RESEARCH ARTICLE

Running Title: GC-MS of Terminalia chebula against Aeromonas hydrophila

Extracting and Exploring of Bioactive Compounds in Terminalia chebula through GC-MS Profiling against fish pathogen Aeromonas hydrophila

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ABSTRACT

Aeromonas hydrophila is a significant pathogen associated with ulcerative disease and high mortality rates in freshwater aquaculture, especially in Channa striata. With the growing issue of antimicrobial resistance (AMR), the search for plant-based alternatives has become increasingly crucial. This research assessed the antibacterial properties of Terminalia chebula fruit extract against a virulent Aeromonas hydrophila strain obtained from naturally infected murrel fish. The identification of the pathogen was confirmed using morphological observations, biochemical testing, and 16S rRNA gene sequencing analysis. The amplified 545 bp 16S rRNA gene fragment showed >99% sequence similarity with Aeromonas hydrophila strains available in the NCBI GenBank database, and the sequence was deposited under accession number PQ495909 (strain CS-Ah001). Antibiogram analyses indicated resistance to ampicillin, while showing considerable sensitivity to ciprofloxacin, gentamicin, doxycycline, meropenem, and ceftazidime. Methanolic extracts of Terminalia chebula exhibited a rich array of phytochemicals, and GC–MS profiling identified 78 bioactive compounds, primarily consisting of phenolics, benzoic acid derivatives, long-chain hydrocarbons, and phytosterols. Agar well diffusion tests displayed a dose-dependent inhibition (12–18 mm), suggesting that phenolic constituents likely contribute to antibacterial mechanisms through oxidative processes and disruption of membranes. These findings suggest that Terminalia chebula has notable antibacterial potential and could be considered a natural and sustainable therapeutic option for controlling Aeromonas hydrophila infections in aquaculture. In summary, this research underscores Terminalia chebula as a promising environmentally friendly phytotherapeutic substitute for managing Aeromonas hydrophila infections in freshwater aquaculture. Further in vivo investigations are required to validate its practical application in fish health management.

Key words: Aeromonas hydrophila, antimicrobial resistance, GC-MS, Channa striata, natural therapeutics.
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Introduction

In aquaculture, where disease outbreaks significantly hinder productivity, there is a growing interest in plant-derived therapeutics (*Chakraborty & Hancz, 2011*). Although aquaculture plays a crucial role in global food security, it remains highly susceptible to bacterial infections that can result in substantial economic losses. One such bacterium, *Aeromonas hydrophila*, is a Gram-negative opportunistic pathogen known for causing Motile Aeromonas Septicemia (MAS), which results in ulcerative lesions, hemorrhaging, and high mortality rates in freshwater fish like *Channa striata* (Murrel) (*Igbinosa et al., 2012; Behera et al., 2018*). The extensive use of antibiotics to manage these infections has led to significant issues, such as antibiotic resistance, disruptions in aquatic microbiota, and the accumulation of residues in fish tissues and the surrounding environment (*Cabello, 2006; Defoirdt et al., 2011*).

The current circumstances have prompted the exploration of eco-friendly, sustainable alternatives to antibiotics in aquaculture. Several studies have indicated that extracts from medicinal plants not only possess direct antibacterial properties against *Aeromonas hydrophila* but also boost the immune responses, growth performance, and

survival rates of fish (*Harikrishnan et al., 2011; Newaj-Fyzul & Austin, 2015*). For instance, compounds such as eugenol (derived from clove), thymol (found in thyme), and quercetin (present in onion and citrus plants) have shown significant inhibitory effects against *Aeromonas* species (*Bhuvaneswari & Balasundaram, 2015; Pachanawan et al., 2008*). This underscores the potential of phytochemicals as natural agents for disease control in managing fish health.

In recent years, herbal therapy has emerged as a promising approach to address these challenges. Medicinal plants are natural reservoirs of bioactive compounds such as alkaloids, flavonoids, phenolics, tannins, terpenoids, and essential oils. These secondary metabolites have been documented to possess antimicrobial, antioxidant, anti-inflammatory, and immunostimulatory properties (*Reverter et al*., 2014; *Hodar et al*., 2019). Unlike synthetic antibiotics, plant-derived compounds are biodegradable, generally non-toxic, and more acceptable to consumers due to their natural origin. In aquaculture, herbal extracts can be administered through water or feed, where they promote fish growth, enhance immunity, and provide resistance against a broad spectrum of pathogens (*Ghosh et al*., 2021). Their affordability and accessibility also make them highly suitable for small and medium-scale farmers, who form the backbone of aquaculture in many Asian countries, including India.

One such medicinal plant of particular interest is *Terminalia chebula* Retz., commonly known as black myrobalan or “Haritaki.” Belonging to the family Combretaceae, *Terminalia chebula* has been extensively used in traditional medicine systems such as Ayurveda, Siddha, and Unani. Its fruits are renowned for their therapeutic effects in treating digestive disorders, skin ailments, urinary tract infections, diabetes, cardiovascular diseases, and even cancer. Phytochemical studies have shown that *Terminalia chebula* fruits contain a wide variety of bioactive molecules, including polyphenols, tannins, glycosides, and flavonoids. Among these, pyrogallol and other phenolic compounds have demonstrated strong antimicrobial and antioxidant properties (*Akimoladan et al.,* 2007). These compounds act by scavenging free radicals, protecting against oxidative stress, and inhibiting the growth of harmful microbes, making them ideal candidates for application in aquaculture disease management.

To effectively utilize of medicinal plants, it is crucial to identify and characterize their bioactive compounds. Advanced analytical techniques like Gas Chromatography-Mass Spectrometry (GC-MS) have transformed the process of phytochemical profiling. GC-MS merges the precise separation of volatile compounds with the capability of mass spectrometry to elucidate structures, enabling the accurate identification of both major and minor components in complex extracts (*Sparkman et al., 2011*). This methodology has been extensively used to discover compounds recognized for their antimicrobial, antioxidant, and immunostimulatory properties, thus confirming the traditional applications of plants and associating them with distinct biological functions (*Kanthal et al., 2014; Ncube et al., 2012*).

Considering the importance of phytochemicals in managing health within aquaculture, the present study aims to explore the antibacterial properties of methanolic extracts obtained from the fruits of *Terminalia chebula* against *Aeromonas hydrophila*, a detrimental bacterium commonly associated with disease outbreaks in striped murrel farming. Through a series of phytochemical assessments, antimicrobial evaluations, and GC-MS analysis, this research aims to identify the bioactive compounds that contribute to the antibacterial properties. By highlighting the positive effects of *Terminalia chebula* extracts as natural alternatives to traditional antibiotics, this study seeks to provide a sustainable approach for disease management in aquaculture. The outcomes may assist in developing plant-based antibacterial formulations or therapeutic options that enhance fish health, lower mortality rates, and reduce reliance on synthetic antibiotics. The results of this research are anticipated to assist in creating natural, cost-efficient, and sustainable methods for addressing bacterial diseases in aquaculture, thereby minimizing the dependence on antibiotics and promoting safer fish production. Ultimately, this approach supports safer fish production, safeguards consumer health, and encourages the sustainability of aquaculture practices.

Materials and Methods:

Collection and Authentication of Specimen

The fruits of *Terminalia chebula* were collected from the nursery garden located in Perumbakkam, Chennai–600100. The plant material was authenticated by the Herbarium, Department of Plant Biology and Plant Biotechnology, Guru Nanak College, Velachery, Chennai, Tamil Nadu–600042, and a voucher specimen was deposited with the authentication certificate number **PBPBT/GNC/2025/12/002**.

Extraction of Plant Material (Fruit Part)

The selected plant's fruits were gathered and dried in the shade at room temperature (about 30°C) under sterile conditions to avoid microbial contamination and loss of phytochemicals. To maintain the integrity of heat-sensitive compounds, exposure to direct sunlight was minimized (*Pandey & Tripathi, 2014*). The dried fruits were ground into a fine powder and passed through a 75 mm mesh to achieve a consistent particle size, promoting efficient solvent penetration during the extraction process (*Harborne, 1998*). The powdered samples

were then stored in sterile zip-lock bags under controlled conditions until they were needed.

For the extraction process, 10 g of the powdered fruit sample was mixed with 100 mL of methanol, a polar solvent widely used for efficient extraction of phytochemicals (Tiwari et al., 2011; Doughari, 2012). The mixture was vortexed thoroughly and kept on a rotary shaker at 32 °C for 24 h to enhance solvent penetration and extraction efficiency (Azwanida, 2015). Following extraction, the mixture was filtered through Whatman No. 1 filter paper to remove insoluble plant residues. The filtrate was subsequently centrifuged at 12,500 rpm for 10 min to obtain a clear supernatant. The methanolic extract was concentrated by evaporation under room temperature conditions until a semi-solid crude extract was obtained. The final crude extract was collected, weighed, and stored under sterile conditions until further phytochemical, antibacterial, and GC–MS analyses.

Qualitative Phytochemical Analysis

The initial phytochemical analysis utilizing crude extracts was performed in accordance with established protocols (*Vishnu, et al., 2019*). The extraction yields were determined using the formula provided below: Yield (%) = W_1/ W_2 x 100 In this equation, W_1 represents the weight of the extract following the evaporation of the solvent, while W_2 denotes the dry weight of the plant sample (*Abbas, A., et al. 2021*).

Bacterial Isolate and Culture Maintenance

The bacterial isolate of *Aeromonas hydrophila* used in the present study was obtained from laboratory stock cultures maintained under sterile conditions. The culture was subcultured onto SCDA agar plates using a sterile inoculation loop to obtain discrete bacterial colonies. The inoculated plates were incubated at 35 °C for 12 h following standard microbiological procedures for bacterial revival and isolation. (*Austin & Austin, 2016; Roberts, 2012; Buller, 2014*).

Confirmation of Bacterial pathogen

The isolated bacterial pathogen was subjected to morphological and biochemical characterization, including Gram staining and carbohydrate fermentation tests. Molecular confirmation of the bacterial isolate was performed through amplification and sequencing of the 16S rRNA gene using Polymerase Chain Reaction (PCR). The amplified PCR product was sequenced, and the obtained nucleotide sequence was analyzed using the NCBI BLAST tool for species-level identification. Phylogenetic analysis was performed using the Neighbor-Joining method in MEGA X software based on 16S rRNA gene sequences aligned using the ClustalW algorithm.

Antibiogram study

The Kirby-Bauer disk diffusion technique using Mueller-Hinton agar was utilized to assess antimicrobial susceptibility, following the most recent guidelines provided by the Clinical and Laboratory Standards Institute (CLSI). The inoculum was uniformly distributed over the agar plates, adjusted to a 0.5 McFarland standard, while antibiotic discs were strategically placed at specific intervals. A variety of antibiotic discs were chosen based on their availability, including Doxycycline hydrochloride (DO), Ceftazidime (CAZ), Ciprofloxacin (CIP), Enrofloxacin (EX), Ampicillin (AMP), Co-Trimoxazole (COT), Oxytetracycline (O), Sulphadiazine (SZ), Cefotaxime (CTX), Furazolidone (FR), Meropenem (MRP), Erythromycin (E), Azithromycin (AZM), and Imipenem (IPM). Following an incubation period of 18 to 24 hours at a temperature ranging from 28 to 30 °C, the diameters of the inhibition zones were recorded and classified as sensitive, intermediate, or resistant based on CLSI breakpoints. Additionally, certain isolates were subjected to minimum inhibitory concentration (MIC) testing using the broth microdilution method in cation-adjusted Mueller–Hinton broth. The Multiple Antibiotic Resistance (MAR) index was determined by calculating the ratio of antibiotics to which the isolate was resistant against the total number of antibiotics tested. Furthermore, a MIC test using the broth microdilution technique in cation-adjusted Mueller-Hinton broth was performed on a selection of isolates, with the MAR index defined as the fraction of resistant antibiotics compared to the total number of antibiotics assessed.

Antimicrobial Assay

The antibacterial activity of the *Terminalia chebula* fruit extract was evaluated against the isolated strain of *Aeromonas hydrophila* using the disc diffusion method on Mueller–Hinton agar (MHA). The bacterial suspension was uniformly swabbed onto sterile MHA plates to obtain confluent growth. Sterile discs impregnated with different concentrations of the methanolic plant extract (25, 50, 75, and 100 mg/mL) were aseptically placed on the inoculated agar surface. Discs loaded with methanol alone served as negative controls. The plates were incubated at 35 °C for 24 h, after which the zones of inhibition were measured in millimeters. All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. The antibacterial activity was interpreted based on the diameter of the inhibition zones according to standard microbiological procedures (Murray et al., 1995).

GC- MS analysis

Preparation of Extract for GC–MS Analysis

A 10 g portion of the fresh dried, finely powdered sample was subjected to solvent extraction using 100 mL of Methanol in an orbital shaker operated at room temperature for 24 h. The extraction was repeated multiple times with fresh portions of the same solvent until the solvent phase appeared clear and colourless, indicating complete

exhaustion of extractable compounds. All extract phases were combined and filtered separately through Whatman No. 41 ashless filter paper to remove particulate matter. The extract was filtered and concentrated under reduced pressure using a rotary evaporator to obtain a dry crude extract, which was stored at −20 °C until GC–MS analysis.

The pooled filtrate was then concentrated under reduced pressure at 60 °C using a Buchi Rotavapor R-300 system to evaporate the ethyl acetate and obtain the crude extract. The resulting dark brown semisolid residue was reconstituted in 2 mL of analytical-grade methanol and used as the working sample for GC–MS analysis.

GC/MS analysis was performed using an Agilent 8890 GC system equipped with a PAL3 Series II RSI 85 Automatic Liquid Sampler and coupled to an Agilent 5977B Series Inert Plus MSD Turbo EI mass spectrometer. Chromatographic separation was achieved on an HP-5ms UI capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). High-purity helium served as the carrier gas at a constant flow rate of 1.0 mL/min.

Samples were injected in split mode (1:10) with an injection volume of 2 µL. The GC oven temperature was programmed as follows: an initial temperature of 110 °C (held for 2 min), ramped to 200 °C at 10 °C/min without a hold, followed by a ramp to 280 °C at 5 °C/min, with a final hold for 9 min. The total analysis time was 36 min, including a 2-min solvent delay.

Mass spectral data were acquired in electron ionization (EI) mode, and the resulting chromatograms were processed for Total Ion Chromatogram (TIC) interpretation. Compound identification was performed by comparing the obtained mass spectra with those available in the NIST 2020 mass spectral library (*National Institute of Standards and Technology, 2020*) and the Wiley Registry of Mass Spectral Data (*Wiley, 2020*), using match quality scores to assign tentative identifications.

Peak identification and data processing

Compounds were preliminarily identified by matching their mass spectra against the NIST and Wiley commercial spectral libraries, adhering to a match-factor threshold of over 60 for acceptance (*NIST, 2017; Heller et al., 2014*). Retention indices (RI) were determined in relation to a homologous series of C_8–C_{40} n-alkane standards and were utilized in combination with spectral similarity to enhance the identification of potential compounds (Kovats, 1958; Adams, 2007). Integrated total ion chromatogram (TIC) peak areas were recorded for each identified compound and were used to rank their relative abundance within the extract (Stein, 1999).

Quality control and repeatability

Each extract underwent analysis in triplicate to confirm analytical consistency, and solvent blanks were incorporated between runs to detect possible contaminants from the column or reagents. Background substances typically linked to laboratory contamination like phthalates and parabens (e.g., methylparaben) were identified and excluded from biological analysis (*Snyder et al., 2012; Wang & Kannan, 2013*). The identification of compounds relied on spectral similarity, where match-factor values of 60 or greater were considered tentative identifications, while scores exceeding 90 were regarded as high-confidence matches in line with established GC–MS standards (*Adams, 2007; Stein, 1999*).

Results

Bacterial Morphology and Biochemical Characterization

Table 1: Biochemical characterization of the isolated Bacterial strain.

S. No	Name of the biochemical test	Results
1.	Citrate	+
2.	Urease	+
3.	Triple sugar iron	+
4.	Indole	-
5.	Methyl red	-
6.	Voges proskauer	-
7.	Nitrate broth	-
8.	Arginine dihydrolase broth	+
9.	Ornithine decarboxylase broth	+
10.	Lysine decarboxylase broth	+
11.	Maltose	+
12.	Fructose	+
13.	Cellobiose	-
14.	Trehalose	-
15.	Salicin	-
16.	Arabinose	+
17.	Mannose	+
18.	Gram Staining test	- (negative)

The colonies that developed on nutrient agar were observed to be pale white, circular, flat, and opaque, with some exhibiting a dull white appearance, smooth edges, and either pulvinate or umbonate shapes, with large and irregular sizes. The isolates were found to be motile, and Gram staining revealed Gram-negative rod-shaped bacterial cells (Figure 1 (b)). Results from biochemical tests (Table 1) were positive for both oxidase and catalase, and there was also a positive hemolytic activity, suggesting the strain's virulent properties (Figure 1 (a) and Figure 2). Therefore, the isolated strain is characterized as *Aeromonas hydrophila*.

(a)

(b)

Figure 1: (a) Hemolytic activity of *Aeromonas hydrophila* on blood agar showing β-hemolysis; (b) Gram staining showing Gram-negative rod-shaped bacterial cells

Figure 2: Biochemical characterization of *Aeromonas hydrophila*

Antibiogram Study

The antimicrobial susceptibility of the isolated strain of *Aeromonas hydrophila* was evaluated using the Kirby-Bauer disc diffusion method (Table 2). The isolate showed considerable sensitivity to gentamicin and ciprofloxacin, while moderate sensitivity was observed for chloramphenicol and tetracycline. In contrast, the isolate displayed resistance to ampicillin, highlighting the increasing concern regarding β-lactam antibiotic resistance in aquatic pathogens. The resistance to ampicillin underscores the need for alternative strategies, such as herbal biopotentiators and probiotic supplementation, to manage diseases in aquaculture (Figure 3). However, the significant sensitivity to ciprofloxacin and gentamicin suggests that these antibiotics remain effective treatment options for *Aeromonas hydrophila* infections in fish.

Table 2. Antimicrobial susceptibility pattern of the isolated bacterial strain.

S. No	Name of the antibiotic	Disc potency (µg/disc)	Zone of interpretation in mm	Interpretation
1.	Ceftazidime (CAZ)	30	21mm	Sensitivity
2.	Co-trimoxazole (COT)	25	22 mm	Sensitivity
3.	Enrofloxacin (EX)	5	34 mm	Sensitivity
4.	Doxycycline hydrochloride (DO)	30	24 mm	Sensitivity
5.	Ciprofloxacin (CIP)	5	34 mm	Sensitivity
6.	Ampicillin (AMP)	10	Zero	Resistance
7.	Oxytetracycline (O)	30	24 mm	Sensitivity
8.	Erythromycin (E)	15	15 mm	Intermediate
9.	Imipenem (IPM)	10	16 mm	Intermediate
10.	Meropenem (MRP)	10	28 mm	Sensitivity
11.	Cefotaxime (CTX)	30	27 mm	Sensitivity
12.	Sulphadiazine (SZ)	300	24 mm	Intermediate
13.	Azithromycin (AZM)	30	27 mm	Sensitivity
14.	Furazolidone (FR_	100	24 mm	Sensitivity

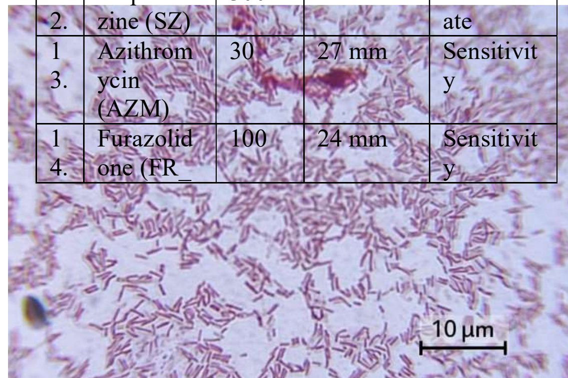
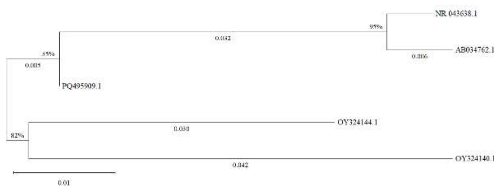


Figure 3: Antibiotic susceptibility profile of *Aeromonas hydrophila* determined by the Kirby–Bauer disc diffusion method on Mueller–Hinton agar.

Molecular Identification

The bacterial isolate was identified at the molecular level through 16S rRNA gene amplification and sequencing. The amplified 16S rRNA gene fragment was sequenced, and the obtained nucleotide sequence was analyzed using the BLAST tool available in the NCBI GenBank database. Sequence analysis revealed more than 99% similarity with previously reported *Aeromonas hydrophila* strains, confirming the identity of the isolate as *Aeromonas hydrophila*. The sequence was

submitted to the NCBI GenBank database under accession number PQ495909 and designated as strain CS-Ah001. Based on morphological, biochemical, and molecular characterization, the bacterial isolate used in the present study was confirmed as *Aeromonas hydrophila*.



The GenBank entry details are as follows:
LOCUS: PQ495909
Length: 545 bp
Organism: *Aeromonas hydrophila*
Gene: 16S ribosomal RNA
Based on the molecular analysis and sequence homology, the bacterial isolate used in the present study was confirmed as *Aeromonas hydrophila*.
Phylogenetic analysis based on 16S rRNA gene sequences demonstrated that the isolate PQ495909.1 (*Aeromonas hydrophila* strain CS-Ah001) clustered closely with reference *Aeromonas hydrophila* strains retrieved from the NCBI GenBank database, confirming the molecular identity of the bacterial

isolate used in the present study (Figure 4).

Figure 4: Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences showing the evolutionary relationship between *Aeromonas hydrophila* strain CS-Ah001 (PQ495909.1) and related *Aeromonas* species retrieved from the NCBI GenBank database.

Extract recovery

The extraction method produced a quantifiable quantity of crude compound from the dried botanical material. Following the complete evaporation of the solvent, the final weight of the crude extract was noted as 2g, signifying the concentrated bioactive portion of the herbal source. The extract appeared as a dark brown semi-solid residue, suggesting the presence of various phytochemicals. This crude extract was then employed for phytochemical screening and antibacterial testing.

Phytochemical analysis

The qualitative assessment of the herbal extracts revealed the presence of various bioactive compounds (Table 3). High concentrations of alkaloids, phenols, and tannins were identified, while terpenoids and proteins were observed in moderate levels (Figure 5). Flavonoids were detected in moderate amounts along with alkaloids, terpenoids, phenols, and tannins in the examined extract. These phytochemicals are well-recognized for their capabilities in fighting microbes, providing antioxidant benefits, and may contribute to biological activity relevant to aquaculture applications, which may improve the extract's effectiveness against harmful bacteria.

Table 3: Phytochemical analysis of Plant compound.

S. No	Test	Presence/Absence	Colour Indication
1	Alkaloid test	Presence (++)	Reddish brown
2	Terpenoids test	Presence (+)	Red brown
3	Flavonoids	Presence (+)	Dark pink
4	Protein	Absence (-)	No significant colour change
5	Phenol & Tannins	Presence (++)	Deep blue

Figure 5: Qualitative phytochemical analysis of *Terminalia chebula* extract

GC–MS Analysis

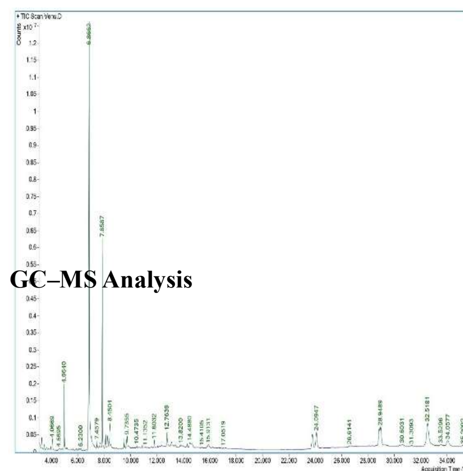


Figure 6: GC–MS chromatogram of *Terminalia chebula* extract showing the identified bioactive compounds.

Table 4: GC–MS evaluation displaying retention duration, peak size, and recognized compounds of *Terminalia chebula*.

Peak	Retention time (S)	Base Peak Area	Match Fac	Chemical Formula	Compound Name
1	3.258917	265043.6	83.97785	C5H6N2O2	2,4(1H,3H)-Pyrimidinedione, 5-methyl-
2	3.45615	109579	83.10775696	C6H6O3	2-Furancarboxylic acid, methyl ester
3	3.62155	33417.38	81.55963786	C6H8O4	2,3-Dimethylfumaric acid
4	3.77425	15736.8	77.51853242	C6H6O3	Levoglucosenone
5	3.946017	32919.27	69.87049264	C6H8O4	2-Acetyl-2-hydroxy-.gamma.-butyrolactone
6	4.0669	108055.8	92.66670346	C6H8O4	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
7	4.5695	99100.98	83.24294338	C6H6O2	(pyro)-catechol
8	4.811267	11554	64.64063141	C15H29ClO2	5-Chloropentanoic acid, 6-ethyl-3-octyl ester
9	4.96395	1170342	97.68544557	C6H6O3	5-Hydroxymethylfurfural
10	5.135733	21713.92	61.40797293	C11H22O3	Decanoic acid, 3-hydroxy-, methyl ester
11	5.212067	14332.49	69.52131013	C6H6OS	2-Mercaptophenol
12	5.511083	5030.054	57.58624135	C7H16N2S	Thiourea, N,N'-dipropyl-
13	5.638333	14292.36	62.78488669	C8H14O3	Methyl 6-oxoheptanoate
14	5.80375	3583.442	68.95794686	C11H22O	2-Undecanone
15	5.867367	32142.27	79.45012796	C6H6O3	Maltol
16	5.98825	2589.862	66.61188747	C7H10N2O4	Acetamide, N-(2-acetyl-3-oxo-4-isoxazolidinyl)-
17	6.115483	16932.61	70.16059617	C9H14O2Si	Resorcinol, TMS derivative
18	6.23	36456.85	85.28492953	C8H6O4	1,2-Benzenedicarboxylic acid
19	6.605367	2251.52	60.99501318	C12H24O2	Dodecanoic Acid
20	6.707167	2530.991	80.36836941	C10H12O2	3-Allyl-6-methoxyphenol
21	6.866217	15527347	98.00735509	C6H6O3	1,2,3-Benzenetriol
22	7.037983	21824.89	82.19742566	C15H30	1-Pentadecene
23	7.457883	76172.52	91.69372239	C9H8O2	2-Propenoic acid, 3-phenyl-
24	7.686917	79474.11	78.34801983	C9H13N3O5	Cytidine
25	7.8587	5514415	99.23170645	C8H8O3	Methylparaben
26	8.113183	390356.7	95.06448684	C7H6O3	Benzoic acid, 3-hydroxy-
27	8.253133	142954.8	79.24742606	C6H6O3	1,2,4-Benzenetriol
28	8.450367	879220.8	97.34285363	C7H6O3	Benzoic acid, 4-hydroxy-
29	8.590333	12402.84	73.57456515	C14H22O	2,4-Di-tert-butylphenol
30	8.806633	4471.329	58.41926337	C13H29BrOSi	10-Bromo-1-decanol, TMS
31	8.92115	8055.042	57.30655042	C19H32	1-Pentyloctylbenzene
32	9.25835	6578.584	63.11318491	C16H26	1-Ethyloctylbenzene
33	9.519183	39266.42	91.4823396	C18H34Cl2O2	Dichloroacetic acid, 4-hexadecyl ester
34	9.6019	7585.387	63.14200648	C24H50S2	Disulfide, di-tert-dodecyl
35	9.7355	155192.8	89.09440771	C7H12O6	1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid
36	9.881833	2810.503	56.01565227	C17H24O2	Panaxydol
37	10.19993	1786.838	71.00048748	C17H28	Benzene, (1-propyloctyl)-
38	10.31445	1120.57	58.25542245	C16H32	Cyclohexane, decyl-
39	10.4735	16525.52	79.46108215	C15H30O	8-Pentadecanone
40	10.59438	4069.257	64.25613869	C8H12O5	Shikimic acid, methyl ester
41	10.86158	43059.41	78.10157262	C17H28	Benzene, (1-methyldecyl)-
42	11.0779	1857.872	56.33515319	C16H24N2O3	N-(3-Nitrophenyl)decanamide
43	11.13515	5122.532	61.69865799	C18H30	Benzene, (1-pentylheptyl)-
44	11.33238	10987.42	63.36008426	C18H30	Benzene, (1-propylnonyl)-
45	11.59322	13123.95	59.86105103	C18H30	Benzene, (1-ethyldecyl)-
46	11.73955	2429.502	50.85304841	C13H20N8O3	1-(4-Amino-furazan-3-yl)-5-(4-methyl-piperazin-1-ylmethyl)-1H-[1,2,3]triazole-4-carboxylic acid ethyl ester
47	11.80317	29817.06	92.72364097	C18H36	1-Octadecene
48	11.87952	2721.445	51.31870329	C12H25Cl3	Silane, trichlorododecyl-
49	12.03857	22387.14	70.31523678	C18H30	Benzene, (1-methylundecyl)-
50	12.28033	8206.448	57.78852998	C19H32	1-Pentyloctylbenzene
51	12.36303	4828.145	56.52217722	C17H24O2	3-(5-Benzyloxy-3-methylpent-3-enyl)-2,2-dimethyloxirane
52	12.76385	360031.6	92.81836759	C8H8O5	Benzoic acid, 3,4,5-trihydroxy-, methyl ester
53	13.10103	67287.28	82.96114331	C23H48	Tricosane
54	13.2919	24035.46	64.08350108	C19H32	Benzene, (1-methyldodecyl)-
55	13.41913	784.2025	68.38669648	C17H34O2	Pentadecanoic acid, 14-methyl-, methyl ester
56	13.73723	8670.721	73.2447985	C18H28O3	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester
57	13.81995	6720.062	68.89938334	C19H38O4	Hexadecanoic acid, 2,3-dihydroxypropyl ester
58	13.98537	15435.02	60.14483786	C16H22O4	Dibutyl phthalate
59	14.28438	16691.81	83.1811026	C19H40O	n-Nonadecanol-1
60	14.48797	196927.4	85.62924959	C27H56	Heptacosane
61	15.3214	1473.837	51.74939446	C18H16O6	(4-(methoxycarbonyl)phenyl)methyl methyl terephthalate
62	15.41047	7291.541	60.86761546	C19H38O	10-Nonadecanone
63	15.77947	1354.151	75.3782114	C23H44O2	trans-9-Octadecenoic acid, pentyl ester
64	15.91307	83074.91	84.21285127	C25H52	Pentacosane
65	16.23753	3080.265	66.51703945	C18H34O2	cis-13-Octadecenoic acid
66	17.05188	1262.797	65.92855802	C22H46O	1-Docosanol
67	17.18548	1124.15	51.54691616	C17H36	Tetradecane, 2,6,10-trimethyl-
68	23.78293	489145.5	93.55949	93.55949297 C29H60	Nonacosane
69	24.09468	496765.6	92.49840396	C29H60	Nonacosane
70	26.61407	1237.823	53.46841601	C39H78O3	Stearic acid, 3-(octadecyloxy)propyl ester
71	28.84715	439374.4	89.45974047	C31H64	Hentriacontane
72	28.94893	158686.9	73.07652047	C34H70	Tetracosane, 11-decyl-
73	30.60307	11895.05	67.21253674	C29H48O	Stigmasterol
74	31.30927	6771.506	50.11318534	C20H34D2O3	12,12-Dideutero-14-oxo-nonadec-10-enoic acid methyl ester
75	32.51805	215149.4	94.59083314	C29H50O	(24R)-Stigmast-5-en-3.beta.-ol
76	33.52962	5943.038	56.12730942	C24H30O8	Gnaphalidin
77	34.05767	242185.8	74.91212731	C30H62	Tetracosane, 2,6,10,15,19,23-hexamethyl-
78	35.2092	10827.11	62.67740013	C29H50O	beta.-Sitosterol

The GC–MS analysis of the phytochemical extract identified a total of 78 compounds (Table 4), characterized by retention times between 3.25 to 35.20 minutes (Figure 6). The compounds classified predominantly included phenolics, benzoic acid derivatives, furans, fatty acids, esters, hydrocarbons, lactones, organosilicons, and phytosterols, reflecting the rich chemical diversity of the extract. Among the metabolites identified, 1,2,3-Benzenetriol (RT 6.86 min) was noted as the principal compound, exhibiting the highest peak area intensity, followed by 5-Hydroxymethylfurfural, various benzoic acid derivatives, Nonacosane, Hentriacontane, and Stigmasterol. These prominent compounds have been well-established for their antibacterial, antioxidant, anti-inflammatory, and immunomodulatory effects, suggesting the extract's potential therapeutic value.

The initial retention time peaks (RT 3.25–6.20 min) mainly included low-molecular-weight phenolic compounds and furan derivatives such as 2,4(1H,3H)-Pyrimidinedione, 2-Furancarboxylic acid methyl ester, Levoglucosenone, and 5-Hydroxymethylfurfural, all of which are known for their antioxidant and antimicrobial properties. The mid-range eluting compounds (RT 6.20–12.80 min) were largely made up of aromatic phenols, benzenetriols, and hydroxybenzoic acids, including 1,2,3-Benzenetriol, Benzoic acid (3- and 4-hydroxy), and Shikimic acid derivatives, which play a crucial role in bacterial killing by destabilizing membranes and denaturing proteins in harmful microbes. The late-eluting, more hydrophobic metabolites (RT 12.80–35.20 min) comprised long-chain alkanes, fatty acid esters, and phytosterols, such as Nonacosane, Hentriacontane, Stigmasterol, Stigmast-5-en-3β-ol, Pentacosane, and β-Sitosterol. These compounds have been reported to possess antimicrobial, antioxidant, and anti-inflammatory properties that may be beneficial in aquaculture-related applications, which are highly beneficial for managing diseases in aquaculture.

Functional Significance of the Bioactive Compounds

The high levels of phenolic compounds and benzoic acid derivatives in the extract suggest a robust antioxidant and antimicrobial capability, likely aiding in the suppression of *Aeromonas hydrophila*. Compounds like 1,2,3-Benzenetriol and hydroxy-benzoic acids are recognized for their ability to induce oxidative stress in bacterial cells, resulting in increased permeability and eventual cell lysis. Furthermore, furan derivatives such as 5-Hydroxymethylfurfural provide additional anti-inflammatory and antibacterial properties, which may help mitigate tissue damage associated with infections. The presence of phytosterols (Stigmasterol, Stigmast-5-en-3β-ol, β-Sitosterol) indicates further advantages in suggesting potential biological relevance in future aquaculture applications, contributing to enhanced resistance to diseases.

GC–MS Result Summary

Table 5: Major Phytocompounds Identified with Their Retention Times and Biological Activities

Major Compound	RT (min)	Biological Relevance
1,2,3-Benzenetriol	6.86	Strong antibacterial & antioxidant
5-Hydroxymethylfurfural	4.96	Antioxidant & anti-inflammatory
3- & 4-Hydroxy Benzoic Acids	8.11–8.45	Antibacterial & antifungal
Stigmasterol / Stigmast-5-en-3β-ol	30.60 – 32.51	Immunomodulatory & anti-inflammatory
Nonacosane / Hentriacontane	23.78 – 28.84	Antimicrobial & structural plant wax components

The major bioactive compounds identified through GC–MS profiling, along with their retention times and biological relevance, are summarized in (Table 5). The key elements found in the methanolic extract demonstrate a variety of chemically unique bioactive groups that are well-recognized for their biological importance in antimicrobial research. The abundance of phenolic compounds, including 1,2,3-benzenetriol and hydroxybenzoic acids, indicates significant redox activity and the potential to disrupt membranes, likely contributing to the noted inhibitory effect on *Aeromonas hydrophila*. It is well-established that phenolic compounds can induce oxidative stress, modify membrane permeability, and lead to the leakage of intracellular components in bacterial cells (*Cowan, 1999; Gyawali & Ibrahim, 2014; Zhou et al., 2019*). The identification of 5-hydroxymethylfurfural reinforces the existence of bioactive substances with antioxidant and antimicrobial characteristics that might disrupt microbial metabolic processes (*Islam et al., 2021*). Furthermore, the presence of phytosterols such as stigmasterol and stigmast-5-en-3β-ol implies potential immunomodulatory and anti-inflammatory effects which are particularly relevant in aquaculture disease management (*Wang et al., 2020; Reverter et al., 2014*). Background substances typically linked to laboratory contamination like phthalates and parabens were identified and excluded from biological analysis.

Conclusion of GC–MS Profiling

The GC–MS analysis confirms that the extract is abundant in pharmacologically active compounds, mainly phenolics and sterols, which together provide significant antioxidant and antimicrobial effects. The composition of these chemicals strongly indicates the potential use of

these phytocompound as a natural treatment for *Aeromonas hydrophila* infections in aquaculture.

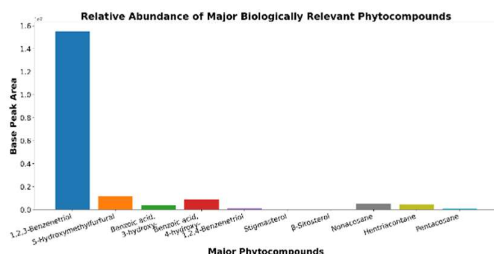


Figure 7: Relative abundance of major biologically relevant phytocompounds identified in *Terminalia chebula* extract through GC–MS analysis.

The bar chart (Figure 7) illustrates the relative abundance of major biologically relevant phytocompounds identified through GC–MS analysis of *Terminalia chebula* extract. The predominance of phenolic compounds, particularly 1,2,3-benzenetriol and hydroxybenzoic acid derivatives, suggests strong antioxidant and antibacterial potential. The presence of phytosterols such as stigmasterol and β-sitosterol further indicates possible anti-inflammatory and immunomodulatory properties relevant to aquaculture disease management.

Interpretation: The notably high amounts of **1,2,3-benzenetriol** and the existence of various hydroxy-benzoic derivatives and HMF suggest a strong phenolic nature and a probable antioxidant/antimicrobial role; the plentiful long-chain alkanes and sterols align with waxy and membrane-active constituents commonly found in plant extracts.

Antibacterial Screening

The methanolic fruit extract of *Terminalia chebula* exhibited notable antibacterial activity against *Aeromonas hydrophila*, as evidenced by clear zones of inhibition around the extract-impregnated discs. The antibacterial activity increased in a concentration-dependent manner, with inhibition zones ranging from 12 ± 0.5 mm at 25 mg/mL to 18 ± 0.7 mm at 100 mg/mL (Figure 8 & Table 6). Methanol-loaded control discs did not exhibit any inhibitory activity, confirming that the observed antibacterial effect was attributed solely to the phytocompounds present in the extract. The results indicate that the phenolic-rich extract possesses substantial antibacterial potential against the fish pathogen *Aeromonas hydrophila*.

Table 6: Antibacterial activity of *Terminalia chebula* extract against *Aeromonas hydrophila* at different concentrations

S. No	Tested Species Against	Extract Concentration	Zone of inhibition
1.	*Aeromonas hydrophila*	25 mg/mL	12 ± 0.5
		50 mg/mL	14 ± 0.6
		75 mg/mL	16 ± 0.4
		100 mg/mL	18 ± 0.7

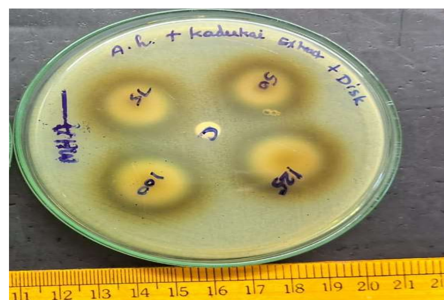


Figure 8: Concentration-dependent antibacterial activity of *Terminalia chebula* methanolic extract against *Aeromonas hydrophila*

Discussion

Aeromonas hydrophila is a significant opportunistic bacterial pathogen in freshwater aquaculture, responsible for motile Aeromonas septicemia (MAS) and resulting in considerable economic losses for cultured fish. The virulence of the isolate examined in this study was verified through biochemical characteristics, molecular identification, and the detection of pathogenic traits, highlighting its potential to induce systemic infections. Previous studies have reported similar results, linking virulence factors like hemolysins and aerolysins to tissue damage and increased mortality in affected fish (*Khajanchi et al., 2010; Tomás, 2012*).

The antibiogram results showed resistance to ampicillin, while the isolate remained susceptible to various broad-spectrum antibiotics like ciprofloxacin and gentamicin. Resistance to β-lactam antibiotics is well-documented in *Aeromonas* species and is primarily linked to the production of chromosomally encoded β-lactamases (*Esteve et al., 2015*). The rising prevalence of antimicrobial resistance in aquaculture settings has raised significant concerns about the efficacy and sustainability of antibiotic treatments (*Reverter et al., 2020*). These challenges highlight the critical need for alternative and environmentally friendly disease management methods, especially those based on plant-derived therapies.

Terminalia chebula is a renowned medicinal plant known for its extensive antimicrobial and antioxidant effects. In the current

study, qualitative phytochemical analysis indicated the presence of phenols, tannins, alkaloids, flavonoids, and terpenoids, all of which are well documented for their antimicrobial properties. Phenolic compounds and tannins exert their antibacterial effects by precipitating microbial proteins, disrupting cellular membranes, and hindering enzymatic functions (*Cowan, 1999; Borges et al., 2013*).

The GC–MS analysis revealed a wide variety of bioactive components, with phenolic compounds and benzoic acid derivatives being the most common chemical classifications. Among the compounds identified, 1,2,3-benzenetriol (pyrogallol), hydroxybenzoic acids, and 5-hydroxymethylfurfural are noted for their significant antibacterial and antioxidant properties. Phenolic compounds are recognized for their ability to induce oxidative stress in bacterial cells, resulting in membrane damage, leakage of intracytoplasmic materials, and ultimately cell death (*Gyawali & Ibrahim, 2014; Zhou et al., 2019*). Furthermore, the identification of phytosterols such as stigmasterol and β-sitosterol suggests potential anti-inflammatory and immunomodulatory properties that may be relevant for future aquaculture applications (*Wang et al., 2020*).

The antibacterial assay revealed a distinct dose-dependent inhibitory effect of *Terminalia chebula* extract on *Aeromonas hydrophila*, with larger inhibition zones occurring at higher extract concentrations. Previous studies have also reported a similar dose-dependent antibacterial effect of *Terminalia chebula* against fish pathogens (*Baskaran et al., 2016; Singh et al., 2017*). The antimicrobial activity observed is likely attributed to the combined effect of various phytochemicals rather than a single active ingredient.

The presence of phenolics, organic acids, and phytosterols together suggests that the extract possesses antibacterial properties and potential biological relevance in aquaculture-related applications. These versatile plant extracts are seen as promising substitutes for synthetic antibiotics because they break down naturally, are less likely to cause resistance, and are safe for the environment (*Reverter et al., 2014*). These phytochemicals may be explored further as natural alternatives for aquaculture disease management following detailed in vivo validation studies.

Despite the current research being confined to in vitro assessments, additional studies that include in vivo challenge tests, toxicity evaluations, and dosage optimization are needed to confirm the practical use of *Terminalia chebula* in aquaculture systems.

Conclusion

The current research showed that *Terminalia chebula* has notable antibacterial activity against the fish pathogen *Aeromonas hydrophila*. Phytochemical analysis confirmed the existence of bioactive substances such as phenols, tannins, and other secondary metabolites. Gas Chromatography-Mass Spectrometry (GC–MS) analysis further identified a variety of bioactive components, especially phenolic compounds and organic derivatives recognized for their antimicrobial effects. The plant extract demonstrated a distinct concentration-dependent inhibitory action against the pathogen, suggesting its potential as a natural antibacterial agent.

With growing concerns about antibiotic resistance in aquaculture, *Terminalia chebula* may serve as a potential environmentally friendly alternative for future aquaculture disease management applications. The identified phytocompounds may contribute to the observed antibacterial activity against *Aeromonas hydrophila*. Nonetheless, additional in vivo research, toxicity assessments, and dosage optimization are necessary to confirm its practical use in aquaculture settings. In summary, the results underscore the potential of *Terminalia chebula* as a sustainable plant-based treatment for addressing *Aeromonas hydrophila* infections and supporting future development of plant-based antibacterial agents for aquaculture applications.

Ethical Statement

No live animal experimentation or challenge studies were conducted during this investigation. The bacterial isolate of *Aeromonas hydrophila* used in the present study was obtained from laboratory stock cultures for microbiological and molecular analyses. Therefore, separate ethical approval was not required.

Authors Contribution

Snehan S. developed the study's concept and design, conducted the experiments, analysed the data, and wrote the initial draft of the manuscript. Venu S. oversaw the research, offered comprehensive guidance throughout the project, and thoroughly reviewed and revised the manuscript. Sathiyaraj G. and Mithunraj contributed to the research by offering technical support, scientific insights, and pertinent information to enhance the study and refine the manuscript. All authors evaluated, reviewed, and consented to the final version of the manuscript for publication.

Conflict of Interest

The authors assert that there are no conflicts of interest pertaining to the release of this document.

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Data Availability

The information backing the conclusions of this research can be obtained from the corresponding author upon a reasonable request.

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