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Natural Co-Infection With *Streptococcus agalactiae* and *Klebsiella pneumoniae* in Cultured Nile Tilapia: Assessing Plant-Based Therapeutics as Sustainable Alternatives to Antibiotics

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ABSTRACT

Nile tilapia (*Oreochromis niloticus*), a key species in global freshwater aquaculture and a major contributor to Bangladesh's fish production, is increasingly affected by bacterial infections that compromise farm productivity. This study investigated natural co-infection of bacterial pathogens in diseased tilapia and assessed the effectiveness of both conventional commercial antibiotics and plant-derived extracts. Clinical and post-mortem examination, phenotypic and biochemical characterisation, molecular diagnostics, histopathological examination and antibiotic susceptibility testing confirmed *Streptococcus agalactiae* and *Klebsiella pneumoniae* as the causative pathogens. Notably, two hallmark clinical manifestations of streptococcal infection—meningoencephalitis and panophthalmitis—were observed. Molecular identification through 16S rRNA gene sequencing (GenBank accessions PP967716, PP967717 and PP967718) revealed high similarity with reference strains—99.86% for *S. agalactiae* (NR_040821) and 99.78% for *K. pneumoniae* (NR_114506). Infected fish showed severe histopathological lesions in the brain, gills, spleen, kidney and liver. Antibigram analysis indicated multidrug resistance among the isolates, while acetonic extracts of six medicinal plants—*Elaeocarpus serratus* (olive fruit), *Phyllanthus emblica* (emblica fruit), *Psidium guajava* (guava leaf), *Allium sativum* (garlic), *Syzygium aromaticum* (clove) and *Nigella sativa* (black cumin)—exhibited strong antibacterial activity. A treatment trial against co-infection demonstrated that olive fruit extract significantly improved cumulative percent survival ($p < 0.05$) compared to amoxicillin and showed comparable efficacy to tetracycline. Kaplan–Meier survival analysis revealed low survival in fish treated with amoxicillin ($16.7 \pm 1.93\%$), while tetracycline ($83.3 \pm 1.93\%$) and olive extract ($80 \pm 1.91\%$) resulted in markedly higher survival ($p < 0.05$). This study provides the first report of natural co-infection by *S. agalactiae* and *K. pneumoniae* in tilapia farms of Sylhet, Bangladesh, and highlights the limitations of conventional antibiotics due to resistance. The findings underscore the promising potential of plant-based therapeutics, particularly olive extract, as sustainable alternatives for the management of bacterial diseases in aquaculture.

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1 | Introduction

Nile tilapia (*Oreochromis niloticus*), a fast-growing and hardy freshwater species, is a cornerstone of global aquaculture, especially in intensive farming systems (Abdel-Latif and Khafaga 2020; Abd El-Naby et al. 2024; Bari et al. 2024). In terms of tilapia production, Bangladesh is ranked fourth in the world and third in Asia (FAO 2020). Large-scale cultivation is essential to providing reasonably priced animal protein for expanding populations. However, the intensification of aquaculture operations has often led to inadequate management techniques and environmental neglect, compromising fish health (Elgendy, Abdelsalam, Kenawy, et al. 2022; Elgendy, Abdelsalam, Mohamed, et al. 2022; Elgendy, Sherif, et al. 2022). Fish under such circumstances experience higher levels of stress, which increases their vulnerability to pathogenic and opportunistic infections (Ali et al. 2020, 2022). As a result, disease outbreaks have become a major constraint to sustainable aquaculture, with epizootics frequently causing substantial mortality and financial loss (Moustafa et al. 2015; Radwan et al. 2022; Elgendy, Ali, Abbas, et al. 2023; Elgendy, Ali, Abdelsalam, et al. 2023). In tilapia farms, bacterial pathogens such as *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Aeromonas dhakensis*, *Aeromonas veronii*, *Aeromonas jandaei* and *Klebsiella pneumoniae* are particularly linked to serious infections and disease outbreaks (Dong et al. 2015, 2017; Al Yahya et al. 2018; Verner-Jeffreys et al. 2018; Ghetas et al. 2021; Vaneci-Silva et al. 2022).

Co-infections, or simultaneous infections by multiple pathogens, are becoming a common concern in aquaculture health management because they frequently lead to more severe clinical outcomes and higher mortality than single-agent infections (Cox 2001; Pakingking et al. 2003; Ma et al. 2019; Achariya et al. 2025). Reports of natural co-infections in tilapia have been increasing globally (Nicholson et al. 2020; Puneeth et al. 2022), yet such cases remain underexplored in Bangladesh, particularly in the Sylhet region. Lack of information frequently leads to incorrect diagnoses or insufficient treatment, which only targets the dominant pathogen and ignores co-infecting agents (Abdel-Latif et al. 2020).

Even while antibiotics are still the major treatment for bacterial infections (Sonkol et al. 2020), abuse of them has had concerning effects, such as deterioration of the environment and the rise of multidrug-resistant (MDR) strains of bacteria (T. Li et al. 2020; Sharma et al. 2015). Such challenges have accelerated the search for alternative therapeutic strategies. Among these, medicinal plants have drawn interest due to their antimicrobial, immunomodulatory and eco-friendly properties (Devasree et al. 2014; Sutuli et al. 2016; Kot et al. 2019). Compounds including alkaloids, flavonoids, and tannins present in herbal extracts offer promising solutions, particularly in regions with limited access to cutting-edge treatments (Pandey and Madhuri 2010; Ravikumar et al. 2010). Moreover, their minimal side effects and reduced potential for resistance development make them attractive options in modern aquaculture (Zhang et al. 2022). The emergence of MDR pathogens such as *S. agalactiae* (Ghetas et al. 2021) and *K. pneumoniae* (Vaneci-Silva et al. 2022) has further underscored the urgent need for sustainable interventions. Although Bangladesh has a rich ethnobotanical heritage (Yusuf

et al. 2009), little is known about how to use native medicinal herbs to control co-infections in aquaculture.

Investigating a disease epidemic in Nile tilapia from the BRAC Carp-Tilapia Hatchery and Brood Fish Development Centre in Moulvibazar, Sylhet, was the primary objective of this study. The causative bacteria were isolated and identified through biochemical and molecular techniques. Their resistance patterns to commercial antibiotics and locally available medicinal plant extracts were also evaluated. Furthermore, experimental infection trials, including co-infections with *S. agalactiae* and *K. pneumoniae*, were conducted to assess the therapeutic efficacy of an extract from *Elaeocarpus serratus* (olive fruit). This study provides a comprehensive insight into co-infection dynamics and highlights the potential of plant-based alternatives for managing antibiotic-resistant bacterial pathogens in tilapia aquaculture.

2 | Materials and Methods

2.1 | Sample Collection and Examination

In September 2023, an outbreak of disease affecting cultured Nile tilapia (*O. niloticus*) was documented at the BRAC Carp-Tilapia Hatchery and Brood Fish Development Centre, situated in Sreemangal Upazila (Latitude: 24°18'56" N; Longitude: 91°41'09" E) within the Moulavi Bazar district of Sylhet division, Bangladesh (Figure 1). This facility operated 48 ponds; each stocked at a density of 40 fish per cubic meter. The farmer first observed fish mortality on 16 September 2023, and the deaths continued daily thereafter. During a site visit, moribund and freshly deceased fish displaying clinical abnormalities and skin erosion were randomly collected ($n = 30$). The specimens were then transported to the Fish Disease Diagnosis and Pharmacology Laboratory in ice-cooled containers to minimise further deterioration.

2.2 | Bacteriological Isolation and Identification

Before taking swabs from the kidneys, spleen, liver, gills and exterior lesions, each fish was surface sterilised with 70% ethanol. After that, the samples were aseptically streaked onto nutrient agar (NA) plates (HiMedia, India) and incubated for 12–18 h at 37°C. Dominant colonies were chosen based on colony morphology, and they were further purified by repeatedly streaking on new NA plates in the same settings. For further examination, the isolated bacterial strains were kept at –40°C in Brain Heart Infusion broth (HiMedia) with 30% glycerol. Identification of the bacterial isolates at the genus level was performed using a series of biochemical tests following the methods described by Puneeth et al. (2022), including Gram staining, motility, catalase activity (3% hydrogen peroxide solution), cytochrome oxidase, indole production, carbohydrate fermentation, and the methyl red and Voges–Proskauer (MR–VP) test. The biochemical characteristics of the isolates were compared with reference strains of *S. agalactiae* and *K. pneumoniae*, as reported in previous studies (Table 1).

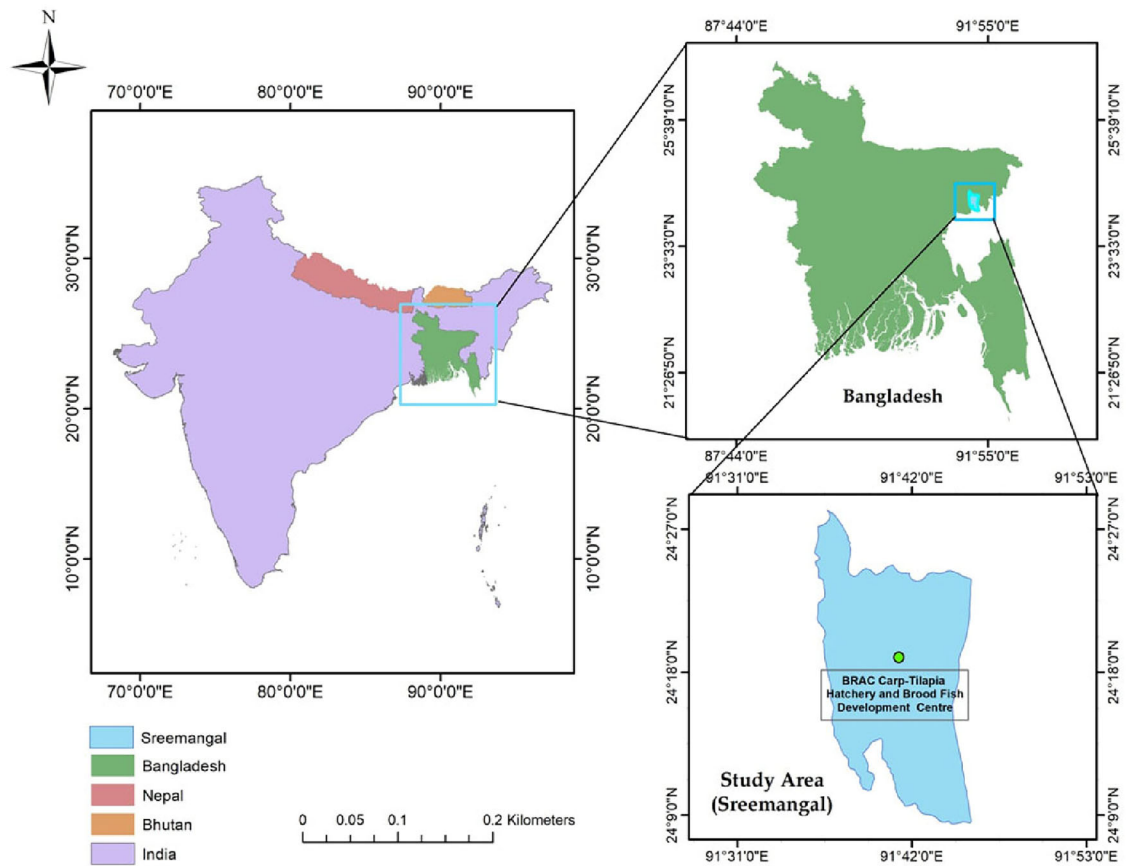


FIGURE 1 | Geographical map of the sampling area (BRAC Carp-Tilapia Hatchery and Brood Fish Development, Sreemangal Upazila [latitude: 24°18'56" N; longitude: 91°41'09" E], Moulavi Bazar, Sylhet, Bangladesh).

TABLE 1 | Biochemical test results for identification of isolated bacterial strains.

Test	<i>Streptococcus agalactiae</i>		<i>Klebsiella pneumoniae</i>	
	Present study	Reference strain (Ghetas et al. 2021)	Present study	Reference strain (Das et al. 2018)
Gram staining	+	+	–	–
Cell morphology	Cocci in chain	Cocci in chain	Rod	Rod
Motility	–	–	–	NA
Catalase	–	–	+	NA
Oxidase	–	–	–	+
Citrate	–	NA	–	–
MR (methyl red)	–	NA	–	–
VP (Voges-Proskauer)	–	–	–	+
Indole	–	–	+	–
Sucrose	+	+	+	+
Maltose	+	+	+	+
Glucose	+	NA	+	+
Mannitol	+	+	+	+
Lactose	+	–	+	+

Abbreviation: NA, not available.

2.3 | Molecular Identification of the Pathogen

Genomic DNA was extracted from the bacterial isolates following the method described in M. Z. Rahman, Hossain et al. (2021), with minor modifications. Briefly, three bacterial colonies were grown in nutrient broth and incubated overnight at 37°C. Then, 1.5 mL of each culture was centrifuged at 13,000 rpm for 10 min to collect the bacterial cell pellets. The pellets were resuspended in 350 µL of lysis buffer along with proteinase K (200 mg/mL) and incubated at 65°C for 30 min to facilitate cell lysis. After cooling, 3 µL of RNase A was added to remove RNA, followed by a 10-min incubation at 37°C.

To remove proteins, an equal volume (373 µL) of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the sample, followed by centrifugation at 14,000 rpm for 10 min. The upper aqueous phase (~200 µL) was then collected and combined with an equal volume of isoamyl alcohol (24:1). After a second round of centrifugation, the resulting supernatant (~150 µL) was gently transferred into a fresh microcentrifuge tube. DNA precipitation was carried out by adding 800 µL of ice-cold ethanol and incubating at -40°C for 1 h. The precipitated DNA was then pelleted by centrifugation (13,000 rpm, 10 min), washed with 1 mL of chilled 70% ethanol containing 20 µL of 7.5 M ammonium acetate, and stored at -20°C for 30 min. The resulting DNA pellet was allowed to air-dry and then dissolved in 50 µL of TE buffer (pH 7.6) by incubating at 37°C for 10 min.

For molecular identification, PCR amplification of the 16S rRNA gene was performed using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), as described by Acinas et al. (2004). Each 25 µL PCR reaction included 10 ng of template DNA, 2 mM MgCl₂, 1 µL of each primer (10 µM), and 1X Taq Master Mix. The expected amplicon size was approximately 1400 bp.

PCR amplification was performed using a thermal cycler (Applied Biosystems GeneAmp 9700) with the following conditions: an initial denaturation at 95°C for 5 min, followed by 32 cycles consisting of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s. This was concluded with a final extension step at 72°C for 10 min, and the reaction was then held at 4°C. A 1 Kb Plus DNA ladder (New England Biolabs, UK) was used to determine the size of the PCR products. The amplified fragments were separated on a 1% agarose gel stained with ethidium bromide and visualised using a gel documentation system (EZEE Clearview UV transilluminator). The PCR products were subsequently purified using a commercial PCR purification kit (UltraClean PCR Clean-Up Kit, MO BIO Laboratories, USA) according to the manufacturer's protocol.

The purified PCR products were sequenced in both directions using the same primers via Sanger sequencing (Sanger et al. 1977) at Wuhan Tianyi Huayu Gene Technology Co. Ltd., China. Raw sequences were processed and edited using BioEdit software, and high-quality consensus sequences were submitted to the NCBI GenBank database. Sequence identity was determined through BLAST analysis (McGinnis and Madden 2004), and phylogenetic relationships were inferred by constructing a neighbour-joining

tree using MEGA 11 (Tamura et al. 2021). The analysis was based on the Tamura-Nei model (Tamura and Nei 1993), with branch confidence evaluated using 1000 bootstrap replicates (Felsenstein 1985).

2.4 | Histopathological Assessment

The histological process was conducted manually following the methodology described by Mamun et al. (2022). Tissue samples (brain, gills, liver, kidney and spleen) were collected and fixed in 10% buffered formalin for 72 h. The samples were then cleared with xylene, infiltrated with melted paraffin and embedded in paraffin blocks. The blocks were trimmed with a sharp knife, and tissue sections of 5-µm thickness were cut using a microtome. Sections were stained with haematoxylin and eosin, mounted with Canada balsam, and examined under a microscope at 10× and 40× magnifications (Carl Zeiss Primostar 3, Germany).

2.5 | Antibiotic Sensitivity Test

The antibiotic sensitivity of *S. agalactiae* and *K. pneumoniae* isolates obtained from diseased fish was assessed using the Kirby-Bauer disc diffusion technique (Bauer et al. 1966). This test was performed on Mueller-Hinton agar (MHA) plates (HiMedia Laboratories Pvt. Ltd., India) following the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI 2017). Two dominant colonies of each isolate were used for the assay. A total of 15 commercially available antibiotic discs were employed in the experiment (Tables 2 and 3). The inoculated plates were incubated at 37°C for 12–18 h, after which clear zones of inhibition were observed. The diameters of these inhibition zones were measured, and the isolates were classified as sensitive, intermediate, and resistant based on CLSI (2017) standards. The multiple antibiotic resistance (MAR) index was determined by dividing the number of antibiotics to which an isolate showed resistance by the total number of antibiotics tested against that isolate (Krumperman 1983).

2.6 | In Vitro Susceptibility to Plant Extracts

To evaluate the antibacterial properties of the *S. agalactiae* and *K. pneumoniae* isolates obtained in the current study, thirteen medicinal plant species were selected for screening (Table S1). As outlined by Ehsan et al. (2023), plant materials were first shade-dried and finely powdered. For extraction, 25 g of each powdered sample was mixed with 100 mL of analytical-grade acetone and placed on an orbital shaker at room temperature for 72 h to extract bioactive components. Afterwards, the extracts were filtered through Whatman filter paper and evaporated at 50°C using a rotary evaporator, yielding crude extracts. The antibacterial activity of each extract was assessed via the disc diffusion method following the procedure of M. Rahman et al. (2017). Sterile filter paper discs saturated with the plant extracts were positioned on agar plates pre-inoculated with the test bacteria. Discs containing only acetone were used as negative controls. To ensure the accuracy and consistency of the results, all experiments were performed in triplicate.

TABLE 2 | Antimicrobial susceptibility analysis of *Streptococcus agalactiae* isolates.

Antibiotics	Concentration (µg/disc)	Interpretative category and zone diameter (mm)			Inhibition zone (mm)	Result
		S	I	R		
Oxytetracycline	30	≥ 23	19–20	≤ 18	23	S
Tetracycline	30	≥ 23	19–20	≤ 18	29	S
Ciprofloxacin	5	≥ 21	16–20	≤ 15	27	S
Oxacillin	10	≥ 15	12–14	≤ 11	6	R
Ceftriaxone	30	≥ 17	14–16	≤ 13	11	R
Co-trimoxazole	25	≥ 19	16–18	≤ 15	29	S
Doxycycline	30	≥ 23	19–22	≤ 18	22	S
Levofloxacin	5	≥ 17	14–16	≤ 13	23	S
Novobiocin	30	≥ 19	14–18	≤ 13	18	I
Erythromycin	15	≥ 21	16–20	≤ 15	6	R
Amoxicillin	30	≥ 17	14–16	≤ 13	6	R
Ampicillin	20	≥ 17	14–16	≤ 13	6	R
Azithromycin	15	≥ 18	14–17	≤ 13	26	S
Streptomycin	10	≥ 17	14–16	≤ 13	21	S
Gentamicin	10	≥ 15	13–14	≤ 12	22	S

Abbreviations: I, intermediate; R, resistant; S, sensitive.

Source: CLSI (2017).

TABLE 3 | Antimicrobial susceptibility analysis of *Klebsiella pneumoniae* isolates.

Antibiotics	Concentration (µg/disc)	Interpretative category and zone diameter (mm)			Zone of inhibition (mm)	Result
		S	I	R		
Oxytetracycline	30	≥ 15	12–14	≤ 11	25	S
Tetracycline	30	≥ 15	12–14	≤ 11	28	S
Ciprofloxacin	5	≥ 21	16–20	≤ 15	29	S
Oxacillin	10	≥ 15	12–14	≤ 11	9	R
Ceftriaxone	30	≥ 23	20–22	≤ 19	14	R
Co-trimoxazole	25	≥ 16	11–15	≤ 10	26	S
Doxycycline	30	≥ 14	11–13	≤ 10	22	S
Levofloxacin	5	≥ 17	14–16	≤ 13	27	S
Novobiocin	30	≥ 19	14–18	≤ 13	18	I
Erythromycin	15	≥ 13	—	≤ 12	6	R
Amoxicillin	30	≥ 17	14–16	≤ 13	6	R
Ampicillin	20	≥ 17	14–16	≤ 13	7	R
Azithromycin	15	≥ 13	—	≤ 12	28	S
Streptomycin	10	≥ 15	12–14	≤ 11	19	S
Gentamicin	10	≥ 15	13–14	≤ 12	23	S

Abbreviations: I, intermediate; R, resistant; S, sensitive.

Source: CLSI (2017).

2.7 | Lethal Dose

To determine the lethal dose (LD₅₀) of *S. agalactiae* and *K. pneumoniae*, two separate pathogenicity trials were conducted using juvenile Nile tilapia (20.0 ± 2 g). The fish were collected from Reliance Aqua Farms, Trishal, Mymensingh, Bangladesh and transported to the laboratory in oxygenated airtight plastic bags to maintain optimal oxygen levels. Upon arrival, the fingerlings were acclimatised to laboratory conditions for 7 days in a 1000-L fibre-reinforced plastic (FRP) tank and fed a commercial floating feed (30% crude protein) once daily. The fish were not obtained from a specific pathogen-free (SPF) facility; however, prior to the challenge experiments, a subset of the fingerlings underwent autopsy and bacterial streaking on NA plates, and no bacterial growth was observed, confirming that they were free of detectable pathogens. For each bacterial strain, six treatment groups ($n = 10$ fish per group) were established, including one phosphate-buffered saline (PBS)-injected control group (G1). Bacteria were cultured on NA, incubated at 37°C for 18 h, and then transferred to nutrient broth for further incubation under the same conditions. Viable cell counts were determined using standard serial dilution and plate count methods (Ducklow et al. 1980). Graded concentrations of bacterial suspensions (G2: 1×10^4 , G3: 1×10^5 , G4: 1×10^6 , G5: 1×10^7 and G6: 1×10^8 CFU/mL) were prepared, and 100 µL of each dose was administered intramuscularly into the dorsal muscle. Fish were anaesthetised using MS-222 (tricaine methanesulfonate) at a dose of 100 mg/L of water prior to injection. The quality of water was monitored using a multiparameter probe (HI 9828, YSI Incorporated, Yellow Springs, OH, USA) and remained within the recommended range for the comfort and well-being of the fish (dissolved oxygen 7.5 ± 0.2 mg/L, temperature $28 \pm 1.5^\circ\text{C}$, and pH 7.4 ± 0.1). Fish mortality in each group was monitored at 24-h intervals for 10 days, and LD₅₀ values were calculated using the Reed–Muench method (Reed and Muench 1938) based on cumulative mortality data. The LD₅₀ was determined using the following formula:

$$\text{Proportionate distance} = \frac{[(\text{mortality at dilution next above } 50\% - 50\%)]}{[\text{mortality next above } 50\% - (\text{mortality next below } 50\%)]}$$

$$\log \text{LD}_{50} = \text{Dilution above } 50\% - (\text{proportionate distance} \times \text{dilution factor})$$

Mortality data were analysed and visualised using cumulative mortality curves to compare the progression of mortality across treatment groups.

2.8 | Therapeutic Evaluation of Olive Extract and Antibiotics Against Co-Infection

To assess the recovery potential of acetonetic olive (*E. serratus*) extract (T3) in Nile tilapia juveniles (20 ± 2 g) co-infected with *K. pneumoniae* and *S. agalactiae*, a 10-day laboratory experiment was conducted using three treatment groups. Each group ($n = 30$; three replicates of 10 fish) was intramuscularly injected on Day 0 with 0.1 mL of a bacterial suspension containing the LD₅₀ dose of both pathogens. Treatments consisted of amoxicillin (T1,

40 mg/kg body weight), tetracycline (T2, 10 mg/kg body weight), or olive extract (T3, 375 mg/kg body weight), each incorporated into commercial feed at 3% of body weight and administered twice daily to apparent satiation. The doses of the antibiotics were selected based on published literature reporting effective and safe therapeutic concentrations for bacterial infections in fish (Bae et al. 2025; Hadfield and Clayton 2021). Feeding was withheld on Days 0 and 1 post-infection to allow establishment of the infection, and treatments were initiated on Day 2 and continued throughout the 10-day experimental period. Amoxicillin and tetracycline were chosen based on their distinct responses in antibiotic sensitivity tests, where the isolates exhibited resistance to amoxicillin and susceptibility to tetracycline. Olive extract was selected as it demonstrated the highest antibacterial efficacy among all tested plant extracts in the disc diffusion assay. The dosage of olive plant extract used in this study was selected from a previous report against virulent strains of *K. pneumoniae* and *S. agalactiae* (M. Rahman et al. 2017). Fish were housed in nine 120-L aquaria with continuous aeration, and water quality was maintained at dissolved oxygen 7.5 ± 0.2 mg/L, temperature $28 \pm 1.5^\circ\text{C}$ and pH 7.4 ± 0.1 .

2.9 | Ethical Approval and Animal Welfare

All experimental procedures involving fish were conducted in accordance with internationally accepted ethical guidelines for the care and use of animals in research. During the collection of moribund fish from the farm, as well as during the bacterial challenge and therapeutic experiments, fish were humanely euthanised to minimise suffering. The study protocol was reviewed and approved by the Animal Ethics Committee of Sylhet Agricultural University under the ethical clearance memo number SAU/AEC/FOF/FHM-697.

2.10 | Statistical Analysis

Data obtained from the antimicrobial activity of plant extract through disc diffusion assay were analysed using one-way ANOVA (Assaad et al. 2014) and visualised in RStudio (version 4.4.1). Kaplan–Meier analysis was applied to evaluate survival probabilities over a 10-day period for treatments with olive extract, tetracycline and amoxicillin. Mortality was coded as 1 and survival as 0. Through SPSS version 26.0, survival curves were compared using the log rank (Mantel–Cox) test for both overall and pairwise differences, with statistical significance set at $p < 0.05$.

3 | Results

3.1 | Clinical Signs and Gross Pathology

At the BRAC Carp-Tilapia Hatchery and Brood Fish Development Centre, a significant mortality event was observed in *O. niloticus*, accompanied by notable clinical signs. The affected fish exhibited signs of lethargy, whirling movement, and a tendency to isolate from the fish school, often congregating near the water surface. Externally, the fish showed purulent accumulations around the

head, a rotten head, darkening skin, swollen lips, abdominal shrinkage, corneal hypopyon, petechiae/erythema on the fin base and pronounced haemorrhages on the body surfaces (Figure 2A–H).

3.2 | Bacterial Isolation and Identification

Bacterial isolates from infected *O. niloticus* were preliminarily identified as *Streptococcus* spp. and *Klebsiella* spp. using standard biochemical tests. The Gram-positive chain-forming cocci of *Streptococcus* spp. (Figure S1A), isolated from brain and kidney tissues, producing transparent white colonies on nutrient agar. It tested negative for indole, oxidase, catalase, MR and VP tests. The Gram-negative *Klebsiella* spp. (Figure S1B), recovered from the kidney and liver, appeared as non-motile rods producing smooth, convex colonies. It was catalase- and indole-positive, but negative for oxidase, citrate, MR and VP tests. Both isolates showed acid production from glucose, sucrose, maltose, mannitol, and lactose fermentation. The biochemical test results are summarised in Table 1 and validated by comparison with findings from previously published studies.

3.3 | Molecular Confirmation of the Isolates

The 16S rRNA gene is commonly recognised as a reliable molecular tool for identifying bacterial species. In this study, PCR amplification of the 16S rRNA gene yielded fragments of approximately 1080 bp and 1437 bp for *Streptococcus* spp. and 1360 bp for *Klebsiella* spp. (Figure S2). The resulting sequences from two *S. agalactiae* isolates and one *K. pneumoniae* isolate were submitted to GenBank with accession numbers PP967716, PP967717 and PP967718, respectively. BLAST analysis revealed that the *Streptococcus* isolates shared 98.96%–99.86% sequence similarity with reference *S. agalactiae* strains, while the *Klebsiella* isolate showed 99.78% similarity with the reference *K. pneumoniae* strain (Table S2), confirming their identity. Phylogenetic analysis further supported these findings, showing that the *S. agalactiae* isolates (PP967716 and PP967717) clustered closely with Indian reference strains OR816117.1 and MH916751.1, respectively (Figure 3). Similarly, the *K. pneumoniae* isolate (PP967718) grouped with Indian reference strains PP291969.1 and OQ789963.1 (Figure 4), highlighting their close evolutionary relationships.

3.4 | Histopathological Observations

Histopathological alterations in brain, kidney, spleen, liver and gill tissues of Nile tilapia (*O. niloticus*) naturally co-infected with *S. agalactiae* and *K. pneumoniae*, showing vascular changes, degenerative lesions, inflammatory cell infiltration, tissue necrosis, and structural disruptions across multiple organs (Figure 5).

3.5 | Antibiotic Sensitivity Test

The antibiotic susceptibility analysis revealed that *S. agalactiae* and *K. pneumoniae* exhibited resistance to oxacillin, ery-

thromycin, ceftriaxone, amoxicillin and ampicillin, while showing intermediate sensitivity to novobiocin. In contrast, both isolates were highly susceptible to a broad spectrum of antibiotics, including oxytetracycline, tetracycline, ciprofloxacin, cotrimoxazole, doxycycline, levofloxacin, azithromycin, streptomycin and gentamycin (Tables 2 and 3). The calculated MAR index for both bacterial strains was 0.33.

3.6 | In Vitro Susceptibility to Plant Extracts

The in vitro antibacterial assay (Figures 6 and 7) demonstrated notable variation in the efficacy of different plant extracts against *S. agalactiae* and *K. pneumoniae*. *E. serratus* (fruit) showed the strongest antibacterial activity, followed by *Phyllanthus emblica* and *Syzygium aromaticum*, all producing significantly larger inhibition zones. Moderate effects were observed for *Nigella sativa*, *Psidium guajava* (leaf), and *Allium sativum*. In comparison, *Tamarindus indica*, *Terminalia arjuna* and *P. guajava* (fruit) exhibited weaker activity. Extracts such as *Moringa oleifera*, *Azadirachta indica*, *Curcuma longa* and *Allium cepa* showed no inhibition beyond the disc diameter (8 mm), indicating no antibacterial effect. According to statistical analysis, the differences in inhibition zones among most extracts were significant ($p < 0.05$), confirming the relative effectiveness of certain plants over others.

3.7 | LD₅₀ Determination and Mortality Analysis of *K. pneumoniae* and *S. agalactiae* in Nile Tilapia

The median LD₅₀ was calculated using the Reed–Muench method based on cumulative mortality data obtained from fish injected with graded doses of pathogenic bacteria. The LD₅₀ values for *K. pneumoniae* and *S. agalactiae* were determined to be 7×10^6 and 5.5×10^6 CFU/mL, respectively. The cumulative mortality curve analysis further confirmed the progressive increase in mortality with increasing bacterial dose for both pathogens. In the case of *K. pneumoniae* (Figure S3), mortality reached 70% at a higher dose (1×10^7 CFU/mL), with 30% mortality observed at 1×10^6 CFU/mL, identifying this range as critical for LD₅₀ estimation. Similarly, for *S. agalactiae* (Figure S4), mortality ranged from 40% to 60% between 1×10^6 and 1×10^7 CFU/mL, supporting the calculated LD₅₀ value. The cumulative mortality curves in Figures S3 and S4 clearly depict a dose-dependent mortality pattern, demonstrating the pathogenicity of both bacterial strains in Nile tilapia juveniles.

3.8 | Therapeutic Effect of Olive Extract on Post-Infection Recovery in Nile Tilapia

Following experimental co-infection, infected Nile tilapia exhibited a range of clinical signs, including lethargy, reduced feeding, resting at the bottom of the tank, abrasive mouth, darkened skin, purulent accumulations around the head, haemorrhages on the body surface and exophthalmia. These symptoms appeared as early as 6 h post-infection and peaked around the third day post-infection. The severity of clinical signs gradually declined over the course of 7 days. No mortality was recorded from Day 8 post-infection until the end of the experiment, indicating the



FIGURE 2 | Clinical manifestations in *Oreochromis niloticus* naturally co-infected with *Streptococcus agalactiae* and *Klebsiella pneumoniae* bacterial pathogens: (A) moribund and dead fish floating near the water surface, (B) panophthalmitis (yellow arrow) and rotten head (black arrow), (C) corneal opacity (black arrow). (D) purulent discharge around the cranial region (black arrow) accompanied by periocular haemorrhage or periocular congestion (yellow arrow), (E) intense reddening and haemorrhages visible at the operculum and base of the pectoral fin (yellow arrow), (F) darkening skin (black arrow), abdominal shrinkage (white arrow), and corneal hypopyon (yellow arrow), (G) petechiae/erythema (yellow arrow) on the fin base and (H) severe accumulation of pus from mouth dorsal fin region (yellow arrow) and swollen lips (white arrow).

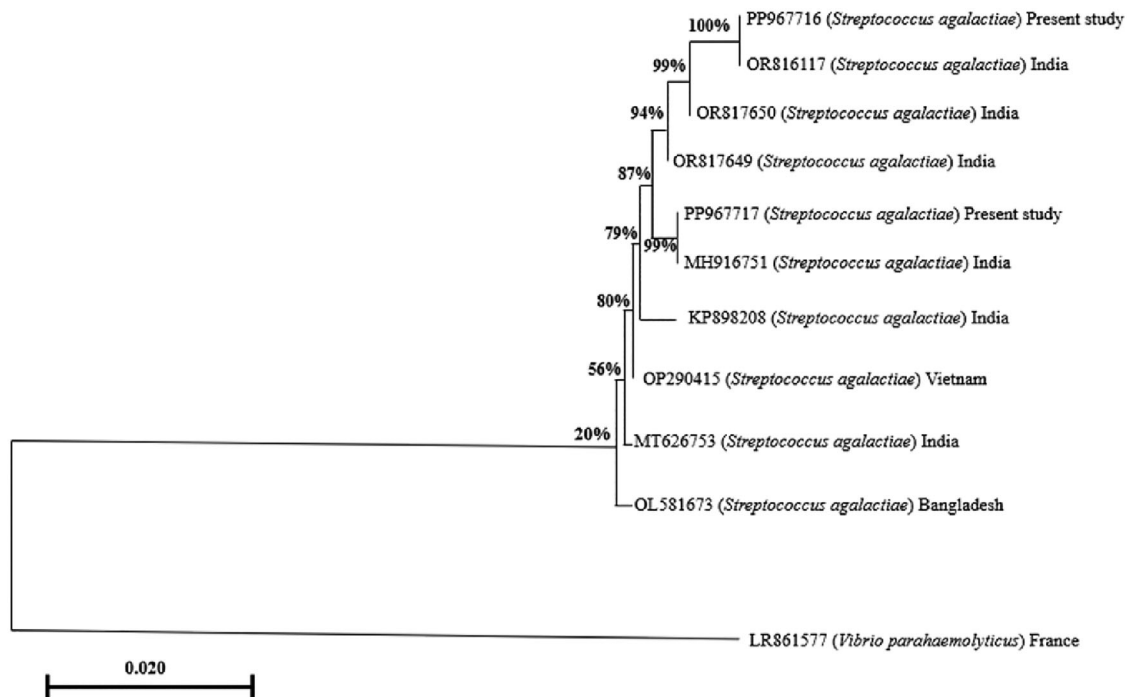


FIGURE 3 | Phylogenetic tree illustrating the genetic relationship between two *Streptococcus agalactiae* isolates from the present study (PP967716 and PP967717) and reference strains from India (OR816117 and MH916751). The phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap replicates. *Vibrio parahaemolyticus* was considered an out-group.

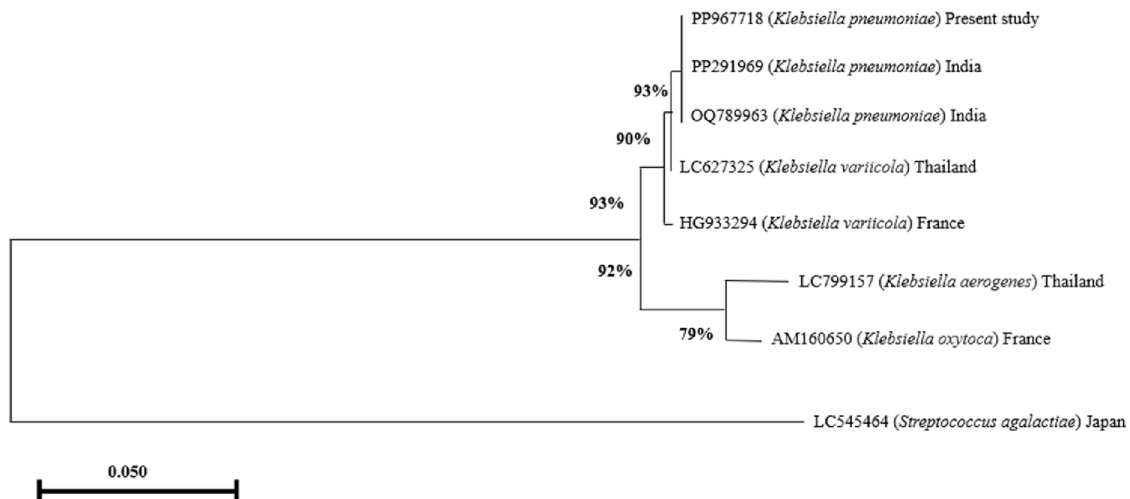


FIGURE 4 | Phylogenetic tree illustrating the genetic relationship between *Klebsiella pneumoniae* isolate from the present study (PP967718) and reference strains from India (PP291969 and OQ789963). The phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap replicates. *Streptococcus agalactiae* was considered an out-group.

effectiveness of the treatments in controlling the disease. The treatment efficacy was evaluated through Kaplan–Meier survival analysis, which demonstrated significant differences ($p < 0.05$) between the groups. The survival rate in the amoxicillin-treated group (T1) was notably low at $16.7 \pm 1.93\%$, while fish treated with tetracycline (T2) showed the highest survival at $83.33 \pm 1.93\%$. Fish receiving olive extract (T3) also demonstrated a high survival rate of $80 \pm 1.91\%$, closely comparable to that of tetracycline (Table S3 and Figure 8), indicating the promising recovery potential of the olive extract against co-infection.

4 | Discussion

In 2023, a significant disease outbreak associated with elevated mortality was observed in Nile tilapia (*O. niloticus*) at the BRAC Carp-Tilapia Hatchery and Brood Fish Development Centre in Sreemangal, Bangladesh. Laboratory investigations identified the involvement of two bacterial pathogens: Gram-positive *S. agalactiae* and Gram-negative *K. pneumoniae*, both recognised for their pathogenicity in intensively farmed tilapia (Abdel-Latif and Khafaga 2020; Basri et al. 2020; Ghetas et al. 2021; Vaneci-

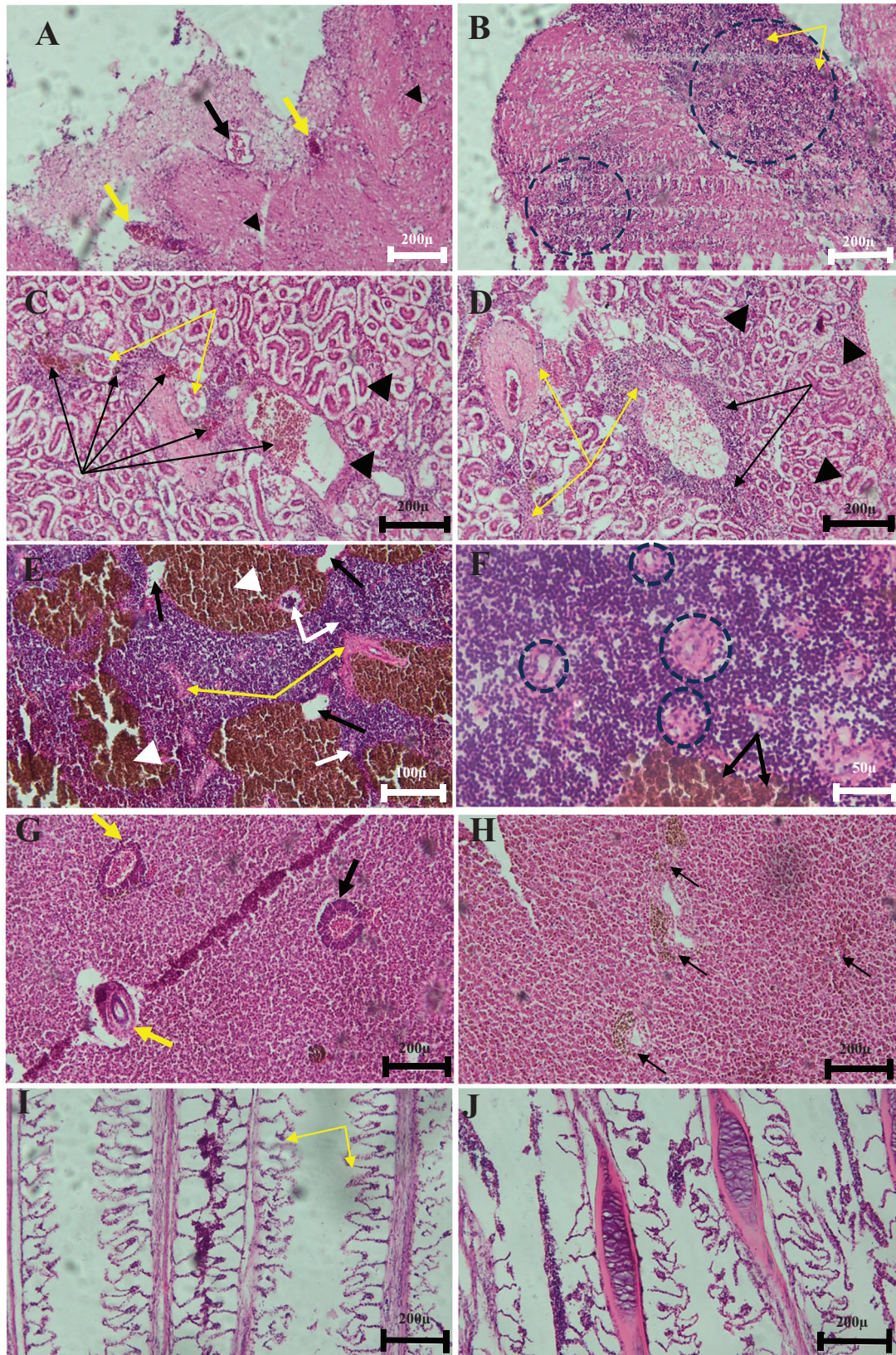


FIGURE 5 | Histopathological alterations in multiple organs of Nile tilapia (*Oreochromis niloticus*) naturally co-infected with *Streptococcus agalactiae* and *Klebsiella pneumoniae*: (A) brain showing hyperaemic blood vessels (yellow arrow) with microabscess formation surrounded by necrotic tissue (black arrow), along with perivascular and pericellular oedema (arrowhead); (B) brain tissue with meningeal congestion (dashed circles) and pronounced haemorrhages (yellow arrow); (C) kidney exhibiting haemorrhage (black arrow), disruption of renal tubules (yellow arrow) and degeneration of glomerulus (arrowhead); (D) kidney with thrombus formation in glomeruli and tubules (yellow arrow), lymphocyte infiltration (black arrow) and atrophy of haematopoietic tissue (arrowhead); (E) spleen showing multifocal haemosiderin deposition (arrowhead), vacuolisation (black arrow), lymphocyte aggregation around periarteriolar macrophage sheaths (PAMSs) (white arrow) and thrombus in splenic blood vessel (yellow arrow);

Silva et al. 2022). Sugiani et al. (2012) and Sumiati et al. (2015) have also documented co-infection scenarios in tilapia involving Gram-negative *A. hydrophila* and Gram-positive *S. agalactiae*. Moreover, *A. hydrophila* and *S. agalactiae* are frequently present in fish infected with Tilapia Lake Virus (TiLV) (Basri et al. 2020). While Amal et al. (2018) found co-infections with TiLV and *A. veronii* in red hybrid tilapia, Dong et al. (2015) documented concurrent infections involving *Flavobacterium* spp., *Streptococcus* spp., *Aeromonas* spp. and *Iridovirus* in cultured tilapia in Thailand. More recently, Vaneci-Silva et al. (2022) confirmed *K. pneumoniae* as a causative agent of disease in cage-cultured Nile tilapia in Brazil. *Alectis nigripes*, *Labeo rohita*, *Nibea japonicus*, *Cyprinus carpio* and *O. niloticus* are among the fish species that have been linked to disease outbreaks caused by *K. pneumoniae*, even though it has historically been thought of as an opportunistic human pathogen (Oliveira et al. 2014; Gopi et al. 2016; Das et al. 2018). Clinical investigations are considered an important tool for identifying specific infections (M. S. Rahman et al. 2025). In the present study, infected *O. niloticus* exhibited lethargy, surface-floating behaviour, whirling movements, corneal hypopyon, purulent discharge from the head and body haemorrhages. Similar clinical signs have been reported in Nile tilapia worldwide (Anshary et al. 2014; Akter et al. 2021; Hadfield and Clayton 2021; M. M. Rahman, Rahman et al. 2021). Corneal hypopyon, described as purulent exudate in the anterior chamber of the eye due to severe inflammation (Pépin et al. 2025), is a key sign of streptococcosis in Nile tilapia (Hadfield and Clayton 2021) and may progress to panophthalmitis, a severe suppurative inflammation affecting all ocular layers and surrounding tissues (Laith et al. 2017). Group B *Streptococcus* (GBS), a Gram-positive pathogen, has the capacity to pass over the blood-brain and spinal barriers (Abdallah et al. 2024), enabling its invasion of the central nervous system and leading to conditions such as meningoencephalitis (Doran et al. 2005;

Abdallah et al. 2024), a finding consistent with our observations. Although *K. pneumoniae* was also detected in the present study, it did not produce distinct pathological features, with nonspecific signs such as anorexia, haemorrhages, and ascites aligning with previous reports (Das et al. 2018, 2019; Vaneci-Silva et al. 2022).

Bacterial isolates from brain and kidney tissues produced small, round, greyish-white colonies identified as Gram-positive *S. agalactiae*, which fermented glucose, sucrose, mannitol, lactose, and maltose, but tested negative for catalase, oxidase, indole, MR, VP, and citrate, consistent with previous reports (Osman et al. 2017; Hamom et al. 2020; M.M. Rahman et al. 2021; M. M. Rahman et al. 2022). Gram-negative *K. pneumoniae* was isolated from kidney and liver tissues, displaying smooth, convex, non-motile rod-shaped colonies, positive for catalase and indole, negative for oxidase, VP and MR, and capable of fermenting the same sugars, in agreement with Das et al. (2019) and Vaneci-Silva et al. (2022).

To achieve more accurate identification beyond phenotypic traits, the dominant pathogens were further confirmed using 16S rRNA gene sequencing following approaches used in earlier studies (Abbott et al. 2003; M. Rahman et al. 2017; Akter et al. 2021; Ehsan et al. 2021; M. M. Rahman, Rahman et al. 2021). The *Streptococcus* isolates showed 98.96%–99.86% similarity to *S. agalactiae* reference strains, while the *Klebsiella* isolate showed 99.78% similarity to *K. pneumoniae*. Phylogenetic analysis further confirmed their close relationship with known strains. Basri et al. (2020) used similar molecular methods to identify *A. hydrophila* and *S. agalactiae* in red hybrid tilapia co-infected with TiLV, and Vaneci-Silva et al. (2022) used both phenotypic and 16S rRNA-based analyses to confirm *K. pneumoniae* in infected Nile tilapia. While the combined use of biochemical assays and 16S rRNA gene sequencing identified preliminary species identification, the inability to perform additional molecular confirmation using

(F) spleen with hypercellular PAMs exhibiting fibrinoid necrosis (dashed circles) and melano-macrophage centres (MMCs) (black arrow); (G) liver displaying thickened vascular walls with inflammatory cell infiltration and oedema (black arrow), and disruption of blood vessels (yellow arrow); (H) liver tissue showing endothelial damage with vasculitis (black arrows) and perivascular extension of inflammatory cells; (I, J) gills exhibiting disorganised structures and disruption of gill lamellae.

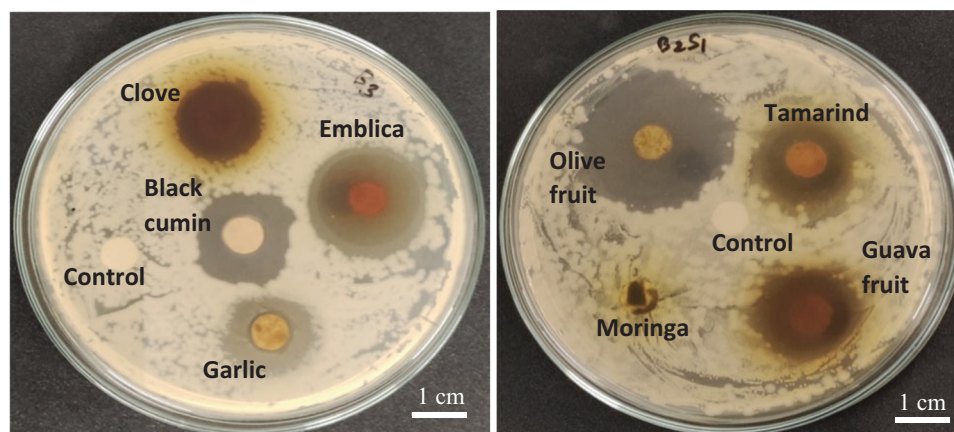


FIGURE 6 | Antibacterial activity of acetonic extracts from selected medicinal plants against *Streptococcus agalactiae* and *Klebsiella pneumoniae* as determined by the agar well diffusion assay. Distinct zones of inhibition were observed around wells containing plant extracts, while no inhibition was detected in the negative control. Scale bar = 1 cm.

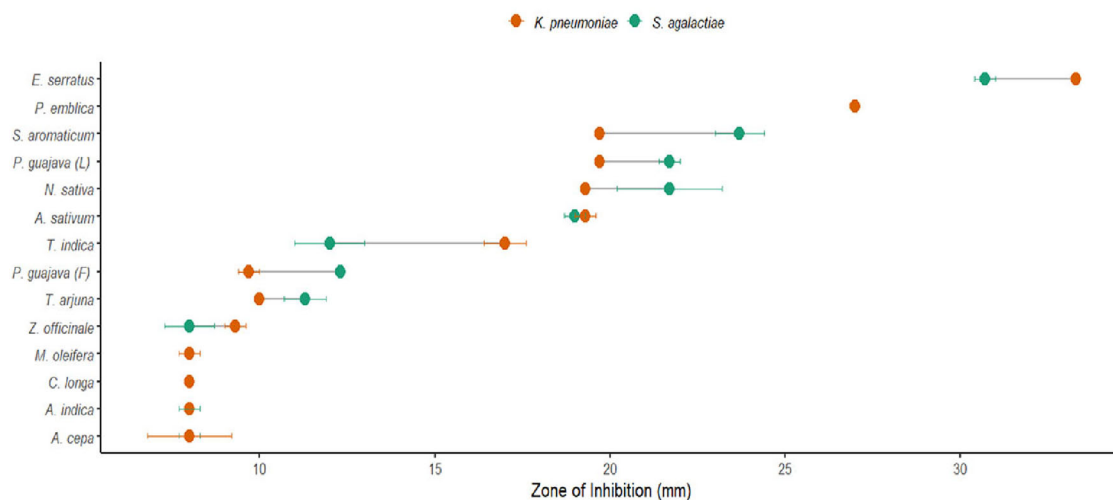


FIGURE 7 | Comparative antibacterial efficacy of various medicinal plant extracts against pathogenic *Streptococcus agalactiae* and *Klebsiella pneumoniae*, expressed as mean zone of inhibition (mm). Data are presented as mean $\pm$ SD. Differences in inhibition zones indicate variable antibacterial potency among plant species.

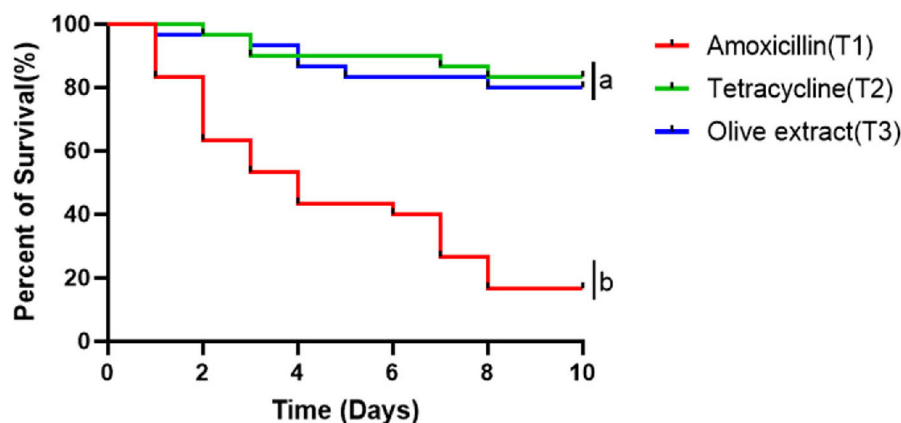


FIGURE 8 | Kaplan–Meier survival analysis of juvenile *Oreochromis niloticus* (20 ± 2 g) co-infected with *Klebsiella pneumoniae* and *Streptococcus agalactiae*. Fish ($n = 30$ per treatment group) were intramuscularly injected on Day 0 with 0.1 mL of a bacterial suspension containing *K. pneumoniae* (7×10^6 CFU/mL) and *S. agalactiae* (5.5×10^6 CFU/mL). Treatments were initiated on Day 2 post-infection and continued for 10 days via commercial feed (3% body weight, twice daily), supplemented with amoxicillin (40 mg/kg body weight), tetracycline (10 mg/kg body weight) or acetic olive (*Elaeocarpus serratus*) extract (375 mg/kg body weight). Different letters (a, b) indicate significant differences among treatment groups (the log rank test, chi-square = 43.89, $p = 0.0001$).

housekeeping genes (e.g., *rpoB*, *gyrA* or *infB*) represents a limitation of the present study. Future investigations should include multi-locus sequence analysis or species-specific PCR to improve taxonomic accuracy.

Following confirmation of the causative pathogens, histopathological examination revealed pronounced lesions across multiple organs in infected Nile tilapia. The brain showed vascular congestion, vacuolar degeneration, oedema, and haemorrhages (Laith et al. 2017; Ferrari et al. 2024). Gills exhibited lamellar disruption, fusion, shrinkage, necrosis and folding (He et al. 2018). Spleen tissues displayed multifocal haemosiderin deposition, vascular congestion, thrombosis, vacuolisation, MMCs, PAMS-associated lymphocyte aggregation and fibrinoid necrosis (He et al. 2018; Ghetas et al. 2021; Ferrari et al. 2024). Kidney lesions included haemorrhage, tubular and glomerular degeneration, thrombosis,

haematopoietic atrophy and inflammatory infiltration (Zapata and Cooper 1990; Laith et al. 2017; Ferrari et al. 2024). Liver histology showed vascular congestion, portal vein thrombosis, endothelial injury, bacterial emboli and perivascular inflammatory infiltration (Dougkias et al. 2021; Ferrari et al. 2024). Overall, these systemic alterations confirm the severe pathological impact of *S. agalactiae* and *K. pneumoniae* co-infection in tilapia (Puneeth et al. 2022).

The bacterial pathogens in this study showed a MAR index of 0.33, suggesting exposure to high-risk sources with frequent antibiotic use (Krumperman 1983; Puneeth et al. 2022). Such extensive and inappropriate antibiotic use can promote the development of resistant strains, posing serious risks to aquatic environments and public health (Smith et al. 1994; Aisyhah et al. 2015; Puneeth et al. 2022). The findings of this study showed that

S. agalactiae and *K. pneumoniae* were resistant to erythromycin, oxacillin, ceftriaxone, amoxicillin and ampicillin, but remained susceptible to several other antibiotics such as streptomycin, co-trimoxazole, ciprofloxacin, doxycycline, levofloxacin, oxytetracycline, gentamycin and azithromycin. Although Basri et al. reported different resistance profiles for *S. agalactiae*, these results are consistent with earlier findings by Osman et al. (2017), Basri et al. (2020), and Das et al. (2019).

Due to the adverse impacts of conventional antibiotic use—such as resistance development, residue accumulation in fish tissues, ecological disruption, and potential health risks—there is growing interest in environmentally friendly alternatives for disease management (Bhat et al. 2022; Jeon et al. 2023). Consequently, phytotherapeutics have gained attention as sustainable options, offering antimicrobial, antioxidant, and immunostimulatory benefits (Silva et al. 2024). In this study, the antibacterial effects of various medicinal plant extracts against *K. pneumoniae* and *S. agalactiae* were evaluated using the disc diffusion method. Among the plants tested—*E. serratus*, *P. emblica*, *S. aromaticum*, *N. sativa*, *P. guajava* (leaf and fruit), *A. sativum*, *T. indica*, *T. arjuna*, *M. oleifera*, *A. indica*, *C. longa* and *A. cepa*—the crude extract of *E. serratus* (olive fruit) showed the strongest antibacterial activity. Significant inhibition zones ($p < 0.05$) were also recorded for *P. emblica*, *S. aromaticum*, *N. sativa*, *P. guajava* (leaf) and *A. sativum*. These effects are attributed to diverse bioactive secondary metabolites with antimicrobial properties (Mawalagedera et al. 2019; Ashraf et al. 2023; Zouine et al. 2024). Specifically, allicin in garlic (*A. sativum*) and eugenol in cloves (*S. aromaticum*) are well-recognised for their bactericidal potential (Starliper et al. 2015; Senarathna and Gunathilaka 2023). Furthermore, the high levels of phenolic compounds, flavonoids, and oleuropein in olive extracts contribute to enhanced immune function and disease resistance in aquaculture species (M. Li et al. 2022; Pal and Lal 2023).

To evaluate the therapeutic effectiveness of acetonc *E. serratus* (olive fruit) extract compared to conventional antibiotics, juvenile Nile tilapia (*O. niloticus*) were experimentally challenged with a combined LD₅₀ dose of *S. agalactiae* and *K. pneumoniae*, based on their determined LD₅₀ values. Among the tested treatments—tetracycline (most effective antibiotic), amoxicillin (resistant antibiotic) and acetonc olive (*E. serratus*) extract (most effective plant extract from the disc diffusion assay)—the *E. serratus* extract significantly improved fish survival (80%), showing comparable efficacy to tetracycline (83.3%) in reducing disease impact, whereas amoxicillin provided minimal protection (Figure 8). The superior performance of tetracycline is attributed to its broad-spectrum efficacy against both pathogens (Evode et al. 2024), while the ineffectiveness of amoxicillin, despite its widespread use (Nakao et al. 2019), reflects the resistance patterns of the isolates observed in the disc diffusion test (Maniyappan et al. 2025). The notable therapeutic effect of *E. serratus* extract can be attributed to its rich composition of bioactive compounds, particularly oleuropein and hydroxytyrosol, which exhibit potent antimicrobial, antioxidant and immunostimulatory properties, enhancing both innate and adaptive immune functions (Nediani et al. 2019). These findings are consistent with earlier studies highlighting the anti-inflammatory and immune-modulating effects of olive-derived compounds such as hydroxytyrosol, tyrosol, and oleuropein (Corona et al. 2009; Centrone et al. 2021;

Pal and Lal 2023; M. Li et al. 2022) and their role in improving gut health and immune defence in fish (Hazreen-Nita et al. 2022). Although previous in vivo studies using plant extracts in tilapia challenged with *Streptococcus* spp. and other bacteria reported promising outcomes (Guo et al. 2019; Haenen et al. 2023; Abdallah et al. 2024), this study is the first to demonstrate the substantial effectiveness of olive (*E. serratus*) extract against co-infection with *S. agalactiae* and *K. pneumoniae* in experimentally infected Nile tilapia. These results advance our understanding of co-infection pathogenesis in *O. niloticus*, as simultaneous exposure to both pathogens led to increased disease severity, indicating a synergistic interaction. In this context, the observed in vitro and in vivo efficacy of the olive extract highlights its potential as a sustainable, plant-based alternative to conventional antibiotics for managing MDR bacterial co-infections in aquaculture. However, it should be noted that the therapeutic evaluation was conducted over a relatively short experimental period, focusing on the acute phase of infection and early recovery, which may differ in long-term outcomes. To establish olive extract as a practical and effective alternative to commercial antibiotics, future research should also focus on optimising its formulation, dosage, and application strategies under field conditions to ensure feasibility and adoption by fish farmers.

5 | Conclusion

Co-infections between *K. pneumoniae* and *S. agalactiae* pose a severe threat to Nile tilapia, and this study emphasises the limitations of traditional antibiotic treatments because of the rise in antimicrobial resistance. The acetonc extract of *E. serratus* (olive fruit) exhibited promising therapeutic efficacy, showing comparable recovery outcomes to tetracycline and presents itself as a potential eco-friendly alternative for managing bacterial diseases in aquaculture. However, as this research was limited to a short-term laboratory trial with a modest sample size, further studies are needed to assess its effectiveness under field conditions, evaluate possible long-term or side effects, and investigate the immune and molecular responses involved to support its broader application in sustainable fish health management.

Author Contributions

M.A.A.M. led the conceptualisation, validation, project administration and contributed to funding acquisition and writing – original draft. M.S.R.S. handled the methodology, software, formal analysis and contributed to writing – original draft. M.K., D.B., M.S.R. and S.R. contributed to the methodology, software, data curation, investigation. S.N. supervised the project and contributed to visualisation, writing – review and editing. M.M.R., S.M.I.K. and R.A.R. provided resources and contributed to writing – review and editing. M. M.M.A. contributed to visualisation and writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The nucleotide sequence data generated in this study have been deposited in the NCBI GenBank database, and the corresponding accession numbers are provided in the manuscript. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary material: aff270205-sup-0001-SuppMat.docx