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Antibacterial Efficacy of *Hormophysa cuneiformis* Ethanolic Extract Against *Aeromonas* spp.

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ABSTRACT

This research investigated the antibacterial efficacy of ethanolic extract from the brown seaweed *Hormophysa cuneiformis* against *Aeromonas eucrenophila*, *A. hydrophila*, and *A. taiwanensis* at three concentrations: 0.10, 0.20, and 0.40mg/ mL. The agar disc diffusion method was employed to assess antimicrobial activity. Results indicated that *H. cuneiformis* extracts did not exhibit any antibacterial activity against *A. eucrenophila* at the tested concentrations, suggesting no inhibitory effect. Conversely, notable antibacterial effects were seen against *A. hydrophila* and *A. taiwanensis*, with the most substantial zones of inhibition measured at 0.10mg/ mL (18.42±1.27mm) and 0.20mg/ mL (24.03±0.56mm), respectively. Varying levels of antibacterial activity were recorded for the control antibiotic, with the highest inhibition zone for *A. hydrophila* (18.42±1.27mm). The findings suggest that the bioactive compounds in the extracts have an optimal range for antibacterial efficacy. Further studies are recommended to explore the mechanisms of inhibition and the potential use of this extract as antimicrobial agent in aquaculture.

INTRODUCTION

Aquaculture is a significant source of protein and a key economic driver, particularly in developing nations **(BFAR, 2022; FAO, 2023)**. Excluding algae, total world fisheries and aquaculture production showed a 45% growth between 2000 and 2021 **(FAO, 2023)**. In the Philippines, total fisheries production in 2023 reached 4.26 million metric ton (MT), with aquaculture sector contributing 56% or 2.38 million MT. In the same year, the aquaculture accounted for 37.7% of the total fisheries value of Php 328.54 billion **(BFAR, 2023)**. In the third quarter of 2024, aquaculture remained the primary contributor to fish production, accounting for 53.07% among the sub-sectors namely municipal and commercial fisheries. However, during the same quarter, all sub-sectors experienced a decrease in production, with aquaculture dropping by 2.78%. Overall, total fisheries production reached 965,715.60 MT, marking 5.07% decline

compared to the production during the same period of the previous year **(BFAR, 2024)**. In the aquaculture production of the Philippines, seaweeds are among the commercially important fisheries commodities and the country is a leading seaweed producer, alongside China and Indonesia **(Ferdouse *et al.*, 2018; BFAR, 2022)**. In 2022, seaweed accounted for 65.76% of the country's fisheries production, yielding 1.54 million MT valued at Php 16.60 billion **(BFAR, 2022)**.

Globally, bacterial infections in aquaculture hinder sustainable production and trade **(Henriksson *et al.*, 2018)**. Pathogenic diseases are identified as a key limiting factor for the aquaculture expansion by 2050 **(Stentiford *et al.*, 2012)** with estimated production losses of about billion-dollars **(Shinn *et al.*, 2018)**. The genus *Aeromonas* comprises over 30 Gram-negative, oxidase-positive *bacilli* in the family Aeromonadaceae, native to aquatic environments **(Pessoa *et al.*, 2019)**. These opportunistic pathogens are common in freshwater and cause aquatic diseases, especially under stress in aquaculture systems **(Aberoum & Jooyandeh, 2010; Salvat & Ashbolt, 2019)**. *A. hydrophila*, *A. euenophile*, and *A. taiwanensis* infections in aquatic animals lead to acute and chronic conditions like hemorrhagic septicemia, skin ulcers, and enteritis, resulting in high mortality in aquaculture **(Lio-Po *et al.*, 1983; Bondad *et al.*, 2005)**. *Aeromonas* species cause significant economic losses in aquaculture due to their high virulence and association with massive fish mortalities **(Gieseke *et al.*, 2022; Golomidova *et al.*, 2024)**. The emergence of antibiotic-resistant *Aeromonas* strains underscores the urgency for sustainable treatment strategies **(Cunha *et al.*, 2022)**.

Seaweeds, or benthic marine algae, are plants found in marine or brackish waters **(Chapman & Chapman, 1980)**. Seaweed bioactive compounds, noted for their antitumor, anticancer, antithrombin, and antioxidant properties, are essential in commercial food products and hold promise as natural therapeutics due to their proven antimicrobial potential against bacterial infections in aquaculture without promoting antibiotic resistance **(Perez *et al.*, 2016; Qari & Khan, 2019; Yogesh *et al.*, 2021; Hamad *et al.*, 2023)**. These compounds include fatty acids, phenolics, lectins, terpenes, alkaloids, and hydrogen peroxide, though further research is needed to purify and characterize them and to test their efficacy against specific bacterial pathogens **(Mohamed *et al.*, 2012; Rayapu *et al.*, 2017)**.

Brown seaweed *Hormophysa cuneiformis*, found in Santa Ana, Cagayan, and other coastal areas in the Philippines, is rich in metabolites like phenolic compounds, polysaccharides, and terpenoids, known for their potent antibacterial properties **(Cox *et al.*, 2010)**. It exhibits antimicrobial activity against bacterial pathogens such as *Aeromonas, Enterococcus faecalis, Staphylococcus aureus,* and *Pseudomonas aeruginosa* **(Abdel-Raouf, *et al.*, 2015; El-Manawy *et al.*, 2019)** in aquatic organisms and humans **(Abdel-Raouf *et al.*, 2015; Mohamed & Saber, 2019; Osman *et al.*, 2023)**. Offering an eco-friendly alternatives to conventional antibiotics, *H. cuneiformis* provides

effective antimicrobial agents without contributing to antibiotic resistance **(Radhika *et al.*, 2015; Vatsos & Rebours, 2015)**.

This study assessed the inhibitory potential of ethanolic extract of *H. cuneiformis* seaweed against three *Aeromonas* species through measuring the zone of inhibition (ZOI) using the Kirby-Bauer diffusion method. By addressing gaps in aquaculture, the research seeked to advance environmental microbiology, promote public health, and offer sustainable alternatives to antibiotics for mitigating *Aeromonas* risks.

MATERIALS AND METHODS

1. Seaweed collection and identification

Seaweed was collected from the coastal area of Sitio Racat, Barangay Rapuli, Sta. Ana, Cagayan Valley, Philippines (18°25'12.60"N; 122°7'30.43"E) (Fig. 1). The thallus part of the seaweeds was manually collected, and afterwards was cleaned and washed with seawater. About 10kg of fresh samples were stored in a covered plastic container, and were transported to the Central Luzon State University for the laboratory part of the study. The collected seaweed species was identified as *Hormophysa cuneiformis* (Fig. 2a) using available references, including the AlgaeBase data system, the work of **Silva *et al.* (1987)**, and seaweeds of Panay by **Ponce *et al.* (1992)**.

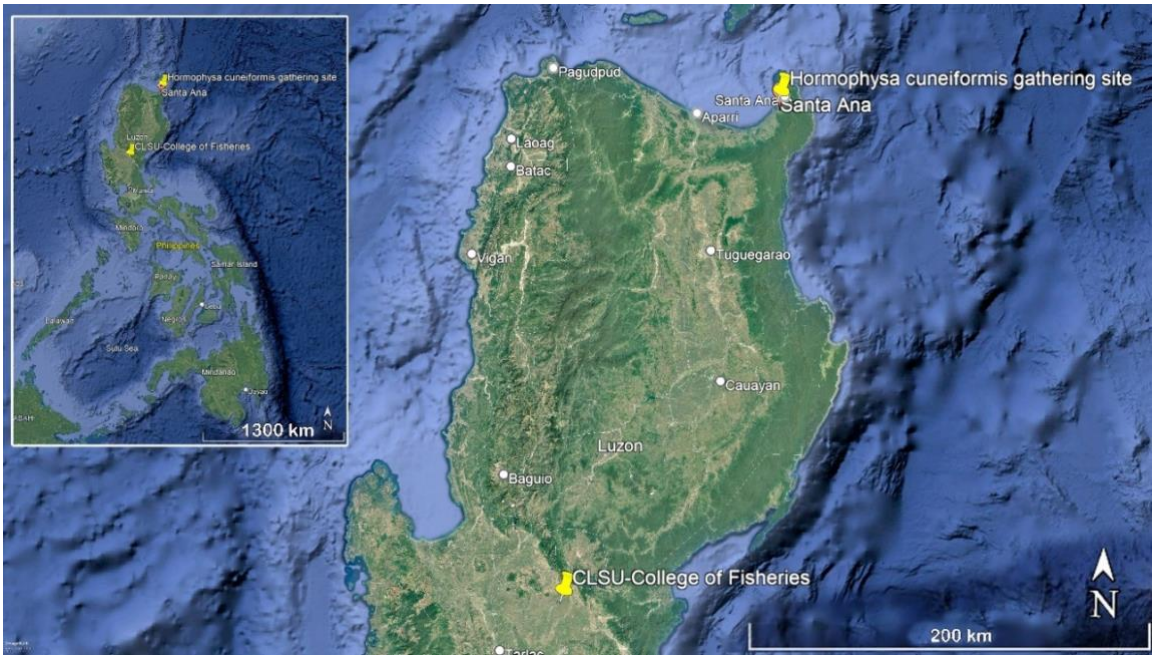


Fig. 1. A Map showing the collection site of *Hormophysa cuneiformis* in Sta. Ana, Cagayan, Philippines and the designated laboratory experiment area in the College of Fisheries-Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines **(Source: Google Earth Pro)**

2. Drying of seaweed and preparation of extract

The seaweed was dried using a mechanical dehydrator set to 50°C (120°F) for 12 hours **(Sulaiman *et al.*, 2020)**. A concrete mortar and a wooden pestle were used to partially reduce its size (Fig. 2b), followed by a portable mechanical grinder to pulverize the dried seaweed. The powdered seaweed was then stored in a plastic container prior to extraction (Fig. 2c).

(a) (b) (c)

Fig. 2. (a) Fresh form; (b) Dried form; and (c) Powdered form of *Hormophysa cuneiformis*

Seaweed crude extraction was performed using the decoction method of **Caetano (2023)**, with slight modifications. In each 500mL beaker, approximately 25g, 50g, and 100g of powdered seaweed were separately packed into 7cm × 5cm tea bags with 250mL of ethanol as the solvent. A hot bath was prepared using an electric stove and pot, and the beakers were heated until the total evaporation of ethanol. After extraction, the seaweed crude extracts were transferred to glass Petri dishes for final dehydration using a mechanical dehydrator set to 60°C (Fig. 3). The powdered extracts were then stored in test tubes for sterilization.

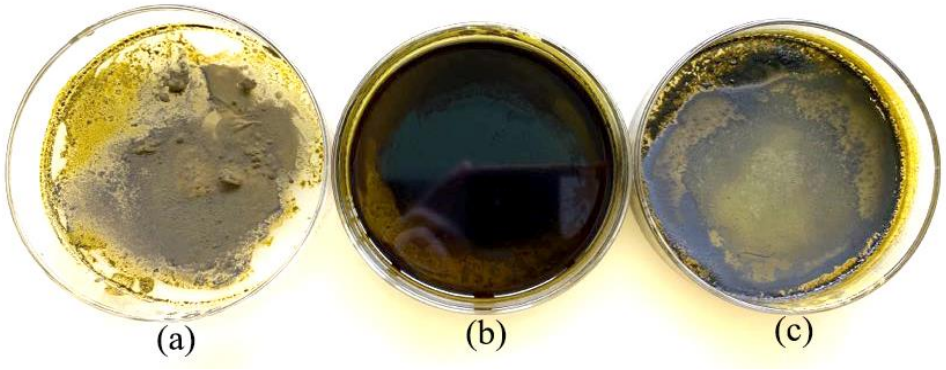


Fig. 3. Images of the macroalga *Hormophysa cuneiformis* after the decoction-extraction process at various ratios of seaweed and ethanol: (a) 25 g:250 mL; (b) 50 g: 250 mL; (c)100 g: 250 mL

3. Preparation of test bacteria

Three species of *Aeromonas* namely *A. eucrenophila*, *A. hydropila*, and *A. taiwanensis* previously isolated from the Nile tilapia were obtained from the Central Luzon State University-College of Fisheries, Science City of Muñoz, Nueva Ecija **(Aquino & Reyes, 2024)**. To ensure their purity, the *Aeromonas* stocks were re-cultured in *Aeromas* selective medium, producing yellow colonies after 24 hours of incubation.

4. Preparation of discs for antibacterial assay

The Kirby-Bauer disk diffusion method was used to assess the antimicrobial potential of the seaweed extracts **(Bauer *et al.*, 1966)**. Powdered seaweed extract at different concentrations (0.10, 0.20, and 0.40g/ mL) were dissolved in 4mL of distilled water. Tetracycline hydrochloride at a dose of 0.03mg/ mL was used as control. Approximately, 25µL of ethanolic seaweed crude extract (assay solutions) and tetracycline (positive control) were dispensed onto improvised paper discs made from Whatman Filter Paper No. 3 with a 7mm diameter. The paper discs were placed in a dehydrator machine to allow them to dry.

The *Aeromonas* stocks were grown until logarithmic phase in an Erlenmeyer flask containing broth. The bacterial suspension was prepared in sterile water to match the 0.5 McFarland turbidity standard (approximately 1×10^8 CFU/mL). From the adjusted bacterial suspension, 0.1mL was pipetted and evenly spread over the surface of the agar plate to create a uniform lawn of bacterial growth. The inoculum was let to dry for few minutes. Using sterile forceps, the seaweed crude extract and antibiotic discs were placed on the agar plate in eight replicates, ensuring sufficient spacing to avoid overlapping of inhibition zones. The discs were gently pressed onto the agar surface using a loop to ensure good contact. Finally, the plates were incubated at 30 to 35°C for 24 hours, allowing the bacteria to grow and the seaweed extracts and antibiotics to diffuse into the agar.

5. Measuring of zones of inhibition

After incubation, the diameters of the clear zones around the crude extracts and antibiotic discs, where bacterial growth was inhibited, were measured using a digital Vernier caliper with 0.01mm precision. The diameter of the inhibition zones were interpreted based on standard susceptibility criteria for seaweed extracts. The zone of inhibition (ZOI) was considered an indicator of antimicrobial activity of the seaweed extract against *Aeromonas* spp. ZOIs larger than 19mm in diameter were considered very active, between 14 to 19mm as active, between 10 and 13mm as partially active, and <10mm as inactive indicators of antimicrobial activity of the crude extract **(Guevarra *et al.*, 2005)**.

6. Statistical analysis

All data were subjected to one-way ANOVA to compare the ZOIs of different concentrations of seaweed extracts and antibiotics against the three species of *Aeromonas*. Results were presented as the means ± SD and significant difference at 5% confidence level was attained using LSD post-hoc analysis test. All statistical analyses were performed using Microsoft Excel 2021 and Statistical Tool for Agricultural Research (STAR) version 2.0.1.

RESULTS

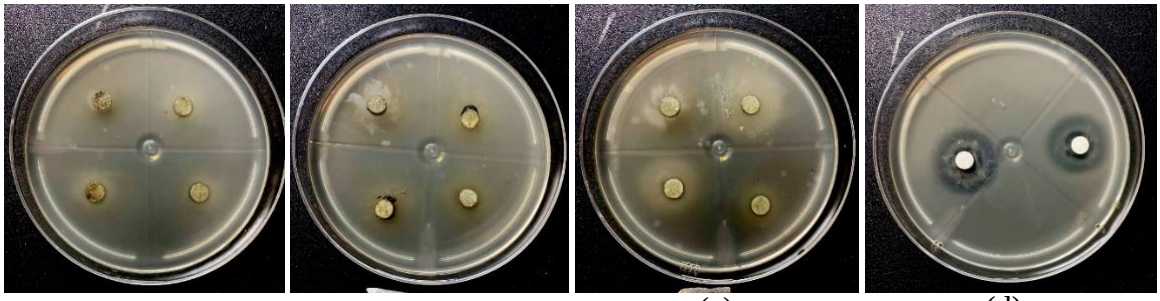
Table (1) shows the mean and standard deviation of inhibitory zones of *H. cuneiformis* extracts at various concentrations (0.10, 0.20, and 0.40g/ mL) against three species of *Aeromonas* (*A. eucrenophila*, *A. hydrophila*, and *A. taiwanensis*). The antimicrobial activity was classified based on the criteria by **Guevarra *et al.* (2005)**, which defines inhibition zones as very active (>19mm), active (14 to 18mm), partially active (10 to 13mm), and inactive (<10mm).

Table 1. Inhibitory zone of *Hormophysa cuneiformis* extracts against *Aeromonas* spp.

Concentration	Zone of Inhibition (Mean±SD)		
g/mL	*A. eucrenophila*	*A. hydropila*	*A. taiwanensis*
0.10	n.a.	21.88±2.56^{a}	23.25±1.51^{b}
0.20	n.a.	18.94±1.26^{b}	24.03±0.56^{a}
0.40	n.a.	16.18±1.72^{c}	23.00±1.77^{b}
Control	17.67±2.06^{a}	18.42±1.27^{b}	15.17±0.79^{c}

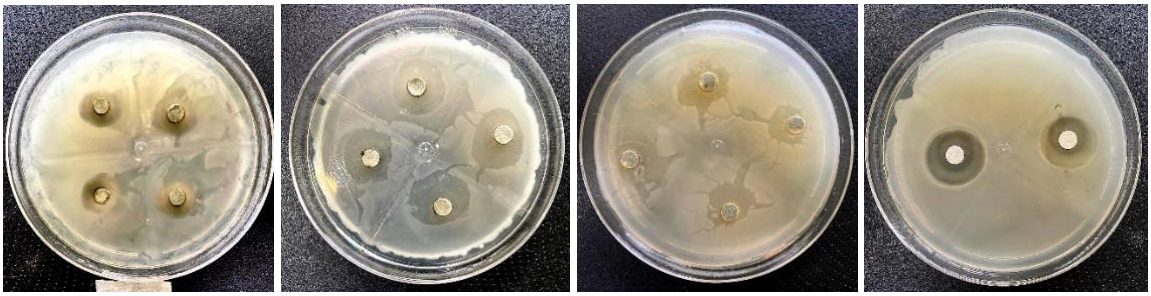
Note: Each value is presented as mean ± SD (n=8), and n.a. represents no activity. Values with the same letters (a-c) in each column are not significantly different (P<0.05). Antimicrobial effect: inhibition zone considered very active (>19mm); active (14 to 18mm); partially active (10 to 13mm); and inactive (< 10mm) (**Guevarra *et al.*, 2005)**.

The *H. cuneiformis* extracts showed no activity (n.a.) against *A. eucrenophila* (Fig. 4) at all tested concentrations, indicating a lack of inhibitory potential against this species. In contrast, significant antimicrobial effects were observed against *A. hydrophila* (Fig. 5) and *A. taiwanensis* (Fig. 6), though the extent of activity varied. For *A. hydrophila*, the ZOI decreased as the concentration increased. At 0.10g/ mL, the extract exhibited very active inhibition with a zone of 21.88±2.56mm. However, the activity declined to 18.94±1.26mm (active) at 0.20g/ mL and further to 16.18±1.72mm (active) at 0.40g/ mL. This decreasing trend may suggest potential interference at higher concentrations, possibly due to compound aggregation or reduced bioavailability.



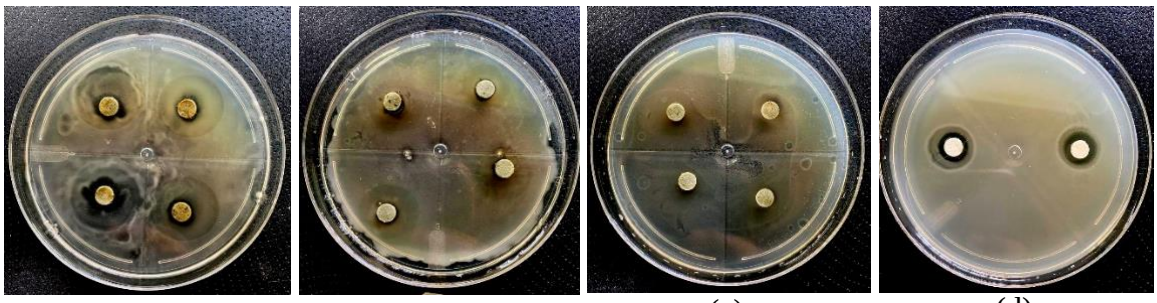
(a) (b) (c) (d)

Fig. 4. Antimicrobial activity of *Hormophysa cuneiformis* extract against *Aeromonas eucrenophila* at (a) 0.10 g/mL, (b) 0.20 g/mL, (c) 0.40 g/mL concentrations, and (d) in tetracycline as positive control



(a) (b) (c) (d)

Fig. 5. Antimicrobial activity of *Hormophysa cuneiformis* extract against *Aeromonas hydrophila* at (a) 0.10 g/mL, (b) 0.20 g/mL, (c) 0.40g/ mL concentrations, and (d) in tetracycline as positive control (d).



(a) (b) (c) (d)

Fig. 6. Antimicrobial activity of *Hormophysa cuneiformis* extracts against *Aeromonas taiwanensis* at (a) 0.10 g/mL, (b) 0.20 g/mL, (c) 0.40 g/mL concentrations, and (d) in tetracycline as positive control

Against *A. taiwanensis*, the extract exhibited very active antimicrobial effects across all concentrations. The highest ZOI (24.03±0.56 mm) was recorded at 0.20g/ mL, while at 0.10 and 0.40g/ mL showed slightly lower but still very active zones of inhibition, measuring 23.25±1.51mm and 23.00±1.77mm, respectively. These results

highlight an optimal concentration for peak activity, particularly against this *Aeromonas* species.

The control antibiotic demonstrated varying activities across the bacterial species. Against *A. eucrenophila*, the inhibition zone was 17.67±2.06mm, categorizing it as active. For *A. hydrophila*, the control exhibited a zone of 18.42±1.27mm, while its activity against *A. taiwanensis* was the lowest at 15.17±0.79mm, but both within the active range. Compared to the control, the extracts demonstrated comparable or superior inhibitory effects, particularly against *A. hydrophila* and *A. taiwanensis*.

DISCUSSION

The antimicrobial activity of *H. cuneiformis* extracts against *Aeromonas* spp. reveals species-specific inhibition, emphasizing the potential of marine-derived compounds as bioactive agents in combating aquaculture pathogens. Significant differences among seaweed treatments were observed after 24 hours of incubation.

1. Inhibitory activity against *A. eucrenophila*

The absence of inhibitory activity (n.a.) against *A. eucrenophila* at all concentrations suggests that the active compounds in *H. cuneiformis* may lack specificity toward this bacterial species. This finding aligns with studies that emphasize species-specific resistance mechanisms in bacteria, including differences in cell wall structures and efflux pumps **(Du *et al.*, 2018)**. The resistance observed could also be attributed to the limited penetration of bioactive compounds through bacterial membranes **(Demirel *et al.*, 2009)**.

2. Inhibitory activity against *A. hydrophila*

The extracts demonstrated a concentration-dependent decline in inhibitory activity against *A. hydrophila*. At 0.10g/ mL, the inhibition zone was 21.88 ± 2.56mm, classified as "very active." However, at higher concentrations (0.20 and 0.40g/ mL), the activity decreased to 18.94 ± 1.26 and 16.18 ± 1.72mm, respectively. This trend suggests that the crude extract may contain antagonistic compounds at higher concentrations, reducing the bioavailability of active components. Similar concentration-dependent activity has been reported in studies investigating other brown algae species (**Abdel-Raouf, 2015; Osman *et al.*, 2023)**. The efficacy of the extracts against *A. hydrophila*, a significant aquaculture pathogen, underscores its potential as an alternative to synthetic antibiotics **(Cunha *et al.*, 2022)**.

3. Inhibitory activity against *A. taiwanensis*

The extracts exhibited consistently high activity against *A. taiwanensis*, with inhibition zones of 23.25 ± 1.51 mm at 0.10 g/mL, 24.03 ± 0.56 mm at 0.20 g/mL, and

23.00 ± 1.77mm at 0.40g/ mL, all within the "very active" range. This highlights the efficacy of *H. cuneiformis* metabolites in targeting this bacterial species. Studies have demonstrated that phenolic compounds, such as phlorotannins found in brown algae, contribute significantly to antimicrobial activity by disrupting bacterial cell membranes and inhibiting enzyme functions, with *H. cuneiformis* extract also showing potential to induce DNA damage and promote apoptotic body formation in cancer cell lines in a dose-dependent manner **(Osman *et al.,* 2020; Ganesan, 2021)**. The significant activity observed against *A. taiwanensis* aligns with findings that brown algae-derived bioactives exhibit strong efficacy against Gram-negative bacteria **(Cox *et al.,* 2010)**.

4. Comparison with the control

The control tetracycline antibiotic showed moderate inhibition against all tested bacteria, with inhibition zones of 17.67 ± 2.06mm for *A. eucrenophila*, 18.42 ± 1.27mm for *A. hydrophila*, and 15.17 ± 0.79mm for *A. taiwanensis*. While the control exhibited lower activity against *A. taiwanensis*, the extract of *H. cuneiformis* demonstrated superior inhibition. This suggests that marine algae-derived compounds could serve as promising alternatives to conventional antibiotics, addressing concerns about antibiotic resistance **(Rayapu *et al.,* 2017; Qari & Khan, 2019)**.

In line with these findings, **Mohamed and Saber (2019)** conducted GC–MS analysis of the crude chloroform extract of *H. cuneiformis*, revealing the presence of 45 different bioactive compounds. Notably, fatty acids such as arachidonic acid (C20:4, ω–6; 16.18%), oleic acid (C18:1, ω–9; 15.61%), palmitic acid (C16:0; 9.18%), and dihomo-γ-linolenic acid (C20:3, ω–6; 8.97%) accounted for 71.48% of the total compounds. These essential fatty acids, known for their anti-inflammatory and antimicrobial properties, support the potential of *H. cuneiformis* as a source of bioactive compounds with therapeutic benefits **(Osman *et al.,* 2023)**.

Additionally, the sulfated polysaccharides found in *H. cuneiformis* also contribute to its bioactivity, demonstrating potential antimicrobial and anti-inflammatory activities **(Hamad, *et al.,* 2023)**. This further strengthens the argument for the utilization of marine algae, particularly *H. cuneiformis*, in the development of alternative antimicrobial agents.

5. Implications and future research

Aly *et al.* (2023) highlighted brown algae as a rich source of primary and secondary metabolites with unique biological activities, noting their diverse secondary metabolites, such as terpenoids (especially diterpenes), polymeric phenolics, phenolic acids, and flavonoids, which make them potential antibacterial agents. The results highlight the potential of *H. cuneiformis* extracts as a natural antimicrobial agent **(El-Manawy *et al.,* 2019)**, particularly against aquaculture pathogens like *A. hydrophila* and *A. taiwanensis*. Further studies are needed to isolate and characterize the bioactive compounds responsible for the observed activity, optimize extraction methods, and evaluate

synergistic effects with other natural compounds **(Cox *et al.*, 2010; Aly *et al.*, 2023)**. The antimicrobial activity depends on the type of extraction solvent and the concentration of seaweed used **(Mohamed & Abdullah, 2018)**. Understanding the mechanisms underlying the lack of activity against *A. eucrenophila* could inform strategies to enhance efficacy, such as combining extracts with permeabilizing agents or using other solvent for seaweeds crude extraction.

CONCLUSION

In summary, *H. cuneiformis* extracts exhibit selective antimicrobial activity. While they were inactive against *A. eucrenophila*, the extracts were highly effective against *A. hydrophila* and *A. taiwanensis*. For *A. eucrenophila*, only tetracycline at 0.03mg/ mL showed a significant ZOI; for *A. hydrophila*, 0.10g/ mL had the highest ZOI; and for *A. taiwanensis*, treatments showed distinct differences, with 0.20g/ mL being the most consistent. The concentration-dependent response suggests that the bioactive compounds in the extracts may have an optimal range for activity. These findings underscore the importance of further exploration into the inhibitory processes and the potential application of these extracts as antimicrobial agents against aquaculture pathogens.

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