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Allelopathic effects of *Padina* sp. and *Sargassum* sp. as biological control agents of harmful algal blooms (HABs) in the Persian Gulf and Gulf of Oman

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Harmful algal blooms (HABs) pose serious threats to coastal ecosystems, public health, and local economies. This study explores the potential of two native macroalgal genera, *Padina* sp. and *Sargassum* sp., collected from the Persian Gulf (Bandar Abbas) and the Gulf of Oman (Chabahar), as biological control agents against *Cochlodinium polykrikoides*. Species identification was achieved through morphological observations and DNA barcoding (rbcL and psbA), ensuring accurate classification despite overlapping traits. Methanolic extracts of *Padina* (sp. A and sp. C) and *Sargassum* (sp. B and sp. D) strains inhibited *C. polykrikoides*, with *Padina* sp. A showing the strongest activity ($LC_{50} = 122.2 \mu\text{g/mL}$) and *Sargassum* sp. D the weakest ($LC_{50} = 371.2 \mu\text{g/mL}$). Fractionation and identification of the most active extracts using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) revealed the presence of terpenoid and phenolic functional groups in the active fractions of *Padina* sp. A and *Sargassum* sp. B, respectively. These extracts exhibited negligible effects on non-target microalgae (*Chlorella vulgaris*, *Isochrysis galbana*) at concentrations $> 1 \text{ mg/mL}$, shrimp larvae (*Litopenaeus vannamei*), and human endothelial cells (HUVEC), highlighting their selectivity and safety. These findings demonstrate that *Padina* and *Sargassum* produce bioactive compounds capable of selectively suppressing harmful microalgae, underscoring their promise as environmentally compatible agents for managing HABs in marine and aquaculture systems.

Keywords Harmful algal blooms, Biological control, Allelopathic macroalgae, *Padina* sp., *Sargassum* sp., *Cochlodinium polykrikoides*

Harmful algal blooms (HABs) continue to pose a persistent and increasingly complex problem for marine and coastal ecosystems worldwide. These events occur when certain phytoplankton or cyanobacterial groups proliferate rapidly under favorable environmental conditions, often resulting in visible water discoloration, including the well-known “red tides.” Their impacts extend far beyond their appearance. HABs can deplete dissolved oxygen, cause large-scale mortality of fish and invertebrates, create strong nuisance odors, and degrade both ecological processes and coastal water quality¹. Although harmful species represent only a small portion of known microalgal diversity, their ecological, economic, and public-health effects are disproportionately severe. Of roughly 5,000 described marine microalgal species, about 300 are associated with water discoloration, and approximately 75 have been confirmed toxin producers (see e.g. <http://www.marinespecies.org/hab/index.php>). Among these, the dinoflagellate *Cochlodinium polykrikoides* has emerged as one of the most destructive bloom-forming species in tropical and subtropical waters. This strain is characterized by chain-forming cells with distinctive morphology, rapid bloom initiation, and the ability to persist under a wide range of salinity and temperature conditions². Unlike many toxin-producing microalgae, *C. polykrikoides* exerts its harmful

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effects primarily through the production of reactive oxygen species (ROS) and hemolytic compounds, which damage gill tissues and lead to massive fish kills. Blooms of this species are often associated with hypoxia, altered microbial community dynamics, and cascading impacts on benthic biodiversity^{3,4}. Its ecological plasticity and capacity for wide dispersal have facilitated its expansion into new geographic regions, including the Persian Gulf and Gulf of Oman, where recurrent outbreaks have been documented.

The severe bloom of *C. polykrikoides* in 2008–2009 across the Persian Gulf and Gulf of Oman exemplifies its destructive potential. This event caused extensive fish mortalities, coral degradation, and major disruptions to desalination operations in Iran, Saudi Arabia, and the United Arab Emirates^{5,6}. The strain's ability to form resting cysts further complicates management, as these cysts act as seed populations for future blooms, making eradication difficult and recurrence likely. Given its ecological resilience and socioeconomic impacts, *C. polykrikoides* is now considered a high-priority target for HAB mitigation research.

A range of mitigation strategies has been explored in response to these impacts, including chemical, physical, and biological interventions. Chemical treatments typically target algal cells directly, whereas physical methods—such as surface skimming, clay flocculation, or ultrasonic disruption—aim to remove or destabilize bloom biomass. Increasing attention, however, has turned to macroalgae as a biological control option because of their ecological compatibility and capacity to exert allelopathic or competitive pressure on harmful species. The effectiveness of macroalgal approaches depends strongly on species selection and local environmental conditions, emphasizing the need for careful experimental design to avoid unintended ecological consequences. Integrating macroalgal interventions with microbial consortia or physical removal strategies may yield synergistic benefits and enhance long-term sustainability⁷.

In this context, the present study investigates the capacity of native macroalgae and their associated microbial consortia to function as biological control agents against *C. polykrikoides* under controlled laboratory conditions. Our research focuses on evaluating the inhibitory activity of two ecologically abundant algal species, *Padina* sp. and *Sargassum* sp., which possess documented allelopathic potential and a history of bioactive compound production. This study aims to characterize the specificity of their inhibitory effects against non-target microalgae and to assess their biosafety for both shrimp larvae and human endothelial cell lines. In parallel, we characterize the metabolites associated with algicidal activity and consider how these results can inform the development of sustainable marine biotechnology applications and environmentally responsible HAB management strategies in the southern coastal waters of Iran.

Materials and methods

Sampling

Two native macroalgal genera, *Padina* and *Sargassum*, were collected from coastal sites in Bandar Abbas (Persian Gulf) and Chabahar (Gulf of Oman). Sampling took place during the winter–spring transition (February–April) in intertidal and shallow subtidal habitats (0–2 m depth). Specimens were collected in triplicate from spatially independent locations, with care taken to avoid areas influenced by pollution or other anthropogenic disturbances. Fresh material was rinsed with ambient seawater to remove debris and epiphytic organisms, placed in sterile polyethylene bags, and transported to the laboratory under cooled, dark conditions (4 ± 1 °C). All samples were then stored at -20 °C until further processing.

Preparation of algal samples

In the laboratory, macroalgal specimens were rinsed with 0.45- μ m filtered seawater to remove sediments and epiphytic organisms, followed by examination under a stereomicroscope to confirm the absence of surface contaminants. Only healthy thalli exhibiting intact morphology and normal pigmentation were retained. Cleaned samples were blotted dry and air-dried in the dark at ambient temperature (25 ± 2 °C) for 48–72 h. The dried material was then ground to a fine powder using a sterile mortar and pestle, transferred to airtight containers, and stored at -20 °C until extraction and subsequent biochemical analyses.

Morphological identification

Morphological identification was conducted following established taxonomic protocols and reference keys for tropical marine macroalgae⁸. Diagnostic features—including thallus architecture, blade morphology, branching patterns, pigmentation, surface texture, and reproductive structures were examined under a compound light microscope (40–100 $\times$ magnification). *Padina* species were identified based on characteristics such as concentric banding, calcified margins, and fan-shaped thalli, whereas *Sargassum* specimens were distinguished by vesicular air bladders, serrated or dentate blades, and well-developed receptacles. Taxonomic determinations were cross-validated using regional floras and photographic atlases. Voucher specimens were prepared for long-term archiving and deposited in the institutional herbarium.

Molecular identification

DNA barcoding complemented morphological observations. Total genomic DNA was extracted from ~ 100 mg dried tissue using a CTAB protocol optimized for polysaccharide- and polyphenol-rich macroalgae. Amplification targeted *rbcL*, *COI*, and *tufA* genes using genus-specific primers. PCR products were purified, sequenced bidirectionally, and chromatograms were edited and aligned using MEGA X with ClustalW. Species-level identity was confirmed via BLAST searches against GenBank and BOLD, using a $\geq 98\%$ sequence similarity threshold. Integrating molecular and morphological data provided a robust framework for accurate species identification, particularly in cases of morphological plasticity or cryptic diversity.

Amplification of *rbcl* and *psbA* genes

Amplification of the *rbcl* and *psbA* genes was performed using gene-specific primer sets: *rbcl*-F (5'-ATGTCA CCACAAACAGAGACTAAAGC-3') and *rbcl*-R (5'-TAAATCAAGTCCACCRG-3') for *rbcl*, and *psbA*-F1 (5'-GTGGCTGCTCACGGTTATTT-3') and *psbA*-R1 (5'-CCAAGCAGCCAAGAAGAAGT-3') for *psbA*. PCR reactions were prepared in 50 µL volumes containing 5 µL of 10× PCR buffer, 3 µL of MgCl₂ (4 mM final concentration), 5 µL of dNTPs (0.2 mM each), 1 µL of each primer (10 pmol), 0.3 µL of Taq DNA polymerase (1 U µL⁻¹), and approximately 1 µL of genomic DNA (~10 pg), with nuclease-free water added to achieve the final volume. Thermal cycling conditions consisted of an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for *rbcl* or 52 °C for *psbA* for 1 min, and extension at 72 °C for 2 min. A final extension step was carried out at 72 °C for 5 min. PCR amplicons were separated on 1.5% agarose gels stained with ethidium bromide and visualized using a gel documentation system. Purified PCR products were subsequently sequenced, and species identity was confirmed by comparison with reference sequences in publicly available databases^{9,10,11}.

Evaluation of anti-microalgal activity of macroalgae

The anti-microalgal activity of the macroalgal extracts was assessed using a stepwise screening framework that integrated both extract-based bioassays and direct co-culture experiments. This dual approach enabled the evaluation of inhibitory effects arising from soluble metabolites, as well as potential allelopathic interactions that occur during direct contact between macroalgae and the target microalgae^{12,13}.

Cultivation of *Cochlodinium polykrikoides*

Pure cultures of *C. polykrikoides* were obtained from the Aquaculture Division of the Persian Gulf and Gulf of Oman Ecology Research Center. Stock cultures were maintained in modified F2 medium¹⁴ at a salinity of 32 ppt. Cultures were incubated at 26 °C under a 12:12 h light–dark cycle using white fluorescent lamps for 12 days, with sampling conducted during the logarithmic growth phase^{15,16}.

Anti-microalgal activity of macroalgal extracts

Dried macroalgal samples were ground into a fine powder. For extraction, 100 g of algal powder was sonicated in 1 L of methanol for 20 min, vortexed for 30 min, and incubated at room temperature for 48 h. The extract was centrifuged at 1500 rpm for 10 min, filtered through Whatman No. 1 paper, and concentrated using a rotary evaporator. Serial dilutions of the crude extracts were prepared at 1000, 500, 250, 125, and 62.5 µg/mL. A volume of 100 µL from each concentration was added to 50 mL of *C. polykrikoides* suspension (5 × 10⁶ cells/mL). After 24 h of exposure, live cell densities were quantified by direct microscopic enumeration. The percentage inhibition of *C. polykrikoides* was calculated using the formula:

$$\% \text{Inhibition} = \frac{N_{\text{control}} - N_{\text{test}}}{N_{\text{control}}} \times 100$$

where N_{test} and N denote the cell concentrations in the treated and untreated cultures, respectively. Median lethal concentrations (LC₅₀) were subsequently estimated using GraphPad Prism 6, following the approach described by Gozari et al.¹⁷.

Direct co-culture assay

To assess potential allelopathic effects under direct-contact conditions, 5 g of purified macroalgal biomass was transferred into 250-mL Erlenmeyer flasks containing modified F2 medium (32 ppt) and inoculated with *C. polykrikoides* at an initial density of 1,000 cells mL⁻¹. Cultures were maintained for 15 days at 26 °C under a 12:12 h light–dark photoperiod, with gentle aeration (approximately 30 bubbles min⁻¹) to ensure adequate mixing without causing mechanical stress. Cell concentrations were quantified every 48 h using a Sedgewick–Rafter counting chamber, and growth inhibition was calculated as previously described. Flasks lacking macroalgal biomass served as experimental controls, and all treatments were conducted in triplicate to ensure statistical robustness^{18,19,20}.

Spectrum of bioactivity

To assess the selectivity and potential ecological risks of the macroalgal extracts, their activity was also evaluated against beneficial microalgae, including *Chlorella vulgaris* and *Isochrysis galbana*. Extracts were applied at the same concentration range used for *C. polykrikoides* (62.5–1000 µg/mL) to 50 mL suspensions of each microalga (5 × 10⁶ cells mL⁻¹). After 24 h of exposure, live cell densities were determined, and inhibition percentages and LC₅₀ values were calculated following the procedures described for *C. polykrikoides*¹⁷.

Bioactivity and toxicity assessment of macroalgal extracts

Preparation of solutions and dilutions

After extraction and complete evaporation of methanol under a gentle nitrogen stream, the dry residue was dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solution. Then, the required dilutions (1000, 500, 250, 125, and 62.5 µg/mL) were prepared using modified F2 culture medium. The final concentration of DMSO in all treatments was kept below 0.1%. Experimental controls included samples without extract and samples containing the same amount of DMSO^{21,22}.

Controls

To validate the bioassay results, three types of controls were included in all experiments: untreated cultures containing only microalgal cells in growth medium, solvent controls containing cells plus the same amount of DMSO used for dilutions but without macroalgal extract, and distilled water controls containing cells with the addition of water only. These controls ensured that any observed effects could be attributed specifically to the macroalgal extracts rather than to the solvent or diluent.

Activity against beneficial microalgae

To evaluate the selectivity of the macroalgal extracts, bioassays were conducted using *Chlorella vulgaris* and *Isochrysis galbana* as model non-target microalgae.

These two species were chosen because they are widely recognized as beneficial microalgae in aquaculture and marine ecosystems: *C. vulgaris* is commonly used as a nutritional supplement and bioresource due to its high protein and lipid content, while *I. galbana* is an important feed organism for bivalve larvae and shrimp hatcheries. Their ecological and economic relevance makes them suitable indicators for assessing potential non-target impacts of algicidal compounds.

Extracts were prepared at concentrations of 1000, 500, 250, 125, and 62.5 µg/mL, and 100 µL of each concentration was added to 50 mL algal suspensions containing 5×10^6 cells/mL. Cultures were incubated under standard growth conditions for 24 h, after which live cell densities were determined. The percentage inhibition was calculated following established protocols, and median lethal concentrations (LC₅₀) were estimated using GraphPad Prism 6¹⁷, providing insights into extract potency and potential ecological impacts.

Toxicity to shrimp larvae

To assess potential non-target effects on aquatic organisms, toxicity assays were performed using post-larvae (PL9) of *Litopenaeus vannamei*. Twenty-five larvae were stocked in 5 L tanks containing 2 L of seawater, and macroalgal extracts were added at the same concentration range used for microalgae. Mortality was monitored daily over six days, and cytotoxicity was calculated using:

$$\% \text{Cytotoxicity} = \frac{N_{\text{control}} - N_{\text{test}}}{N_{\text{control}}} \times 100$$

Where N_{test} and N_{control} represent the number of live larvae in treated and untreated tanks, respectively. Each treatment was performed in triplicate¹⁷.

Cytotoxicity against human cells

Biosafety was further evaluated using human umbilical vein endothelial cells (HUVEC; C554) obtained from the Pasteur Institute of Iran. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 IU/mL penicillin, and maintained at 37 °C under 5% CO₂. Cytotoxicity was assessed using the MTT assay, which measures mitochondrial dehydrogenase activity in viable cells. Treated and control cells were incubated with MTT solution, allowing viable cells to reduce MTT into purple formazan crystals. Cytotoxicity was calculated as:

$$\% \text{Cytotoxicity} = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

Where A_{test} is the absorbance of treated wells, A_{control} of untreated wells, and A_{blank} of medium-only wells.

Purification of algicidal compounds

The chemical composition of the bioactive extracts was investigated using thin-layer chromatography (TLC). Extracts were applied to fluorescent silica gel plates and developed in solvent systems comprising various ratios of n-hexane, chloroform, and methanol. Following development, plates were examined under ultraviolet (UV) light at 254 nm, and the positions of separated compounds were recorded as retention factor (Rf) values. For chemical screening, TLC plates were treated with chromogenic reagents including anisaldehyde, ninhydrin, vanillin, and ferric chloride (FeCl₃) in both aqueous and acidic forms to identify functional groups. The composition of these reagents and their target functional groups are summarized in Tables 1, 2, 3 and 4²³. This approach allowed the characterization and tentative identification of the chemical classes present in the macroalgal extracts.

Macroalgal Strain	LC ₅₀ ± SE (µg/ml)	95% Confidence Interval (µg/ml)
<i>Padina</i> sp. A	122.2 ± 0.17	111–134.5
<i>Padina</i> sp. C	228.5 ± 0.098	217.3–240.4
<i>Sargassum</i> sp. B	182.6 ± 3.58	169.3–196.9
<i>Sargassum</i> sp. D	371.2 ± 11.74	313.8–439.1

Table 1. Assessment of anti-algal activity of macroalgal extracts against the microalga *Cochlodinium polykrikoides*.

Macroalgal Strain	LC ₅₀ (µg/ml) – <i>Chlorella vulgaris</i>	LC ₅₀ (µg/ml) – <i>Isochrysis galbana</i>
<i>Padina</i> sp. A	> 5000	> 2500
<i>Padina</i> sp. C	> 5000	> 2500
<i>Sargassum</i> sp. B	> 5000	> 5000
<i>Sargassum</i> sp. D	> 5000	> 5000

Table 2. LC₅₀ values of anti-algal activity of tested macroalgal extracts against beneficial microalgal species.

Macroalgal Strain	LC ₅₀ (µg/ml) – Shrimp Larvae
<i>Padina</i> sp. A	> 2500
<i>Padina</i> sp. C	> 5000
<i>Sargassum</i> sp. B	> 5000
<i>Sargassum</i> sp. D	> 5000

Table 3. LC₅₀ values of macroalgal extract toxicity against Shrimp Larvae.

Seaweed Extract	IC ₅₀ (µg/ml)	Standard Error (SE)	95% Confidence Interval (µg/ml)
<i>Padina</i> sp. A	9.27	± 3.671	8.728–9.613
<i>Padina</i> sp. C	9.65	± 3.934	10.004–10.865
<i>Sargassum</i> sp. B	74.17	± 12.28	13.85–107.1
<i>Sargassum</i> sp. D	95.70	± 13.53	15.54–115.1

Table 4. Determination of the IC₅₀ of the cytotoxic activity of organic algal extracts against the HUVEC cell line.

High-performance liquid chromatography

Purification of bioactive compounds from crude macroalgal extracts was achieved using High-Performance Liquid Chromatography (HPLC) equipped with a C18 reverse-phase column. A 10 µL aliquot of each extract was injected, and separation was performed using gradient elution with solvent systems comprising water, methanol, and water: acetonitrile mixtures. Detection was carried out at multiple wavelengths (210, 230, 254, and 320 nm) to encompass a broad range of metabolite classes, with a flow rate of 1 mL/min maintained²⁴.

Statistical analyses

All experimental data from anti-microbial, toxicity, and cytotoxicity assays are presented as mean ± standard deviation (SD). Dose–response relationships were evaluated across multiple concentrations, and inhibition efficiencies were calculated accordingly. Statistical analyses were performed using GraphPad Prism 6. Differences between treated and control groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, with significance accepted at $P < 0.05$. Anti-microbial activity is reported as LC₅₀ ± standard error (SE), while cytotoxicity on HUVEC cells is expressed as IC₅₀ ± SE. All bioassays were conducted in triplicate to ensure reproducibility and statistical reliability.

Results

Identification of the studied macroalgae

Morphological observations indicated that samples A and C belonged to the genus *Padina*, characterized by erect, flattened thalli with a parenchymatous structure and terminal growth. Samples B and D were assigned to *Sargassum*, exhibiting a discoid holdfast, short stipe, and branched axes bearing leaf-like appendages and receptacles. Detailed morphological features are provided in Figs. 1 and 2.

To confirm taxonomic identity, molecular analyses were performed using rbcL (*Padina*) and psbA (*Sargassum*) gene markers. Phylogenetic analysis revealed that *Padina* sp. A showed high similarity (98.86%) to *Padina australis*, confirming genus-level classification (Supplementary data).

Anti-microbial activity of macroalgal extracts

Evaluation of the algal extracts revealed that all four tested samples exhibited inhibitory effects against the microalga *Cochlodinium polykrikoides* at varying concentrations. The dose–response curves demonstrated that the anti-algal activity of the extracts was concentration-dependent and followed a typical dose–response pattern (Fig. 3). Determination of LC₅₀ values showed that the methanolic extract from *Padina* sp. A achieved 50% inhibition of algal cells at a concentration of 122.2 ± 0.17 µg/ml. This was followed by *Sargassum* sp. B, which exhibited an LC₅₀ of 182.6 ± 3.58 µg/ml. The LC₅₀ values for *Padina* sp. C and *Sargassum* sp. D. were recorded as 228.5 ± 0.098 µg/ml and 371.2 ± 11.74 µg/ml, respectively. All LC₅₀ values were calculated at a 95% confidence interval (Table 1).

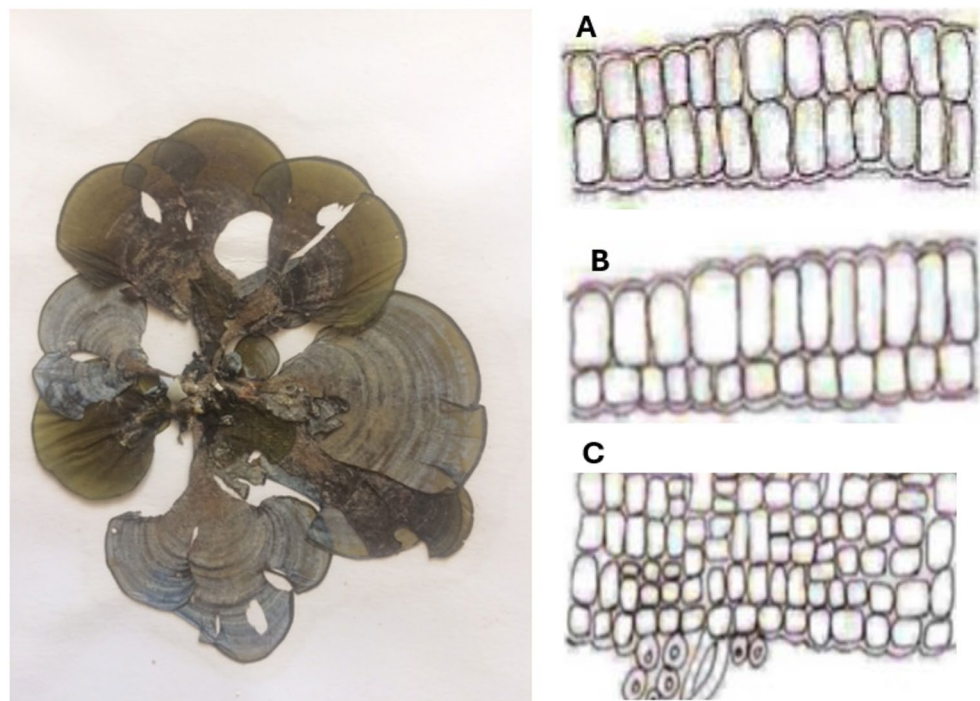


Fig. 1. Morphology of the alga *Padina* sp. (A) Cross-section of the upper layers; (B) Cross-section of the middle layer; (C) Cross-section of the cell layers adjacent to the rhizoids. Scale bar: 300 μ m (Herbarium code: 2487).

Anti-microbial activity of macroalgae in direct co-culture

The evaluation of macroalgal activity against *Cochlodinium polykrikoides* in direct co-culture revealed that significant algicidal effects first occurred between days 4 and 6 (Figs. 4 & 5). Analysis of the algicidal activity curve for *Padina* sp. A confirmed that by day 8, this strain had eliminated over 60% of *C. polykrikoides* cells. By day 10, it had completely eradicated all microalgal cells in the culture, demonstrating 100% algicidal activity. Similarly, *Padina* sp. C showed 55% inhibition by day 8 and reached full algicidal activity by day 12 (Fig. 4). In contrast, *Sargassum* sp. B exhibited a slower response, eliminating only 65% of microalgal cells by day 10 and achieving complete inhibition by day 14 (Fig. 4). The algicidal activity of *Sargassum* sp. D began to intensify from day 10 onward, culminating in 100% inhibition of *C. polykrikoides* by day 14 (Fig. 4). Microscopic observations provided valuable evidence supporting the algicidal progression. Recorded images captured the dominant morphology of *C. polykrikoides* cells at various time points, and these visual assessments corroborated the cell density-based inhibition curves.

Spectrum of macroalgal effects

Anti-microbial activity of macroalgae against beneficial microalgae

The algicidal activity of macroalgal extracts—previously shown to be effective against *Cochlodinium polykrikoides* was evaluated against beneficial microalgae, including *Isochrysis galbana* and *Chlorella vulgaris* (Fig. 6). The results indicated minimal cytotoxicity of the tested extracts toward these beneficial species. All extracts demonstrated algicidal effects against *Chlorella vulgaris* only at concentrations exceeding 5000 μ g/ml. The algicidal activity of *Padina* sp. extracts against *Isochrysis galbana* was observed at concentrations above 2500 μ g/ml, while *Sargassum* sp. B required concentrations exceeding 5000 μ g/ml to exhibit similar effects (Table 2).

Toxicity of extracted macroalgal extracts against shrimp larvae

The toxicity of the tested macroalgal extracts was evaluated against shrimp larvae. The results indicated that the extract from *Padina* sp. A exhibited no toxicity at concentrations below 2500 μ g/ml. In contrast, the extracts from *Padina* sp. C, *Sargassum* sp. B, and *Sargassum* sp. D showed no observable toxicity at concentrations below 5000 μ g/ml (Table 3). These cytotoxic activities were significantly lower than cytotoxicity doses against *C. polykrikoides* (p -value < 0.05).

Cytotoxic activity against human cells

Given the importance of biocontrol agents in relation to human cell safety, the cytotoxic effects of the extracted macroalgal compounds were evaluated against the human umbilical vein endothelial cell line (HUVEC) (Figs. 7 and 8). The results indicated that the macroalgal extracts exhibited low cytotoxicity toward human cells. The methanolic extract of *Padina* sp. A showed concentration-dependent cytotoxicity, with an IC_{50} value of 476 ± 24.77 μ g/ml against HUVEC cells. Similarly, the extract from *Padina* sp. C demonstrated an IC_{50} of



Fig. 2. Morphology of the studied *Sargassum* alga (Herbarium code: 2483).

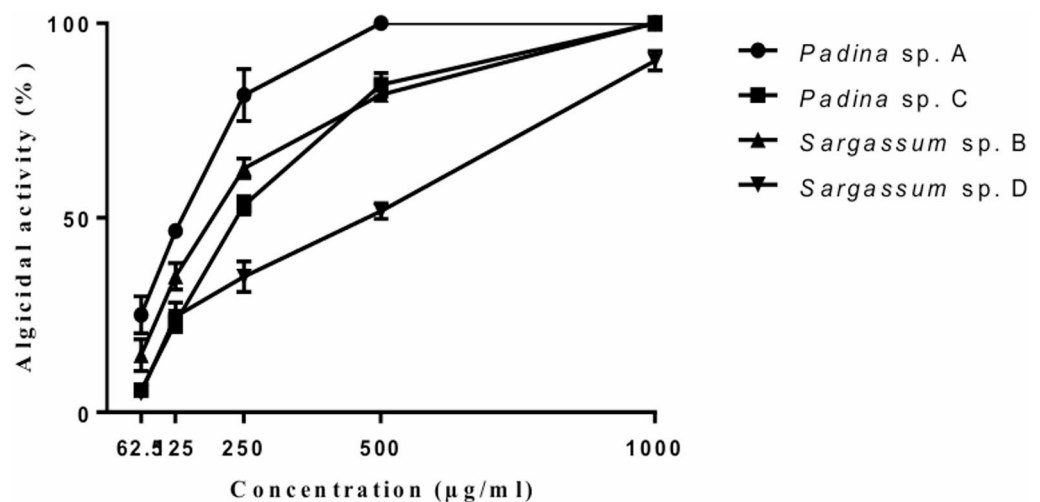


Fig. 3. Trend of anti-algal activity of macroalgal extracts against the microalga *Cochlodinium polykrikoides*.

476 ± 24.77 µg/ml. In contrast, *Sargassum* sp. B exhibited lower cytotoxicity, with an IC₅₀ of 672 ± 73.27 µg/ml, and *Sargassum* sp. D showed comparable low toxicity with an IC₅₀ of 672 ± 73.27 µg/ml (Table 4). Microscopic examination of treated versus untreated cells confirmed a noticeable reduction in viable cell count (p-value < 0.05) and morphological alterations, supporting the quantitative findings.

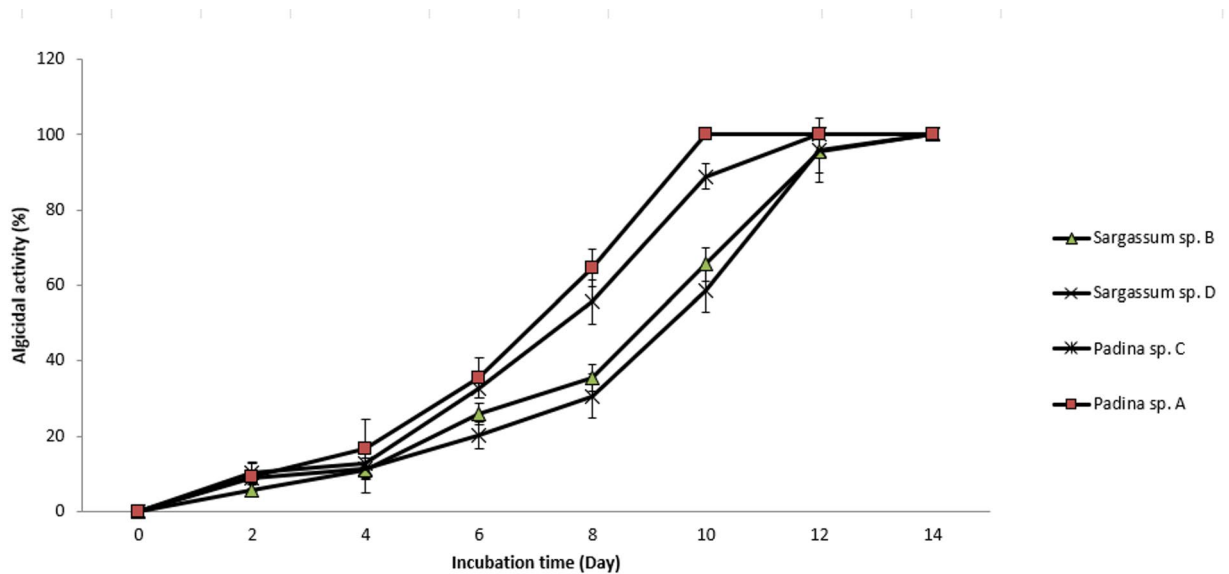


Fig. 4. Morphological progression of algal activity of macroalgal extracts against *Cochlodinium polykrikoides*. representative micrographs showing time-dependent cellular alterations induced by *Padina* sp. A, *Padina* sp. C, *Sargassum* sp. B, and *Sargassum* sp. D (rows). Columns indicate time points after inoculation: (A) 1 h; (B–D) subsequent days, depending on the strain—Days 4, 6, and 8 for *Padina* spp.; Days 6, 8, and 10 for *Sargassum* sp. B; and Days 6, 8, and 12 for *Sargassum* sp. D. Progressive morphological changes, including cell deformation, structural damage, and eventual lysis of *C. polykrikoides* cells, are observed over time.

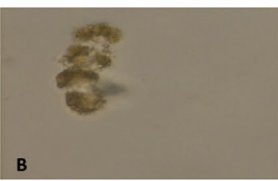
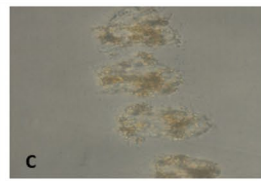
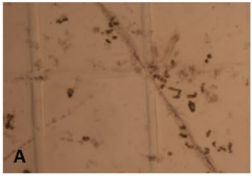
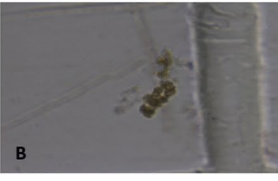
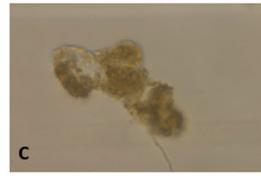
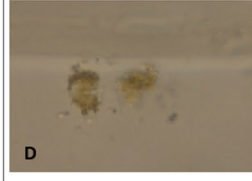
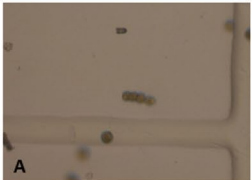
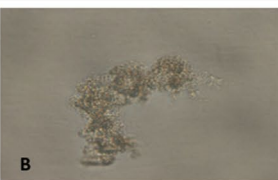
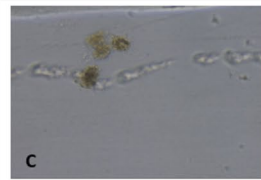
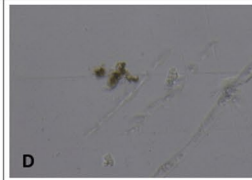
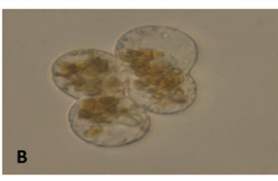
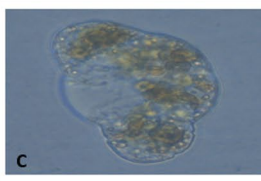
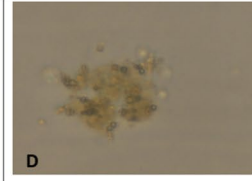
	1h	Day4	Day6	Day8
Padina sp. A				
Padina sp. C				
	1h	Day6	Day8	Day10
Sargasum sp. B				
Sargasum sp. D				

Fig. 5. Time-course of anti-algal activity of studied macroalgal strains against *Cochlodinium polykrikoides* in co-culture.

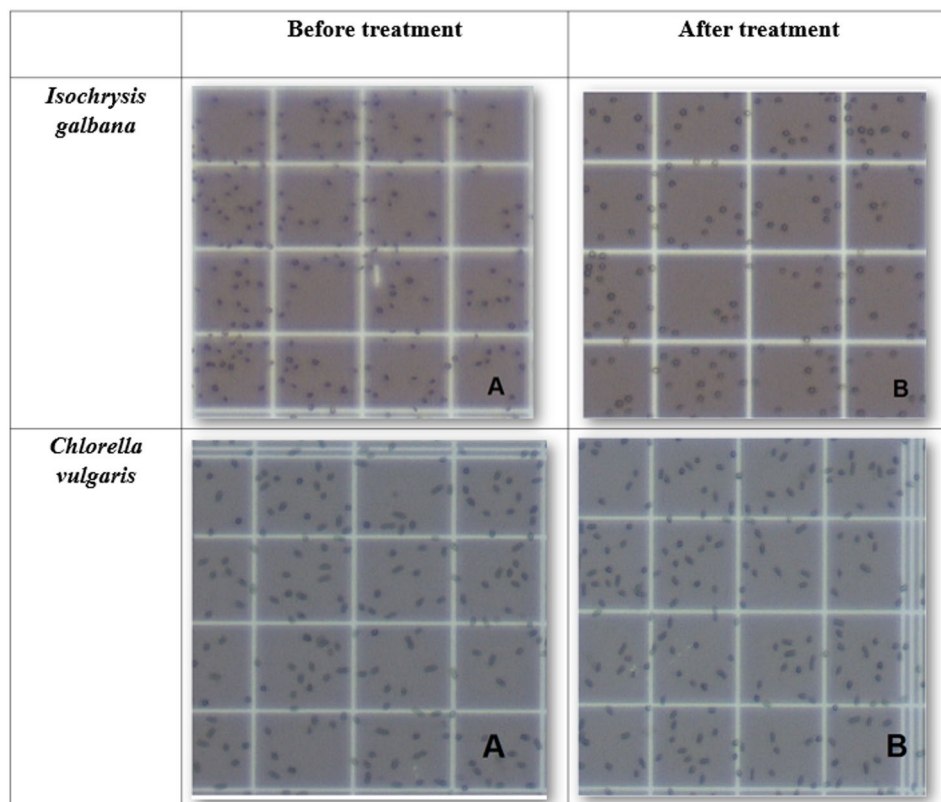


Fig. 6. Evaluation of algicidal activity of macroalgal extracts against the microalga *Isochrysis galbana* and *Chlorella vulgaris*. **A:** Cell density of *I. galbana* and *C. vulgaris* before treatment **B:** Cell density of *I. galbana* and *C. vulgaris* after treatment (magnification $\times 100$).

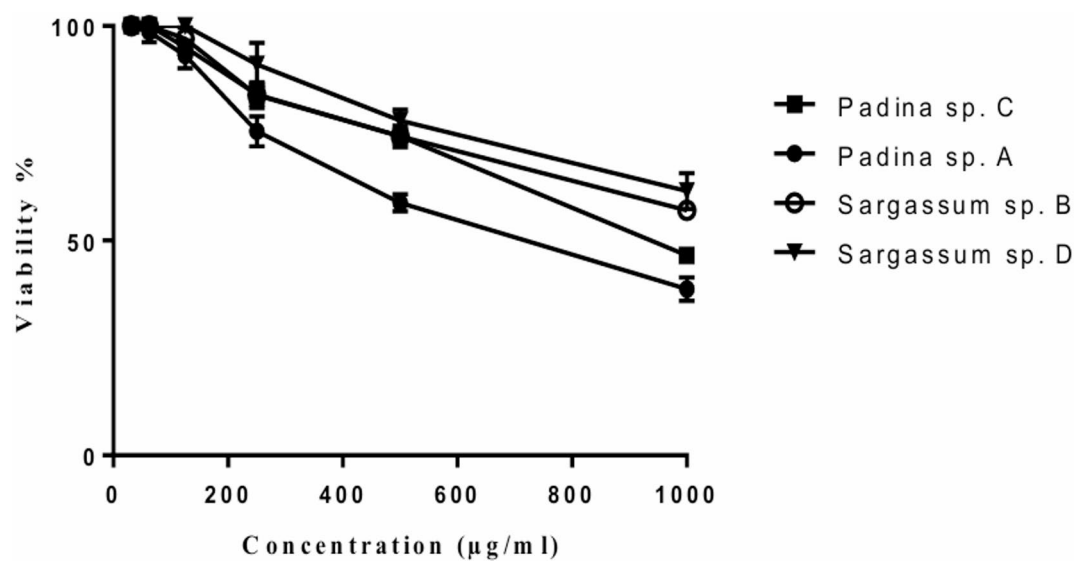


Fig. 7. Cytotoxic activity profile of macroalgal extracts against hUVEC cell line.

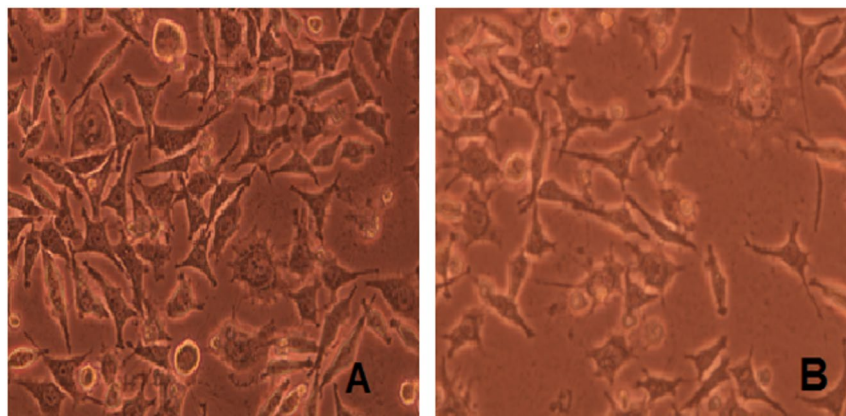


Fig. 8. Cytotoxic effects of algal extracts on the morphology and density of the HUVEC cell line (A) before and (B) after treatment. The micrographs indicate that the macroalgal extracts exhibited low cytotoxicity toward human cells.

This figure illustrates the dose-dependent cytotoxic effects of organic macroalgal extracts on human umbilical vein endothelial cells (HUVEC), highlighting the IC_{50} values and the morphological changes observed under microscopy.

Discussion

Harmful algal blooms (HABs) have shown a growing trend in recent years, largely driven by increasing marine pollution and climate change. This has highlighted the urgent need for effective strategies to prevent and mitigate their impacts. One innovative approach is Integrated Harmful Algal Bloom Management (IHABM), which combines satellite monitoring, field surveillance, and various control measures. Within this framework, biological control plays a central role, though it must be applied cautiously to avoid disrupting ecosystem dynamics. Among biological methods, the use of macroalgae appears particularly promising, as their evolutionary interactions with harmful microalgae can reduce ecological risks and increase the likelihood of achieving safe and effective management of HABs.

Screening of anti-microalgal activity of selected macroalgal extracts

The initial step in identifying biological control agents involves screening the anti-microalgal activity of dominant native macroalgal species from regions affected by HABs. In this study, a stepwise screening strategy was employed to ensure rigorous evaluation. This approach enabled the identification of macroalgal species with potential for biological control of microalgal blooms. Failure to apply such a strategy or reliance on improper screening methods could lead to overlooking the latent potential of macroalgae or result in scientifically inaccurate assessments²⁵. *Cochlodinium polykrikoides* was selected as the target microalga for screening due to its frequent bloom occurrences along the Persian Gulf coast, particularly in the study area. As a member of the dinoflagellates, a major group of phytoplankton responsible for HABs. This species provides a relevant model for assessing macroalgal efficacy against other harmful taxa²⁶. Several previous studies have also targeted *C. polykrikoides* in their screening protocols. For instance, Nagayama et al.²⁷, investigated the effect of phlorotannins isolated from the brown alga *Ecklonia kurome* against multiple strains of *C. polykrikoides*. Similarly, Oh et al.²⁸, demonstrated that methanolic extracts of the red macroalga *Corallina pilulifera* exhibited inhibitory effects on this species. Moezi et al.²⁹, reported that extracts from *Enthromorpha intestinalis*, *Gracillaria cortica*, and *Colpomenia sinuosa* negatively affected the growth of *C. polykrikoides*. Nan et al.³⁰, showed that *Ulva lactuca* extract had allelopathic inhibition against *Alexandrium tamarense*, another dinoflagellate species. Recent literature further supports the use of macroalgae in HAB mitigation. Anabtawi et al.³¹, emphasized the role of macroalgal secondary metabolites, such as phycobilins and phenolics—in suppressing HAB-forming microalgae, highlighting their ecological safety and sustainability. The WHOI HAB Response Program (2023) also acknowledged the limitations of physical mitigation methods like clay flocculation and advocated for integrated approaches, including biological control. These findings align with the Integrated Harmful Algal Bloom Management (IHABM) framework, which promotes multi-tiered strategies combining satellite monitoring, field surveillance, and biological interventions. In the present study, initial screening results revealed that the tested macroalgal extracts eliminated 100% of *C. polykrikoides* cells in culture (Fig. 3). Comparative analysis showed that *Padina* sp. A and *Sargassum* sp. B collected from the Bandar Abbas coast exhibited stronger anti-algal activity than their counterparts from the Chabahar coast. Although taxonomically identical, this variation warrants further investigation. Geographic isolation and ecological differences such as salinity, temperature, and pollutant levels between the Persian Gulf (Bandar Abbas) and the Gulf of Oman (Chabahar) may contribute to the observed bioactivity divergence. These regional distinctions represent only a fraction of the environmental variability influencing macroalgal metabolite profiles. Since macroalgal bioactivity is closely linked to environmental conditions, understanding these differences is essential for optimizing biological control strategies. Quantitative analysis of LC_{50} values for the tested extracts showed that bioactivity was evident at concentrations below 1 mg/

mL. This potency further supports the effectiveness of the selected macroalgae. For comparison, Nagayama et al.²⁷, reported that phlorotannins exhibited strong algicidal activity against *C. polykrikoides* at concentrations as low as 50 mg/L, with complete inhibition observed at 100 mg/L.

Screening of anti-microalgal activity of macroalgae in direct exposure

In the second step of the stepwise screening strategy, the anti-microalgal activity of macroalgae was evaluated through direct exposure to *Cochlodinium polykrikoides* cells. Studying the kinetics of algicidal activity during co-culture provided deeper insight into the biological control process. Laboratory-based investigation of this interaction yielded tangible evidence applicable to natural conditions, particularly in enclosed coastal ecosystems³². Understanding macroalgal growth kinetics enables assessment of their impact on blooming microalgae. Co-cultivation of macroalgae and target microalgae also facilitates examination of intercellular signaling and its influence on population dynamics. The results of this assay confirmed anti-microalgal activity and revealed its progression during simultaneous cultivation. The onset of algicidal activity by *Padina* sp. A was observed on day four post-inoculation. By day eight, over 60% of *C. polykrikoides* cells were eliminated, suggesting peak production of algicidal metabolites. These compounds are likely secondary metabolites, such as phlorotannins, which have been shown to interact with microalgal proteins and disrupt membrane integrity²⁷. However, the involvement of primary metabolites like enzymes cannot be excluded. A similar trend was observed for *Padina* sp. C, with a slight delay. On day eight, it eliminated 55% of microalgal cells, completing its algicidal activity by day twelve. Despite taxonomic similarity, this temporal difference may reflect variations in metabolite polarity, solubility, and mechanism of action. Enhanced solubility improves dispersion and efficacy, while reduced solubility may limit impact. Growth rate and metabolic adaptation also influence algicidal performance. Since all samples were cultured under identical conditions, initial adaptation likely affected metabolite yield and activity progression. This may explain the slower response of *Sargassum* sp. B, which eliminated 65% of cells by day ten and achieved complete lysis by day fourteen. *Sargassum* sp. D followed a similar trajectory, with a sharp increase in activity on day ten and full elimination by day fourteen. Overall, *Padina* species exhibited stronger and earlier algicidal effects than *Sargassum* species. The onset and duration of action were more pronounced in *Padina* strains, indicating their potential as rapid-response biological control agents. Morphological changes in *C. polykrikoides* cells during the algicidal process were notable (Fig. 4). Cells transitioned through distinct phases: motile and intact, stationary, undergoing lysis, and fully lysed. Images representing dominant degradation phases were selected for presentation. As shown in Figs. 4 and 6, the algicidal process progressed in stages. Loss of motility preceded gradual lysis, likely initiated by cell wall disruption and release of cellular contents. In cells exposed to *Sargassum* sp. B, lysis began with swelling, possibly due to osmotic imbalance from disrupted sodium-potassium pump function, leading to rupture and protoplasm release. Recent studies confirm that macroalgal compounds, especially phlorotannins, interact with *C. polykrikoides* proteins and membrane channels, disrupting osmotic regulation and inducing cell death²⁷. Co-culture experiments have also shown that macroalgae host algicidal bacteria on their surfaces, contributing to red tide mitigation³². Despite documented degradation stages, full elucidation of the algicidal mechanism requires further investigation, including electron microscopy. Strong correlations between macroalgal bioactives and microalgal cellular proteins have been reported, supporting their role in targeted control. In this context, Abdolalian et al.³³, demonstrated that *Enthromorpha intestinalis*, *Gracillaria cortica*, and *Colpomenia sinuosa* negatively affected *C. polykrikoides* in direct co-culture. Similarly, Nan et al.³⁰, confirmed inhibitory effects of fresh *Ulva lactuca* tissue on dinoflagellate growth.

Evaluation of the bioactivity spectrum of studied macroalgae

Identifying biological control agents with selective anti-algal activity against harmful microalgal species remains a central objective in HAB-related research. The narrower and more selective the bioactivity spectrum, the greater the ecological safety and field applicability of the control agent³⁴. Selective agents are particularly valuable in aquaculture systems, where maintaining beneficial microalgal populations is essential for ecosystem stability and productivity.

To evaluate selectivity, complementary assays were conducted to assess the bioactivity spectrum of the studied macroalgae against two beneficial microalgal species: *Chlorella vulgaris* and *Isochrysis galbana*. These species are widely recognized for their nutritional value and foundational roles in aquatic food chains, especially in shrimp hatcheries and larval fish diets^{35, 36}. The results demonstrated that metabolites extracted from the four macroalgal species, *Padina* sp. A, *Padina* sp. C, *Sargassum* sp. B, and *Sargassum* sp. D did not exhibit algicidal effects at concentrations effective against *Cochlodinium polykrikoides*. This outcome suggests a high degree of selectivity, likely due to structural and physiological differences between the harmful dinoflagellate and the beneficial microalgae. Dinoflagellates possess unique cell wall compositions and membrane proteins that may be more susceptible to macroalgal secondary metabolites such as phlorotannins, while chlorophytes and haptophytes may lack these vulnerable targets²⁷.

This selectivity is critical for biosafety, particularly in aquaculture applications. One of the long-term goals of this project is to implement macroalgal-based biological control strategies in shrimp hatcheries and grow-out ponds. In such systems, regulating microalgal population dynamics requires agents that suppress harmful blooms while preserving or enhancing beneficial ones. Macroalgae that negatively affect nutritionally important species must be excluded from application to avoid compromising larval health and feed quality³⁶. Moreover, the absence of adverse effects on *C. vulgaris* and *I. galbana* supports the hypothesis that macroalgal metabolites act through targeted mechanisms, possibly involving membrane disruption or oxidative stress pathways specific to dinoflagellates. This aligns with previous findings that phlorotannins and other polyphenolic compounds selectively inhibit red tide species without affecting green or golden microalgae^{27, 34}. In conclusion, the studied macroalgae exhibit a favorable bioactivity spectrum, combining strong algicidal effects against *C. polykrikoides*

with minimal impact on beneficial microalgae. This selectivity enhances their potential as safe and effective biological control agents in integrated HAB management and sustainable aquaculture.

Toxicity assessment of metabolites produced by selected strains

For practical and optimal application of biological control agents in field conditions, *biosafety* is a critical prerequisite. Consequently, the use of native macroalgal populations from the target region is prioritized in HAB control strategies to minimize ecological disruption and enhance compatibility with local biota³⁷. To evaluate toxicity, larvae of the Pacific white shrimp (*Litopenaeus vannamei*) were selected as indicator organisms. Shrimp larval bioassays are widely recognized for their sensitivity, cost-effectiveness, and relevance to aquaculture applications³⁸. In this study, the metabolites extracted from *Padina* sp. A, *Padina* sp. C, *Sargassum* sp. B, and *Sargassum* sp. D exhibited no observable significant toxic effects on shrimp larvae at concentrations effective against *Cochlodinium polykrikoides* (p-value < 0.05). These findings support the safe use of these macroalgae in shrimp hatcheries and grow-out ponds during HAB events.

To further assess biosafety, a second tier of toxicity evaluation was conducted using human umbilical vein endothelial cells (HUVEC). This assay aimed to determine the cytotoxic potential of macroalgal secondary metabolites on human cell lines, providing insight into potential risks to human health and environmental exposure³⁹. The results revealed that *Padina* sp. A and *Padina* sp. C exhibited IC₅₀ values of 671.3 and 934.3 µg/mL, respectively, against HUVEC cells. These values were significantly higher than the concentrations required for algicidal activity (p-value < 0.05), indicating a wide safety margin between effective and cytotoxic doses. Similarly, *Sargassum* sp. B and *Sargassum* sp. D showed IC₅₀ values of 1228 and 1253 µg/mL, while their LC₅₀ values against *C. polykrikoides* were 182.6 and 371.2 µg/mL, respectively. This significant difference (p-value < 0.05) between cytotoxic and algicidal thresholds confirms the preferential impact of macroalgal metabolites on microalgal cells over human or animal cells. Such selectivity is likely due to specific interactions between macroalgal compounds such as phlorotannins and sulfated polysaccharides and dinoflagellate membrane proteins, which are absent or structurally distinct in metazoan and human cells^{27,38}. Moreover, previous studies have demonstrated that macroalgal extracts can enhance shrimp immunity and growth when used as dietary supplements, further supporting their compatibility with aquaculture systems³⁷. These findings collectively reinforce the potential of the studied macroalgae as safe, selective, and ecologically viable biological control agents for HAB mitigation in aquaculture environments.

Purification and identification of extracted compounds

Due to the complexity and challenges associated with the purification and identification of bioactive algicidal compounds from marine organisms, only a limited number of such compounds have been fully characterized to date. The vast metabolic diversity of marine macroalgae presents both an opportunity and a challenge: successful isolation and identification require a comprehensive understanding of their biosynthetic potential and chemical ecology^{40, 41, 42, 43}. Identified algicidal compounds from macroalgae typically fall into three major categories: peptides and proteins⁴⁴, pigments such as chlorophyll derivatives and carotenoids⁴⁵, and biosurfactants, including glycolipids and lipopeptides⁴⁶. These compound classes differ in polarity, solubility, and mode of action, necessitating tailored extraction and separation protocols. In this study, extracts from the two most active macroalgal strains, *Padina* sp. A and *Sargassum* sp. B were subjected to thin-layer chromatography (TLC). The chloroform: methanol solvent system at a 10:90 ratio yielded optimal separation of bioactive fractions (Figs. 6 and 8). Chromatographic behavior, retention factors (Rf), and HPLC retention times indicated the presence of polar and semi-polar compounds, consistent with known classes of macroalgal secondary metabolites⁴⁷. Chemical screening assays of *Padina* sp. A revealed that fraction No. 5, which exhibited strong algicidal activity, contained functional groups associated with terpenoids, flavonoids, sugars, and phenolic compounds (Table 4). These compound classes are frequently implicated in allelopathic and antimicrobial activity, with terpenoids and phenolics known to disrupt algal cell membranes and interfere with photosynthetic pathways⁴⁸. The extract from *Sargassum* sp. B displayed a similar chemical profile, though the active fraction was more strongly associated with phenolic groups. Phenolic compounds, particularly phlorotannins, are abundant in brown algae and have been widely documented for their antioxidant, antimicrobial, and algicidal properties²⁷. Comparative HPLC chromatograms of the two active fractions confirmed distinct chemical signatures, suggesting that different macroalgal species may produce structurally diverse yet functionally convergent algicidal agents. These findings underscore the importance of integrating chromatographic and spectroscopic techniques in the identification of bioactive compounds. Further structural elucidation using advanced tools such as LC-MS/MS and NMR spectroscopy is recommended to confirm compound identity and explore structure–activity relationships.

Comparative discussion of phylogenetic findings

The phylogenetic tree constructed in this study using *rbcl* and *PSbA* gene sequences places *Padina* sp. A close to *Padina australis* (98.86% homology). These results align well with prior molecular phylogenetic studies that used plastid and organellar genome data to resolve species relationships within the *Padina* and *Sargassum* genera. In the case of *Padina* sp., previous studies have shown that *P. australis* and *P. boergesenii* form distinct but closely related clades based on *rbcl* and *cox3* gene sequences. The identification of *Padina iranica* sp. in this phylogeny tree, clustering near *P. australis*, is consistent with findings by Boo et al. and other phycologists who have described cryptic diversity and regional endemism within *Padina* species in the Indo-Pacific and Persian Gulf regions. Importantly, this tree includes multiple accessions and outgroup species (*Dictyota dichotoma*, *Stypopodium* sp.), which strengthens the resolution and confirms the monophyly of the studied genera. The bootstrap values in your tree (mostly >90%) are comparable to those reported in plastid genome studies, indicating robust support for the inferred relationships. In summary, the studied phylogenetic results are in strong agreement with published molecular data, validating the identity of the studied strains and supporting

their use in ecological and biotechnological applications. This genetic confirmation enhances the credibility of your bioactivity, toxicity, and compound profiling results by linking them to well-characterized evolutionary lineages.

Conclusion

This study successfully identified native macroalgal strains exhibiting targeted anti-microalgal activity against the harmful bloom-forming species *Cochlodinium polykrikoides*. Through a stepwise screening approach, two strains, *Padina* sp. and *Sargassum* sp., were collected from the Persian Gulf and Gulf of Oman and confirmed as effective candidates for biological control. Both strains demonstrated selective algicidal activity, inhibiting *C. polykrikoides* effectively without significant adverse effects on beneficial microalgae commonly utilized in aquaculture. Toxicological assessments confirmed the safety of their metabolites, as no detectable toxicity was observed toward human cells or shrimp larvae. The species were identified through both morphological and molecular analyses, and their algicidal kinetics under co-culture conditions were characterized, further bolstering the reliability of the results^{34,49}.

In summary, the macroalgal strains studied fulfill critical criteria as environmentally safe biocontrol agents, making them promising candidates for applications in marine ecosystems, aquaculture systems, and harmful algal bloom (HAB) management strategies^{50,51}.

Data availability

The datasets generated and/or analysed during the current study are available in the NCBI GenBank repository under the accession number MN694968.1. The data can be accessed at the following persistent link: <https://www.ncbi.nlm.nih.gov/nuccore/MN694968.1>. All datasets are publicly available.

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Author contributions

STJ and NB conceptualized and designed the study. MG performed data collection and experimental work. SP analyzed the data and contributed to the interpretation of results. STJ and NB wrote the initial draft of the manuscript. All authors reviewed, edited, and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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