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Co-infection with Tilapia Lake Virus, *Aeromonas hydrophila*, and *Streptococcus agalactiae* in Nile Tilapia: Mortality Investigation and Antibacterial Evaluation of *Tinospora cordifolia*

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ABSTRACT

The present study investigated mass mortality events in Nile tilapia (*Oreochromis niloticus*), with daily mortalities ranging from 70 to 100 fish/day a period of 15 days, reported during March, 2024 from two commercial tilapia farms in the Krishna district of Andhra Pradesh, India. Infected tilapia exhibited lethargy, exophthalmia, skin ulceration, scale loss, anorexia, and fin rot. Sample were screened for tilapia viruses and bacterial pathogens using standard procedures. Phytochemical analysis and in vitro antibacterial activity of *Tinospora cordifolia* was evaluated. TiLV confirmed by RT-PCR, while *Aeromonas hydrophila* and *Streptococcus agalactiae* were confirmed by conventional microbiological methods and species-specific PCR. *A. hydrophila* shown resistance to oxytetracycline (30 µg), doxycycline (30 µg), co-trimoxazole (25 µg) whereas *S. agalactiae* resistant to oxytetracycline (30 µg), doxycycline (30 µg) and multiple antibiotic resistance index was 0.4 and 0.2, respectively. Regarding virulence genes, *A. hydrophila* harbored *aerA*, *alt*, *act*, and *enol*, and *S. agalactiae* harbored *cpsG* genes. *T. cordifolia* extract showed a strong antibacterial activity in 50 mg ml^{-1} , 75 mg ml^{-1}, 100 mg ml^{-1}, 125 mg ml^{-1} concentration and zone of inhibition ranging from 8±0.19 to 25±1.2 mm for *A. hydrophila* and 12±0.32 to 32±1.4 mm for *S. agalactiae*. These findings underscore the need for stringent biosecurity measures, adoption of best management practices, use of high-quality seed and feed, and incorporation of phytochemicals to mitigate disease outbreaks, reduce antibiotic dependency and promote sustainable tilapia aquaculture.

KEYWORDS: Aquaculture, tilapia, co-infection, virulence, phytotherapy

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1. INTRODUCTION

Aquaculture is the fastest-growing food production sector globally, contributing significantly to food security, nutrition, and employment opportunities (Munguti et al., 2023). Tilapia has experienced remarkable growth over the last two decades and is currently one of the most widely farmed fish worldwide (Arumugam et al., 2023). Global tilapia production has exceeded 6 million metric tons (MMT), making it the second most cultured freshwater fish after carp, with projections reaching 7.3 mmt by 2030. Major producing countries include China, Indonesia, and Egypt (Jansen et al., 2019). Nile tilapia (*Oreochromis niloticus*) is particularly valued for its rapid growth, omnivorous feeding habit, adaptability to diverse farming systems, and tolerance to varying environmental conditions, making it an important source of animal protein and essential nutrients (Nuryanto et al., 2022; Puneeth et al., 2022). Despite its advantages, the intensification of tilapia farming has increased susceptibility to infectious diseases, resulting in significant economic losses worldwide (Bigarre et al., 2009; Vineetha et al., 2021). Disease outbreaks and antimicrobial resistance (AMR) have emerged as major constraints to sustainable aquaculture production (Suresh and Pillai, 2023; Suyamud et al., 2024). Stressful farming conditions, including overcrowding, poor water quality, and nutritional imbalances, compromise fish immunity and increase vulnerability to opportunistic pathogens and mixed infections (Cabello et al., 2016). Tilapia Lake Virus (TiLV) is an emerging negative-sense RNA virus associated with severe disease outbreaks in both wild and cultured tilapia populations. Since its first report in Israel in 2014, TiLV has spread to 17 countries and is responsible for mortality rates reaching up to 90% in affected stocks (Abbadi et al., 2023). Infected fish typically exhibit lethargy, anorexia, exophthalmia, skin discoloration, abdominal distension, scale protrusion, and abnormal swimming behavior (Behera et al., 2018). Alongside viral diseases, bacterial pathogens such as *Aeromonas hydrophila* and *Streptococcus agalactiae* are major threats to tilapia aquaculture. *A. hydrophila* causes motile Aeromonas septicemia (MAS), characterized by hemorrhages, ulcerations, abdominal dropsy, exophthalmia, and fin erosion (Suresh and Pillai, 2023; Semwal et al., 2023). *S. agalactiae* is responsible for streptococcosis, leading to septicemia, meningoencephalitis, and sudden mortality in freshwater and estuarine fish species (Alazab et al., 2022; Preenanka et al., 2023). The pathogenicity of these bacteria is largely mediated by numerous virulence factors (Rasmussen-Ivey et al., 2016). In *A. hydrophila*, genes encoding aerolysins, cytotoxins, hemolysins, lipases, elastases, proteases, and biofilm-associated factors contribute significantly to disease development (Suresh and Pillai, 2023). Similarly, adhesins, invasins, and immune-evasion genes play crucial roles in the virulence of *S. agalactiae* (Alazab et al., 2022). The emergence of antimicrobial resistance among aquatic pathogens further complicates disease management, as the misuse and overuse of antibiotics in aquaculture accelerate the dissemination of resistant bacteria and resistance genes, posing risks to animal, environmental, and public health (Suresh et al., 2023; Suresh and Pillai, 2024a,b). Fish diseases are often multifactorial, involving interactions between primary and secondary pathogens. Co-infections have become increasingly common in intensive aquaculture systems and are frequently associated with greater disease severity and mortality than single-pathogen infections (Kotob et al., 2017; Rao et al., 2021). Several studies have reported TiLV co-infections with bacterial pathogens, including *Aeromonas* spp., *Flavobacterium* spp., and *Streptococcus* spp., resulting in aggravated clinical manifestations and higher mortalities (Basri et al., 2020; Nicholson et al., 2020; Piewbang et al., 2022; Puneeth et al., 2022). However, the epidemiology and pathogenic significance of such co-infections remain poorly understood. Phytotherapy have gained increasing attention as sustainable alternatives to conventional antibiotics. *Tinospora cordifolia* is a medicinal plant rich in bioactive compounds, including alkaloids, phenolics, glycosides, diterpenoid lactones, steroids, and β-sitosterol, which exhibit antibacterial and immunomodulatory properties (Semwal et al., 2023). Extracts of *T. cordifolia* have demonstrated activity against several pathogenic bacteria, including *Aeromonas* spp., *Streptococcus* spp., *Escherichia coli, Staphylococcus aureus,* and *Klebsiella pneumoniae* (Kuebutornye and Abarike, 2020; Shivakumar et al., 2022). Therefore, the present study investigated a natural co-infection involving TiLV, *A. hydrophila*, and *S. agalactiae* in Nile tilapia. The study further characterized the bacterial isolates through antimicrobial susceptibility testing and virulence gene profiling using PCR, while also evaluating the in vitro antibacterial activity of *T. cordifolia* extracts against *A. hydrophila* and *S. agalactiae*.

2. MATERIALS AND METHODS

2.1. Case history

In March 2024, Mass mortalities were reported in two tilapia farms in Krishna district (16.4245°N, 80.9978°E) in Andhra Pradesh, India. The affected fish farms adopted semi-intensive culture and the stocking density of monosex tilapia is 5 fish/m^2. The infected fish weighed around was 500-700 g and had a body length of 20–30 cm. Between 70–100 fish deaths were recorded daily in each farm over 15 days, with mortality rates peaking on these days and then a gradual decrease in mortalities was noticed after treatment was initiated. Fish farms and water source are good and were not connected to sewage channels. No aerators were used in these farms. Concurrently, the mean±standard deviation of water quality parameters was monitored during this study,

such as temperature, dissolved oxygen, pH, and unionized ammonia were 28.4±0.3, 4.56±0.48, 7.76±0.30, 0.33±0.16, respectively.

2.2. Fish sampling

A total of 20 moribund fish from two farm (n=10 from each farm) that showed signs of disease such as lethargic, abnormal swimming pattern, skin haemorrhage, exophthalmia and fin rot were categorized as diseased group. Moribund fish were randomly collected, packed in sterile bags, and sent to the laboratory in 1–2 h and post-mortem investigation was performed (Austin and Austin, 2007). Tissue samples from the brain, kidney, liver and spleen were collected, pooled and preserved in RNAlater and 70% ethanol for virus nucleic acid extraction for the detection of tilapia viruses. For bacterial isolation and identification, swabs of liver, kidney and spleen were streaked directly onto Tryptic Soy Agar (TSA- HiMedia, India) with 5% sheep blood and Aeromonas selective agar (M1890; HiMedia, India) and incubated at 32°C for 24 h.

2.3. Phenotypic and molecular identification of bacteria

For bacterial isolation and identification, five distinct colonies were aseptically chosen from each plate, sub-cultured on the TSA and ASA and incubated under the same conditions. Subsequently, purified bacterial cultures were subjected to Gram staining, oxidase, and catalase test. For further identification and characterization, API®20E and API®20Strep kits were used according to the manufacturer's instructions. The bacterial genomic DNA was extracted using the boiling method (Kpoda et al., 2018). Briefly, 3–4 individual bacterial colonies were suspended in 200 µl of TE buffer and boiled at 95°C for 10 min in a dry bath and then centrifuged at 10000 rpm for 10 min, and the collected supernatant for the PCR confirmation. Furthermore, *16s rRNA* gene of *A. hydrophila* and the *cpsG* gene of *S. agalactiae* were identified and characterized using species-specific PCR primers (Table 2). The identified bacterial strains were kept at -80°C in TSB broth containing 20% glycerol until further use.

2.4. Antibiotic susceptibility test

The antibiotic susceptibility testing of representative *A. hydrophila* and *S. agalactiae* that were isolated from this disease outbreak was performed using by Kirby-Bauer disc diffusion method in accordance with the Clinical Laboratory Standards Institute guidelines (Anonymous, 2022). The list of antibiotic disks (HiMedia, India) and their concentration used in this study were given in Table 3. Overnight-grown bacterial cultures were adjusted to 0.5 McFarland standard, and 0.1 ml of bacterial culture was spread on to Mueller-Hinton agar (MHA- HiMedia, India) and kept for drying. Antibiotic discs were placed on MHA using HiMedia antimicrobial disc dispenser and incubated at 32°C for 24 h. After incubation, the zone of inhibition (diameter) was measured and interpreted according to Anonymous, 2022. Further, isolates that showed resistance to three or more antimicrobial agents belongs to three different antibiotic classes were considered as multi-drug resistant (MDR) bacteria (Abdel-moneam et al., 2025; Magiorakos et al., 2012). The multiple antibiotic resistance index (MAR) was calculated according to Krumperman (1983). MAR=a/b, where *a*=number of antibiotics to which isolate is resistant; *b*=total antibiotics tested).

2.5. Nucleic acid extraction

Tissue organs such as the brain, kidney, liver and spleen of the moribund fish samples used for nucleic acid extraction. The samples were pooled and 100 mg of tissue was taken for nucleic acid extraction using Guanidium Thiocyanate lysis buffer (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The obtained pellet containing nucleic acids was resuspended in nuclease free water. The purity of the extracted nucleic acids was measured using a UV-vis spectrophotometer (Nano drop 2000, Thermo Fisher Scientific, USA). For screening RNA viruses, 1 µg of extracted nucleic acid was then used to synthesis cDNA using Prime script RT reagent kit (Takara, Japan) according to the manufacturer's protocol.

2.6. Detection of tilapia lake virus

PCR was performed to identify TiLV and other viral infections as described according to earlier reports (Table 2). Briefly, PCR was carried out in a 25 µl reaction mixture containing 12.5 µl of Taq polymerase Master Mix (Ampliqon, Denmark), 1 µl of each primer (20 µmol), 8.5 µl of nuclease free water, and 2 µl of template. The reaction was performed in an Applied Biosystems™ Proflex™ thermal cycler (Thermo Fisher Scientific, USA). Specific primer sequences (Sigma-Aldrich, USA), expected amplicon sizes, and PCR conditions were given in Table 1. The amplified products were electrophoresed in 1.2% agarose gel stained with ethidium bromide and visualized under Gel Documentation System (Bio-Rad, USA). A 100 bp DNA ladder (Ampliqon, Denmark) was used to determine the fragment sizes.

2.7. Virulence genes profiling for A. hydrophila and S. agalactiae

The presence of virulence genes in the *A. hydrophila* and *S. agalactiae* isolated from Nile tilapia was examined using PCR with primers specific to the virulence genes, namely aerolysin (*aerA*), cytotonic enterotoxins (*alt*), cytotoxic enterotoxins (*act*), enolase (*enol*), polar flagella (*fla*), Hemolysin (*hly*), fibrinogen-binding protein (*fbsA*), CAMP factor (*cfb*), penicillin-binding protein (*pbp1A/ponA*), which were amplified as described by Alazab et al., 2022; Suresh and Pillai, 2023. The oligonucleotide sequences, PCR conditions and expected amplicon size were summarized in Table 1. The PCR reaction was performed in a similar manner as that described in the previous section.

Table 1: List of primers, PCR conditions and PCR amplification of different genes used in this study

Target	Primer name	Primer sequence	Amplicon size (bp)	Tm (°C)	Cycles	Reference
16S rRNA gene *A. hydrophila*	A.h 16S rRNA-F	AGGTTGATGCCTAATAC-GTA	683	58	35	Basri et al., 2020
	A.h 16S rRNA-R	CTGGCTGGCAA-CAAAGGACAG				
cpsG gene *S. agalactiae*	cpsG-F	ACATGAACAG-CAGTTCAACCGT	352	56	28	
	cpsG-2-3-6-R	TCCATCTA-CATCTTCAATCCAAGC				
Tilapia larval encephalitis virus	TLEV-1F	TCGTGGGCCTTATCCC-GCGT	170	48	38	Shlapobersky et al., 2010
	TLEV-1R	GAGACCAGAAAGT-GCTTCTC				
Betanodavirus	F2	CGTGTCAGTCATGTGTC-GCT	294	53	40	Bigarre et al., 2010
	R3	CGAGTCAACACGGGT-GAAGA				
Tilapia lake virus	Ext-1	TATGCAGTACTTTCCCT-GCC	415	58	32	Eyngor et al., 2014
	ME1	GTTGGGCA-CAAGGCATCCTA				
	7450/150R/ME2	TATCACGTGCGTACTC-GTTCAGT	250	56	35	
	ME1	GTTGGGCA-CAAGGCATCCTA				
Tilapia parvovirus	TiPV-2452-F	TGGCTTTATGGACTTT-GCTGAT	734	60	35	Yamkasem et al., 2021
	TiPV-3185-R	CATCCCTCCTGCTCTTG-GTT				
	TiPV-1607-F	GAGATGGTGTGAAAAT-GAACGGG	534	58	35	Liu et al., 2020
	TiPV-2141-R	CTATCTCCTCGTTGCTCG-GTGTATC				
	TiPV-1743-F	TAGCCGAGCAAGAGATA-AAG	215	58	35	Rajendran et al., 2023
	TiPV-1958-R	TGGGAGGCTCTCGGTT-GTGG				
Infectious spleen and kidney necrosis virus	MCP-F	CGTGAGACCGTGCGTATG	562	52	30	Wang et al., 2007
	MCP-R	AGGGTGACGGTCGATATG				
Aerolysin gene	aerA-F	CAAGAA-CAAGTTCAAGTGGCCA	309	59	30	Suresh and Pillai, 2023
	aerA-R	ACGAAGGTGTGGTTC-CAGT				

Target	Primer name	Primer sequence	Amplicon size (bp)	Tm (°C)	Cycles	Reference
Cytotonic enterotoxins	alt-F	TGACCCAGTCCTGGCAC-GGC	442	59	32	
	alt-R	GGTGATCGATCACCAC-CAGC				
Cytotoxic enterotoxins	act-F	GAGAAGGTGACCAC-CAAGAACA	232	62	30	
	act-R	AACTGACATCGGCCTT-GAACTC				
Enolase	enol-F	CGCCGACAACAACGTC-GACATC	598	61	32	
	enol-R	CTTGATGGCAGC-CAGAGTTTCG				
Polar flagella	fla-F	TCCAACCGTYTGACCTC	608	57	30	
	fla-R	GMYTGGTTGCGRATGGT				
Hemolysin	hly-F	GGCCGGTGGCCCGAA-GATACGGG	597	62	32	
	hly-R	GGCGGCGCCGGACGAGA-CGGGG				
Adhesion gene	fbsA-F	AACCGCAGCGACTTGTTA	278	52	35	Alazab et al., 2022
	fbsA-R	AAACAAGAGCCAAGTAG-GTC				
Invasin gene	cfb-F	GGATTCAACTGAACTC-CAAC	600			
	cfb-R	GACAACTCCACAAGTG-GTAA				
Immune evasion gene	pbp1A/ponA-F	AGGGGTAGTAGCATTAC-CAT	939			
	pbp1A/ponA-R	CAACTATAT-GACTGGGATCG				

2.8. Estimating the antibacterial activity of Tinospora cordifolia

The inhibitory activity of methanol extract of *T. cordifolia* medicinal plant, selected based on its medicinal properties (Ezhilarasu et al., 2023; Semwal et al., 2023), was evaluated against the isolated *A. hydrophila* and *S. agalactiae* pathogens.

2.8.1. T. cordifolia extract preparation and phytochemical screening

For this purpose, a commercially available *T. cordifolia* leaves were purchased from a local Ayurveda store in Chennai, India. The leaves were washed with sterilized distilled water, shade dried, finely powdered, and packed in sealed food-grade polythene bags for further studies. To know more details about the active principles in the plant extracts, extracts were screened for presence of phytochemicals (Kaur et al., 2016) and fractioned with methanol (Ezhilarasu et al., 2023) and then an in vitro assay was performed against these pathogenic isolates using the disk diffusion method. For extraction, 50 g of dried plant material was soaked in 200 mL methanol with stirring for 48 h, filtered again through double layers of muslin cloth, centrifuged at 9000 rpm for 10 min and finally filtered again through Whatman filter paper No. 41 to attain a clear filtrate. The filtrate was evaporated and dried at 45°C under reduced pressure using a rotatory vacuum evaporator. The extract yields were weighed, stored in a small glass bottle in fridge at 5°C.

2.8.2. In vitro application

Bacterial strains isolated from co-infected fish were sub-cultured in SCDA broth overnight at 32°C in a shaker incubator. The bacterial growth was harvested using 5 ml sterile saline water, its absorbance was adjusted at 580 nm

and diluted to attain a viable cell count of 10^7 CFU ml^{-1} using spectrophotometer. The paper disc diffusion method was used to screen antimicrobial activity and performed by using MHA in accordance with the CLSI guidelines (Anonymous, 2022). The plant extract residues (1000 mg) were re-dissolved in 2.5 ml of DMSO, sterilized through a Millipore filter (0.22 µm) and made to a final concentration of 400 mg ml^{-1}. From this, 50 mg ml^{-1}, 75 mg ml^{-1}, 100 mg ml^{-1}, and 125 mg ml^{-1} concentrations were prepared for antibacterial test against *A. hydrophila* and S. *agalactiae*. Ciprofloxacin (5 µg) disc were used as positive controls (not represented in Figure 2). The test is performed in triplicates. The inoculated plates were incubated at 32°C for 24 h. After incubation, the diameter (mm) of the inhibition zones was measured; the values of the antimicrobial activity were expressed as the mean of inhibition zones (mm) with three replicates. The presence of inhibition zones was measured by Vernier caliper, recorded and considered as indication for antimicrobial activity. Extracts were classified as sensitive (>18 mm), intermediate (13-18 mm), or resistant (<13 mm) based on their zone of inhibition diameters (Barry et al., 1970; Cruikshank et al., 1975).

3. RESULTS AND DISCUSSION

3.1. Clinical signs and pathology

The most common clinical signs of diseased fish were lethargic, loss of appetite, exophthalmia, swimming at the water surface, erratic swimming, dark discoloration of body, skin ulcerations, scale loss, anorexia, and fin and tail rot (Figure 1). The daily mortality was around 70–100 fish/day continued for 15 days. On post-mortem examination, ascites in the peritoneal cavity, enlarged gall bladder and kidney, and damaged internal organs was observed.

Figure 1: Pathological changes of moribund fish. Exophthalmia, scale loss, fin and tail rot (arrows)

3.2. Bacterial identification

The presumptive isolates grown on Aeromonas selective agar exhibited round and reddish orange colour, clear to opalescent were presumptively confirmed as *Aeromonas* spp. Similarly, colonies exhibited white, mucoid appearance with small, pinhead-sized dimensions, Gram-positive cocci, and catalase negative isolates were presumptively confirmed as *Streptococcus* spp. Biochemical and PCR method confirmed the presence of *A. hydrophila* (683 bp) and *S. agalactiae* (352 bp) in affected fish (Table 2).

Table 2: Phenotypic and biochemical characteristics of *A. hydrophila* and *S. agalactiae* in the present study

A. hydrophila		*S. agalactiae*	
Test	Present study	Test	Present study
Gram stain	- (rod shaped)	Gram stain	+ (cocci)
O/129	+ (Resistant)	Oxidase	-
Oxidase	+	Catalase	-
Catalase	+	Motility	-
Indole	+	Haemolysin	+
Motility	+	Acetoine production	+
Haemolysin	+	B-glucosidase hydrolysis	-
Citrate utilization	+	α-galactosidase	-
β-galactosidase	+	β-glucuronidase	-
Argine dihydrolase	+	β-galactosidase	-
Lysine decarboxylase	-	Alkaline phosphate	+
Ornithine decarboxylase	-	Leucine aminopeptidase	+
Urease	-	Arginine dihydrolase	-
H_2S production	-	Ribose acidification	+
Gelatinase	+	Arabinose	-
Glucose fermentation	+	Mannitol	-
Mannitol	+	Sorbitol	-
Arabinose	+	Lactose	-
Sorbitol	-	Trehalose	+
Inositol	+	Insulin	-
		Raffinose	-
		Amidon	-
		Glycogen	-

3.2.1. Virus confirmation

The subsequent RT- PCR revealed that 100% of fish were positive for TiLV (415bp) and co-infected with *A. hydrophila* and *S. agalactiae*. Furthermore, none of the infected tissue samples tested positive for TLEV, Betanodavirus, TiLV, TiPV, or ISKNV. RT- PCR confirmed that, 100% (20/20) infected fish were positive for TiLV. Among 100% of infected fish, 70% (14/20) were co-infected with TiLV, *A.*

hydrophila and *S. agalactiae*, while the remaining 30% were infected with TiLV and *A. hydrophila*. None of the other tilapia viruses such as TLEV, Betanodavirus, TiPV and ISKNV was detected by PCR amplificat.

3.3. Antibiotic susceptibility test

Antibiotic resistance patterns of *A. hydrophila* and *S. agalactiae* were assessed and illustrated in Table 3. *A. hydrophila* shown resistance to oxytetracycline, doxycycline, co-trimoxazole whereas *S. agalactiae* resistant to oxytetracycline, doxycycline. In contrast, *A. hydrophila* and *S. agalactiae* displayed intermediate resistance to gentamicin and co-trimoxazole, respectively. The multiple antibiotic resistance index for both *A. hydrophila* is 0.4 and *S. agalactiae* was determined to be 0.2.

3.4. Virulence associated genes in A. hydrophila and S. agalactiae

In the present study, five representative isolates of *A. hydrophila* and *S. agalactiae* were examined for virulence factors. PCR analysis revealed the presence of the *aerA, alt, act*, and *enol* virulence genes in *A. hydrophila*, while *cpsG* gene was detected in *S. agalactiae*. The *cpsG* gene served both as a marker for identification of species and virulence.

3.5. Phytochemical screening and antibacterial activity

Phytochemical analysis of *T. cordifolia* extracts has identified the presence of alkaloids, glycosides, flavonoids, phenols, saponins, terpenoids, and tannins as key natural product classes (Table 4), which contribute to a wide range of pharmacological activities. To validate this, extracts of the *T. cordifolia* plant were tested against *A. hydrophila* and

Table 3: Antibiotic susceptibility pattern of *A. hydrophila* and *S. agalactiae*

Antibiotic disk	Concentration (µg)	Resistance pattern (Zone of Inhibition, mm)		S	I	R
		A. hydrophila	*S. agalactiae*			
Oxytetracycline	30	R (10)	R (10)	≥ 17	12-14	≤11
Doxycycline	30	R (10)	R (11)	≥ 17	12-14	≤11
Co-trimoxazole	25	R (8)	I (13)	≥ 16	11-15	≤10
Ciprofloxacin	5	S (25)	S (24)	≥ 21	16-20	≤15
Enrofloxacin	10	S (24)	S (24)	≥ 21	16-20	≤15
Cefotaxime	30	S (24)	S (26)	≥ 23	15-22	≤14
Ampicillin	10	S (20)	S (22)	≥ 17	14-16	≤13
Gentamicin	10	I (14)	S (16)	≥ 15	13-14	≤12

S: Susceptible, I: Intermediate, R: resistant

S. agalactiae isolated from infected fish. The methanolic extract of *T. cordifolia* exhibited significant antibacterial effects against *A. hydrophila* and *S. agalactiae* as shown in Figure 2. The inhibition zones for the methanolic extract of *T. cordifolia* varied with concentration, measuring between 8±0.19 to 25±1.2 mm for *A. hydrophila* and 12±0.32 to 32±1.4 mm for *S. agalactiae* (Table 4).

In Aquaculture, stressors such as overcrowding, overfeeding, lack of vital nutrients, poor water quality, transportation, infectious agents, and pollution make fish vulnerable to diseases, frequently with several infections occurring simultaneously (Munguti et al., 2023). Co-infections with tilapia lake virus (TiLV), streptococcosis, and motile aeromonad septicaemia (MAS) have been reported across

Table 4: Phytochemical screening and antibacterial activity of *T. cordifolia* extract

A. Phytochemical test		B. Antibacterial activity		
Alkaloids (Mayer's test)	+ ve	Bacterial strain	*S. agalactiae*	*A. hydrophila*
Glycosides (Legal's test)	+ ve	Test sample	Zone of inhibition (mm)	
Flavonoids (Alkaline reagent test	+ ve	50 mg ml^{-1}	12±0.32	8 ±0.19
Phenols (Ferric chloride test)	+ ve	75 mg ml^{-1}	15±0.43	12 ±0.56
Saponin (Saponin test)	+ ve	100 mg ml^{-1}	23±0.9	17 ±0.8
Terpenoids (Salkowski's test)	+ ve	125 mg ml^{-1}	32±1.4	25 ±1.2
Tannin (Ferric chloride test)	+ ve	Ciprofloxacin (5 µg as control, not mentioned in Figure 2)	25±1	26 ±1

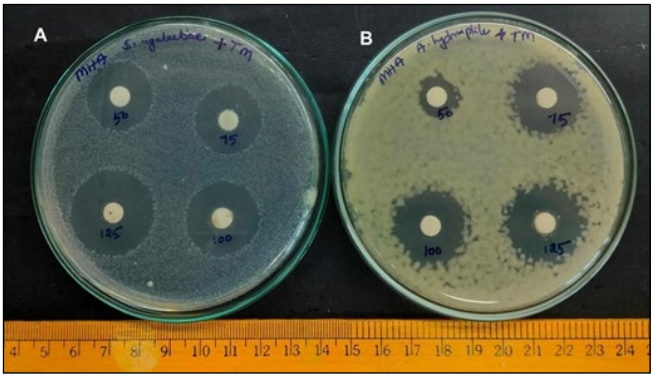

Figure 2: Antibacterial activity of *T. cordifolia* extract against A. hydrophila and *S. agalactiae*

various tilapia farming regions worldwide (Bwalya et al., 2020; Ndashe et al., 2023). The present study has identified a natural co-infection of TiLV, *A. hydrophila*, and *S. agalactiae* in Nile tilapia (*O. niloticus*) from Andhra Pradesh, India, as validated by both phenotypic and molecular methods. TiLV is an emerging and transboundary disease menace to tilapia, with mortality rates reaching 90-100% in fingerlings and juveniles (Behera et al., 2018). In the current study, infected tilapia farms had a mortality rate of 70-100 fish per day, clinical signs and pathological assessment of the affected fish in this study resembled typical signs of TiLV, *A.hydrophila* and *S.agalactiae* infected fish. The post-mortem study revealed the presence of ascites in peritoneal cavity and kidneys, as seen in earlier studies of fish co-infected with TiLV, *Aeromonas* spp and *Streptococcus* spp (Nicholson et al., 2020). Co-infections, caused by simultaneous or sequential infections with multiple pathogens, can worsen disease severity and increase morbidity and mortality (Abdel-Latif et al., 2020). Recent reports of TiLV and bacterial co-infections in Nile tilapia in India and other Asian countries support our findings (Nicholson et al., 2020; Rao et al., 2020).

Antimicrobial resistance presents a significant challenge within the aquaculture industry (Suyamud et al., 2024). The expansion of intensive farming systems, the prevalence of opportunistic bacterial pathogens, and suboptimal husbandry conditions have collectively driven extensive antimicrobial usage. Consequently, this has led to the accumulation of antimicrobial compounds in aquatic environments and facilitated the emergence and dissemination of antibiotic-resistant bacterial strains. (Suresh et al., 2023; Suresh and Pillai, 2024a, b). It is reported that *A. hydrophila* and *S. agalactiae* are antibiotic-resistant bacteria in many fish farming countries (Basri et al., 2020; Nhinh et al., 2021; Suresh and Pillai 2023). In this study, *A. hydrophila* shown resistance to oxytetracycline (30 µg), doxycycline (30 µg), co-trimoxazole (25 µg) whereas *S. agalactiae* resistant to oxytetracycline (30 µg), doxycycline (30 µg). These findings are consistent with our previous research into the presence and distribution of virulence genes in antibiotic-resistant *A. hydrophila* from the same farming region (Suresh and Pillai, 2023). Other investigations have demonstrated that *S. agalactiae* from Nile tilapia has various antibiotic resistance patterns (Basri et al., 2020; Alazab et al., 2022). The multiple antibiotic resistance index for *A. hydrophila* and *S. agalactiae* was found to be 0.4 and 0.2 which is similar in comparison to other fish pathogen isolates from the same aquaculture area (Suresh et al., 2023; Suresh and Pillai, 2024b).

Aeromonas hydrophila, A. veronii, A. caviae, A. bestiarum, A. schuberti, and *A. jandaei* are common bacterial pathogens in farmed Nile tilapia, capable of inflicting large mortality and economic losses (Nicholson et al., 2020). *A. hydrophila* is highly virulent and causes substantial mortality in a variety of freshwater fishes including tilapia (Suresh and Pillai 2023). The pathogenicity and toxicity of *A. hydrophila* are multifaceted and linked to several virulence factors such as structural lipopolysaccharides, outer-membrane proteins, pili and flagella, type secretion systems, and extracellular components comprising enzymes and toxins (Rasmussen-Ivey et al., 2016). Exotoxins such as aerolysin (*aerA*), cytotonic enterotoxin (*alt*), cytolytic enterotoxin (*act*), and hemolysin (*hly*), as well as enzymes lipase (*lip*), serine proteases (*ahy*), enolase (*enol*), and nucleases (*exu*), appear to play important roles in pathogenesis. In the current study, *A. hydrophila* from co-infected fish were found to carry *aerA, alt, act,* and *enol,* which are responsible for tissue damage, fluid build-up in intestinal epithelial cells, enterotoxic, cytotoxic, and hemolytic effects in the host (Nhinh et al., 2021). Similarly, *S. agalactiae* is a major threat causing large mortality and jeopardizing the sustainability of global tilapia production (Li et al., 2015; Alazab et al., 2022). It has previously been reported that *S. agalactiae* produces an extracellular virulence factor, which includes adhesins (*fbsA, fbsB, pavA, Imb*, and *scpB*), invasins (*cylE, cfb, spB1*, *hylB, rib,* and *bca*), and immune evasion genes (*bac, cspA,* and *pbp1A/ponA*), all of which play a role in pathogenicity. In this study, cpsG virulence gene have identified in *S. agalactiae*, is responsible for attachment, penetration, inhibiting phagocytic death by the host, and cell lysis in the host.

The importance of phytotherapy in aquaculture has grown in recent years due to their antibacterial, antifungal, antiparasitic, and antiviral characteristics, as well as their capacity to promote growth and stimulate immunological response to infections (Van Hai, 2015; Tadese et al., 2022). *T. cordifolia* effectiveness is attributed to its rich variety of polysaccharides, phenols, alkaloids, glycosides, steroids, aliphatic compounds, and diterpenoid lactones, which have antibacterial, antioxidative, anti-inflammatory, and immunomodulatory properties in fish (EI-Basuini et al.,

2022). In this investigation, *T. cordifolia* extract showed substantial antibacterial activity against *A. hydrophila* and *S. agalactiae*. The inhibitory zone of diameters varied by concentration, ranging 8±0.19 to 25±1.2 mm for *A. hydrophila* and 12±0.32 to 32±1.4. These findings are consistent with Barry et al., 1970 and Cruikshank et al., 1975, who classified plant extracts as sensitive (>18 mm), intermediate (13–18 mm), or resistant (<13 mm) based on zone of inhibition diameters. Here, the antibacterial activity was evaluated at multiple concentrations (50–125 mg ml^{-1}) to assess dose-dependent effects and to identify an effective concentration suitable for farm-level application. Previous research has shown that *T. cordifolia* has antibacterial activity and is effective against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, A. hydrophila, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella* sp., *Enterobacter aerogenes, streptococcus sp.,* and *Candida albicans* (Semwal et al., 2023). Phytochemicals mitigate bacterial infections in aquaculture by targeting multiple mechanisms. For instance, flavonoids and alkaloids inhibit quorum sensing, reducing virulence factor expression and biofilm formation, while phenolics and terpenoids disrupt bacterial membranes and enzyme function. Polysaccharides and glycosides enhance host immunity, promoting pathogen clearance (Nik Mohamad et al., 2022). Furthermore, introducing *T. cordifolia* into diets can improve growth performance, improve digestive enzyme activity, boost immunological and antioxidative responses, and enhance Nile tilapia resistance to hypoxic environments and bacterial infections in aquaculture (El-Basuini et al., 2022).

5. CONCLUSION

Co-infection of Nile tilapia with Tilapia Lake Virus (TiLV), *Aeromonas hydrophila*, and *Streptococcus agalactiae* caused high mortality and severe pathological changes, indicating synergistic disease effects. The bacterial isolates showed antimicrobial resistance and possessed important virulence genes, highlighting their pathogenicity. *Tinospora cordifolia* exhibited notable antibacterial activity against both pathogens, suggesting its potential as a natural therapeutic or preventive agent. Despite limitations, including a small sample size and lack of challenge studies, the findings emphasized the need for co-infection monitoring and phytotherapeutic approaches in sustainable tilapia aquaculture.

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