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Research Article

Antimicrobial efficacy of *Ayapana triplinervis* chloroform extract against fish pathogenic *Aeromonas* strains

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Abstract

The increasing prevalence of antibiotic-resistant *Aeromonas* spp. in aquaculture necessitates alternative treatments for motile *Aeromonas* septicemia (MAS). This study evaluated the antibacterial potential of *Ayapana triplinervis* chloroform leaf extract against pathogenic *Aeromonas hydrophila*, *A. veronii*, and *A. caviae*. GC-MS analysis identified key bioactive compounds, including thymohydroquinone dimethyl ether (12.59%), ayapanin (17.27%), β-selinene (19.95%), and linolenic acid (12.55%), known for antimicrobial and membrane-disrupting properties. Antibiotic susceptibility testing revealed resistance to clindamycin, cefepime, and vancomycin but sensitivity to fluoroquinolones and tetracyclines, highlighting emerging multidrug resistance. The chloroform extract demonstrated dose-dependent antibacterial activity in well diffusion assays, exhibiting maximum inhibition zones of 15.0±0.8 mm to 20.0±0.8 mm at 40 µL/well, with *A. veronii* and *A. caviae* showing the highest sensitivity. *A. hydrophila* demonstrated lower susceptibility, possibly due to efflux pumps or biofilm formation. Pathogenicity assessment in *Labeo rohita* confirmed Aeromonas virulence (LD_{50}: 1.28×10^6 – 2.3×10^7 CFU/fish). The extract's efficacy is likely due to synergistic interactions among phytochemicals, particularly non-polar compounds solubilized by chloroform. These findings position *A. triplinervis* as a promising phytotherapeutic agent against MAS. Future studies should isolate active compounds and validate in vivo safety and efficacy for sustainable aquaculture disease management.

Keywords: Aeromonas, Antimicrobial resistance, *Ayapana triplinervis*, GC-MS, Phytochemicals

Highlights

- *A. triplinervis* chloroform extract shows strong anti-*Aeromonas* activity (≥18mm zones at 40µL)
- GC-MS reveals key antimicrobial phytochemicals (TQDE, ayapanin, β-selinene, caryophyllene)
- Dose-dependent inhibition ($p<0.05$) against *Aeromonas*, including multi drug resistant strains
- Synergistic phytochemical action disrupts membranes/enzymes, offering aquaculture alternative
- Extract outperforms polar solvents, solubilizing non-polar antimicrobial compounds effectively

INTRODUCTION

Ayapana triplinervis (syn. *Eupatorium triplinerve*), commonly known as "Ayapana" or "Vishalyakarani," is a perennial herb of the Asteraceae family. Originally native to South America-particularly Brazil-it has become naturalized across various tropical regions, including India. In traditional medicine systems such as Ayurveda, *A. triplinervis* is widely used for its antipyretic, anti-inflammatory, and antimicrobial properties. Its leaves are traditionally applied in the treatment of wounds and are noted for their antinociceptive and healing effects (Maity *et al.*, 2021; Rodrigues *et al.*, 2022; de Sousa *et al.*, 2024).

Phytochemical investigations have identified several key bioactive constituents within *A. triplinervis*, including thymohydroquinone dimethyl ether (TQDE), β-selinene, and the coumarin derivative ayapanin, all of which contribute to the plant's pharmacological activities (Gauvin and Marodon, 2008). Extracts prepared using various organic solvents-such as methanol, ethanol, acetone, and petroleum ether-have demonstrated broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria (Unnikrishnan *et al.*, 2014; Matos *et al.*, 2015; Krishnan and Nair, 2016).

In the context of Indian aquaculture, species of *Aeromonas* are among the most significant bacterial pathogens, responsible for motile Aeromonas septicemia

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(MAS), ulcerative lesions, and substantial mortality in freshwater cultured fishes (Saharia *et al.*, 2021). Pathogenic species such as *A. hydrophila*, *A. veronii*, and *A. caviae* are known to produce various virulence factors, including hemolysins, enterotoxins, and biofilm-forming enzymes (Majeed *et al.*, 2023). The increasing prevalence of antibiotic-resistant *Aeromonas* strains has highlighted the urgent need for alternative, sustainable antimicrobial strategies (Semwal *et al.*, 2023).

Numerous medicinal plants have been explored for their antibacterial activity against *Aeromonas* spp., with varying results depending on the extraction solvent and assay conditions. For example, ethanol extracts of *Curcuma longa* (turmeric), *Ocimum sanctum* (holy basil), and *Azadirachta indica* (neem) have shown inhibition zones ranging from 8 to 11 mm against *A. hydrophila* at 10 µl concentrations in disc diffusion assays (Harikrishnan and Balasundaram, 2008). In comparison, chloroform extracts of *A. triplinervis* have demonstrated equal or superior antibacterial efficacy, likely due to the presence of potent phytochemicals such as methyl thymohydroquinone (MTQ) and ayapanin (Matos *et al.*, 2015; Unnikrishnan *et al.*, 2014).

Considering the escalating challenge of antibiotic resistance in aquaculture pathogens, the present study aims to evaluate the chemical composition and antibacterial potential of chloroform leaf extract of *Ayapana triplinervis* against multiple strains of *Aeromonas*. The objective is to establish a phytotherapeutic foundation for its use as a natural antibacterial agent in fish health management.

MATERIALS AND METHODS

Plant collection and authentication: Fresh leaves of *Ayapana triplinervis* were collected during summer from Habibpur Farmer's Producer Company Limited, Boxinagar, Malda (24°57′52″ N, 88°14′48″ E). The plant was identified and authenticated by the Central National Herbarium, Botanical Survey of India, Shibpur, Howrah. Leaves were shade-dried, ground to a fine powder, and stored in airtight containers.

Extract preparation: Leaf powder (100 g) was subjected to chloroform extraction in a Soxhlet apparatus (60°C, four cycles). The extract was concentrated using a rotary evaporator (Hei-VAP/HB/HL/G1, Germany), dried, weighed to calculate yield, and stored at 4°C in amber bottles.

Phytochemical screening and GC-MS analysis: GC-MS analysis was performed using a Thermo MS-TSQ 9000 triple-quadrupole mass spectrometer with a DuraGuard capillary and TG-5 MS column (30 m × 0.25 mm × 0.25 µm). Operating conditions included: injector temp 260°C; helium carrier gas at 1 mL/min; oven program from 50°C to 300°C; EI mode at -70eV; full scan range 40-550 m/z. Compound identification was based on NIST 2020 library using Chromeleon CDS software (Adams, 2017).

Bacterial strains: Six motile Aeromonas strains comprising one *A. hydrophila* (BAH; PV450193), three *A. veronii* (B10: PV450195, H09: PV450197, 012: MZ366333), and two *A. caviae* (CSRMS2: PP087423, SSG: ON969256), were aseptically revived from cryopreserved stocks of the Department of aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, using Brain Heart Infusion Broth (BHIB) at 28°C. Strain viability and purity were verified through Gram-negative staining characteristics and standardized biochemical identification using the API 20E system.

Pathogenicity assessment: An experiment assessed the pathogenicity of a bacterial isolate by determining its median lethal dose (LD_{50}) using the Reed and Muench, (1938) method. The isolate was cultured, harvested, washed, and resuspended in saline, then diluted to concentrations ranging from 1×10^8 to 1×10^9 CFU/mL. Groups of 30 healthy *Labeo rohita* fish were injected intramuscularly with 0.1 mL of each dilution, while controls received sterile saline. Fish were kept in aerated tanks at 25±2°C, and mortality/morbidity was monitored for 5 days. Necropsies and bacterial re-isolation were conducted on affected fish to confirm infection. It's important to note that all animal experiments adhered to guidelines set forth by the Committee for Control and Supervision of Experiments on Animals, Department of Animal Husbandry and Dairying, Government of India.

Antibiotic susceptibility testing: Antibiotic susceptibility was tested via the Kirby-Bauer disk diffusion method. Bacterial suspensions were plated on Mueller-Hinton agar, and 17 antibiotic discs were placed evenly. After 24-hour incubation at 28°C, inhibition zone diameters were measured and interpreted as sensitive, intermediate, or resistant based on standard breakpoints.

Antibacterial assay: Antibacterial activity was evaluated via soft agar overlay well diffusion assay (Hockett and Baltrus, 2017). Overnight Brain Heart

Infusion Broth (Himedia, India) cultures of the test strains were mixed with soft agar (0.5% agar) and overlaid on Brain Heart Infusion Agar (Himedia, India) plates preloaded with 10-40 µL of the extract in 6 mm wells. Plates were incubated at 32°C for 24 h, and inhibition zones were measured. All tests were performed in triplicate.

Statistical analysis: Data were expressed as mean±SD. Inhibition zones were analyzed using one-way ANOVA, followed by Tukey's post hoc test (Ricker, 1945; Zar, 1999). Statistical significance was set at $p<0.05$ using IBM-SPSS version 22.0.

RESULTS

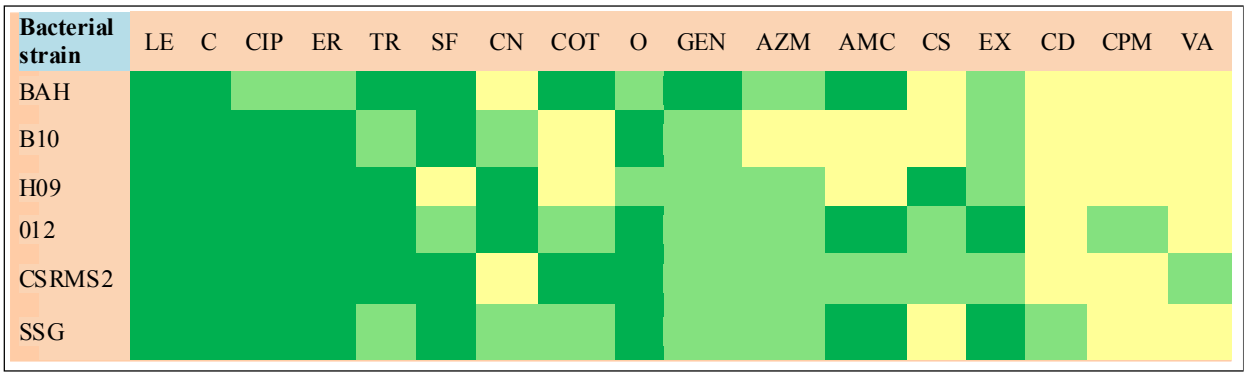
GC-MS Phytochemical Profiling: Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the chloroform extract of *Ayapana triplinervis* leaves revealed a complex blend of bioactive constituents (Table 1). A total of 15 major compounds were identified based on retention time and relative abundance compared to the NIST 2020 library. Among them, β-selinene (19.95%), ayapanin (17.27%), thymohydroquinone dimethyl ether (12.59%), linolenic acid (12.55%), and n-hexadecanoic acid (8.93%) represented the most abundant compounds.

Other notable constituents included caryophyllene (7.97%), linoelaidic acid (5.62%), and caryophyllene oxide (4.37%). The presence of lupeol acetate (3.88%) and neophytadiene (1.17%) further indicated the bioactive diversity of the extract. These compounds are widely documented for their anti-inflammatory,

Table 1. GC-MS identified antimicrobial compounds in *A. triplinervis* chloroform extract.

Sl No.	Compound name	Retention Time (min)	Relative Area(%)	Properties	Reference
1	Cypera-2,4-diene	15.601, 15.809	1.26	Anti-ulcer,anti-inflamatory	Qu *et al*., 2021
2	Beta-elemene	16.061	0.95	Antimicrobial, anti-inflammatory, anticancer, anti-stress	Xie *et al*., 2020
3	Beta-Maaliene	16.262	1.22	Anti-oxidant,anti-inflammatory	Destryana *et al*., 2014
4	Thymohydroquinone dimethyl ether	16.490	12.59	Anti-inflammatory, antibacterial, antinociceptive	Bhavikatti *et al*., 2024
5	Caryophyllene	16.567	7.97	Antibacterial,Anti-inflammatory	Santos *et al*., 2021 Mardiana *et al*., 2020
6	Beta-Selinene	17.647	19.95	Antimicrobial, anti-inflammatory and wound healing activity	Mardiana *et al*., 2020
7	Caryophyllene oxide	19.139	4.37	Analgesic and anti-inflammatory	Chavan *et al*., 2010
8	Ayapanin	21.420	17.27	Antimicrobial, anticeptive, anti-inflammatory, antihelminthic, hepatoprotective, antioxidant	Bhattacharyya *et al*., 2023
9	Neophytadiene	22.433	1.17	Anxiolytic-like and anticonvulsant activities	Gonzalez-Rivera *et al*., 2023
10	Phytol	22.768, 23.016	1.79	Antioxidant, antinociceptive, immune-modulating, antimicrobial effects	Usman *et al*., 2022
11	n-Hexadecanoic acid	24.150	8.93	Anti-Inflammatory antioxidant and antibacterial agent	Ganesan *et al*., 2024
12	Linoelaidic acid	26.641	5.62	Anti-cancer	Dutta *et al*., 2023
13	Linolenic acid	26.752	12.55	Antioxidant, antimicrobial activity	Odchimar *et al*., 2016 Krishnaveni *et al*., 2014
14	7-Methyl-Z-tetradecen-1-ol acetate	27.034	0.46	"-"	"-"
15	Lupeol acetate	45.934	3.88	Antimicrobial, anti-inflammatory, antioxidant, and anticancer	Bako *et al*., 2023 Usman *et al*., 2022

Table 2. Antibiotic resistance profiles of six *Aeromonas* strains.



[**LE:** Levofloxacin, **C:** Chloramphenicol, **CIP:** Ciprofloxacin, **ER:** Erythromycin, **TR:** Trimethoprim, **SF:** Sulphafurazole (sulfisoxazole), **CN:** Cefalexin (Cephalexin), **COT:** Co-trimoxazole, **O:** Oxytetracycline, **GEN:** Gentamycin, **AZM:** Azithromycin, **AMC:** Amoxyclav, **CS:** Colistin sulphate, **EX:** Enrofloxacin, **CD:** Clindamycin, **CPM:** Cefepime, **VA:** Vancomycin] [Yellow: Resistant, Light Green: Intermediate, Green: Sensitive]

Table 3. Zones of growth inhibition (mm) showing antibacterial activity of *Ayapana triplinervis* chloroform extract in soft agar overlay well diffusion assay.

Bacterial strains	Concentration (µL/well)			
	10	20	30	40
	Zone of inhibition (mm)			
A. hydrophila (PV450193) BAH	3.3±0.5^{aA}	8.7±0.5^{bA}	11.3±0.5^{cA}	15.0±0.8^{dA}
A. veronii (PV450195) B10	3.7±0.5^{aA}	10.3±0.5^{bB}	13.3±0.5^{cB}	16.0±0.8^{dA}
A. veronii (PV450197) H09	6.3±0.5^{aB}	11.3±0.9^{bB}	15.0±0.8^{cC}	20.0±0.8^{dB}
A. veronii (MZ366333) 012	7.0±0.0^{aB}	11.7±0.5^{bB}	16.3±0.5^{cC}	18.3±0.5^{dB}
A. cavie (PP087423) CSRMS2	6.3±0.5^{aB}	11.3±0.5^{bB}	14.7±0.5^{cB}	19.3±0.5^{dB}
A. cavie (ON969256) SSG	3.7±0.9^{aA}	8.2±0.8^{bA}	11.7±0.5^{cA}	16.0±0.8^{dA}

Values are presented as mean±standard deviation of three observations. Values showing different small alphabets within a row differed significantly (p<0.05). Values showing different large alphabets within a column differed significantly (p<0.05).

antioxidant, and antimicrobial properties.

Antibiotic susceptibility test: The antibiotic susceptibility profiles of the six bacterial isolates against 17 antibiotics are presented as a heatmap in Table 2. All isolates were uniformly sensitive to levofloxacin, chloramphenicol, ciprofloxacin, erythromycin and trimethoprim. In contrast, clindamycin, cefepime, and vancomycin were the least effective antimicrobial agents. Majority of the isolates exhibited multi drug resistance.

Pathogenicity assessment: The bacterial strains BAH, B10, H09, 012, CSRMS2, and SSG demonstrated pronounced virulence in *Labeo rohita*, eliciting dose-dependent mortality within 1-5 days post-infection (dpi), with complete lethality (100%) observed at a high challenge dose (1×10^9 CFU/mL). The median lethal doses (LD_{50}) were quantified as 1.28×10^7 (BAH), 8.9×10^6 (B10), 1×10^7 (H09), 2.3×10^7 (012), 1.2×10^7 (CSRMS2), and 6.4×10^6 (SSG) CFU/fish, indicating strain-specific variations in pathogenicity. In stark contrast, negative control specimens administered sterile physiological saline remained asymptomatic and exhibited zero mortality throughout the experimental period. Post-mortem microbiological assessment confirmed successful re-isolation of the pathogens from hepatic and renal tissues, with subsequent morphological and biochemical analyses corroborating their identification and pathogenic role.

Antibacterial assay: The antibacterial activity of *A. triplinervis* chloroform extract was assessed against six strains of *Aeromonas* spp., namely three strains of *A. veronii*, two of *A. caviae*, and one of *A. Hydrophila* (Table 3). The assay revealed a clear significant (p<0.05) dose-dependent inhibition pattern across all strains tested. At the lowest concentration (10 µL/well), minimal inhibitory activity was observed, with inhibition zones ranging from 3.3 to 7.0 mm whereas at higher doses (40 µL/well) zones were as large as 20.0 mm. Among all

tested strains, *A. veronii* (PV450197), *A. veronii* (MZ366333) and *A. caviae* (PP087423) were the most sensitive. *A. hydrophila* showed the lowest sensitivity at 10 µL (3.3±0.5 mm) and gradually increased to 15.0±0.8 mm at 40 µL (Fig. 1).

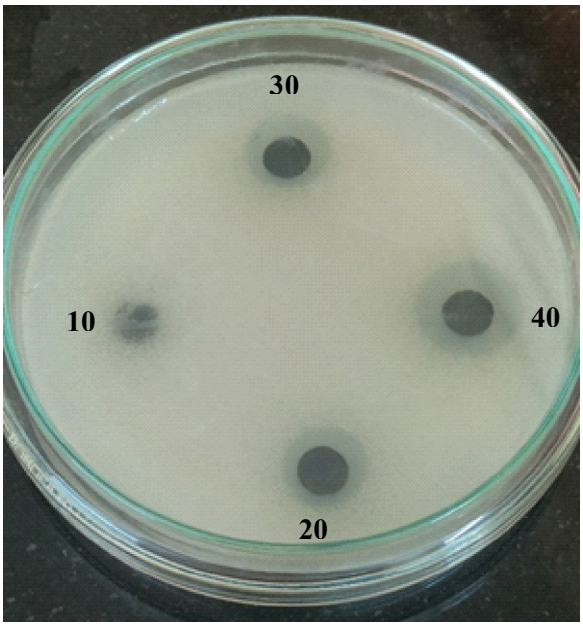


Fig. 1. Agar overlay well diffusion assay of *Ayapana triplinervis* chloroform leaf extract against *Aeromonas hydrophila.*

DISCUSSION

This study demonstrates the significant antibacterial activity of *Ayapana triplinervis* chloroform leaf extract against fish-pathogenic *Aeromonas* spp., highlighting its potential as a natural and sustainable alternative for managing motile Aeromonas septicemia (MAS) in aquaculture. The promising results align with prior evidence supporting the antimicrobial efficacy of *A. triplinervis* and underscore the value of phytotherapeutic approaches in aquaculture health management.

The chemical profile of *A. triplinervis* can vary considerably depending on phenological and environmental conditions, as is commonly observed in aromatic plants (Gauvin and Marodon, 2008). Variations in phytoconstituents across geographical regions have been documented. For example, Gupta *et al.*, (2004) identified selin-4 (15), 7(11)-dien-8-one (36.6%), β-caryophyllene (14.7%), and σ-elemene (5.9%) in essential oil extracted from leaves collected in Lucknow, India. Conversely, Unnikrishnan *et al.* (2014) reported high levels of dimethyl ether thymoquinone (TQDE), ranging from 80.3% to 86.9%, in samples from Kerala. The presence of ayapanin, a coumarin compound, has also been confirmed in multiple studies (Viet Huong *et al.*, 2020; Gauvin and Marodon, 2008). Gauvin-Bialecki and Marodon (2008) found that the essential oil composition of *Ayapana triplinervis* varied by geographic location, suggesting climatic influences on its chemical profile. Unnikrishnan *et al.* (2014) further reported seasonal fluctuations in oil yield and constituent composition across plant parts.

The GC-MS analysis revealed the presence of key phytochemicals such as TQDE, ayapanin, β-selinene, caryophyllene, linolenic acid, and n-hexadecanoic acid-several of which are known to possess antimicrobial, membrane-disrupting, or enzyme-inhibiting activities (Maity *et al.*, 2021; Adams, 2017). Notably, coumarins such as ayapanin (also known as herniarin) have demonstrated antimicrobial and anti-inflammatory properties, as well as inhibitory effects on various enzyme systems (Murray *et al.*, 1982). The strong antibacterial activity observed in this study, especially against *A. veronii* and *A. caviae* (≥18 mm at 40 µL), may be attributed to the synergistic interactions among these bioactive constituents.

Previous studies have shown that methanol and ethanol extracts of *A. triplinervis* inhibit several pathogenic bacteria, including *E. coli*, *S. aureus*, *S. typhi*, and *P. aeruginosa* (Unnikrishnan *et al.*, 2014; Matos *et al.*, 2015). The enhanced antibacterial efficacy observed with the chloroform extract in this study could be due to its ability to solubilize non-polar compounds like TQDE and â-selinene, which are less extractable in polar solvents. These compounds are known for their potent biological activities, including disruption of microbial membranes and inhibition of nucleic acid synthesis (Gauvin and Marodon, 2008). On the other hand Fernezelian *et al*. (2023) suggested that *Ayapana triplinervis* exhibits potential for mitigating metabolic disorders and related complications, as evidenced in zebrafish studies, with leaf extracts showing no developmental toxicity at elevated doses. Similarly, Haddad *et al*. (2019) reported that thymohydroquinone dimethyl ether, a key phytoconstituent of *A. triplinervis*, induced no toxicity in zebrafish.

Concurrently, this study assessed the antibiotic susceptibility of *Aeromonas* isolates, revealing high sensitivity to levofloxacin, ciprofloxacin, chloramphenicol, and tetracyclines, consistent with prior findings (Sonkol *et al.*, 2020; Mohanty *et al.*, 2008). However, resistance to clindamycin, cefepime, and vancomycin was observed, likely due to selective pressure from antibiotic misuse in aquaculture (Santos and Ramos, 2018). Moreover, emerging resistance to co-trimoxazole, gentamicin, colistinsulfate,

azithromycin, and amoxicillin-clavulanate underscores the urgent need for alternative treatments to curb multidrug-resistant (MDR) strains (Piotrowska and Popowska, 2015).

The extract exhibited significant ($p<0.05$) dose-dependent inhibition across all tested *Aeromonas* strains, suggesting a concentration-driven mechanism of action. Interestingly, *A. hydrophila* showed relatively lower sensitivity, which may reflect inherent resistance mechanisms such as efflux pump activity or biofilm formation (Majeed *et al.*, 2023). Nevertheless, inhibition zones up to 15 mm were recorded even against this species, indicating meaningful antibacterial potential.

In conclusion, the findings from this study reinforce the potential of *A. triplinervis* chloroform leaf extract as an effective antibacterial agent against *Aeromonas* spp. in aquaculture. The presence of multiple bioactive constituents with complementary mechanisms likely contributes to the observed efficacy. Future studies should aim to identify and isolate specific active compounds, investigate their combined effects, and assess the extract's effectiveness and safety when administered through feed to fish. This includes examining potential toxicity risks to ensure the development of safe, plant-based treatments for managing fish health.

This study reveals that *Ayapana triplinervis* chloroform extract effectively combats drug-resistant Aeromonas pathogens through synergistic action of its bioactive components (TQDE, ayapanin, β-selinene, caryophyllene). These compounds collectively damage bacterial cell structures, block essential enzymes, and bypass resistance pathways, marking this extract as a potential aquaculture treatment. However, while traditional herbal remedies like oregano, garlic, and neem enjoy widespread aquaculture use and documentation (Ng *et al.*, 2024), *A. triplinervis* faces several knowledge gaps. Current limitations include undefined regulatory status, unoptimized administration methods, and insufficient comparative data against existing treatments - all requiring thorough investigation before practical implementation in aquatic disease control.

Conflict of interest: The authors declare that they have no conflict of interest.

Data availability statement: All the data are available with the author and can be provided if required.

Author's contribution: AS: Conceptualization, material preparation and laboratory analysis; SB: Conceptualization, laboratory analysis and data collection; PM: Conceptualization, design and writing original draft; DM: Performed laboratory analysis; NC: Performed interpretation of results; AS: Performed laboratory analysis; BB: Performed laboratory analysis and material preparation; GD: Performed editing of manuscript; TJA: Performed editing of manuscript; SK: Performed statistical analysis of data.

Use of artificial intelligence tools: No artificial intelligence tools were used in this research.

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