% methanol and 100% methanol fractions were purified by HPLC.

### 2.4. LC-MS and HPLC analyses

For LC-MS profiling, vacuum-dried extracts were diluted in 50% acetonitrile before injection into the LC-MS. Chromatographic separation was performed by an Agilent 1100 series HPLC system coupled to a Bruker Daltonics micrOTOF-HS mass spectrometer. The separation was performed on a Cadenza CD-C18 column (2 × 150 mm, 3 μm particle size). The mobile phase consisted of solvent A: water with 0.1% formic acid, and solvent B: acetonitrile with 0.1% formic acid. The LC column was set at 25 °C, and the solvent was flowed at 0.2 mL/min [28]. Gradient elution was applied from start to 30 min with a gradient from 40% to 95% solvent B, followed by isocratic elution at 95% solvent B from 30 to 40 min.

The 80% methanol and 100% methanol fractions (45.2 mg and 80.7 mg, respectively) were purified by reversed-phase HPLC with isocratic elution in 25% acetonitrile containing 0.05% trifluoroacetic acid in a Cosmosil Cholester 4.6 × 250 mm (Nacalai Tesque, Inc., Japan), with a flow rate of 2.0 mL/min. The isolation process yielded 9.9 mg and 19.1 mg from the 80% and 100% methanol fractions, respectively.

### 2.5. Ultra-high-performance liquid chromatography-elevated energy mass spectrometry (UHPLC-MS$^E$)

#### 2.5.1. Chromatography

Reversed-phase chromatography was run through Waters ACQUITY® I-Class System in a binary pump, UPLC® BEH C18 column with dimensions 1.7 μm, 2.1 × 100 mm (Waters Corp., Milford, USA). The liquid chromatography system comprises a binary solvent manager pump, a CH-A column compartment heater set at 30 °C, and a sample manager-flow-through needle (SM-FTN) autosampler. The *M. bilineata* HPLC isolate was eluted with a linear gradient following the method of Lacson et al. [29] with modifications and Simora et al. [20]. The isolate was run from 2.5% acetonitrile with 0.1% formic acid to 100% acetonitrile with 0.1% formic acid over 10 min, and 2.5% acetonitrile with 0.1% formic acid from 10.01 min to 12 min, with a flow rate of 0.2 mL/min. The sample was diluted in 2.5% acetonitrile and injected with a 1-μL aliquot into the column.

#### 2.5.2. Sample preparation for UHPLC-MS$^E$

Sample preparation was done following the method of Simora et al. [28]. A 6.5 mg HPLC isolate was dissolved in 1300 μL of 50% acetonitrile in water to make a 5 mg/mL solution. A 5 μL of solution was added to 95 μL acetonitrile. A 10 μL solution was added to 90 μL water and diluted to obtain a 50 μg/mL solution. A 1-μL solution was drawn and injected into the mass spectrometer.

#### 2.5.3. Mass spectrometry

Elevated mass spectrometry (MS$^E$) was done following the method of Simora et al. [20] and Lacson et al. [29] in a positive ion mode on Waters Xevo G2-XS QTof® (Waters MS Technologies, Manchester, UK). The mass spectrometer was set with a source temperature of 120 °C, desolvation temperature of 450 °C, 10 L/h cone gas flow, and desolvation gas flow of 800 L/h using nitrogen gas and argon as the collision gas. It was calibrated to the manufacturer's specifications. Leucine-enkephalin as a lock mass was set to *m/z* 556.2766. The acquisition range was default from *m/z* 50 to *m/z* 1600 in MS$^E$ (sensitivity mode) with 1 kV capillary voltage, cone voltage of 40 *V*, collision energy (CE) of 6 eV for MS, and 15 eV–60 eV (ramp) for MS$^E$. The chromatograms were processed from the Waters UNIFI Scientific Information System (Waters MS Technologies, Manchester, UK).

### 2.6. UV–Vis absorption measurement

The light absorption of the two fractions was measured using a UV–Vis spectrophotometer (SpectroStar Nano, BMG Labtech, Germany) following the method of Ashenafi et al. [30]. Each fraction was prepared at a concentration of 5 mg/mL in 50% acetonitrile, and 200 μL of the solution was loaded into a 96-well microplate. The solvent served as the blank. Spectral measurements were recorded in the range of 400–515 nm with a resolution of 1 nm. For accuracy, spectra were obtained from 2 to 3 replicate scans and averaged.

### 2.7. Cytotoxicity

The MCF-7 human breast cancer cells were preserved in RPMI-1640 medium (Wako, Japan) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Cytotoxic activity of the purified fractions was assessed by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described by Denizot and Lang [31]. Cells were seeded in 96-well plates at a $1 \times 10^4$ cells/well density and incubated overnight at 37 °C in a humidified atmosphere containing 5% $CO_2$. Following incubation, the cells were treated with varying concentrations of the purified fractions for 72 h at 37 °C. Cisplatin was the positive control, while 1% filtered ethanol was the vehicle (negative) control. After the treatment, MTT stock solution was added to each well, and the plates were incubated for 3 h. Subsequently, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the resulting formazan crystals. Absorbance was measured at 540 nm using a Multiskan JX microplate reader (Thermo Labsystems). A decrease in absorbance relative to control wells indicated a reduction in cell viability, corresponding to cytotoxic effects of the test samples.

% Cell inhibition = 100 − (% cell viability); where % cell viability = {(At - Ab)/ (Ac - Ab)} × 100

Where "At" is the absorbance value of the test compound, "Ab" is the absorbance value of the blank (three wells/plates were used as blanks), and "Ac" is the absorbance value of the control.

### 2.8. Antimicrobial activity

The antibacterial activity of the purified fractions was evaluated by determining the minimum

inhibitory concentration (MIC) using the standard two-fold broth microdilution method [32]. Inhibitory activities were tested against six bacterial strains, comprising three Gram-positive species, *Staphylococcus aureus* ATCC 12600, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, and *Bacillus subtilis* ATCC 6051, and three Gram-negative species, *Pseudomonas aeruginosa* NBRC 12689, *Escherichia coli* BIOTECH 1634, and *Klebsiella pneumoniae* BIOTECH 1754. Dilutions of the test samples were prepared in 96-well microplates using Mueller-Hinton broth to yield final concentrations of 1250, 625, 312.5, 156.3, 78.2, 39, and 19.5 μg/mL. Each well was inoculated with 100 μL of bacterial suspension at $1 \times 10^6$ CFU/mL and incubated at 37 °C for 24 h. Bacterial growth was quantified by measuring the optical density at 570 nm using a Multiskan JX microplate reader (Thermo Labsystems). The MIC was defined as the lowest concentration of the compound that visibly inhibited bacterial growth.

### 2.9. Antioxidant activities

#### 2.9.1. 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH)

The antioxidant activity of the purified fractions was evaluated based on their DPPH radical scavenging capacity, following the method of Blois [33] with slight modifications. Briefly, 100 μL of each sample extract was mixed with 100 μL of DPPH solution (0.1 mM in 96% ethanol) in a 96-well microplate. The reaction mixtures were gently shaken and incubated in the dark at room for 30 min. The absorbance at 570 nm was scanned using a Multiskan JX microplate reader. Ethanol was the blank control, and Trolox was the positive control. The DPPH radical scavenging activity was computed using the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{Control}} \times 100$$

Where "$Abs_{control}$" is the absorbance of the reference solution (100 μL of ethanol instead of the test sample) and "$Abs_{sample}$" is the absorbance of the test solution/positive control.

#### 2.9.2. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity (ABTS)

ABTS radical scavenging activity was assessed using the method described by Re et al. [34]. The ABTS radical cation ($ABTS^+$) was made by incubating a mixture of 2.45 mM potassium persulfate and 7 mM ABTS for 16 h. The resulting $ABTS^+$ solution was diluted with 5 mM phosphate-buffered saline (PBS) at pH 7.4 until an obtained absorbance of 0.7 ± 0.02 at 734 nm. The assay was initiated by adding 100 μL of sample or standard solution to 100 μL of the $ABTS^+$ solution in a 96-well microplate (Costar 96, USA). The mixture was incubated in the dark at room temperature for 15 min. The absorbance of the wells was measured at 734 nm by a UV–Vis spectrophotometer (SpectroStar Nano, BMG Labtech, Germany). Trolox, a vitamin E analog, was used as the positive control. The radical scavenging activity was calculated using the following equation:

$$ABTS\ radical\ scavenging\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100,$$

Where "$Abs_{control}$" is the absorbance of the reference solvent and "$Abs_{sample}$" is the absorbance of the test solution/positive control.

### 2.10. Anti-inflammatory activity

The protein denaturation inhibition assay was performed according to Gambhire et al. [35]. Fresh egg albumin was used as the protein source for the assay. Standard and test samples were prepared by mixing 0.2 mL egg albumin, 2.8 mL phosphate-buffered saline (PBS), and 2 mL distilled water for the control. Test compounds were incubated at 37 ± 2 °C for 15 min, followed by heating at 70 °C for 5 min. The absorbance was measured at 660 nm. The percentage inhibition of protein denaturation was calculated using the following formula:

$$Inhibition\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100,$$

Where "$Abs_{control}$" is the absorbance of the reference solvent and "$Abs_{sample}$" is the absorbance of the test solution/positive control.

### 2.11. Statistical analysis

Data from the biological assays were analyzed using one-way analysis of variance (ANOVA) to assess significant differences among the samples. Tukey's post hoc test was then applied to identify specific groups that differed significantly. All statistical analyses were conducted using R software version 4.1.2. A $p$-value of less than 0.05 was considered statistically significant.

## 3. Results and discussion

### 3.1. Carotenoids identified from M. bilineata

The HPLC chromatograms showed one major peak from the 80% and 100% methanol fractions (Fig. 1). These peaks were collected and further optimized for LC-MS and UHPLC-MS$^E$. The main peaks isolated from the 80% methanol fraction (Fig. 1A) and 100% methanol fraction (Fig. 1B) were identified as carotenoids by UHPLC-MS$^E$. The UV–Vis spectral data of the two fractions were obtained to confirm their identity as carotenoids (Fig. 1). As shown in Table 1, the $\lambda_{max}$ values of naturally extracted zeaxanthin and lutein reported in literature were presented and compared to the

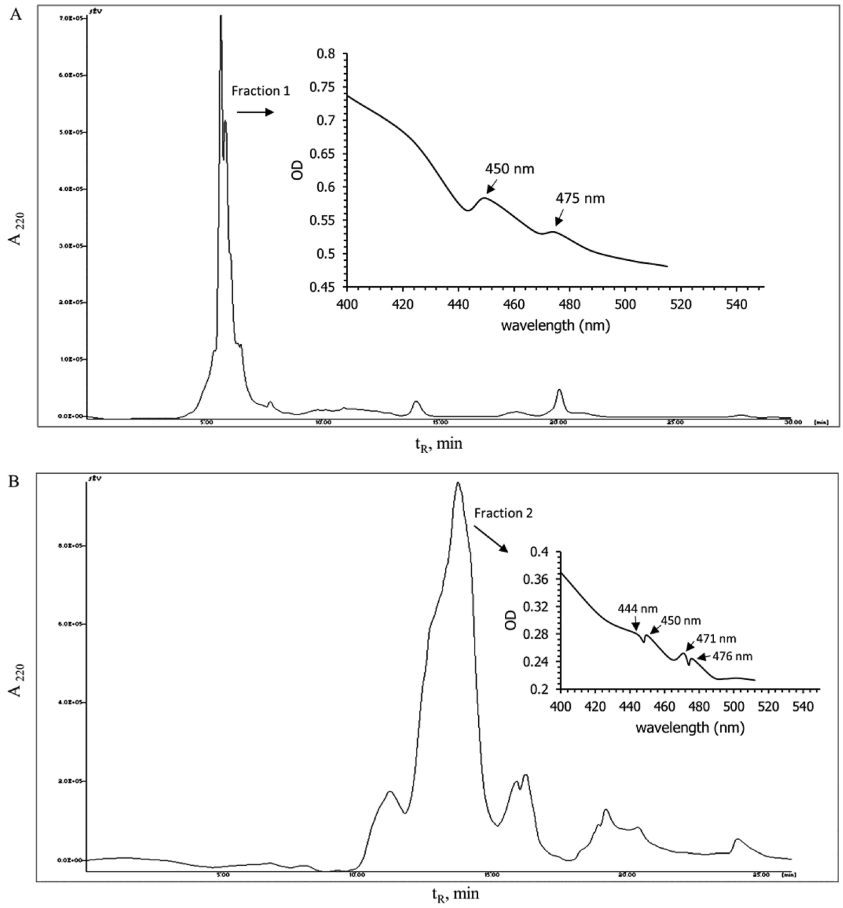


*Fig. 1. **HPLC profile of 80% (A) and 100% methanol fractions (B) from powder residue of Magallana bilineata along with their respective UV–Vis spectrum.** Reversed-phase HPLC isocratic elution of the main peaks in 25% acetonitrile containing 0.05% trifluoroacetic acid in a Cosmosil Cholester (4.6 × 250 mm) with a flowrate of 1.0 mL/min).*

*Table 1. MS and UV–Vis spectral data for carotenoids identified in oyster Magallana bilineata powder residue.*

| Fraction | MS spectra | | UV–Vis | |
|---|---|---|---|---|
| | Fragment ions (*m/z*) reported | Fragment ions (*m/z*) found | $\lambda_{max}$ (reported)/nm | $\lambda_{max}$ (found)/nm |
| 1 (zeaxanthin) | 533.4, 550.9, 532.9, 476.4, 463, 434, 366, 338, 175, 145 [39,70,71] | 476, 434 | 425, 450, 475 [70] | 450, 475 |
| 2 (zeaxanthin/lutein isomeric mixture) | 533.4, 550.9, 532.9, 476.4, 463, 434, 366, 338, 175, 145 (zeaxanthin) [39,70,71]<br>551, 533, 495.3, 477, 463, 459, 429.4 (lutein) [39,70–72] | 477, 459, 435 | 425, 450, 475 (zeaxanthin) [70]<br>420, 444, 472 (lutein) [70] | 444, 450, 471, 476 |

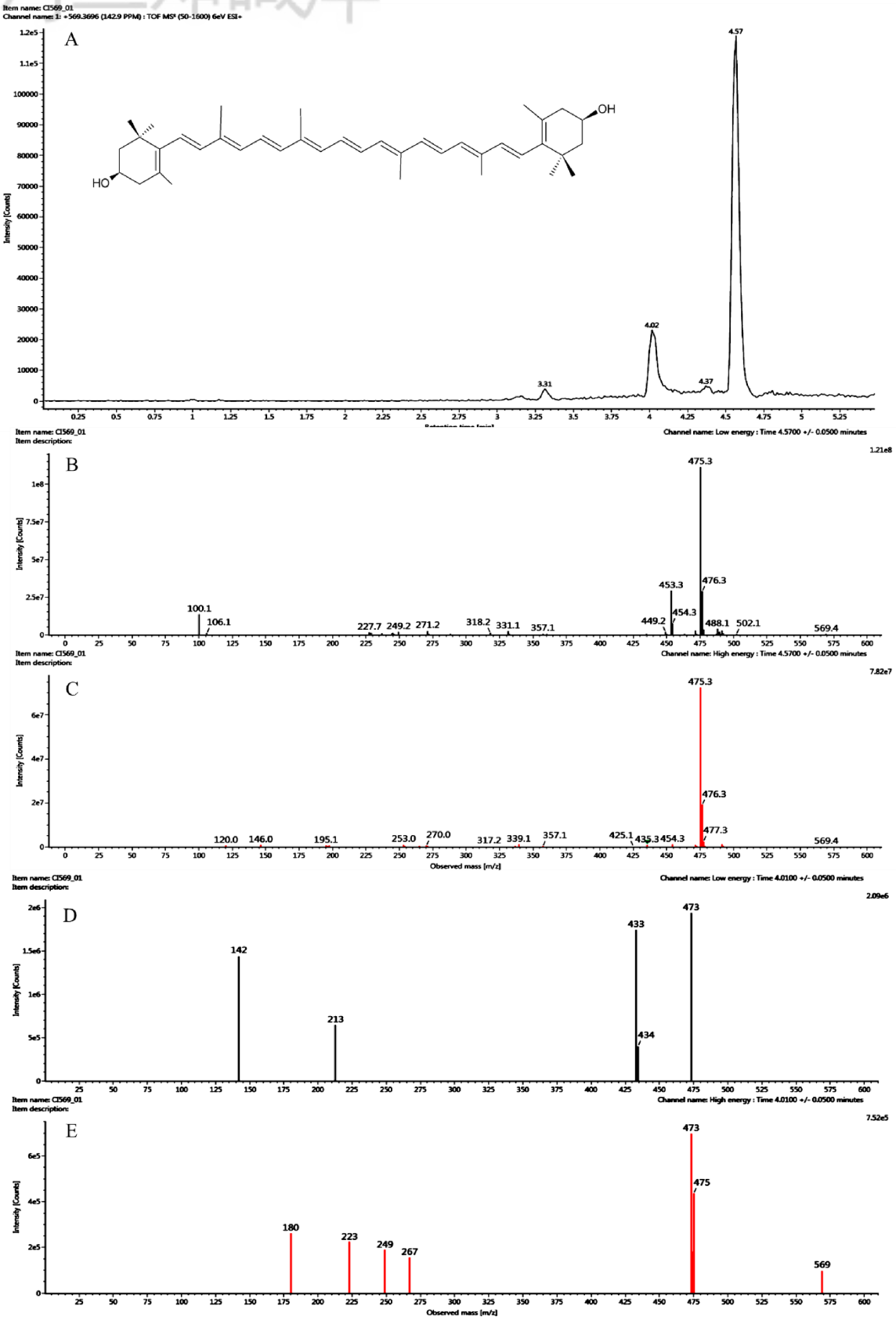


*Fig. 2.* ***UHPLC-MS$^E$*** *of fraction 1 identified as zeaxanthin (compound 1) from Magallana bilineata powder residue. (A). Extracted ion chromatogram (XIC); (B). $t_R$ 4.57, low CE; (C). $t_R$ 4.57, high CE; (D). $t_R$ 4.01, low CE; (E). $t_R$ 4.01, high CE.*

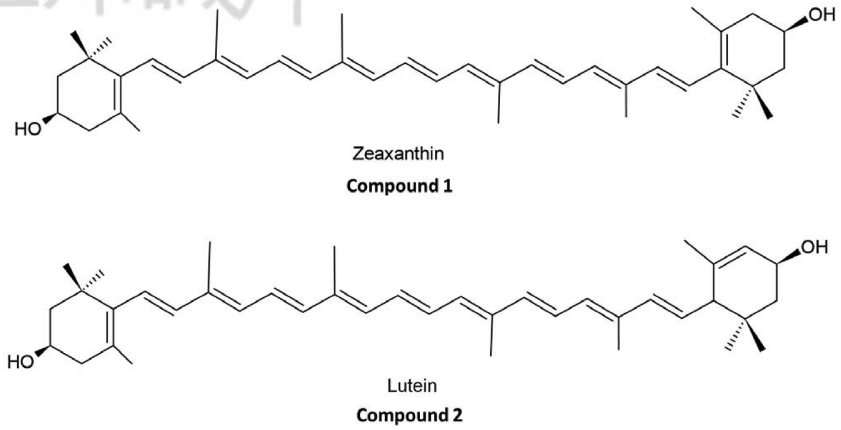


*Fig. 3. Carotenoids detected with m/z 569, zeaxanthin (Compound 1) and lutein (Compound 2) are isomers.*

$\lambda_{max}$ values found in this study. In Fraction 1 (Fig. 1A), only two maximum peaks were observed, corresponding to 450 nm (max 1) and 475 nm (max 2). The characteristic 425 nm shoulder of zeaxanthin was not apparent. This disappearance can be due to variations in polarity or the presence of other compounds in the natural extract that might alter the spectral features of zeaxanthin, potentially shifting or masking minor peaks such as the 425 nm shoulder [36]. Additionally, the shoulder peak of zeaxanthin may be obscured by the overlapping absorption bands from closer regions of the spectrum [37] as shown by the increasing absorbance trend toward the shorter wavelength. In contrast, the main maxima at 450 and 475 nm remain clearly visible because they correspond to higher molar absorptivity and represent the dominant spectral features in the mixture. Fraction 2 (Fig. 1B), on the other hand, represents an isomeric mixture of zeaxanthin and lutein. As a result, the UV–Vis spectrum of this fraction exhibits multiple maxima that correspond to the overlapping absorption peaks of both xanthophylls. The presence of these two structurally similar carotenoids in the same fraction leads to a spectral profile in which the individual peaks of zeaxanthin and lutein merge, producing a broader and more complex pattern [38] compared to fraction 1, yet the dominant peaks of each compound are still detectable at their respective λ max values.

This study utilized an untargeted approach to identify the bioactive compounds present in the oyster powder residue. Tandem mass spectrometry is widely utilized in untargeted metabolomics due to its ability to provide structural information about metabolites in complex mixtures [23]. In this study, we aimed to exclusively use UHPLC-$MS^E$ to provide molecular weight and structural fragments allowing the identification of bioactive compounds in complex mixtures such as those found in marine organisms. Under optimal UHPLC-$MS^E$ conditions, mass spectra were obtained for the HPLC isolates. HPLC fraction 1 is identified as zeaxanthin (compound **1**) with $[M+H]^+$ 569 (Fig. 2A), as reflected in the low CE $MS^E$ spectrum (Fig. 2B). An $[M+H]^+$ is predominantly observed in hydroxycarotenoids like zeaxanthin [39] (Fig. 2C). Zeaxanthin or β, β-carotene-3,3′-diol, lutein or β, ϵ-carotene-3,3′-diol (Fig. 3), and lactucaxanthin or ϵ, ϵ-carotene-3,3′-diol, exhibited a molecular formula of $C_{40}H_{56}O_2$, and an *m/z* 569. However, lactucaxanthin was rejected as a probable compound because MS/MS analysis depicted the absence of *m/z* 475 [40], which compound **1** and lutein possessed when subjected to high CE analysis. Further discrimination between the two fractions proved the presence of zeaxanthin rather than lutein due to the diagnostic peak *m/z* 433 present in zeaxanthin rather than *m/z* 430 present in lutein (Fig. 2D). Maoka [41] proved by MS/MS the presence of both compounds with a 3-ppm mass difference. The presence of *m/z* 569 $[M+H]^+$ indicates the presence of zeaxanthin (compound **1**) (Fig. 2E). The presence of the $t_R$ 4.02 may be due to the solvent effect of zeaxanthin during the elution process.

HPLC fraction 2 was subjected to UHPLC-$MS^E$. The two peaks were observed with $t_R$ 5.32 to $t_R$ 5.38 as peak 1 and $t_R$ 5.79 to $t_R$ 5.84 as peak 2 were observed (Fig. 4A). Peak 1 was assigned as all-*trans*-lutein (compound 2) with *m/z* 477, and *m/z* 459 [42] as diagnostic fragments (Fig. 4B). The presence of *m/z* 435 [48] as a zeaxanthin proved that the isolate is a mixture of isomers of compounds 1 and 2 (Fig. 3). The *m/z* 569 $[M+H]^+$ is a zeaxanthin, and the presence of *m/z* 475 and *m/z* 435 further proved it (Fig. 4C). Fraction 2 is an isomeric mixture of

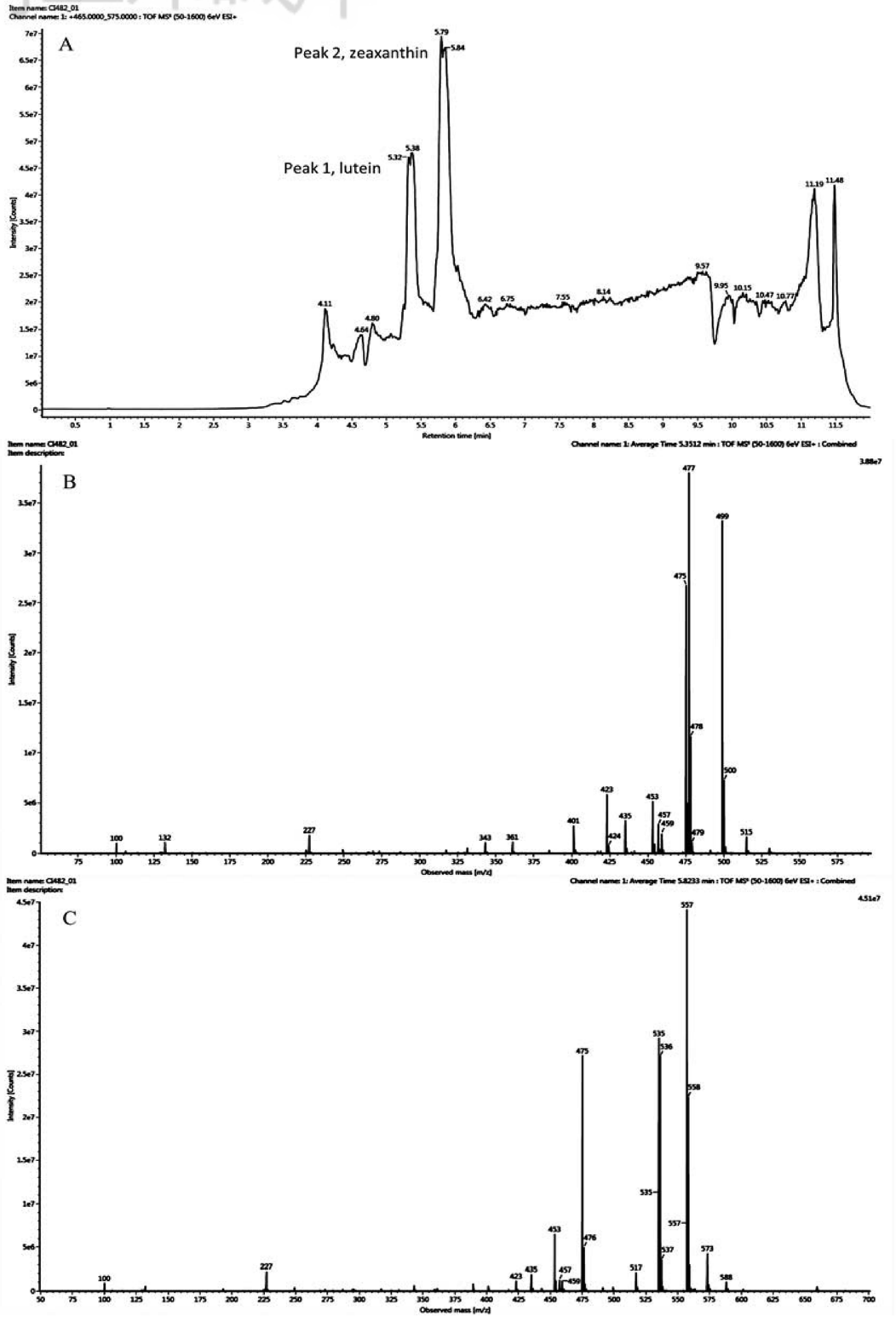


*Fig. 4. UHPLC-MS$^E$ of fraction 2 identified as zeaxanthin/lutein (isomeric mixture) from Magallana bilineata powder residue. (A). Extracted ion chromatogram (XIC); (B). $t_R$ 5.32- $t_R$5.35, low CE; (C). $t_R$ 5.79 to 5.84, low CE.*

zeaxanthin and lutein. Table 1 summarizes the fragment ions (*m/z*) of natural extracts of zeaxanthin and lutein reported in the literature, and we compared these with the fragment ions (*m/z*) found in this study. It is apparent that the fragment ions (*m/z*) found in this study coincided with the fragment ions reported for zeaxanthin and lutein. The MS spectral data further confirmed the identity of the carotenoids isolated and purified from fractions 1 and 2 of the oyster powder residue. Thus, fractions 1 and 2 were subsequently used in all the bioassays conducted.

### 3.2. Biological activities of carotenoids from M. bilineata

#### 3.2.1. Cytotoxicity activity

The cytotoxic potential of zeaxanthin and zeaxanthin/lutein isomeric mixture from oyster powder residue against MCF-7 breast cancer cells, along with the half-maximal inhibitory concentration ($IC_{50}$) is shown in Table 2. The mean $IC_{50}$ represents the drug concentration required to attain a 50% reduction in viable cell count under *in vitro* conditions. The lower $IC_{50}$ value implies more potent cytotoxic effects towards cancer cells [43]. The cytotoxicity of the carotenoids against MCF-7 cells was evaluated relative to the activity of cisplatin as the reference standard. Zeaxanthin/lutein isomeric mixture showed an $IC_{50}$ value of 93.29 ± 0.07 μg/mL, lower than that of cisplatin (97.56 ± 0.10 μg/mL), suggesting better anticancer potency than the control. The observed cytotoxicity can be attributed to the bioactive carotenoids in the powder residue of *M. bilineata* which corroborates with results of the previous studies on the effectiveness of zeaxanthin and lutein as anticancer compounds [44]. Cha et al. [44] reported that microalgae *Chlorella ellipsoidea* and *Chlorella vulgaris* containing active carotenoids including lutein and zeaxanthin, have been shown to effectively inhibit the proliferation of HCT116 human colon cancer cells, yielding $IC_{50}$ values of 40.73 ± 3.71 and 40.31 ± 4.43 μg/mL, respectively. In the study of Elkhalifa et al. [45], lutein derived from vegetable okra was able to reduce the MCF-7 cell viability by 59.84% at a 1 μM concentration. Lutein extracted from alfalfa (*Medicago sativa*) demonstrated anticancer effects comparable to the chemotherapy drug doxorubicin, showing an $IC_{50}$ value of 3.10 ± 0.47 μg/mL in MCF-7 breast cancer cells. It also showed strong cytotoxic effects against HepG2 liver cancer cells, with an $IC_{50}$ of 6.11 ± 0.84 μg/mL [46].

*Table 2. Cytotoxicity of carotenoids identified from oyster Magallana bilineata powder residue against MCF-7 breast cancer cells.*

| Fractions | $IC_{50}$ (μg/mL), Mean ± SD |
|---|---|
| Zeaxanthin (compound 1) | 114.00 ± 0.02[a] |
| Zeaxanthin/Lutein (isomeric mixture) | 93.29 ± 0.07[b] |
| Cisplatin | 97.56 ± 0.10[b] |

[a, b] Statistically significant at $p < 0.05$. All samples were tested in triplicate ($n = 3$).

Lutein and zeaxanthin have shown potential in slowing the growth of breast cancer cells by increasing intracellular levels of reactive oxygen species (ROS) [47]. When acting as pro-oxidants, these carotenoids elevate ROS concentrations beyond the tolerance threshold of cancer cells, leading to oxidative damage to DNA, lipids, and proteins, ultimately triggering apoptosis. This selective pro-oxidant activity contributes to the targeted elimination of cancer cells [48]. The molecular structure of these carotenoids, particularly the polar hydroxyl groups at their terminal positions, enhances their ability to span the phospholipid bilayer and interact with membrane headgroups through hydrogen bonding. This promotes membrane permeability, facilitating carotenoid entry into cancer cells, where they induce apoptosis and inhibit cell proliferation [48]. These insights are supported by epidemiological data showing that higher circulating levels of dietary carotenoids, including lutein and zeaxanthin, are significantly associated with a reduced risk of breast cancer, especially the estrogen receptor-negative (ER−) subtype [49,50].

#### 3.2.2. Antibacterial activity

The antibacterial activity of zeaxanthin and the isomeric mixture was evaluated by the MIC assay (Table 3). The results demonstrated that the antibacterial efficacy of the fractions varied across different bacterial strains. Both exhibited strong inhibitory activity against *K. pneumoniae* and *E. coli*, respectively. Moderate antibacterial activity was observed for zeaxanthin against *E. coli*, as well as for the isomeric mixture against MRSA, *B. subtilis*, and *K. pneumoniae*. Both fractions exhibited weak inhibition against *S. aureus*, and no antibacterial activity was detected against *P. aeruginosa* at the highest concentration tested. The observed antibacterial activity of *M. bilineata* powder residue aligns with findings in the study of Kusmita et al. [51] where carotenoids from the soft coral *Sinularia* sp. demonstrated antibacterial effects against methicillin-resistant *S. aureus* and multidrug-resistant (MDR) *E. coli*.

As filter feeders, oysters are constantly exposed to a variety of microbial communities that include both transient and resident bacteria [8]. Due to their

*Table 3. Antibacterial activities of carotenoids identified from oyster Magallana bilineata powder residue.*

| `Sample | MIC (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | Gram-positive | | | Gram-negative | | |
| | *S. aureus* | MRSA | *B. subtilis* | *P. aeruginosa* | *E. coli* | *K. pneumoniae* |
| Zeaxanthin | + | ++ | ++ | + | +++ | ++++ |
| Zeaxanthin/Lutein (isomeric mixture) | ++ | +++ | +++ | + | ++++ | +++ |
| Oxacillin | 3.12 | 3.12 | | | | |
| Gentamicin | | | 12.5 | | | |
| Chloramphenicol | | | | 12.5 | | |
| Ciprofloxacin | | | | | 12.5 | 6.25 |

Note: +: greater than 1250 μg/mL; ++: 1250 μg/mL, +++: 625 μg/mL, ++++: 313 μg/mL. All samples were tested in triplicate (n = 3). MRSA stands for methicillin-resistant *S. aureus*, and MIC stands for minimum inhibitory concentration.

distinct physical structures, oysters provide numerous surfaces and microhabitats that support microbial colonization. Therefore, it is not surprising that oysters contain bacterial concentrations higher than those found in the surrounding aquatic environment [10]. The immune system of oysters is a combination of hemolymph-mediated responses and antimicrobial effectors produced by epithelial cells found in the gills, mantle, digestive gland, and intestine. In addition to the natural immune system of the oyster, carotenoids acquired through filter feeding exhibit antibacterial properties that can enhance the immune system machinery of bivalves [52], by functioning as an antibacterial agent. Their mechanism of action involves interaction with porin proteins, transmembrane channels located in the outer membrane of bacterial cell walls, particularly in Gram-negative bacteria like *E. coli* [53]. Carotenoids bind to these porins and form stable polymeric complexes, leading to structural disruption and porin degradation. Hydroxyl regions in zeaxanthin anchors to the outer part of the bacterial membrane, disturbing membrane architecture and integrity [54]. This interaction leads to membrane permeabilization, leakage of cellular contents such as ions, ATP and proteins, and ultimately bacterial death [52]. In the present study, the extracted carotenoids from *M. bilineata* powder residue, may have demonstrated a similar inhibitory mechanism, indicating promising antibacterial potential.

### 3.2.3. Antioxidant activity

The antioxidant potential of *M. bilineata* powder residue was evaluated based on its ability to scavenge free radicals using DPPH and ABTS assays. The interaction between the sample and DPPH radicals resulted in a concentration-dependent decrease in absorbance at 517 nm, indicating free radical neutralization and the presence of anti-radical activity (Fig. 5A). Generally, the identified carotenoids from *M. bilineata* showed a dose-dependent radical scavenging effect, with the highest activity exhibited by zeaxanthin/lutein mixture at 47.55% in 5 mg/mL concentration. Trolox, consistently demonstrated high radical scavenging efficiencies, ranging from 55.20% to 73.53%. Similar findings were also obtained in the ABTS assay (Fig. 5B), revealing that the carotenoid-rich powder residue exhibited significant scavenging effects across all concentrations tested. At 1 mg/mL, the scavenging activities of both fractions and Trolox did not differ significantly, suggesting comparable antioxidant capacities. Interestingly, the ABTS radical inhibition results were generally higher than those observed in the DPPH assay, highlighting assay-dependent variability in antioxidant performance.

These findings are consistent with previous studies highlighting the antioxidant potential of carotenoids from various marine organisms. In particular, carotenoids synthesized from microalgae such as lutein and zeaxanthin exhibited potent antioxidant effects by scavenging reactive oxygen species and reducing oxidative cellular damage [55]. Additionally, Liu et al. [56] reported that carotenoid extracted from the marine bacterium *Brevundimonas scallop*, exhibited remarkable radical scavenging capacity, primarily attributed to its conjugated bond structure and hydroxyl functional groups that enhance reactivity with free radicals. Zeaxanthin and lutein also play important roles in defending against age-related macular degeneration (AMD), as their consumption has been linked to enhanced macular pigment density and the regulation of intracellular glutathione (GSH) levels. These carotenoids exhibit various antioxidant activities, including neutralizing reactive oxygen species (ROS), safeguarding DNA from damage, and preventing the oxidation of proteins and lipids [55].

Zeaxanthin and lutein have been identified as potent antioxidants because of their ability to quench singlet oxygen and neutralize free radicals [52,55]. They contain multiple conjugated double bonds, unsaturated ketones, and hydroxyl groups, which can neutralize free radicals through highly

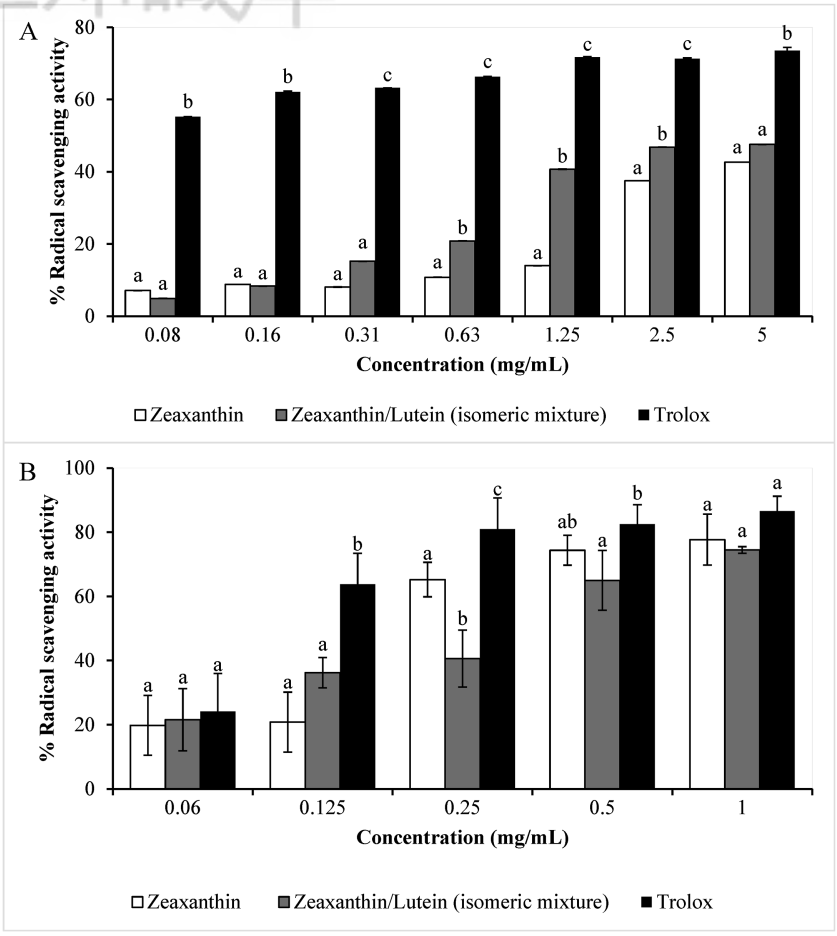


*Fig. 5. **DPPH (A) and ABTS (B) radical scavenging activities of zeaxanthin (compound 1) and zeaxanthin/lutein (isomeric mixture) from Magallana bilineata powder residue.** Values are expressed as mean ± standard deviation (n = 3). Letters (a, b, c) indicate significant differences per compound concentration ($p < 0.05$).*

reactive electron interactions [57]. Specifically, the presence of conjugated double bonds throughout the polyene chain allows zeaxanthin and lutein to capture electrons from reactive species and subsequently neutralize free radicals [55]. Despite having similar hydrocarbon length, zeaxanthin differs from lutein by only one additional conjugated double bond and this subtle structural variation results in distinct antioxidant capacities [58]. Studies have shown that the quenching efficiency of singlet oxygen by carotenoids increases with the number of conjugated double bonds and hydrophobicity, making zeaxanthin a more potent antioxidant than lutein [59]. However, although zeaxanthin is more hydrophobic and has a slightly longer conjugated double bond system, the lutein and zeaxanthin mixture has somewhat different antioxidant behaviors. Lutein can contribute to antioxidant capacity in ways that complement zeaxanthin, such as scavenging different radical species or regenerating zeaxanthin radicals, resulting in an overall enhanced radical scavenging effect in the mixture [60]. This difference in antioxidant capacity of zeaxanthin and zeaxanthin/lutein mixture explains the significant increase in DPPH radical scavenging activity in various sample concentrations in the present study.

The effectiveness of ABTS radical scavenging of *M. bilineata* powder residue may be attributed to its hydrophilic antioxidant component. Unlike DPPH, the ABTS assay measures the ability of antioxidants to neutralize aqueous-phase radicals, making it more suited for evaluating hydrophilic compounds [61]. Zeaxanthin and lutein exhibit enhanced antioxidant activity in aqueous environments due to their hydrophilic nature. The presence of polar hydroxyl groups in lutein and zeaxanthin structures is what makes them more hydrophilic, enabling them to interact with oxygen in the aqueous phase and more effectively scavenge reactive oxygen species (ROS) [60]. The hydroxyl groups at the 3 and 3′ ends of ionone rings serve as hydrogen donors that stabilize free radicals [59]. These

characteristics align with the current findings of the study, wherein Fractions 1 and 2 demonstrated greater inhibitory activities in the ABTS assay compared to the DPPH assay, confirming the influence of compound polarity and medium compatibility on antioxidant activity.

### 3.2.4. *Anti-inflammatory activity*

The anti-inflammatory activity of zeaxanthin and the isomeric mixture was evaluated using the egg albumin denaturation assay (Fig. 6). Their ability to inhibit heat-induced albumin denaturation was compared to aspirin as a reference standard. Zeaxanthin (compound 1) demonstrated no significant difference in inhibition (43.68 ± 0.11%) as compared to aspirin (43.49 ± 0.17%), indicating a comparable inhibitory capacity. In contrast, zeaxanthin/lutein mixture exhibited a slightly lower inhibition value at 42.76 ± 0.32% (1 mg/mL) as compared to zeaxanthin. These results highlight the significant anti-inflammatory potential of oyster powder residue, with superior potency compared to the tested commercial drug.

In the study, the basis of the egg albumin denaturation assay lies in the principle that compounds capable of stabilizing proteins and preventing their thermal denaturation are indicative of anti-inflammatory properties [62]. The current findings suggest that the inhibition of protein denaturation may be attributed to bioactive carotenoids, specifically zeaxanthin and lutein, present in the oyster powder residue. These carotenoids are known to possess structural features such as multiple conjugated double bonds and reactive hydroxyl groups that allow them to scavenge reactive oxygen species (ROS) and prevent lipid peroxidation, ultimately contributing to the suppression of inflammation [63]. This antioxidant property plays an essential role in the albumin denaturation assay by helping maintain the structural and functional integrity of protein under stress conditions such as heat exposure [64]. A recent study suggests that lutein may help slow the development of atherosclerosis, primarily because of its potent antioxidant and anti-inflammatory effects on aortic tissue, as well as its inverse relationship with levels of oxidized LDL in the blood [65]. Mora-Gutierrez et al. [66] reported that the binding of lutein with proteins enhances solubility, provides protection against oxidative degradation, and improves the chemical stability of protein–carotenoid complexes. Zeaxanthin also demonstrates a protective effect by quenching singlet oxygen and capturing ROS, thereby preventing oxidative damage to proteins [59]. Aside from neutralizing ROS, zeaxanthin can also modulate antioxidant systems within cells, particularly the synthesis and maintenance of intracellular glutathione (GSH). This function supports redox homeostasis and reduces the susceptibility of the cell to hydrogen peroxide ($H_2O_2$)-induced damage [67]. Through these mechanisms, free radical scavenging and enhancement of antioxidant defenses, zeaxanthin and lutein in *M. bilineata* powder residue contribute significantly to the anti-inflammatory effects observed in this study.

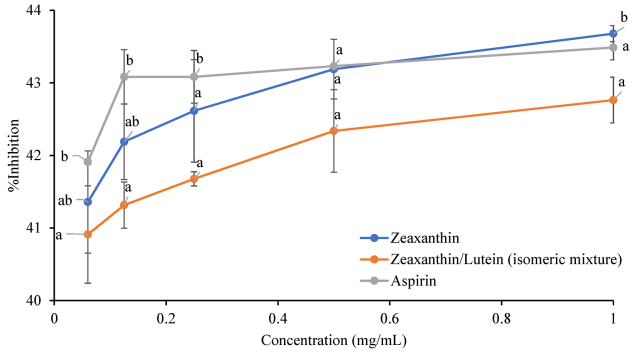


*Fig. 6. **Anti-inflammatory capacity of zeaxanthin (compound 1) and zeaxanthin/lutein (isomeric mixture) from Magallana bilineata powder residue.** Values are expressed as mean ± standard deviation (n = 3). Different lowercase (a, b) indicates significant differences per compound concentration (p < 0.05).*

### 3.3. *Bioactive carotenoids in marine organisms*

The current findings on the biological activities of carotenoids from *M. bilineata* show that this oyster species has great potential as a valuable source for biomedical applications. This advancement not only contributes to sustainable waste utilization but also broadens the spectrum of aquatic organisms recognized as rich sources of functional carotenoids, which are increasingly sought after for their health-promoting properties. Many marine invertebrates, including oysters, are unable to synthesize these compounds and instead obtain them through their diet through filter feeding or by metabolically converting precursor substances [8]. In these organisms, carotenoids play vital roles in protecting against harmful ultraviolet radiation and neutralizing free radicals, functions that are especially crucial in shallow-water environments exposed to intense sunlight [68]. Furthermore, carotenoids contribute to animal nutrition by providing provitamin A activity and supporting immune system function [69], underscoring their ecological and biomedical importance and the broader implications of our research.

*Table 4. Summary of biological activities of carotenoids extracted from aquatic organisms.*

| Aquatic Organism | Scientific Name | Carotenoid | Bioactivity | Reference |
|---|---|---|---|---|
| Tunicates and shellfish | | Mytiloxanthin | Antioxidant | [68] |
| Scallop | *Chlamys nobilis* | Astaxanthin, hydroxy-astaxanthin | Antioxidant | [73] |
| Shrimp | *Parapenaeus longiristris* | Astaxanthin | Antioxidant, anti-proliferation, anticancer | [74] |
| Kelp | *Undaria pinnatifida* | Fucoxanthin | Antioxidant, anticancer, anti-obesity, cardioprotective | [75,76] |
| Kelp | *Laminaria japonica* | Fucoxanthin | Anticancer | [77] |
| Sea squirt | | Halocynthiaxanthin | Anticancer | [78] |
| Sea squirt | *Halocyntia roretzi* | Halocynthiaxanthin, fucoxanthin | Anticancer | [79] |
| Seaweed | *Hijikia fusiforme* | Fucoxanthin | Anticancer | [80] |
| Dinoflagellate | *Heterocapsa triquetra* | Perinidin | Anticancer | [81] |
| Microalgae | *Haematococcus pluvialis* | Astaxanthin | Antioxidant, anti-inflammatory, anticancer | [82] |
| Microalgae | *Chlorella ellipsoidea* | Zeaxanthin | Anticancer Antiproliferative | [83] |
| Microalgae | *Chlorella vulgaris* | Lutein | Anticancer | [48] |
| Marine bacterium | *Gramella aceani sp.* | Zeaxanthin | Antioxidant | [84] |
| Marine bacterium | *Brevundimonas scallop* | Astaxanthin, hydroxy-astaxanthin | Antioxidant | [59] |
| Oyster | *Magallana bilineata* | Zeaxanthin, lutein | Anticancer, antibacterial, antioxidant, anti-inflammatory | This study |

The significant roles of carotenoids in human health and marine life demonstrate their potential for use in supplements and other health products. This understanding encourages more research into using marine organisms and their by-products as sources of natural compounds that can benefit health and wellness. Several marine sources, including tunicates, shellfish, seaweeds, microalgae, and marine bacteria, are documented with bioactive carotenoids (Table 4). This summary supports our claim that *M. bilineata* powder residue exhibits promising biological activities, such as anticancer, antibacterial, antioxidant and anti-inflammatory, largely due to the presence of zeaxanthin and lutein. This underscores the potential of marine-derived carotenoids as natural bioactive compounds for applications in health, nutrition, and disease prevention. This is the first report of biologically active carotenoids present in slipper-shaped oyster *M. bilineata* processing by-product, highlighting the practical implications of our research.

## 4. Conclusions

This study introduced an alternative approach for the isolation and characterization of carotenoids from oyster *M. bilineata* powder residue, utilizing column chromatography and UHPLC-MS$^E$ analysis. Carotenoids from oyster processing by-product were successfully isolated and identified using this approach. Characterization using UHPLC-MS$^E$ confirmed the presence of zeaxanthin and zeaxanthin/lutein isomeric mixture and validated the biochemical origin of the observed bioactivities. Bioassays revealed that the carotenoids exhibited potent cytotoxic, antimicrobial, antioxidant, and anti-inflammatory activities. The significant bioactivities can be attributed to the presence of the mentioned carotenoid constituents. These findings establish that oyster powder residue is a promising source of biologically active compounds for potential biomedical applications, opening new avenues for research and application in the field of marine biochemistry.

## Data availability statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

### Conflicts of interest

The authors declare no competing financial interest.

## CRediT authorship contribution statement

Rhoda Mae C. Simora, Tatsufumi Okino: Conceptualization; Rhoda Mae C. Simora, Raymund B. Parcon, Andrea Roxanne J. Anas: Methodology & experimentation; Rhoda Mae C. Simora, Raymund B. Parcon, Andrea Roxanne J. Anas:

Analysis & data interpretation; Rhoda Mae C. Simora, Raymund B. Parcon, Andrea Roxanne J. Anas: Writing and original draft preparation; Rhoda Mae C. Simora, Tatsufumi Okino: Writing-reviewing & editing.

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## Conflicts of interest

The authors declare no competing financial interest.

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