Bioassay-guided isolation of anti-algal constituents from *Inula helenium* and *Limonium myrianthum*

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In honor of Professor Atta-ur-Rahman on his 65th birthday

Abstract

Over 70 crude plant extracts, primarily from Kazakhstan, were screened against the freshwater phytoplanktons *Oscillatoria perornata* and *Selenastrum capricornutum*. Extracts from *Limonium myrianthum* and *Inula helenium* both demonstrated selective inhibition against the odor-producing cyanobacterium *O. perornata* compared to activity against the green alga *S. capricornutum*. Bioassay-guided fractionation of the *L. myrianthum* dichloromethane extract resulted in the identification of nepodin, torachrysone, chrysophanol, and physcion. Nepodin demonstrated the desired selective inhibition with lowest-complete-inhibition concentration values of 100 µg/mL and >100 µg/mL towards *O. perornata* and *S. capricornutum*, respectively. Similarly, chrysophanol also demonstrated the desired selective inhibition with lowest-complete-inhibition concentration values of 10 µg/mL and >100 µg/mL, respectively. Bioassay-guided fractionation of the *I. helenium* hexane extract resulted in the isolation of alantolactone, isoalantolactone, and 11αH,13-dihydroisoalantolactone. Activities of these three isolated constituents as well as those of synthetic isomers are reported.

Keywords: Cyanobacteria, 2-methylisoborneol, isoalantolactone, alantolactone, nepodin, torachrysone, chrysophanol, physcion

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Introduction

Phytoplankton blooms are a common occurrence in eutrophic freshwater lakes, reservoirs, and ponds. Cyanobacterial blooms (blue-green algae) are the most undesirable type of freshwater phytoplankton blooms, especially in ponds used to culture channel catfish (*Ictalurus punctatus*) in the southeastern United States of America. Among the various negative attributes of cyanobacterial blooms,[1] certain species of cyanobacteria found in catfish aquaculture ponds produce "off-flavor" compounds that can be absorbed into the flesh of the catfish. The most common off-flavor is "musty" and is caused by 2-methylisoborneol (MIB). Catfish producers are not able to market the off-flavor catfish until they lose the musty-taint which can take days or weeks. Such delays in harvest have been estimated to cost catfish producers as much as U.S. $60 million annually.[2]

Most of the pond-raised channel catfish production in the USA occurs in Mississippi and Alabama. The MIB-producing cyanobacterium *Oscillatoria perornata* has been attributed as the primary cause of MIB-related off-flavor in pond-raised catfish in west Mississippi,[3] and *O. perornata* is also prevalent in catfish ponds in east Mississippi and west Alabama.[4] The most common management approach used by catfish farmers to manage musty off-flavor is the application of algaecides such as copper-based products (e.g., copper sulfate, chelated-copper compounds) and diuron [*N*'-(3,4-dichlorophenyl)-*N*,*N*-dimethyurea] to reduce the abundance of *O. perornata*. These products have several negative characteristics including the following: 1) high persistence in the environment; 2) broad-spectrum toxicity towards non-target phytoplankton that can lead to a deterioration of water quality (e.g., dramatic reduction of dissolved oxygen levels) and subsequently endanger fish health; and 3) public's negative perception to the use of synthetic compounds in food-fish production ponds. The discovery of environmentally-safe, selective algaecides would greatly benefit the catfish aquaculture industry. In order to discover novel compounds with potential use to manage the abundance of *O. perornata* in catfish aquaculture ponds, our laboratory has been evaluating extracts from plants for anti-algal activity using a rapid bioassay. Recently, haplamine that was isolated from *Haplophyllum sieversii*, a plant endemic to Kazakhstan, was found to possess selective toxicity towards certain cyanobacterial species including *O. perornata*.[5] In this study, we present the additional discoveries of anti-algal compounds from other plants found primarily in the Republic of Kazakhstan.

Results and Discussion

Crude plant extracts primarily from Kazakhstan were screened against the freshwater phytoplanktons *Oscillatoria perornata* and *Selenastrum capricornutum* (Table 1). Over 70 crude extracts from 17 separate species were evaluated with extracts from *Limonium myrianthum* (Schrenk) Ktzl. (Limoniaceae) and *Inula helenium* L. (Asteraceae) both demonstrating selective

inhibition against the odor-producing cyanobacterium *O. perornata* compared to activity against the green alga *S. capricornutum*. Specifically, the dichloromethane extract of the aerial portions of *L. myrianthum* as well as the hexane extract of the roots of *I. helenium* both demonstrated selective inhibitions with lowest-complete-inhibition concentration (LCIC) values of 10 µg/mL and 100 µg/mL against *O. perornata* and *S. capricornutum*, respectively.

The results of evaluation of the extract fractions are provided in Table 2. Fractions B and C from the dichloromethane extract of the aerial portions of *L. myrianthum* did reveal selective toxicity and LCIC values of 10 µg/mL for *O. perornata*, and, therefore, these extracts were selected to pursue bioassay-guided isolation of the active constituent(s). Further fractionation and purification of fractions B and C using HPLC resulted in the isolation of four compounds, nepodin (**7a**), torachrysone (**7b**), chrysophanol (**8a**), and physcion (**8b**) (Figure 1). Structure elucidation for all compounds was performed using combinations of GC-MS, high-resolution MS, and one- and two-dimensional NMR spectroscopy. Final structure confirmation was accomplished by comparing ^{1}H and ^{13}C NMR data with that reported in the literature.[6-9] Chrysophanol (1,8-dihydroxyanthraquinone) was the most active of the four compounds isolated from fractions B and C from the *L. myrianthum* extract with a LCIC of 10 µg/mL towards *O. perornata* and at least 10X more toxicity when compared with the LCIC results (>100 µg/mL) of *S. capricornutum*.

Although fraction C from the hexane extract of the roots of *I. helenium* (LCIC of 10 µg/mL towards *O. perornata*) did not show selective toxicity, bioassay-guided isolation for active compounds was performed anyhow to determine if the isolated pure compounds might reveal selective toxicity. The LOEC and LCIC values for Copper Control were 1 µg/mL for each test organism, thereby indicating the lack of selective toxicity for the positive control. The isolated pure compounds from fraction C of the crude extract from *I. helenium* were alantolactone (**1**), isoalantolactone (**4**), and 11αH,13-dihydroisoalantolactone (**6**) (Figure 1). These compounds did not reveal selective toxicity towards *O. perornata* and their lowest-observed-effect concentration (LOEC) and LCIC values were at or above 100 µg/mL (Table 3). Furthermore, none of the synthetic compounds evaluated were selectively toxic towards *O. perornata* (Table 3). These synthetic compounds [11,13-dihydroxyalantolactone (**3**), 5α-epoxyalantolactone (**2**) and 4(15)α-epoxyisoalantolactone (**5**)] were included in the screening process since they are isomers of several of the isolated pure compounds from *I. helenium* and were readily available from a previous study.[9]

Table 1. Algal inhibition screening results for crude plant extracts

			LCIC [b] (µg/mL)	
Species (Authority)	Plant part	Extraction solvent[a]	*O. perornata*	*S. capricornutum*
Achillea millefolium L.	Aerial	PE	>100	>100
Asteraceae		H:EtAc:H2O	>100	>100
		EtOH	>100	>100
		H_2O	>100	>100
Allium coeruleum Pall.	Aerial	DCM	>100	>100
Liliaceae		EtOH	>100	>100
		H_2O	>100	>100
	Roots	DCM	>100	>100
		EtOH	>100	>100
		H_2O	>100	>100
Echinops albicaulis Kar. et Kir.	Aerial	DCM	>100	>100
Asteraceae		EtOH	>100	100
		H_2O	>100	>100
	Roots	DCM	100	100
		EtOH	100	100
		H_2O	>100	>100
Echinops ritro L.	Aerial	DCM	100	>100
Asteraceae		EtOH	100	>100
		H_2O	100	100
Echinops spherocephalus L.	Aerial	DCM	100	>100
Asteraceae		EtOH	100	100
		H_2O	100	100
Echinops transiliensis Golosh.	Roots	DCM	100	100
Asteraceae		EtOH	100	>100
		H_2O	>100	>100
	Aerial	DCM	>100	>100
		EtOH	>100	>100
		H_2O	>100	>100
Eremurus fuscus (O. Fedtsch.) Vved.	Aerial	PE	>100	>100
Asphodelaceae		H:EtAc:H2O	>100	>100
		EtOH	>100	>100
		H_2O	>100	>100
Euphorbia latifolia Meyer ex Lebeb	Aerial	PE	>100	>100
Euphorbiaceae		H:EtAc:H2O	>100	>100
		EtOH	>100	>100
		H_2O	100	>100
Ferula leiophylla Korov.	Seed and Racemes	DCM	>100	100

			LCIC [b] (μg/mL)	
Species (Authority)	Plant part	Extraction solvent[a]	*O. perornata*	*S. capricornutum*
Apiaceae		EtOH	>100	>100
		H_2O	>100	>100
Ferula soongarica Pall. Ex Spreng.	Roots	PE	100	>100
Apiaceae		H:EtAc:H2O	100	>100
		EtOH	>100	>100
		H_2O	>100	>100
Geranium transversale Kar et. Kir.	Aerial	DCM	>100	>100
Geraniaceae		EtOH	100	>100
		H_2O	>100	>100
Inula helenium L.	Roots	Hexane	10	100
Asteraceae		DCM	100	100
		MeOH	>100	>100
Limonium myrianthum Shrenk.	Aerial	DCM	10	100
Plumbaginaceae		EtOH	100	>100
		H_2O	100	100
	Roots	DCM	100	100
		EtOH	100	100
		H_2O	>100	>100
Linaria pedicellata Kuprian.	Aerial	DCM	>100	>100
Plantaginaceae		EtOH	>100	>100
		H_2O	>100	>100
Tragopogon ruber S.G. Gmel.	Aerial	DCM	>100	>100
Asteraceae		EtOH	>100	>100
		H_2O	>100	>100
	Roots	DCM	>100	>100
		EtOH	>100	>100
		H_2O	>100	>100
Verbascum orientalis	Aerial	DCM	>100	>100
Scrophulariaceae		EtOH	100	>100
		H_2O	>100	>100
Zygophyllum brachypterum Kar. et Kir.	Aerial	DCM	>100	>100
Zygophyllaceae		EtOH	>100	>100
		H_2O	>100	>100

[a] PE = petroleum ether; H:EtOAc:H_2O = hexane:ethyl acetate:H_2O, 54:44:2; DCM = methylene chloride; EtOH = ethanol. [b] Lowest-complete-inhibition concentration.

Table 2. Evaluation of column chromatography fractions from *I. helenium and L. myrianthum* for antialgal activity

Species	Plant part	Solvent	Fraction	LCIC [a] (µg/mL)	
				O. perornata	*S. capricornutum*
I. helenium	Roots	Hexane	A	>100	>100
			B	>100	>100
			C	10	10
			D	>100	>100
			E	>100	100
L. myrianthum	Aerial	DCM	A	100	>100
			B	10	>100
			C	10	>100
			D	100	100
			E	100	100
			F	>100	>100
			G	>100	>100

[a] Lowest-complete-inhibition concentration.

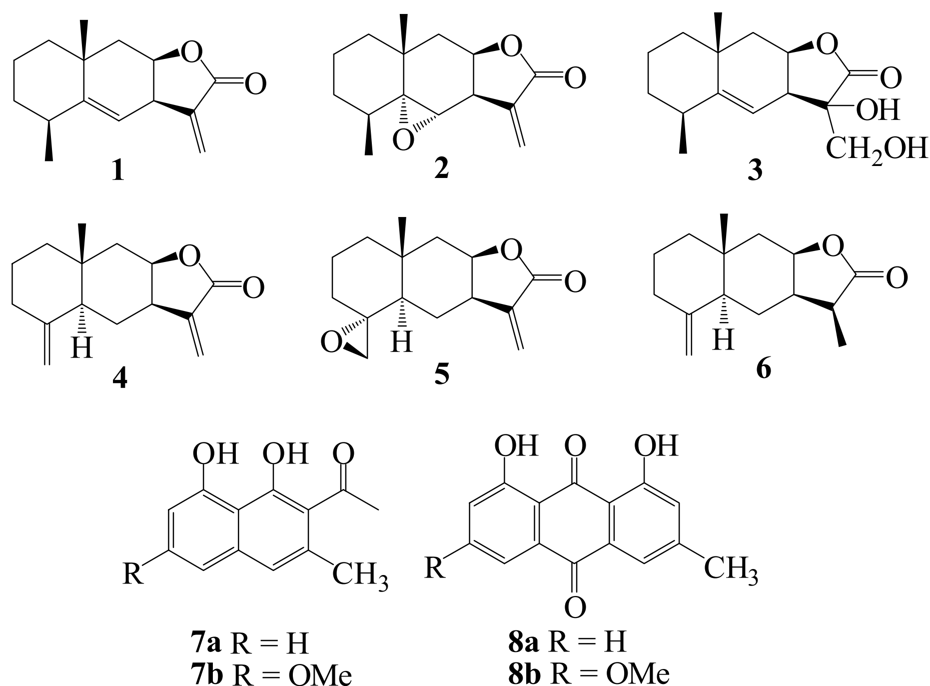

Figure 1. Isolated and semi-synthetic compounds from *L. myrianthum* and *I. helenium*.

Table 3. Evaluation of pure compounds isolated from *I. helenium* and *L. myrianthum* for antialgal activity

Source	Compound	*O. perornata*		*S. capricornutum*	
		LOEC[a] (µg/mL)	LCIC[b] (µg/mL)	LOEC[a] (µg/mL)	LCIC[b] (µg/mL)
I. helenium	alantolactone (**1**)	>100	>100	>100	>100
	isoalantolactone (**4**)	100	100	100	100
	11αH,13-dihydroisoalanto-lactone (**6**)	100	100	100	100
L. myrianthum	nepodin (**7a**)	10	100	100	>100
	torachrysone (**7b**)	100	100	100	>100
	chrysophanol (**8a**)	10	10	>100	>100
	physcion (**8b**)	>100	>100	>100	>100
Synthetics	11,13-dihydroxyalantolactone (**3**)	>100	>100	>100	>100
	5α-epoxyalantolactone (**2**)	100	100	100	100
	4(15)α-epoxyisoalantolactone (**5**)	100	100	100	100

[a] LOEC = Lowest-observed-effect concentration.
[b] LCIC = Lowest-complete-inhibition concentration.

Previously, various anthraquinones have been found to be moderately to highly toxic towards *O. perornata*.[11,12] Schrader reported that chrysophanol was toxic towards *O. perornata* with a LCIC of 100 µM (24 µg/mL), but toxicity was not observed at 10 µM (2.4 µg/mL).[12] The moderate toxicity of chrysophanol is less than other anthraquinone-based compounds that have undergone efficacy testing in catfish ponds. Several water-soluble anthraquinone-based compounds evaluated by Schrader et al. have LCIC values of 0.1 µM (below 1.0 µg/mL).[11] Therefore, chrysophanol is not as preferable as these other anthraquinone-based compounds for use as a selective algaecide in catfish aquaculture due to the greater amount of compound that would be required for reducing the abundance of *O. perornata* in catfish ponds, thereby resulting higher economic costs to catfish producers. Other considerations before pursuing efficacy testing in catfish aquaculture ponds of compounds such as chrysophanol include the development of a formulation or modification of the chemistry of the compound to impart water solubility and the determination of any potential mutagenic and/or antibiotic properties or other unfavorable characteristics for a compound being considered for use as an algaecide in food-fish production ponds.

In summary, extracts from *Inula helenium* and *Limonium myrianthum* have yielded anti-algal compounds. We continue to evaluate extracts from plants obtained from Kazakhstan to discover selective algaecides to help manage musty off-flavor in pond-raised channel catfish.

Experimental Section

General Procedures. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 400 MHz spectrometer (Billerica, Massachusetts, USA). All ^{13}C multiplicities deduced from 90º and 135º DEPT experiments. Column chromatography was performed using a Biotage, Inc. (Charlottesville, Virginia, USA) Horizon™ Pump equipped with a Horizon™ Flash Collector and fixed wavelength (254 nm) detector. High-resolution mass spectra were obtained using an Agilent 1100 HPLC coupled to a JEOL AccuTOF (JMS-T100LC) (Peabody, Massachusetts, USA). HPLC method development work was performed using an Agilent 1100 system equipped with a quartenary pump, autosampler, diode-array detector, and vacuum degasser. Semi-preparative HPLC purifications were performed using a Waters Delta-Prep system (Milford, Massachusetts, USA) equipped with a diode-array detector and a binary pump.

GC-MS analysis. Isolated and synthetic compounds were analyzed by GC-MS on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS. GC was equipped with a DB-5 column (30 m x 0.25 mm fused silica capillary column, film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240 ºC; column temperature, 60-240 ºC at 3 ºC/min then held at 240 ºC for 5 min; carrier gas, He; injection volume, 1 μL (splitless). MS ionization energy was set to 70 eV.

High-resolution LC-MS analysis. All isolated compounds were prepared in methanol and injected directly into a 0.3 mL/min stream of a 20% H_2O/80% MeOH solution containing 1 μg/mL *L*-tryptophan. Mass drift compensations were performed relative to PEG $[M + Na]^+$ adducts for positive ion analysis while mass drift compensations were performed relative to *L*-tryptophan $[M + H]^+$ and/or $[2M + H]^+$ ions for negative ion analysis.

Plant material. Roots of *I. helenium* L. were collected on June 25, 1995, and obtained from Mr. George Sturtz of Aromagen, 31787 Peoria Road, Albany, Oregon 97321, USA. A voucher (Sturtz-Fischer No. 570) is deposited at the Louisiana State University Herbarium in Baton Rouge, Louisiana, USA.

Roots and aerial parts of *L. myrianthum* were collected on June 19, 2004, at the southern shoreline of Kapchagai reservoir, where representatives of Chenopodiaceae family dominated. Accompanying species were *Ferula ovina, Nitraria schobertii, Artemisia species, Atriplex hastate, Petrosimonia brachiata,* and *Bassia seoloides.* A voucher (6351/25-1960) is deposited at the Institute of Botany and Phytointroduction Herbarium, Almaty, Kazakhstan.

All other plant material was collected in a manner similar to that described above for *L. myrianthum*. Voucher specimens for all other plants have been deposited in the Institute of Botany and Phytointroduction Herbarium, Almaty, Kazakhstan.

Crude extractions. Small pieces of fresh roots (4.5 kg) of *I. helenium* were dried at room temperature for two weeks and then soaked in hexane (8.5 L) for 24 hours. The solvent was decanted from the plant residue and evaporated *in vacuo* to yield 15.2 g of crude extract. The residual plant material was re-soaked for 24 hours in CH_2Cl_2 (7.8 L) and subsequently extracted for 24 hours in MeOH (7.8 L), yielding 23.3 g and 85.2 g, respectively.

Aerial parts (0.5 kg) were air-dried followed by grinding in a Willey-Mill plant grinder. Ground plant material was extracted at room temperature using 2.3 L of CH_2Cl_2 providing 7.4 g of extractables after evaporation of solvent. Dried marc was subsequently extracted using 2.2 L of ethanol (95%) providing 11.7 g of extractables following evaporation of solvents. Lastly, extraction with H_2O provided 20.7 g of extractables after lyophilization to remove H_2O. All other plant material has been extracted in a manner similar to that described above for *L. myrianthum*.

Isolation and identification of *Inula helenium* compounds. A portion of the *I. helenium* hexane roots extract (1.503 g) was adsorbed to silica gel and applied to a silica gel chromatography column (40-63 μm, 40 x 150 mm, 60 Å). Elution of the column was performed using increasing polarity mixtures of hexane/EtOAc in a series of three linear gradient steps. Step 1 consisted of 100/0 to 90/10 using 1599 mL with step 2 consisting of 90/10 to 75/25 using 399 mL. Step 3 consisted of 75/25 to 0/100 using 399 mL and the column was washed with 1152 mL of EtOAc. Column eluate was collected in 24-mL test tubes and, based on TLC similarities, recombined into 5 fractions [A, 1-74, 23 mg; B, 75-83, 32 mg; C, 84-96, 1.176 g; D, 97-108, 223 mg; E, 109-150, 48 mg]. Further purification of the bioactive fraction C was accomplished using repeated VLC procedures on silica gel, as described previously,[14,15] to yield pure compounds **1**, **4**, and **6**. Identification was accomplished by comparison of spectroscopic data with that reported in the literature.[14,15]

Derivatization of *I. helenium* compounds. Compounds **1** and **4** were converted to their respective mono-epoxide isomers 5α-epoxyalantolactone (**2**) and 4(15)α-epoxyisoalantolactone (**5**) using previously described methods.[9,16] Similarly, compound **1** was oxidized to its respective diol, 11,13-dihydroxyalantolactone (**3**), using previously described methods and procedures.[9] Identification of all compounds was performed by comparison of spectral data with that reported in the literature.[9,16]

Isolation and identification of *Limonium myrianthum* compounds. A portion of the *L. myrianthum* CH_2Cl_2 roots extract (3.010 g) was adsorbed to silica gel and applied to a silica gel chromatography column (40-63 μm, 40 x 150 mm, 60 Å). Elution of the column was performed using increasing polarity mixtures of hexane/EtOAc in a series of three linear gradient steps. Step 1 consisted of 100/0 to 90/10 using 1152 mL with step 2 consisting of 90/10 to 50/50 using 801 mL. Step 3 consisted of 50/50 to 0/100 using 399 mL and the column was washed with 398 mL of MeOH. Column eluate was collected in 24-mL test tubes and based on TLC similarities, recombined into 7 fractions [A, 1-8, 152 mg; B, 9-24, 686 mg; C, 25-60, 26 mg; D, 61-69, 776 mg; E, 70-73, 153 mg; F, 74-100, 574 mg; G, MeOH wash, 153 mg].

Fraction B (220 mg) was further purified using HPLC utilizing an Agilent Zorbax SB-C18 column (21.2 x 250 mm, 7 μm). Mobile phase consisted of an isocratic solvent system (75% acetonitrile (ACN)/25% water containing 0.1% trifluoroacetic acid) flowing at 20 mL/min for 20 minutes. A total of four peaks [**7a** (3.80 min); **7b** (4.53 min); **8a** (6.67 min), and **8b** (7.79 min)] were collected while monitoring at 254 nm and making multiple injections. Solvent was removed by rotary evaporation followed by lyophilization to remove water resulting in 33 mg of **7a**, 9 mg

of **7b**, 62 mg of **8a**, and 31 mg of **8b**. Fraction C was also analyzed as described above for fraction B revealing the presence of compounds **7a**, **7b**, **8a**, and **8b**.

Nepodin (7a). GC-EI-MS *m/z* 216.0 (100), 201 (39); ^{1}H (400 MHz)[6] and ^{13}C NMR (100 MHz)[7] data ($CDCl_3$) were in complete agreement with that which has been reported previously.

Torachrysone (7b). High resolution positive ion ESI-MS *m/z* 247.0955 $[M + H]^+$, calculated for $C_{14}H_{15}O_4$, 247.0970. ^{1}H NMR (400 MHz in $CDCl_3$) data were in complete agreement with that which has been reported previously.[8]

Chrysophanol (8a). GC-EI-MS *m/z* 254.0 (100), 226 (12); ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data ($CDCl_3$) were in complete agreement with that which has been reported previously.[9]

Physcion (8b). GC-EI-MS *m/z* 284.0 (100), 255 (10); ^{1}H NMR (400 MHz in $CDCl_3$) data were in complete agreement with that which has been reported previously.[9]

Anti-algal bioassay experimental. An isolate of *O. perornata* was obtained from water samples collected from Mississippi catfish ponds.[17] The green alga *Selenastrum capricornutum* was obtained from Dr. J.C. Greene, United States Environmental Protection Agency, Corvallis, Oregon, USA, and was used as a representative of green algae in the bioassay to determine selective toxicity of extracts, fractions, and pure compounds. In order to provide a source of cells growing at a fairly constant rate for the bioassay, cultures of each microorganism were maintained in continuous culture systems using the conditions of Schrader et al.[18]

The same procedures outlined by Cantrell et al. were used to conduct the rapid bioassay.[5] Reusable 96-well quartz microplates (Hellma Cells, Inc., Forest Hills, New York, USA) were used for samples and compounds that were not soluble in water, methanol, or ethanol. Polystyrene microplates (Corning Inc., Corning, New York, USA) were used to test extracts, fractions, and pure compounds that were soluble in water, methanol, or ethanol. Copper Control, a chelated-copper compound produced by Argent Chemical Laboratories (Redmond, Washington, USA), was used for a positive control.

Anti-algal bioassay data analysis. Mean values and standard deviations of absorbance measurements were calculated and graphed to determine the lowest-observed-effect concentration (LOEC) and lowest-complete-inhibition concentration (LCIC). Crude extracts with LCIC values of 10 µg/mL or less for *O. perornata* and with at least 10X higher LCIC values for *S. capricornutum*, thereby indicating selective toxicity towards *O. perornata*, were considered to be suitable for pursuing bioassay-guided isolation of active constituents. Fractions with LCIC values of 10 µg/mL or less for *O. perornata* were considered to be suitable for pursuing bioassay-guided isolation of active constituents. Pure compounds with LCIC values of 10 µg/mL or less for *O. perornata* and with at least 10X higher LCIC values for *S. capricornutum* were considered to be active compounds.

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