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EVALUATION OF THE ANTIOXIDANT ACTIVITY OF CORAL MUCUS ISOLATED FROM *Porites lobata IN VITRO*

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ABSTRACT

Marine-derived natural products have attracted much interest from scientists (chemists and pharmacologists), since many of their potential bioactivities are still unexplored. Among marine natural products discovered to date, 56% are anticancer, 13% are antibacterial, 5% are antifungal, and 3% are antiviral compounds. These compounds come from green algae (1%), red algae (4%), brown algae (5%), sponges (31%), corals (24%), and marine microorganisms (15%). Coral reefs, especially *Porites lobata*, secrete surface mucus layers (SMLs) that serve as essential barriers in host defense and microbial regulation. However, environmental stressors such as bleaching may alter the biochemical composition of this mucus, compromising its biological functions. This study aimed to compare the antioxidant properties of mucus obtained from healthy and bleached *P. lobata* corals to assess the impact of bleaching on their natural protective capabilities. The DPPH assay and flow cytometry with dihydroethidium (DHE) staining were used to evaluate the free radical scavenging activity and antioxidant capacity of coral surface mucus layer samples. The antioxidant activity of healthy coral mucus was significantly higher than that of bleached mucus, with a 2-fold increase at 1/5 and 1/10 dilutions, and nearly a 2.8-fold increase at 1/20 dilution. The results indicate that mucus from healthy corals exhibited significantly higher antioxidant activity than that from bleached corals. DPPH analysis showed stronger radical scavenging ability in healthy mucus extracts, while flow cytometry demonstrated a marked reduction in reactive oxygen species (ROS) accumulation in treated HCT116 cells compared to both bleached mucus and control groups. These findings suggest a decline in the coral's natural defense mechanisms post-bleaching. The mucus from healthy corals has the ability to reduce the accumulation of intracellular ROS in HCT116 cells, indicating its potential to against oxidative stress-related diseases. The robust antioxidant activity of healthy coral mucus highlights its potential as a source of novel marine-derived antioxidants. This study supports further investigation into the bioactive compounds from the surface mucus layer of healthy

Porites spp. corals for potential therapeutic applications against oxidative stress-related diseases.

Keywords: Antioxidant activity, coral mucus, healthy and bleached coral, Porites lobata, ROS.

INTRODUCTION

Marine ecosystems are a rich source of bioactive compounds, many of which have shown promising biological activities with potential applications in pharmaceuticals, agriculture, and biotechnology. Natural products derived from marine organisms, including corals, sponges, algae, and marine invertebrates, have been extensively studied for their diverse therapeutic properties. These compounds often have unique chemical structures that are not found in terrestrial organisms, making them valuable resources for drug discovery. Coral reef ecosystems are among the most important on Earth, yet they are increasingly threatened by climate change, environmental pollution, and the rising acidity of oceans. Coral bleaching, caused by environmental stressors like rising sea temperatures, results in the loss of symbiotic algae (Symbiodinium), weakening coral health. Recent investigations have reported the presence of various bioactive substances from both soft and hard corals, including diterpenoids and sterols (6-epi-Yonarasterol B) with antioxidant properties (Chang *et al.*, 2017; Chung *et al.*, 2012), as well as terpenes, secosterols, and steroids demonstrating anti-inflammatory activity (Elkhateeb *et al.*, 2014; Huang *et al.*, 2016; Tseng *et al.*, 2016; Wei *et al.*, 2013), potential antiviral compounds (Abdelfattah *et al.*, 2024). Furthermore, certain diterpenoids and diterpenes have shown cytotoxicity, anticancer activity against human cell lines (Chao *et al.*, 2022; Lin *et al.*, 2014; Yang *et al.*, 2022; Zhang *et al.*, 2022). Despite these advances, most research has focused on isolating bioactive compounds from corals broadly, often overlooking the rich and diverse bioactive potential present in coral mucus. This matrix has been found to have bioactive compounds. Coral mucus, synthesized by mucocytes within the epidermal layer, constitutes a complex assemblage of proteins, carbohydrates, and lipids that serves as a key adaptive mechanism enabling corals to cope with diverse environmental stressors (Brown & Bythell, 2005). Coral mucus serves as a protective barrier and contains bioactive compounds with antimicrobial and antioxidant properties (Sang *et al.*, 2019). Recently, the coral mucus layer has been extensively studied for its composition and the diversity of microorganisms living within it (Bui *et al.*, 2024; Mahmoud & Kalendar, 2016). Furthermore, the mucus of healthy corals has been shown to exhibit higher biological activity, such as antibacterial and anticancer properties, compared to the mucus of bleached corals (Bui *et al.*, 2024). The study of bioactive compounds from corals is a crucial field focused on exploring and harnessing the pharmacological potential of marine resources. The ongoing exploration and study of marine organisms will likely lead to the discovery of new drugs and treatments for a wide range of diseases, including cancer, infections, inflammatory disorders, and neurodegenerative diseases. Recently, many studies have focused on isolating and evaluating the biological activity of natural compounds from hard corals and their associated symbiotic organisms.

Comparing with bioactive compounds from coral skeleton or from other marine organisms, the bioactive compounds from microorganisms (bacteria, fungi) living in a symbiotic or parasitic relationship with other organisms of the coral holobiont, calledcoral mucus-associated microorganisms, were also reported but less frequently. For example, derivatives of diphenyl ethers and isocoumarins from the coral fungus (*Phoma* sp.) possessed antibacterial activity (Shi *et al.*, 2017), secondary metabolites of the fungus (*Curvularia trifolii*) exhibiting anticancer property (Couttolenc *et al.*, 2016; Hou *et al.*, 2015), aqabamycins A-G from coral bateria (*Vibrio* spp.) showed antibacterial activity (Al-Zereini *et al.*, 2010). However, numerous studies have been conducted, with the majority concentrating on the community structure and diversity of microorganisms linked to coral mucus (Carlos *et al.*, 2013; Kuang *et al.*, 2015; Mahmoud & Kalendar, 2016), their role in the ecosystem and the emergence of coral diseases (Hadaidi *et al.*, 2017; Roder *et al.*, 2014; Wilson *et al.*, 2012), changes of microbial community composition and structure in response to temperature change or environmental factors, etc (Gajigan *et al.*, 2017; Zhang *et al.*, 2015). However, publications primarily concentrate on antibacterial, anticancer, and anti-inflammatory properties, with no studies describing the presence of antioxidant compounds in coral mucus.

Bui *et al*. (2024) analyzed the bacterial communities of the mucus layer from both bleached and healthy populations of *Porites lobata* collected from Nha Trang Bay using 16S rRNA amplicon sequencing (Bui *et al.*, 2024). We investigated the antitumor and antibacterial properties of the surface mucus layer (SML) extracted from both bleached and healthy *P. lobata* corals collected at four sites during the rainy and dry seasons in Nha Trang Bay, Khanh Hoa. Compared to the SML from bleached corals, the mucus from healthy corals exhibited stronger antibacterial effects against the coral bleaching pathogen *Vibrio coralliilyticus* and showed enhanced antitumor activity against HCT116 cells. This was accompanied by increased levels of cleaved PARP and a faster induction of nuclear apoptosis in the treated cells. This study continues to assess the biological activity of the mucus layer isolated from healthy and bleached *P. lobata* corals, specifically focusing on antioxidant activity.

MATERIALS AND METHODS

Collection of coral mucus sample

SML samples were separately collected from bleached and healthy colonies of *Porites lobata* corals in Nha Trang Bay, Khanh Hoa province, Vietnam, during March (dry season) of 2020. At each sampling site, *P. lobata* colonies were examined and divided into two groups: bleached and healthy. Mucus samples were collected from 3 to 5 coral fragments or nubbins. The SCUBA diving collection technique is described in our earlier studies (Bui *et al.*, 2024; Thao *et al.*, 2023). Coral fragments were removed from the water and exposed to air for a duration of 3 to 5 minutes, inducing secretion of mucus that formed long, gel-like threads. To prevent impurities and dilution caused by seawater, the first 20 seconds of mucus secretion was discarded. Mucus from each nubbin was pooled and homogenized to achieve a final volume of approximately 30 mL. Lastly, all mucus samples were divided into cryotubes and

promptly frozen in liquid nitrogen for further examination.

Determination of antioxidant activity

The antioxidant activity was assessed using the DPPH (2,2′-diphenyl-1-picrylhydrazyl) assay (Kedare & Singh, 2011). The DPPH assay is based on the ability of antioxidants to donate an electron or hydrogen atom to the DPPH radical, resulting in a color change from deep violet to yellow.

DPPH was diluted with methanol (99.8%) to obtain a 0.1 mM DPPH solution, kept in a cold and dark place. In a test tube, 3.9 mL DPPH solutions were combined with 100 µL of different ratios of bleached and healthy surface mucus layer to cultures, 1/5, 1/10, and 1/20 (v/v). After that, the tubes were incubated at room temperature for 30 minutes in the dark. Measurement of absorbance was carried out at 517 nm using a UV-Vis spectrophotometer. Calculate the percentage of DPPH radical scavenging activity using the formula:

$$\% \text{ of antioxidant activity} = [(A_{control} - A_{sample}) \div A_{control}] \times 100$$

where: $A_{control}$: Absorbance of DPPH solution without sample;

A_{sample}: Absorbance of DPPH solution with sample

A standard curve was constructed using Trolox (Sigma), and the antioxidant capacity of the sample was expressed as micromoles of Trolox equivalents per gram of dry weight (µmol TE/g DW).

$$\mu\text{mol TE/g DW} = (C \times V)/m$$

where: C: Equivalent Trolox concentration (µmol/L)

V: Volume of extract (L)

M: Weight of dry sample (g)

Determination of intracellular reactive oxygen species levels

The HCT116 human colorectal cancer cell line (American Type Culture Collection, CCL–247TM) was initially cultured in Dulbecco's Modified Eagle Medium (DMEM) medium enriched with 10% fetal bovine serum (FBS), supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were incubated at 37°C for 24 h in a humidified atmosphere containing 5% CO_2. Then, HCT116 cells were plated in a 12-well plate at a density of 200,000 cells/well and cultivated in standard condition for 24 h and treated with different ratios of bleached and healthy SML to cultures, 1/5, 1/10, and 1/20 (v/v). Cells were collected by trypsinization and centrifugation at 200 g (1500 rpm) for 5 minutes, and resuspended in FACS buffer (1% BSA in PBS) containing 5 µM DHE (Sigma-Aldrich). Following a 15-minute incubation at room temperature in the dark, the cells were rinsed with FACS buffer and promptly examined using a BD FACSCalibur™ flow cytometer in combination with CellQuest™ Pro software (FACS, BD Biosciences). Excitation was set at 488 nm, and fluorescence emission was detected in the 564–606 nm range using the FL2 filter. The fluorescence signal is proportional to the reactive oxygen species (ROS) level. A representative result is presented, which is consistent with results from at least three independent experiments.

Statistical analysis method

All tests and measurements were conducted a minimum of three times to ensure reproducibility. Differences between means were analyzed using Duncan's Multiple Range Test with SAS 9.0 software (SAS

Institute, Cary, NC, USA). Results with p-values < 0.05 were considered statistically significant. Data is presented as the mean of three replicates, and means sharing the same letter indicate no significant difference.

RESULTS AND DISCUSSION

Antioxidant activity in bleached and healthy surface mucus layer

Trolox (Vitamin E) is the standard antioxidant commonly used for comparison. Antioxidant activity is expressed as Trolox equivalents (TE) based on a DPPH calibration curve. The DPPH standard curve is shown in Figure 1.

Antioxidant activity is demonstrated by the donation of a hydrogen atom from the antioxidant to reduce the purple free radical DPPH to the yellow DPPH-H. This reduction is quantified by spectrophotometry at a wavelength of 517 nm.

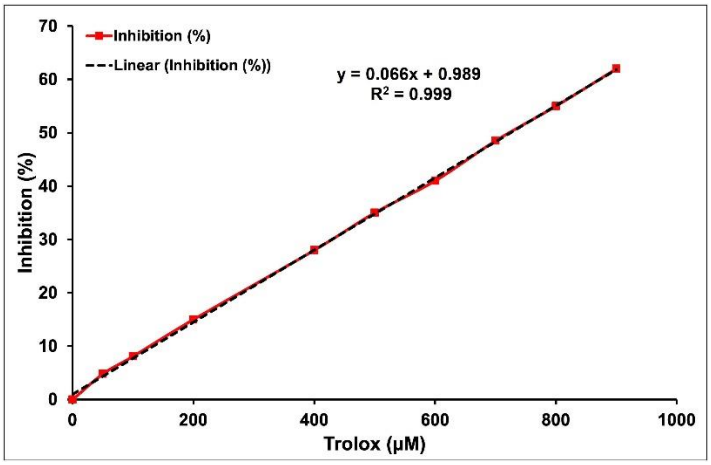

Figure 1. Calibration curve of Trolox. A linear calibration curve was produced with R = 0.999 and used for evaluation of antioxidant activity.

The DPPH assay revealed a significant difference in antioxidant capacity between the SML of healthy and bleached *P. lobata* corals at all tested dilutions (1/5, 1/10, and 1/20, v/v) (Figure 2). The highest radical scavenging activity was observed in the healthy SML at a 1/5 dilution, reaching approximately 1200 µM Trolox equivalent/g DW, which was significantly higher ($p < 0.05$) than the bleached counterpart at the same dilution (approximately 600 µM TE/g DW). This trend persisted at the 1/10 dilution, where healthy mucus demonstrated around 915 µM TE/g DW versus 453 µM TE/g DW in bleached samples, again reflecting a 2-fold enhancement. Notably, at the 1/20 dilution, healthy coral mucus showed approximately 710 µM TE/g DW, representing nearly a 2.8-fold increase relative to bleached mucus, which measured about 255 µM TE/g DW. These results clearly indicate that the antioxidant capacity of coral mucus is substantially diminished following bleaching. A clear concentration-dependent trend was observed, where increasing dilution resulted in decreased antioxidant activity in both groups. However, the healthy coral SML consistently exhibited significantly higher DPPH scavenging ability across all conditions. These findings suggest that bioactive molecules such as phenolics, flavonoids, and other secondary

metabolites in healthy coral SML are diminished or degraded in bleached corals (Downs *et al.*, 2002; Farag *et al.*, 2021; Williams *et al.*, 2021). Coral bleaching, typically resulting from environmental stress such as elevated sea temperatures, leads to the loss of symbiotic zooxanthellae or their pigments, which are major contributors to the coral's metabolic and antioxidant capacity (Downs *et al.*, 2002; Palmer *et al.*, 2010). The reduction in DPPH activity in bleached SML samples may reflect this metabolic impairment, particularly in the production or retention of ROS-scavenging molecules.

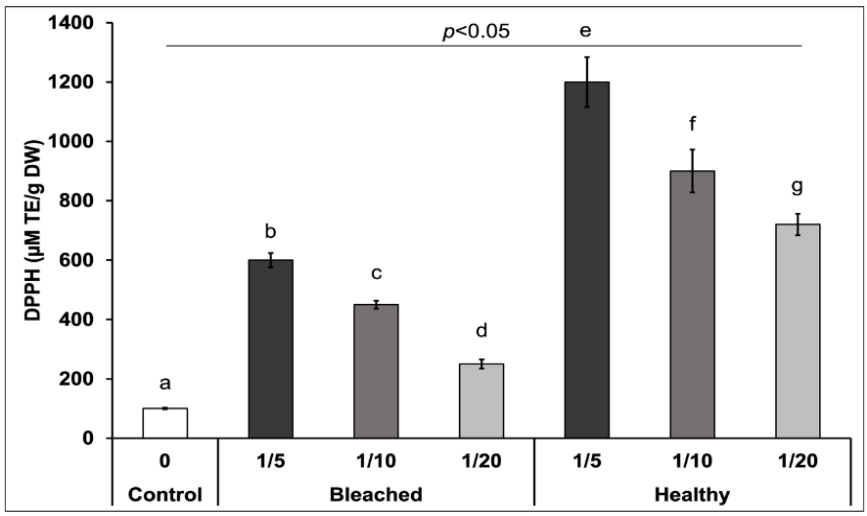

Figure 2. *In vitro* antioxidant activity of healthy and bleached coral mucus. The different ratios of bleached and healthy SML (1/5, 1/10, and 1/20, v/v) are added to the DPPH solution (0.1 mM), thoroughly mixed, and incubated in the dark for 30 minutes. The control is the DPPH solution (0.1 mM) without mucus. The absorbance is measured spectrophotometrically at a wavelength of 517 nm. The differences between the means were analyzed by Duncan's Multiple Range Test and one-way ANOVA, means with the same letter are not significantly different ($p < 0.05$) and vice versa.

Furthermore, the SML serves not only as a physical barrier but also as a critical biochemical defense system against oxidative stress and microbial invasion. Healthy coral mucus is rich in antioxidants that can neutralize free radicals and maintain cellular homeostasis (Brown & Bythell, 2005). The diminished antioxidant capacity in bleached corals may compromise this defense, rendering corals more susceptible to secondary stressors and infections, thereby impeding recovery and survival.

Palmer *et al.* demonstrated that the activity of key antioxidant enzymes such as superoxide dismutase (SOD) and catalase was markedly reduced in thermally stressed and bleached corals (Palmer *et al.*, 2010). Similarly, Rosic *et al.* reported a significant decline in gene expression related to antioxidant pathways following bleaching events (Rosic *et al.*, 2011). These reports support the observed reduction in non-enzymatic antioxidant capacity (as shown by the DPPH assay) in the present study, indicating a systemic loss of oxidative stress resilience in bleached coral samples. However, our research results differ from those of Vilas Bhagwat (2023). Vilas Bhagwat *et al.* conducted research on antioxidant enzymes, including SOD,

catalase, and peroxidase activity, which were observed in both diseased and healthy coral mucus (Vilas Bhagwat *et al.*, 2023). Free radical scavenging of mucus was screened using hydrogen peroxide. This study indicates that the ability of coral mucus to scavenge the DPPH radicals was observed predominantly more in diseased coral mucus (pink line syndrome). This discrepancy may be due to variations in sampling location, coral species, or experimental time points between the studies. These findings indicate that further in-depth research is needed to clarify the mechanisms and conditions influencing this outcome.

Comparison of ROS levels in healthy and bleached coral mucus

The determination of intracellular ROS levels using DHE is a widely utilized method in cellular biology (Kumar & Gullapalli, 2024). Similarly, in this study, we used a DHE fluorescent probe to quantify total ROS production. Flow cytometry analysis was performed to assess intracellular ROS levels of healthy and bleached surface mucus layers in human colorectal cancer cells (HCT116) following 24 h treatment with control (sterile distilled water). After incubation with DHE, fluorescence was measured in the FL2-H channel (564–606 nm) to detect ROS accumulation (Figure 3).

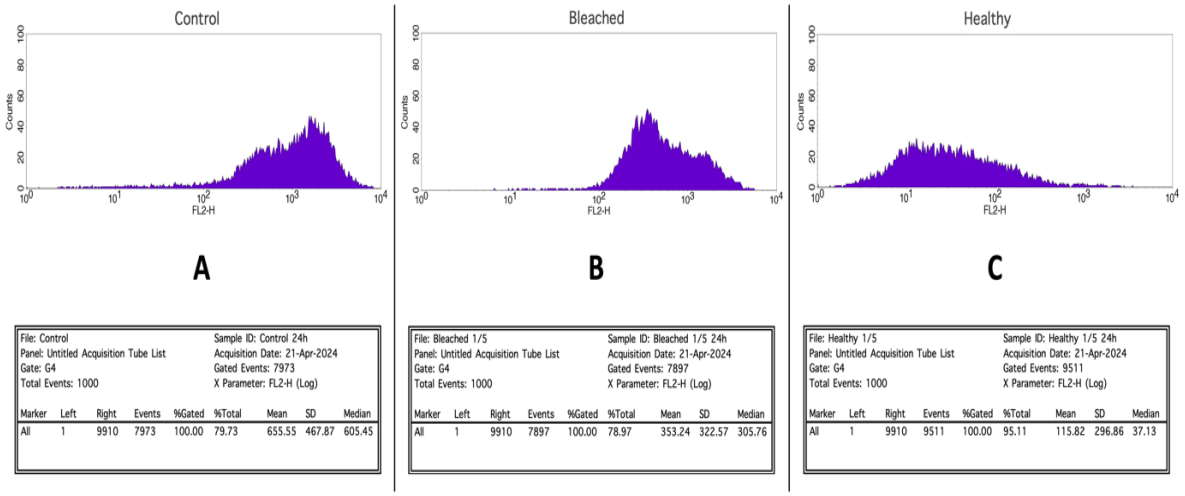

Figure 3. Exemplar of flow cytometry histogram. HCT116 cells were treated with sterile distilled water (control, A), bleached (B) and healthy coral mucus (C) and incubated for 24 h. After 15 minutes incubation with DHE, fluorescence signal was detected at 488 nm and 564–606 nm (FL2 filter) using FACS analysis. Mean value was used to calculate for fluorescence intensity.

The control group exhibited a broad fluorescence peak, representing baseline ROS levels. In contrast, cells treated with bleached coral mucus showed a marked decrease in fluorescence intensity, while the healthy coral mucus group demonstrated a significantly lower fluorescence signal compared to the bleached group and control, suggesting a strong reduction in intracellular ROS levels.

Cells treated with mucus from bleached corals exhibited significantly higher intracellular ROS accumulation compared to cells treated with mucus from healthy coral mucus at all ratios (1/5, 1/10, 1/20, v/v) (Figure 4). The observed decrease in ROS

levels upon treatment with coral mucus indicates the antioxidant potential of coral-derived compounds. Notably, healthy coral mucus demonstrated a significantly stronger ROS-scavenging effect compared to bleached coral mucus and control, reducing ROS levels by over 80% relative to the control. Bleached coral mucus exhibited only a moderate antioxidant activity, reducing ROS levels by approximately 20% to 40% compared to the control. However, the lower efficacy relative to healthy mucus aligns with previous reports showing that bleaching alters coral symbiosis and disrupts the production of bioactive metabolites (Weis, 2008). This indicates that mucus from healthy corals caused a more pronounced reduction in cell viability and exhibited greater anti-proliferative activity against HCT116 cells compared to mucus from bleached corals.

ROS are naturally generated as by-products during cellular aerobic metabolism. Besides mitochondria, various enzymes contribute to ROS production, including NADPH oxidases, xanthine oxidase, nitric oxide synthase, and components within peroxisomes (Magnani & Mattevi, 2019). Additionally, ROS can be formed through exposure to ionizing and ultraviolet radiation, as well as through the metabolism of numerous drugs and xenobiotics (Pizzino *et al.*, 2017). Under normal physiological conditions, steady-state levels of ROS are essential for maintaining cellular functions such as signaling and homeostasis. However, when ROS are produced excessively or when cellular antioxidant defenses fail to neutralize them effectively, oxidative stress arises, leading to cellular damage (Chandimali *et al.*, 2025; Juan *et al.*, 2021).

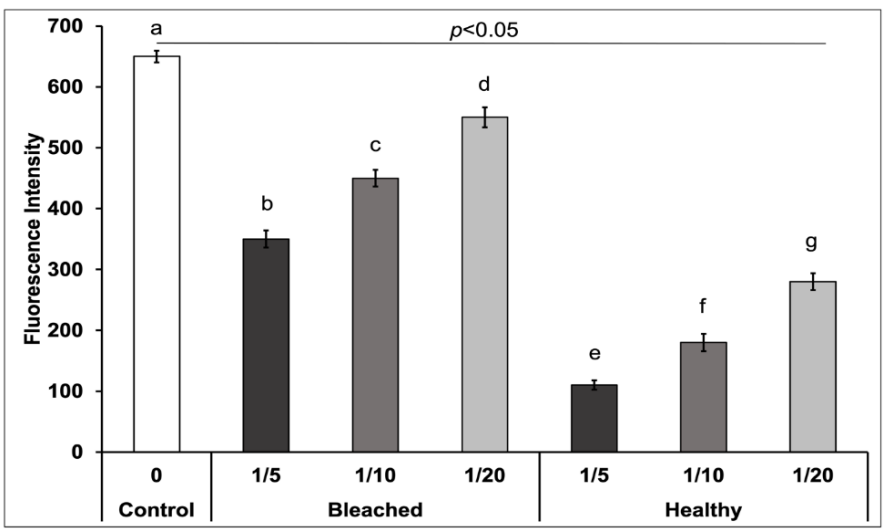

Figure 4. Effect of coral mucus treatment on intracellular ROS accumulation. HCT116 cells were treated with sterile distilled water (control) and different ratios of healthy and bleached coral mucus/cultures, 1/5, 1/10, and 1/20 (v/v). ROS level was determined by the fluorescence signal of DHE analyzed by FACS and proportional to ROS level. All measurements were performed at least 3 times for reproducibility. Mean differences were evaluated using Duncan's Multiple Range Test, and values labeled with the same letter (a–g) were not significantly different at $p < 0.05$.

When the sample concentration is reduced from a ratio of 1/5 to 1/20 by volume, the intracellular ROS accumulation increases for both healthy and bleached coral samples.

This result is entirely consistent with the antioxidant activity findings of healthy and bleached coral mucus samples. A decrease in sample concentration leads to a reduction in the cell's antioxidant capacity (due to a decrease in the concentration of antioxidants), which can make cells more susceptible to damage by ROS, resulting in an increase in the accumulation of intracellular ROS. The mucus from healthy corals has the ability to reduce the intracellular accumulation of ROS in HCT116 cells, indicating its potential to protect cells from oxidative stress-induced damage. This suggests that mucus from healthy corals may contain antioxidants that help neutralize free radicals and minimize cellular damage caused by oxidative processes, thereby contributing to the maintenance of cellular stability and function. In contrast, the mucus from bleached corals either loses this capability or exhibits a significant reduction in its effectiveness. Metagenomic studies have demonstrated that coral bleaching induces significant shifts in the composition and functional potential of coral-associated microbiomes, which in turn alters their biological activities, including antioxidant functions. Specifically, bleaching disrupts the balance and diversity of beneficial microbial communities that contribute to ROS scavenging, leading to a decrease in overall antioxidant activity within the coral holobiont (Bui *et al.*, 2024; Cheng *et al.*, 2023).

CONCLUSION

This study demonstrates that the surface mucus layer of healthy *P. lobata* corals possesses significantly stronger antioxidant properties compared with the bleached ones. The enhanced free radical scavenging ability and reduced ROS accumulation in treated cells highlight the critical role of coral mucus in natural defense against oxidative stress. The robust antioxidant activity observed in healthy coral mucus suggests it as a promising source of novel marine-derived antioxidants with potential therapeutic applications against oxidative stress-related diseases. Therefore, further studies should aim to isolate and characterize distinct bioactive compounds and evaluate their potential applications in biomedicine.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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