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RESEARCH

Therapeutic effect of *Bouea macrophylla* Griffith in protecting Koi fish from *Enterococcus faecalis* infection and acute ammonia toxicity

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Abstract

This study evaluates the potential of dietary supplementation with *Bouea macrophylla* Griffith leaf ethanolic extract (TD1 extract) as an antibiotic alternative for koi carp (*Cyprinus carpio*) within the One Health framework. Fish were fed diets supplemented with 0.5–1.5% TD1 extract and compared with doxycycline-treated and control groups. Disease resistance was tested by challenging fish with haemolytic *Enterococcus faecalis*, and protection against acute ammonia toxicity was also assessed. Fish receiving 0.5% TD1 extract or doxycycline supplementation showed approximately 60% survival after bacterial challenge, while 1% TD1 supplementation resulted in complete survival. In prevention tests, fish fed doxycycline or 1.5% TD1 extract-supplemented feed had reduced natural *Vibrio* infections, with survival rates of 65 and 60%, respectively, compared to 30% in controls after 10 days. Against acute ammonia exposure, fish receiving 1% TD1 extract showed no mortality, while doxycycline and control groups exhibited moderate (43%) and high (90%) mortalities, respectively. Serum glutamic pyruvate transaminase (SGPT) levels remained stable in the group of fish fed TD1 extract-supplemented feed but increased marginally in fish doxycycline-supplemented feed after ammonia exposure. These findings suggest that *B. macrophylla* TD1 extract is a promising and eco-friendly alternative to antibiotics. However, further long-term and large-scale studies are essential to validate safety and efficacy across diverse aquaculture species. Additionally, exploring potential synergistic effects with probiotics or immunostimulants will be important for optimizing its application in commercial aquaculture systems.

Keywords Eco-friendly approach · Koi carp · Toxicity tolerance · Disease resistant · *Enterococcus faecalis*

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Introduction

Ornamental aquaculture contributes to biodiversity, economic prosperity, and cultural significance. However, unsustainable practices such as chemical pollution pose serious risks to ecosystems and human well-being. Pet fish transported across areas without sufficient safety standards disseminate infectious organisms, induce stress, and result in high mortality rates (Cardoso et al. 2019). Inadequate biosecurity controls foster disease outbreaks that reduce aquaculture productivity and output, thereby restricting trade and limiting commerce. Stress factors such as poor water quality, overcrowding, transport, and diet contribute significantly to bacterial outbreaks in ornamental fish. For example, poor aquaculture management can elevate water ammonia levels, posing serious threats to fish health (Tsui et al. 2002).

Pathogenic infections in aquaculture result not only in high mortality rates but also in the emergence of multi-drug

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resistant bacteria. The use of antibiotics in both fish farming and ornamental aquaculture has contributed to the development of antibiotic-resistant pathogens, posing threats to animal and human health. Poor water quality, overcrowding, transportation stress, and inadequate food are key factors that trigger bacterial outbreaks in including in ornamental fish (Trust and Bartlett 1974; Rahman et al. 2017; Walczak et al. 2017). The trade of ornamental fish and contact with contaminated materials contribute to the spread of pathogens, increasing the risk of human infection. Among them, Enterococci, especially *Enterococcus faecalis*, are common opportunistic pathogens in humans, causing severe diseases such as endocarditis, bacteremia, and urinary tract infections (Werner et al. 2008; Teixeira and Merquior 2013; O'Driscoll and Crank 2015).

Bacterial infections exhibiting high resistance to key antibiotics have become prevalent in ornamental aquaculture (e.g., Saengsitthisak et al. 2020; Hossain and Heo 2021; Anjur et al. 2021; Preena et al. 2020; Walczak et al. 2017). In particular, *Vibrio* spp., *Pseudomonas* spp., and *Citrobacter* spp. have been identified, with *Aeromonas* spp. showing resistance rates exceeding 75% for amoxicillin, oxytetracycline, and erythromycin (Saengsitthisak et al. 2020; Bhat and Altinok 2023). In aquaculture, *E. faecalis* is recognized as an opportunistic fish pathogen, causing mass mortality in various fish species worldwide (e.g., Petersen and Dalsgaard 2003; Ahmed and El-Refaey 2013). Studies (e.g., Rahman et al. 2017; Ha et al. 2023) have revealed that *E. faecalis* is a frequent cause of opportunistic infections in hospitals and communities due to antibiotic misuse in clinical environment and animal husbandry. These bacteria can infiltrate the food chain through water transmission, occasionally transferring drug-resistant genes (Bhat and Altinok 2023; Ha et al. 2023).

Environmentally friendly measures, such as proper waste management, substitution of hazardous chemicals, and the use of plant-based natural antibiotics, play a crucial role in risk reduction (Bhat and Altinok 2023; Mishra et al. 2023; Rahman et al. 2017). These sustainable practices not only protect habitats and native aquatic species but also help mitigate disease outbreaks, enhancing biodiversity and improved global public health. Alternative to antibiotics in fish farming is a key strategy for preventing aquatic diseases, minimizing antibiotic resistance, and promoting better environmental and public health outcomes (Ahmadifar et al. 2020; Harikrishnan et al. 2010). Plant-derived compounds offer a sustainable, cost-effective alternative to synthetic antibiotics, with fewer adverse effects and a lower risk of drug resistance (Lewis and Ausubel 2006; Cheesman et al. 2017). However, the effectiveness and modes of plant extracts may vary depending on the fish species (e.g. Ahmadifar et al. 2019; Sadeghi et al. 2021; Mehrinakhi et al. 2021). Furthermore, prior research seldom addressed the integrated challenge of both bacterial infection and environmental toxicity, particularly acute ammonia stress.

Bouea macrophylla Griffith, a tropical fruit plant in the Anacardiaceae family, is known for its rich phytochemical composition including polyphenols, flavonoids, tannins, saponins, glycosides, and essential oils (Hai Ha et al. 2022; Nguyen et al. 2020). Traditionally, it has been used in Southeast Asian folk medicine for its antimicrobial, antioxidant, and anti-inflammatory effects. Previous studies (e.g. Hai Ha et al. 2022; Nguyen et al. 2020; Van Vo et al. 2022) demonstrate its notable antioxidant potency and antibacterial activity against numerous pathogens. This extensive bioactivity supports its potential as a sustainable natural antibiotic alternative in aquaculture. However, there have been no investigations assessing *B. macrophylla* extract for its in vivo ability. The present study is therefore unique in exploring the use of *B. macrophylla* extract as a dietary supplement specifically in koi carp to protect host from *E. faecalis* nor its role in mitigating mortality in response to ammonia exposure. Also, this study evaluates the efficacy of *B. macrophylla* Griffith extract in enhancing koi carp health, promoting it as an eco-friendly antibiotic substitute within the One Health perspective, which has contributed significantly to the development and spread of antibiotic resistance in aquaculture to global AMR concerns (via environmental dissemination and zoonotic transfer) would establish a stronger connection between ornamental aquaculture practices and human health.

Materials and methods

Ethics approval

The experiments were conducted at a research regulation operated by the Falculty of Animal Sciences at HUTECH University. All animal studies adhered to the recommendations of the Department of Animal Health (reference number TCVN 8400:2019), with experimental methods fully approved by the Institute of Animal Sciences for Southern Vietnam.

Bacterial strain

In this study, haemolytic *E. faecalis* isolate KOI-T (10^5 CFU/fish), identified as a virulent bacterium isolated from *Cyprinus carpio* in our previous study (Nguyen 2019), was used for challenge experiments. Bacterial suspensions were prepared using saline solution (0.85% NaCl) and diluted serially ten-fold to obtain the desired final bacterial concentration (CFU/ml). Colony-forming units (CFU) were quantified through the plate dilution method, which involved plating bacterial cell suspensions onto Tryptic Soy Agar (TSA) plates and counting the resulting colonies. The type strain *E. faecalis* ATCC 29212 was utilized as a reference for biochemical comparisons.

Fish

Two hundred koi carp (average weight 6±1 g fish^{-1}) with vibrant colors and active responses were procured from Minh Loc fish farm, Thu Duc City, Vietnam. The fish were acclimated for one week in a 300-L tank system with continuous aeration at the veterinary laboratory. For the pathogenicity tests, each fish was intraperitoneally injected with 0.1 ml of the bacterial suspension and maintained at a temperature of 28±1 ºC. Control fish received injections of 0.1 ml sterile saline (0.85% NaCl). Over a 14-day period, fish were monitored daily under aerated conditions, and mortalities were attributed to the inoculated isolate only if the bacterium was recovered in pure culture from the spleen and liver of dead fish or identified through biochemical tests. Experimental setups included (1) feeding safety and efficacy testing with 30 fish per tank and three replicates per group; (2) infection challenge tests with 10 fish per tank and three replicates; and (3) ammonia toxicity tests with 10 fish per tank and three replicates.

Medicinal plant extraction preparation

Our preliminary experiment demonstrated that the leaf extract of *B. macrophylla* Griffith TD1 exhibited antibacterial activity against *E. faecalis*, which was isolated from various diseased ornamental fish species, including *Cyprinus carpio*, *Cyprinus carpio* Linnaeus, and *Carassius auratus auratus* (Nguyen 2019), or against strain *E. faecalis* ATCC 29212 isolate from urine (Nguyen et al. 2020). This study further investigates the effects of *B. macrophylla* Griffith leaf extract on the health of *Cyprinus carpio*. The leaves, collected during the dry season (February to April) from Vinh Long Province, Vietnam, were botanically identified by Assoc. Prof. Tran Hop at the University of Science, Vietnam National University Ho Chi Minh City. The extraction process followed methods outlined in a previous study (Hai Ha et al. 2022). Briefly, fresh leaves were thoroughly rinsed with distilled water and deionized water, dried at 50ºC until reaching consistent weight, and ground into fine powder using an electric grinder. The powdered material was stored at room temperature in sealed polyethylene bags within plastic containers. The ethanolic extract was prepared by soaking the powder twice in ethanol (EtOH) at 50ºC with a mass-to-volume ratio of 1:10, shaking the mixture at 150 rounds per minute for 24–48 h, and repeating the process three times. The mixture was then filtered multiple times to obtain the crude extract, which was stored at 4ºC for further analysis and fractionation.

The percentage of extract yeild (EY) is calculated according to the following formula (e.g. Ngamkhae et al. 2022):

$$\mathrm{EY}\ (\%) = \frac{\mathrm{M_E}}{\mathrm{M}_{dl} - (\mathrm{M}_{dl} * W_{dl})} \times 100$$

In which: M_E: Mass of extracted extract (g)

M_{dl}: Weight of raw materials (g)

W_{dl}: moisture content of raw material (g)

The chemical composition of TD1 extract was analyzed using the dissolution method, which separates compounds in medicinal herbs based on solvents with varying polarities. Components of the extract were then qualitatively and quantitatively identified through specific chemical reactions, following standard procedures (Ciulei 1982; Sarla et al. 2012). These methods were enhanced by the Department of Pharmacology, Faculty of Pharmacy, University of Medicine and Pharmacy, Ho Chi Minh City (2015) to detect carbohydrates, saponins, flavonoids, amino acids, anthraquinone glycosides, steroids, polyphenols, and tannins.

Evaluating the safety of plant extracts on fish and their efficacy in protecting fish from natural lethal agents

In this experiment, acclimated koi carp (average weight 6±1 g fish^{-1}) were randomly assigned to 20L tanks with aeration. Fish (30 fish per tank, 3 replicates per group) were divided into three groups including control group, antibiotic-treated group, and TD1 extract-treated group. Koi carp were subjected to a preliminary screening process to ensure they were free from bacterial infections. Three fish were chosen at random for kidney isolation and streaked on TCBS medium (Himedia, India), after which they were incubated at 28 °C for 24 h and bacterial growth was detected. The control group was fed a commercial diet, while experimental groups received doxycycline-supplemented feed (100 mg/kg fish weight) or 1.5% TD1 extract-supplemented feed (1.5% kg^{-1} baseline diets). All fish were fed at 2% of their biomass per day, with feedings conducted twice daily (9 am and 5 pm) for two weeks. External symptoms of disease and abnormal behaviors in treated fish were monitored throughout the study.

Evaluating the effectiveness of plant extracts in protecting fish from artificially challenged

The experiment aimed to evaluate the effectiveness of *B. macrophylla* TD1 extracts compared to commercial antibiotics.

After intraperitoneal infection with *E. faecalis* isolate KOI-T (10^5 CFU fish^{-1}), koi carp were randomly assigned to five groups (10 fish per tank, 3 replicates per group). Groups N2 and N3 received 0.5% and 1% (w/w) TD1 extract- supplemented feed, respectively, as therapeutic treatments. Group N4 was fed a diet supplemented with doxycycline (Sigma-Aldrich) at 2% kg^{-1} baseline feed (2 g doxycycline per 98 g basal meal) (Kang et al. 2013), while group N5, the treatment control, was fed a basal diet. Non-challenged fish (injected with 0.85% NaCl solution) were fed a baseline diet and served as controls. All fish were fed twice daily (9 am and 5 pm) at 2% of their body weight in seven days following challenge. The cumulative mortality rates were monitored for seven days, and dead fish were tested to confirm the presence of typical bacteria in the kidney.

Evaluating the in vivo efficacy of plant extracts in protecting fish against ammonia toxicity

The experiment aimed to evaluate the effectiveness of *B. macrophylla* TD1 ethanolic plant extracts in protecting fish from acute toxicity caused by ammonia nitrogen over 24 h. Acclimated koi carp were randomly assigned to three groups (10 fish per tank, 3 replicates per group). Experimental groups were fed diets supplemented with either doxycycline (Sigma-Aldrich) at 2% of the basal diet (2 g doxycycline per 98 g basal diet, group N2) or 1% TD1 extract (group N3). The control group (N1) received a basal diet free of drugs. All fish were fed twice daily at 2% of their body weight at room temperature for 20 days.

In the ammonia challenge experiment, 100 g/L ammonium chloride (NH_4Cl) (Sigma-Aldrich) was dissolved in 1L of distilled water, following the method outlined by Zuffo et al. (2021). This stock solution was then diluted with de-chlorinated tap water to reach the desired ammonia concentrations for testing. The toxicity pretest on koi fish was performed before the formal test. Preliminary trials showed that fish mortality increased as ammonia nitrogen levels rose, with exposure to 0.5 mg/L ammonia nitrogen causing 90% mortality within 24 h. Therefore, the final challenge trial used the 0.5 mg/L ammonia nitrogen concentration deliberately to impose a stringent test condition, providing a valid assessment of the protective effects of treatments.

Water quality and sample collection

Water quality parameters such as teamperature, pH, conductivity, total dissolved solids (TDS), and dissolved oxygen (DO) were monitored daily throughout the experiment using an automatic analyzer. The concentrations of total ammonia nitrogen (TAN) were measured using the Nessler method. The levels of unionised ammonia were then calculated by applying the TAN values, along with measured pH and water temperature, to the Emerson formula, which estimates the equilibrium between unionised ammonia and ammonium based on these parameters (El-Shafai et al. 2004).

After 20 days in the feeding experiment (before ammonia exposure), blood samples were collected from the caudal vein of the fish (n = 5 per group) using 1-mL disposable heparinized syringes. The samples were centrifuged at 3000 g for 5 min at 4 °C to separate serum. Serum enzyme activity levels of glutamic oxalate transaminase (SGOT) and glutamic pyruvate transaminase (SGPT) were analyzed using the Kind-King method in conjunction with a commercial clinical diagnostic kit (Randox Laboratories, UK). This assay was performed at the Ho Chi Minh City Department of Animal Husbandry and Veterinary Medicine, Vietnam. For the next five days, the fish continued to be fed according to the experimental design before u ammonia toxicity testing. On day 25, experimental fish groups were challenged with ammonia by exchanging fresh water containing ammonia to reach a final concentration of 0.5 mg/L of water. Experiments were monitored and the survival of fish were recorded within 24 h. Blood samples were collected from surviving fish and analyzed for SGOT and SGPT as described above. Fish were not fed during the 24-h challenge period.

Statistical analysis

All the data obtained above were measured and calculated using Excel 2019. Statistical analysis was performed using two-way analysis of variance (ANOVA) to find significant differences in various parameters between the in vivo experimental groups. The numbers of dead fish in the experimental groups were compared using Fisher's exact test. All data are expressed as the mean ± standard error (SE). A p-value < 0.05 was considered to be statistically significant.

Results

In-vitro antibacterial activity of ethanolic extract of *B. macrophylla.*

Our preliminary findings (Nguyen 2019) indicate that the ethanolic extract of *B. macrophylla* TD1 exhibits inhibitory activity against the growth of *E. faecalis* isolates KOI-T obtained from ornamental fish. In particular, the TD1 extract exhibited strong resistance (> 12 mm inhibition zone) against the *E. faecalis* KOI-T compared to doxycycline (30 µg), which produced a zone of 11.00 ± 1.73 mm. In contrast, the KOI-T isolate showed sensitivity to the antibiotics Ciprofloxacin (5 µg) and Ofloxacin (5 µg) (Suplementary Table 1), with inhibition zones measuring 29.33 ± 1.15 mm and 24.67 ± 2.52 mm, respectively.

Table 1 Water parameter changes before and after a 24-h exposure to ammonia. Different letters indicate statistically significant differences ($p < 0.05$)

Parameters	Fresh water	Before NH3 exposure	After NH3 exposure		
			Group N1	Group N2	Group N3
Conductivity (μs)	106.33 ± 1.53	208.67 ± 0.58^{a}	440.33 ± 2.52^{b}	322.33 ± 1.53^{c}	361.00 ± 1.73^{d}
Temperature (°C)	31.30 ± 0.46	31.03 ± 0.06^{a}	30.77 ± 0.06^{a}	31.07 ± 0.25^{a}	31.13 ± 0.29^{a}
TDS: total dissolved solids (ppm)	53.00 ± 0.00	104.67 ± 0.58^{a}	221.33 ± 2.08^{b}	161.33 ± 0.58^{c}	181.00 ± 1.73^{d}
pH	7.63 ± 0.12	5.97 ± 0.06^{a}	10.23 ± 0.15^{b}	6.7 ± 0.01^{c}	7.50 ± 0.01^{d}
DO (mg/l)	5.20 ± 0.10	4.50 ± 0.10^{a}	4.97 ± 0.06^{b}	3.4 ± 0.20^{c}	3.80 ± 0.01^{d}

Group N1: control; Group N2: antibiotic doxycycline; Group N3: with 1% extract of *B. macrophylla* TD1

The qualitative analysis of the chemical composition revealed that the ethanolic extract of *B. macrophylla* TD1 contained various secondary compounds, including polyphenols, glycosides, flavonoids, triterpenoids, and essential oils (Suplementary Table 2). In particular, the concentrations of polyphenols (110 mg GAE/g) and flavonoids (426.88 mg RE/g) in the ethanolic extract were higher compared to those in the water extract, which measured 56.61 mg GAE/g and 124.24 mg RE/g, respectively (data not shown).

Efficacy of ethanolic extract of B. macrophylla TD1 in protecting koi fish from pathogen

Therapeutic agent

In the therapeutic experiment, each group consisted of 30 fish, distributed as 10 fish per tank across three replicates. Fish receiving 0.5% TD1 extract (N2) or doxycycline (N4) exhibited a 60% survival rate, higher than the negative control (group N1, 30%) and infected control (group N5, 40%). Fish in group N3 (1% TD1 extract) showed complete survival (100%). Mortality in treated groups N2 and N4 was delayed by two days post-infection relative to untreated groups N1 and N5. Bacteria recovered from the kidney of moribund fish were practically pure *E. faecalis* cultures with distinct biochemical characteristics.

Disease prevention

In this experiment, koi fed doxycycline-supplemented feed or 1.5% TD1 extract-supplemented feed had survival rates of 65% and 60%, respectively, which were notably higher than those (30%) in controls after 10 days of supplementation. In addition, fish in the treated groups showed a reduced density of *Vibrio* spp., as detected on TCBS medium, in kidney tissue samples (Suplementary Fig. S1).

Efficacy of ethanolic extract of B. macrophylla TD1 in protecting fish against ammonia toxicity

As demonstrated in the preliminary study addressing ammonia stress, the protective efficacy of the ethanolic extract of *B. macrophylla* TD1 against ammonia toxicity was assessed using a deliberately high concentration of 0.5 mg/L ammonia nitrogen. This concentration was selected to impose a severe stress condition that enables a rigorous assessment of treatment effects. Fish fed doxycycline-supplemented feed had a mortality rate of 43.33 ± 5.77%, compared to 90.00 ± 1.00% in the control group. In particular, no mortality was observed in the group receiving 1% TD1 extract-supplemented feed. Mortality resulting from ammonia exposure was typically accompanied by clinical signs such as gill redness and inflammation, hemorrhaging, increased opercular movement indicative of rapid respiration at the water surface.

Following ammonia exposure, water quality parameters improved significantly in both treatment groups compared to the control. Conductivity values were 322.33 ± 1.53 µS and 361.00 ± 1.73 µS for the doxycycline and TD1 groups, respectively, versus 440.33 ± 2.52 µS in the control. Total dissolved solids (TDS) were reduced to 161.33 ± 0.58 ppm (doxycycline) and 181.00 ± 1.73 ppm (TD1), compared to 221.33 ± 2.08 ppm in the control. Dissolved oxygen (DO) levels declined to 3.4 ± 0.20 mg/L in the doxycycline group and 3.8 ± 0.01 mg/L in the TD1 group, versus 4.97 ± 0.06 mg/L in the control. In particular, while the control group showed a marked increase in pH (10.23 ± 0.15), pH was more stable in the treated groups, maintained at 6.7 ± 0.01 (doxycycline) and 7.5 ± 0.01 (TD1). These results indicate that both treatments mitigated water quality deterioration and reduced physiological stress in fish.

Efficiency of ethanolic extract of B. macrophylla TD1 supporting for liver function

Before ammonia exposure, control fish had significantly higher SGOT concentrations ($p < 0.05$) compared to fish fed doxycycline or TD1 extract. After exposure, SGPT levels increased significantly in control and doxycycline groups (Fig. 2). Fish fed TD1 extract showed no significant change ($p > 0.05$) in SGPT levels before and after exposure. The SGPT concentration in the doxycycline group increased significantly ($p < 0.05$) after ammonia exposure compared to pre-exposure levels in the TD1 group. In particular, a marginal increase in SGPT ($p=0.07$) was observed in the doxycycline group following ammonia challenge.

Discussion

This study demonstrates the antibacterial and anti-ammonia toxicity effects of ethanolic extract from *B. macrophylla* TD1 on koi fish. Chemical analysis revealed that flavonoids were more abundant than phenolics (Suplementary Table 2), consistent with previous findings (Sukalingam 2018), which conducted on *B. macrophylla* Grifth leaves in Kundang, Malaysia. When methanol was used for extraction, alkaloids, total phenols, and tannins predominated. The primary contributors to bioactivity include polyphenols, flavonoids, caryophyllene, phytols, and transgeranylgeraniol (De Wolf et al. 2017). These compounds contribute to plant resistance traits, acting individually or synergistically through mechanisms such as enzyme inhibition, disruption of microbial cell permeability (particularly by phenolics), and interaction with membrane proteins. In addition, they can bind to and inactivate proteins (e.g. quinones, flavonoids, tannins) while forming complexes with bacterial cell walls (e.g. flavonoids) (Hintz et al. 2015). In consistent with previous studies (e.g., Fu'adah et al. 2022; Nguyen et al. 2020), our findings showed that the ethanolic leaf extract may be a promising candidate for natural antibacterial drugs or as an aid against acute metabolic disorders.

On the other hand, *E. faecalis* isolates are well recognized for their widespread antibiotic resistance across many countries (Arias and Murray 2012). They have been frequently detected in human and animal feces, flies, and various food products from local markets (Thi An Ha et al. 2023; Ha et al. 2023). In our study, the *E. faecalis* isolate KOI-T displayed sensitivity to fluoroquinolones such as ciprofloxacin and ofloxacin (Supplementary Table 1), which contrasts sharply with previous reports from other regions showing high resistance rates (>75%) to amoxicillin, oxytetracycline, and erythromycin in *Aeromonas* spp. and *Enterococcus* spp. (Saengsitthisak et al. 2020; Bhat and Altinok 2023). These differences highlight substantial variability in resistance patterns, which can be attributed to several factors such as genetic variation among isolates (Raoof et al. 2019) and geographic origin (Thi An Ha et al. 2023). For example, while KOI-T was fluoroquinolone-susceptible, a recent study reported that 17.6% of *E. faecalis* isolates were resistant to levofloxacin and 19.5% to ciprofloxacin (Thi An Ha et al. 2023). Since ciprofloxacin and ofloxacin are banned in aquaculture, our study compared TD1 extract with doxycycline, a permitted antibiotic. Moreover, aquaculture practices strongly shape resistance, with intensive antibiotic use fostering resistant strains, while sustainable approaches, such as plant-based alternatives like *B. macrophylla* extract, can reduce selective pressure (Harikrishnan et al. 2010; Bhat and Altinok 2023).

In our agar diffusion assays (Nguyen 2019; Nguyen et al. 2020), the extract produced a 12 mm inhibition zone, slightly larger than the 11 mm zone observed for doxycycline. The ethanolic extract of *B. macrophylla* TD1 also exhibited strong in vivo activity against multidrug-resistant *E. faecalis* KOI-T, confirming its antibacterial efficacy consistent with earlier studies. These findings underscore the potential of TD1 extract as a promising alternative to conventional antibiotics in ornamental aquaculture. In our challenge trials, koi carp infected with KOI-T and fed a diet containing 0.5% TD1 extract (group N2) achieved 60% survival, a rate similar to that of the doxycycline-treated group (N4) (Fig. 1). Notably, complete survival was recorded in fish fed 1% TD1 extract (N3), with no mortalities observed. The antibacterial action of extract is likely driven by polar secondary metabolites that impair bacterial proliferation, as supported by prior profiling and bioactivity studies. Consistent with previous research (Nguyen et al. 2020; Van Vo et al. 2022), phytochemical analysis confirmed that the ethanolic extract contains a wide array of compounds, including polyphenols, flavonoids, tannins, glycosides, triterpenoids, and essential oils, with polyphenols and flavonoids being the most abundant (Supplementary Table 2). These metabolites act through multiple mechanisms, such as disrupting bacterial membranes and inhibiting key enzymes, thereby reinforcing the strong antibacterial activity of the extract.

This research contributes to ongoing efforts to identify effective plant-derived antibacterial agents and highlights the importance of evaluating different extract concentrations to determine optimal treatment levels for disease prevention and control. For instance, koi carp challenged with *E. faecalis* and fed 1% TD1 extract achieved complete survival (100%), compared with 60% survival in both the 0.5% TD1 extract and doxycycline-treated groups, and only 30–40% survival in the controls. These results notably exceed those reported in previous study (Rahman et al. 2017), where tilapia treated with methanolic garlic extract achieved approximately 70.8% survival, comparable to 75.8% survival in azithromycin-treated fish, and 60–63% survival in clove extract-treated groups. Specifically, tilapia challenged with highly virulent *E. faecalis* isolates and treated with methanolic extracts of *Allium sativum* (375 mg kg^{-1} fish) achieved a survival rate of about 70.8%, while azithromycin-treated groups reached 75.8%, and *Syzygium aromaticum* extract treatments yielded 60–63%. Other studies (e.g., Raoof et al. 2019; Ehsan et al. 2021) have primarily investigated the in vitro antibacterial activity of medicinal plant extracts against *E. faecalis*, underscoring increasing concerns about the emergence of streptococcosis.

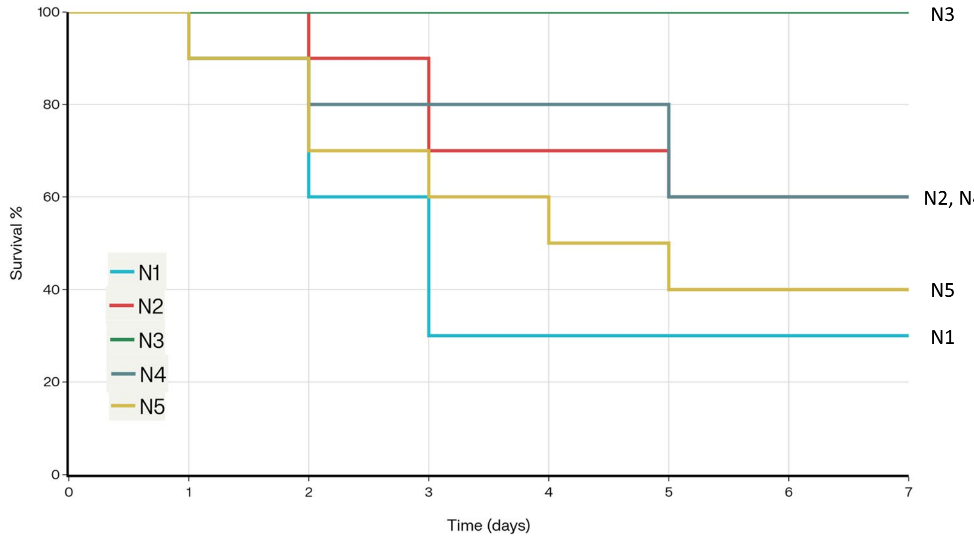

Fig. 1 Fish mortality rate observed in the challenge experiment evaluating the therapeutic efficacy of TD1 extract. Experiment groups included N1, a non-challenged control group fed a commercial diet; N2 and N3, challenged groups receiving feed supplemented with TD1 extract at 0.5% and 1%, respectively; N4, challenged group fed a diet supplemented with doxycycline at 2% kg^{-1} of the baseline feed; N5, challenged group receiving a basal diet as the treatment control

For preventive purposes, dietary supplementation with 1.5% TD1 extract effectively reduced *Vibrio* spp. infection rates. Koi carp fed either doxycycline or 1.5% TD1 extract-supplemented feed exhibited higher survival (65% and 60%, respectively) compared with 30% in the control group over a 10-day feeding period. Thus, these findings demonstrate the superior protective efficacy of TD1 extract under the current experimental conditions and suggest its potential as a promising alternative to conventional antibiotics.

Several studies have demonstrated that ammonia presence significantly impairs fish health, leading to reduced productivity in aquaculture systems (Zuffo et al. 2021; Pedrotti et al. 2018; Wilkie 1997). Enhancing fish resilience and health is vital to counteract the variable and often stressful conditions in aquaculture environments. In this study, fish treated with doxycycline exhibited a significantly lower ($p<0.05$) mortality rate ($43.33\pm5.77\%$) compared to the control group ($90.00\pm1.00\%$), indicating partial protection against lethal ammonia exposure. The use of 0.5 mg/L ammonia nitrogen in the final ammonia challenge was intentionally selected to simulate acute toxicity and provide a rigorous test of fish tolerance and treatment efficacy. In particular, fish fed a diet supplemented with 1% TD1 extract showed no mortality under ammonia stress. However, prolonged antibiotic use raises concerns due to possible bioaccumulation, liver toxicity, and antibiotic resistance (Ha Pham Thi et al. 2022). Also, antibiotics can disrupt aquatic microbiota, impairing their ability to maintain crucial biological functions (Carvalho et al. 2019). Therefore, alternatives to antibiotics with reduced environmental impact are highly desirable.

Supplementation with 1% TD1 extract reduced changes in water parameters and dissolved oxygen (Table 1) more effectively than antibiotics. It was observed that the pH value in the control group was highly elevated, coinciding with a 90% mortality rate, indicating that ammonia exposure can directly and indirectly influence pH fluctuations through fish excretory processes. In contrast, fish fed diets supplemented with either antibiotics or TD1 extract maintained relatively lower pH levels (pH = 6.7 and pH = 7.5, respectively) compared to pre-exposure water conditions. The findings suggest the use of TD1 extract as an option to reduce environmental toxicity.

The analysis of SGPT and SGOT levels revealed distinct differences between treatment groups following ammonia exposure, highlighting the comparative protective effects of TD1 extract and doxycycline. Fish fed with doxycycline-supplemented feed exhibited a marginally significant increase in SGPT concentration ($p=0.07$) after ammonia challenge (Fig. 2), indicative of hepatic stress and potential liver damage, which was associated with a mortality rate of 43.33%. As above mentioned, prolonged antibiotic use can exacerbate liver damage, thereby increasing susceptibility to ammonia toxicity and mortality. The combined negative impacts of elevated ambient ammonia, resulting from poor aquaculture management (Tsui et al. 2002), and antibiotic-induced hepatotoxicity pose serious risks to fish health. This demonstrates the critical necessity for sustainable aquaculture strategies to prevent these risks.

In contrast, fish receiving the TD1 extract maintained stable SGPT levels with no significant change before and after exposure, suggesting a protective effect on liver function under toxic ammonia stress. Parallel results were observed in tilapia (data not shown), where mortality remained low in the TD1 group compared with markedly higher mortality in antibiotic-treated and control groups. Specifically, tilapia treated with cefalexin and erythromycin exhibited mortality rates of 30% and 70%, respectively, while the control group, fed only commercial feed, experienced 100% mortality. These results indicate that the TD1 extract contains bioactive compounds that

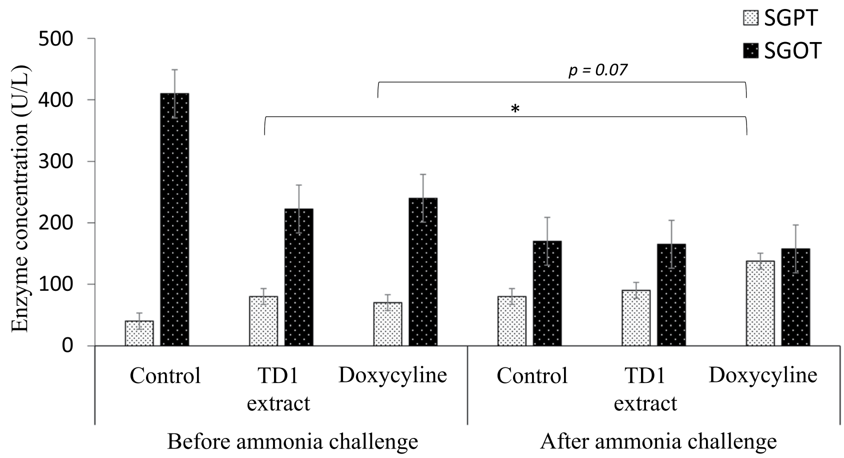

Fig. 2 Effect of exposure of koi carp to ammonia for 24 h on level of SGPT and SGOT in the blood. Results are presented as mean and standard deviation (n = 5 per group). Asterisk indicated significantly different ($p < 0.05$)

strengthen liver cell health and reduce the harmful effects of acute ammonia exposure. The ethanolic extract of *B. macrophylla* TD1, with its rich content of polyphenols and flavonoids, likely contributes to mitigating oxidative stress and preserving hepatic cellular integrity. In contrast, doxycycline, while somewhat effective at reducing mortality, appeared to increase hepatic stress, as indicated by elevated SGPT levels (Fig. 2). Taken together, these findings suggest that TD1 extract is a promising and safer alternative to conventional antibiotics for enhancing fish resilience against environmental toxins such as ammonia.

Fish mainly respire through their gills, directly exposing them to environmental ammonia (Wilkie 1997). Ammonia must pass through the branchial and cutaneous epithelia before entering the bloodstream and reaching vital organs such as the brain. Previous studies have shown that environmental ammonia disrupts branchial ion transport in various fish species, including rainbow trout (Avella and Bornancin 1989) and goldfish (Maetz 1973). In this study, mortality rates of fish exposed to 0.5 mg/L ammonia nitrogen for 24 h were 43.33 ± 5.77% in the doxycycline-supplemented group and 90.00 ± 1.00% in the control group, both exhibiting typical clinical signs reported in earlier studies (Liu et al. 2021), such as gill redness and inflammation, hemorrhaging, and increased opercular movement indicative of rapid respiration at the water surface. Ammonia exposure initially stimulates the expression of antioxidant genes and boosts enzyme activity to mitigate oxidative stress. However, continued exposure leads to a decline in superoxide dismutase and catalase expression after 48 h, likely due to enzyme damage. This suppression compromises antioxidant gene expression, weakens the overall antioxidant defense system, and leads to oxidative damage in gill tissues, disrupting respiration, and potentially resulting in mortality (Topal et al. 2017). The remarkable antioxidant properties of *Bouea macrophylla* Griffith extract (as described in the introduction), along with its hepatoprotective effects following ammonia exposure, highlight its potential to enhance antioxidant capacity and preserve tissue integrity (Ip and Chew 2010). Overall, these results suggest that bioactive compounds such as polyphenols and flavonoids may play a key role in providing these protective effects through their antioxidant and antimicrobial activities, thereby strengthening fish resistance to ammonia toxicity and bacterial infections.

The use of *B. macrophylla* extract in aquaculture offers a sustainable alternative to antibiotics by effectively reducing environmental antibiotic residues, promoting fish health, and aligning with global regulations that restrict antibiotic use. It improves fish resistance to infections and toxicity while maintaining water quality and fish welfare. However, current research has limitations, including the lack of molecular or histological confirmation of liver and gill protection, absence of long-term safety studies, and variability in extract composition due to external factors. Future research should focus on testing *B. macrophylla* extract in real aquaculture conditions, isolating specific bioactive compounds, and exploring synergistic effects with probiotics or immunostimulants to optimize health benefits and practical applications.

Conclusion

Dietary supplementation with *B. macrophylla* extract at 1% concentration achieved 100% survival of koi fish challenged with *E. faecalis*, demonstrating superior protection compared to doxycycline and control groups. Also, fish fed the extract were fully protected against acute ammonia toxicity, with no mortality observed, whereas significant mortality occurred in doxycycline-treated and control fish. These results highlight the strong antibacterial and anti-ammonia toxicity properties, emphasizing its potential as a sustainable and eco-friendly alternative to antibiotics in ornamental aquaculture. Additional research is essential to confirm the long-term safety of *B. macrophylla* extract, ensure consistent standardization of its composition, and validate its efficacy through field trials. Future investigations should also evaluate its effects across various aquaculture species and explore potential synergistic benefits when combined with probiotics or immunostimulants to optimize its practical use in commercial aquaculture systems.

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Data availability Data is provided within the manuscript or supplementary information files.

Declarations

Competing interests The authors declare no competing interests.

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