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Immunomodulatory Effects of Trihedral Ethanol Extracts on Disease Resistance and Immune Function in *Labeo rohita* Against *Pseudomonas fluorescens*

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Abstract

The study investigates the effects of ethanol extract from *Azadirachta indica*, *Eichhornia crassipes* and *Avicennia marina* on the innate immune responses of *Labeo rohita*, focusing on parameters such as respiratory burst activity, myeloperoxidase activity, serum antiprotease levels, enzymatic activity, hematological parameters and disease resistance against *Pseudomonas fluorescens*. Fish were treated with varying doses of the triherbal extracts (100-1000 mg/kg body weight) for 60 days, followed by an intraperitoneal challenge with *P. fluorescens*. On day 70 post infection, significant improvements ($P < 0.05$) in immune parameters, including NBT levels, myeloperoxidase activity, serum lysozyme and serum antiprotease activity, were observed in the triherbal extract treated groups compared to the control group. Notably, fish treated with 100 and 200 mg/kg of the triherbal extracts demonstrated an 80% relative percentage survival against the infection. These findings highlight the beneficial effects of triherbal ethanol extracts in enhancing immune function and disease resistance in *L. rohita*. Additionally, molecular docking analysis identified Phytol, 17-Pentatriacontene and β-D-glucopyranosyl as potential inhibitors of the AprX protein, a critical virulence factor in *P. fluorescens*. These results suggest that these bioactive compounds may represent promising candidates for the development of antibacterial treatments for aquaculture.

Keywords*: Triherbal extract, Labeo rohita, Pseudomonas fluorescens, Innate immune responses.*

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Introduction

Aquaculture is widely recognized as the world fastest growing food production sector, with global fish production reaching approximately 80Mm tonnes annually (1). As the global demand for fish continues to rise, aquaculture has emerged as a critical industry in feeding the world's population (2). In India, fish culture is a deeply ingrained practice with a rich historical tradition. The country stands as the second largest fish producer globally, contributing significantly to both the domestic and international seafood markets (3). Despite this remarkable growth, the aquaculture sector faces increasing challenges, particularly with respect to the emergence of diseases that threaten both fish health and the sustainability of the industry (4). The rapid expansion of the aquaculture industry, particularly in recent years, has led to intensified farming practices that involve higher stocking densities and less optimal living conditions for the fish (5). These overcrowded and stress filled environments have serious implications for fish health. The stress from such conditions suppresses the immune systems of the fish, rendering them more vulnerable to various pathogens (6). In regions like Tamil Nadu, India, where intensive aquaculture is prevalent, fish infected with bacterial pathogens such as *Aeromonas hydrophila* and *Pseudomonas fluorescens* have experienced alarmingly high mortality rates, ranging from 60% to 80% (7). These bacterial infections are particularly severe, causing significant stress in the fish and leading to clinical manifestations such as exophthalmia, protrusion of the eyes, external ulcers and internal organ damage, including gallbladder rupture. Such infections not only impact fish health but also lead to substantial economic losses for fish farmers (8).

To mitigate the challenges posed by emerging diseases in aquaculture, the use of immunomodulators or immunostimulants has emerged as a promising strategy (9). These substances can enhance the immune responses of fish, improving their ability to resist infections and promoting overall health (10). Immunomodulators are especially valuable in aquaculture because they offer a natural and sustainable approach to disease prevention. Plant derived extracts, in particular, have garnered significant attention as eco-friendly solutions for controlling microbial contamination in aquaculture systems (11). These extracts are not only safe for the environment but also water degradable, cost effective and devoid of resistance issues commonly associated with synthetic antibiotics (12). Furthermore, plant based immunostimulants can be integrated into fish feed, making them an ideal tool for promoting long term fish health and sustainability in aquaculture practices. India rich biodiversity offers a wealth of medicinal plants that hold enormous potential in preventing and treating bacterial infections in aquaculture species (13). The present study focuses on exploring the effects of herbal drug containing feed on the immunity of the freshwater fish *Labeo rohita*, a species of significant economic importance in Indian aquaculture. The study aims to investigate how incorporating triherbal leaf extracts into fish feed can enhance the fish's immune responses, improve disease resistance and fulfil their dietary requirements for optimal growth. The selected plants for this study *Azadirachta indica*, *Eichhornia crassipes* and *Avicennia marina* have long been recognized for their medicinal properties and their bioactive compounds may play a crucial role in enhancing the immunocompetence of fish.

The study also aims to delve deeper into the molecular mechanisms underlying the potential benefits of these herbal extracts. One of the key focuses is the AprX protein in *Pseudomonas species*, which is a critical virulence factor contributing to bacterial pathogenicity. The study will employ *in silico* molecular docking analysis to explore the interactions between bioactive compounds from *Avicennia marina*, *Azadirachta indica* and *Eichhornia crassipes*

and the AprX protein. By identifying compounds that bind effectively to the AprX protein, the research seeks to pinpoint potential inhibitors that could serve as the foundation for developing treatments to combat bacterial pathogens in aquaculture. In summary, this study aims to provide valuable insights into the role of plant derived immunostimulants in enhancing fish health and disease resistance in aquaculture. By incorporating herbal extracts into fish feed, the research strives to promote more sustainable and effective practices in fish farming, with the potential to reduce the reliance on antibiotics and minimize the risks of antibiotic resistance. Moreover, through molecular docking studies, the research opens up new avenues for the development of targeted therapies against bacterial pathogens, contributing to the long term sustainability and health of aquaculture systems (14).

Materials and Methods

Experimental animal and their maintenance

Healthy *Labeo rohita* (Rohu) fish, weighing 45.15 ± 1.85 g, were obtained from a local farm in Saliyamangalam, Tamil Nadu, India. The fish showed no signs of disease, as confirmed by gross and microscopic examinations of their skin, gills, intestines and kidney tissues. Additionally, they had no previous history of parasitic infections. The fish were acclimatized for 15 days in 300 L plastic tanks containing dechlorinated tap water before the experiment. During this period, 10% of the water was replaced daily, along with the removal of any leftover feed and fecal matter. Water quality parameters were monitored every 5 days to ensure optimal conditions throughout the experiment. The temperature ranged from 27 to 29°C, dissolved oxygen was maintained at 5.04 ± 0.36 mg/L, pH was 7.25 ± 0.8, nitrite levels were 0.011 ± 0.008 mg/L and ammonia levels were 0.114 ± 0.016 mg/L. The fish were fed a basal diet at 4% of their body weight (15).

Sample collection and clinical examination

Fish infected with *Pseudomonas fluorescens* were collected and their skin was sampled using sterile containers. The tissue was ground with a mortar and pestle and then centrifuged at 2,000 ×g for 10 min. The supernatant was discarded and the deposit was suspended in 1 mL of phosphate buffered saline (PBS). A 50 µL portion of the concentrate was inoculated onto nutrient agar plates and incubated at 37°C for 24 h. The resulting bacterial colony was identified as *P. fluorescens* (JQ247720).

Triherbal extract preparation

Leaves of *Azadirachta indica* (A. Juss.), *Eichhornia crassipes* (Mart.) Solms and *Avicennia marina* (Forssk.) Vierh were collected in June 2016 from Saliyamangalam (Thanjavur district) and Muthupet Mangrove (Thiruvarur district), Tamil Nadu, India. The plants were botanically identified and the leaves were washed with sterile distilled water, shade dried, powdered and stored at -20°C until further use. The extraction was performed using the method outlined by (16). Equal amounts of each leaf powder (10 g) were mixed in a 1:1:1 ratio (w/w) and placed in sterilized 100 ml conical flasks. Each flask was filled with 100 ml of ethanol and agitated daily for 7 days at room temperature. After extraction, the mixture was filtered through sterile muslin cloth. The solvent was evaporated using a rotary vacuum evaporator (Buchi SMP) and the residue was dissolved in sterile ethanol at a concentration of 0.25% (w/v) in a sterilized screw cap glass container for further use (17).

Fourier transform infrared spectroscopy (FTIR) analysis

The chemical bonds present in the substance were identified using Fourier Transform Infrared Spectroscopy (FTIR). Prior to analysis, the sample was dried, thoroughly mixed, and combined with potassium bromide (KBr), then weighed. The measurements were conducted

using a Nicolet iS50 FTIR spectrometer (Thermo Scientific, USA). The infrared absorption spectra were recorded over the range of 400 to 4000 cm^{-1}, enabling the identification of functional groups within the substance.

Gas Chromatography–Mass Spectrometry (GC-MS) analysis

GC-MS analysis was performed using a Shimadzu GCMS-QP2020 system with an SH-Rxi-5% Sil MS silica column (30 m × 0.25 mm × 0.25 µm). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The injector was maintained at 280°C, and 1 µL of the sample was injected. The oven temperature started at 40°C and was increased to 280°C at a rate of 10°C/min, with a 3-minute hold time. The ion source temperature was set to 230°C, using electron impact ionization at 70 eV. Spectra were recorded over a mass range of 40–550 Da with a scan time of 0.2 s and interval of 0.1 s. Compound identification was achieved by comparing mass spectra with entries in the NIST-2017 library.

Experimental design

A total of 216 *L. rohita* fish were selected for the study and distributed into 18 tanks, with each tank containing 12 fish. Three replicate tanks were used per treatment. The control group was fed a basal diet, while the other groups received triherbal extract incorporated into the feed at concentrations of 100 mg/kg (T1), 200 mg/kg (T2), 400 mg/kg (T3), 800 mg/kg (T4) and 1000 mg/kg (T5) for 60 days. On day 60, the fish were infected with *P. fluorescens* via intraperitoneal injection at a dose of 1.5×10^4 cells/ml. After 10 days of infection, hematological, immunological and biochemical parameters were measured on day 70 to assess the relative percentage survival rate and disease resistance against *P. fluorescens*.

Composition of the mixture to supply for 1kg dry weight

The mixture, sourced from Virbac Animal Health, India, contains the following composition such as Vitamin A (700,000 IU), Vitamin D3 (70,000 IU), Vitamin E (250 mg), Nicotinamide (1,000 mg), Cobalt (150 mg), Copper (1,200 mg), Iodine (325 mg), Iron (1,500 mg), Manganese (1,500 mg), Potassium (100 mg), Selenium (10 mg), Sodium (5.9 mg), Sulfur (0.72 mg), Zinc (9,600 mg), Calcium (25.5 mg) and Phosphorus (12.75 mg) (18).

Proximate analysis of supplementary fish feed

The proximate analysis of the fish feed was conducted using established standard methods at the Fish Nutrition Laboratory, CIFE (Central Institute of Fisheries Education) (19). This analysis aimed to determine the key nutritional components of the feed, providing essential information on the dietary profile for fish.

Immunological Parameters

Neutrophil Activity (NBT)

The neutrophil activity of the phagocytes was assessed using the nitroblue tetrazolium (NBT) assay, following the method, which was later modified by Stasiack and Bauman (20). This assay measures the respiratory burst of neutrophils, a critical immune response that reflects the fish's ability to fight bacterial infections.

Serum lysozyme activity

Lysozyme activity, a crucial enzyme for bacterial defense was evaluated through a turbidimetric assay with a microplate adaptation developed. The lysozyme enzyme is known to break down bacterial cell walls, providing a measure of the fish's innate immune defense.

Myeloperoxidase activity

Total myeloperoxidase activity in the serum was determined following the method with subsequent modifications. Myeloperoxidase is a key enzyme in neutrophil antimicrobial activity

and its levels are an important indicator of the fish immune response to infection.

Serum antiprotease activity

Serum antiprotease activity was measured based on the procedure described by (21). Antiproteases play a critical role in modulating immune responses by inhibiting proteolytic enzymes that could potentially damage the fish tissues during inflammation.

Enzyme assay
Acid phosphatase and alkaline phosphatase

The levels of acid phosphatase and alkaline phosphatase were estimated using the King method (22). These enzymes are involved in various physiological processes, including the breakdown of phosphates and their activity levels can serve as biomarkers for immune and metabolic functions in fish.

Hematological analyses
Red Blood Cell (RBC) and White Blood Cell (WBC) counts

The RBC and WBC counts were determined using an improved Neubauer hemocytometer, following standard protocols (23). These counts are essential for assessing the overall health and immune status of the fish, as changes in blood cell numbers often indicate underlying infections or stress.

Hemoglobin percentage (HB %)

The hemoglobin percentage was estimated using a hemoglobinometer, providing insight into the oxygen carrying capacity of the fish's blood, which is critical for overall metabolic health (24).

Challenge test

At the conclusion of the experiment, 10 fish from each treatment group were selected and intraperitoneally (i.p.) challenged with 0.2 ml of PBS containing 1.5 × 10^4 cells/ml of virulent *Pseudomonas fluorescens*. The fish were closely monitored for any signs of disease, including behavioral abnormalities such as reduced swimming or erratic movement. Mortality rates were recorded over a 96 h period following infection. To confirm the infection, *P. fluorescens* was reisolated from the deceased fish (25). This challenge test aimed to evaluate the efficacy of the treatments in enhancing disease resistance and the overall health of the fish.

Relative percentage survival (RPS)

The survival rate at the end of 10 days post infection was calculated using the following formula such as RPS = ((Number of surviving fish after challenge) / (Number of fish that died due to bacterial infection)) × 100

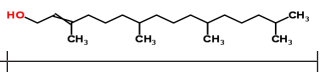
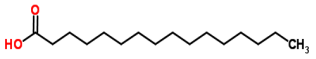
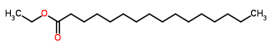
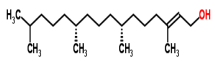
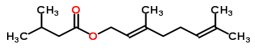
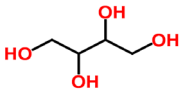
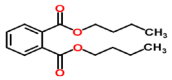
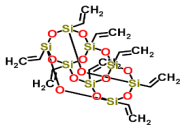
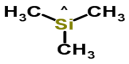
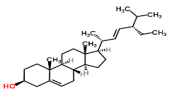
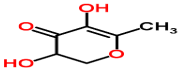
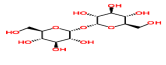
Preparation of protein, ligand and molecular docking analysis

The crystal structure of the AprX protein used in this study was obtained from the RCSB Protein Data Bank. Ligands were sourced from the ChemSpider database (http://www.chemspider.com/) and were derived from bioactive compounds found in *Avicennia marina*, *Eichhornia crassipes* and *Azadirachta indica* (Table 1). Docking studies were conducted based on the binding mode of these bioactive compounds using Hex software (http://www.hex.loria.fr/dist50/). The docking results for all reference inhibitory compounds and the natural compounds under study are summarized in Table 2. From the selected medicinal plants, 13 compounds were chosen for docking calculations. Among these, three compounds exhibited the highest molecular binding energies: Phytol from *A. marina* (-231.39 kcal/mol), 17-Pentatriacontene from *E. crassipes* (-267.35 kcal/mol) and β-D-glucopyranosyl from *A. indica* (-223.37 kcal/mol) (Figure 1). These compounds were identified as potential lead molecules for the development of antibacterial drugs targeting fish pathogens, particularly *Pseudomonas sps.* and further exploration of their mechanism of action in *in vivo* and *in vitro* models is planned for future research (26).

Table 1. Molecular binding energy of docked bioactive compounds

S. No	Name of the plant derivatives	Name of the Plants	Binding energy (kcal/mol)
1.	Tetramethyl-2-hexadecen-1-ol	A.marina	-218.83
2.	n-Hexadecanoic acid	A.marina	-213.85
3.	Ethyl ester	A.marina	-221.27
4.	Phytol	A.marina	-231.39
5.	Butanoic acid, 3-methyl-, 3,7-dimethyl-2,6-octadienyl ester	A.marina	-177.92
6.	2(R),3(S)-1,2,3,4-Butanetetrol	A.marina	-120.21
7.	Dibutyl phthalate	E. crassipes	-188.27
8.	Octasiloxane	E. crassipes	-195.37
9.	1-Monolinoleoylglycerol trimethylsilylethel	E. crassipes	-77.41
10.	17-Pentatriacontene	E. crassipes	-267.35
11.	Stigmasterol	E. crassipes	-252.65
12.	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	A.indica	-107.19
13.	à-D-Glucopyranoside,à-D-glucopyranosyl	A.indica	-223.37

Table 2. List of Ligands from plant derivative

S. No	Name of the plant derivatives	Name of the Plants	Molecular Structure
	Tetramethyl-2-hexadecen-1-ol	*A. marina*	
	n-Hexadecanoic acid	*A. marina*	
	Ethyl ester	*A. marina*	
	Phytol	*A. marina*	
	Butanoic acid, 3-methyl-, 3,7-dimethyl-2,6-octadienyl ester	*A. marina*	
	2(R),3(S)-1,2,3,4-Butanetetrol	*A. marina*	
	Dibutyl phthalate	*E. crassipes*	
	Octasiloxane	*E. crassipes*	
	1-Monolinoleoylglycerol trimethylsilylethel	*E. crassipes*	
	17-Pentatriacontene	*E. crassipes*	
	Stigmasterol	*E. crassipes*	
	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	*A.indica*	
	à-D-Glucopyranoside,à-D-glucopyranosyl	*A.indica*	

Statistical analysisExperimental data are presented as mean ± SD and analyzed using one-way ANOVA followed by Tukey's test to compare means between individual treatments. Statistical analysis was performed using SPSS with a significance level of $p < 0.05$.

Results and Discussion

The results of this study demonstrated significant ($p < 0.05$) differences in the non-specific immune responses of *L. rohita* supplemented with dietary triherbal extract at various concentrations on the 20th, 40th and 60th days of sampling showed in Table 3. A marked improvement in neutrophil burst activity, assessed using the nitroblue tetrazolium (NBT) assay and was observed across the experimental groups compared to the control group. The highest NBT activity was recorded in the T5 group in 1 g of triherbal extract on all three sampling days at 20th, 40th and 60th. Additionally, a noticeable increase in NBT activity was seen between day 20 and 40, indicating an enhanced immune response as the concentration of the triherbal extract increased. The number of NBT positive cells, which are indicative of an active immune response also increased with higher doses of the extract. Post pathogen infection, a peak in NBT positive cells was observed in both control and experimental groups, lasting for about 10 days in Table 4. Fish fed with 0.8 g and 1 g leaf extract concentrations exhibited a significant enhancement in the number of NBT positive cells showed in Figure 1. On day 70 post infection, the lowest NBT activity was recorded in the T1-T3 groups, while the T4 and T5 groups exhibited the highest NBT values, suggesting a sustained immune response in these groups (27).

Table 3. Ingredients of supplementary feed Used for Experimental Fish *L. rohita*

Ingredients (g kg^{-1})	**(Control)**	**T (1)**	**T (2)**	**T (3)**	**T (4)**	**T (5)**
Rice bran	200	200	200	200	200	200
Wheat flour	200	200	200	200	200	200
Corn flour	200	200	200	200	200	200
Ground nut oil cake	90	89	88	86	82	80
Soybean meal	200	200	200	200	200	200
Fish meal	100	100	100	100	100	100
Vitamin and mineral mix[2]	10	10	10	10	10	10
Tri herbal extract (g kg^{-1})	0	1	2	4	8	10

Table 4. Proximate analysis of supplementary feed used for Experimental Fish *L. rohita*

Ingredients (g kg^{-1})	**(Control)**	**T (1)**	**T (2)**	**T (3)**	**T (4)**	**T (5)**
CHO	57.0	57.10	57.32	57.38	54.0	54.12
Crude protein	29.8	29.8	29.9	29.9	30.0	30.0
Crude lipid	7.6	76	7.8	7.8	7.8	7.9
Ash	6.5	6.3	6.5	6.4	6.5	6.5
Crude fiber	5.6	5.8	5.9	5.6	5.7	5.9
Moisture	6.90	7.0	7.03	7.05	7.06	7.07

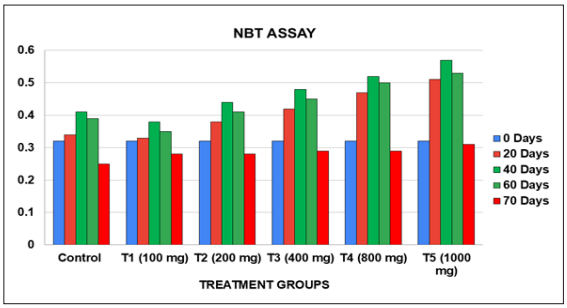


Figure 1. NBT assay of ethanolic leaf extract

Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR spectroscopy results (Figure 2; Table 5) revealed the presence of seven distinct functional groups within the sample. Notable among these were the O–H stretching vibration typical of alcohols and phenols, observed at 3416.48 cm^{-1}, and the C–H stretching in alkenes detected at 2108.06 cm^{-1}. Additionally, the C=O stretching vibration associated with aliphatic compounds appeared at 1626.27 cm^{-1}, while an absorption band at 1399.37 cm^{-1} indicated the presence of an α,β-unsaturated ketone. These findings are consistent with characteristic infrared absorption frequencies reported in recent literature (28).

Table 5. FTIR peak values and functional groups

S. No	Wave number (Cm^{-1})	Intensity	Bond responsible	Functional Groups
1	617.72	Medium	C–H	Alkenes
2	862.67	Medium	C–C	Aliphatic primary amine
3	1117.50	Strong	C–H	Amines
4	1399.37	Medium	C–H	Vinyl ether
5	1626.27	Strong	C=O	α,β-unsaturated ketone
6	2108.06	Medium	C–H	Alkenes
7	3416.48	Strong	C–H	Alkenes

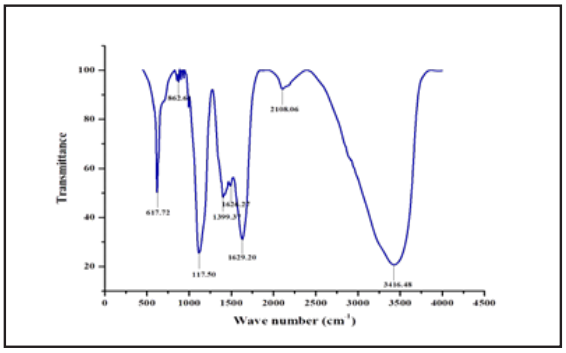
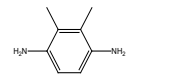
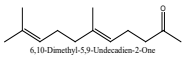
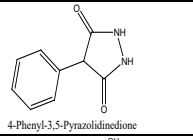
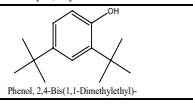
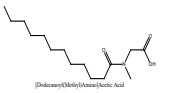
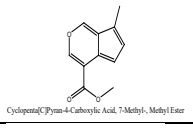
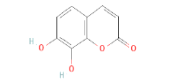
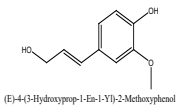
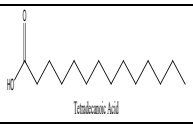


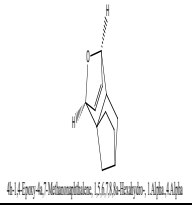
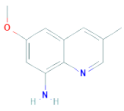
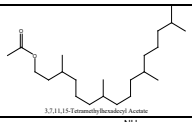
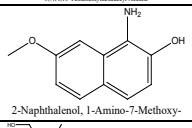
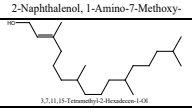
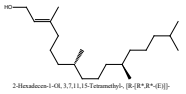
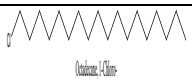
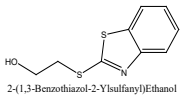
Figure 2: FTIR peak values and functional groups of triherbal extract.

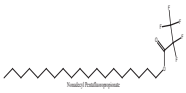
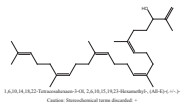
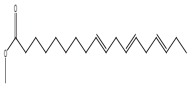
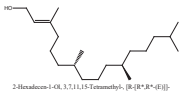
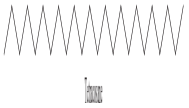
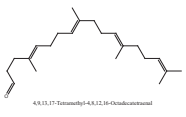
Gas Chromatography–Mass Spectrometry (GC-MS) analysis of the ethanolic extract

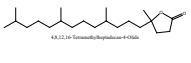
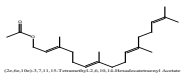
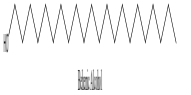
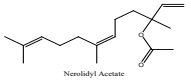
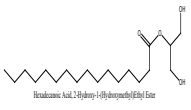
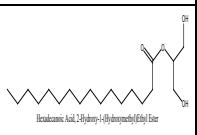
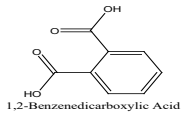
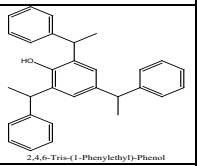
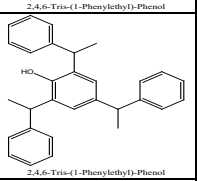
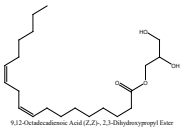
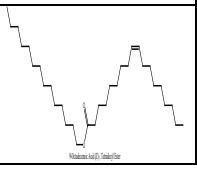
Gas Chromatography–Mass Spectrometry (GC-MS) is a powerful analytical technique widely utilized in phytochemical studies to identify and quantify bioactive constituents in plant extracts. In the present study, GC-MS analysis of the ethanolic leaf extract revealed the presence of 48 bioactive compounds. These compounds were identified based on their retention times and mass spectral data, matched against standard libraries. Among the major constituents detected were N-Hexadecanoic Acid (palmitic acid), Hexadecanoic Acid, Ethyl Ester, and 9,12-Octadecadienoic Acid (Z, Z)-, 2,3-Dihydroxypropyl Ester, all of which are known for their pharmacological and antioxidant properties (Table 6). The presence of these compounds suggests potential therapeutic applications of the extract, including anti-inflammatory, antimicrobial, and lipid-lowering effects, consistent with findings from recent phytochemical studies (29).

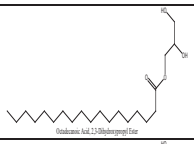
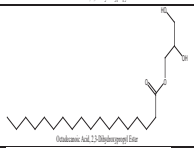
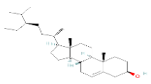
Table 6. GCMS analysis of various bioactive compounds present

S. no	Retention time	Retention End time	Name of the compound	Molecular formula	Compound structure	Biological activity
1	11.055	11.065	1-Pentadecene	$C_{15}H_{30}$		Antibacterial activity
2	11.74	11.75	1,4-Benzenediamine, 2,3-Dimethyl-	$C_{18}H_{24}N_2$		Antifungal, Antibacterial activity
3	11.79	11.8	6,10-Dimethyl-5,9-Undecadien-2-One	$C_{13}H_{22}O$		Antibacterial, Antioxidant activity
4	12.33	12.34	4-Phenyl-3,5-Pyrazolidinedione	$C_9H_8N_2O_2$		Antiarrhythmic, Antibacterial, Anticancer activity
5	12.56	12.57	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	$C_{17}H_{30}OSi$		Antibacterial, Antioxidant
6	13.22	13.23	(Dodecanoyl(Methyl)Amino)Acetic Acid	$C_{15}H_{29}NO_3$		Antiviral, Anticancer activity
7	13.665	13.675	Cyclopenta(C)Pyran-4-Carboxylic Acid, 7-Methyl-, Methyl Ester	$C_{11}H_{10}O_3$		Ant seizure, Anti-inflammatory, Antidiuretic activity
8	14.435	14.445	4-Methyldaphnetin	$C_{10}H_8O_4$		Anti-diabetic and ameliorative activity
9	14.815	14.825	Eicosane	$C_{20}H_{42}$		Anti-inflammatory
10	15.295	15.305	(E)-4-(3-Hydroxyprop-1-En-1-Yl)-2-Methoxyphenol	$C_{10}H_{12}O$		Anti-diabetic, Antioxidant activity
11	15.53	15.54	Tetradecanoic Acid	$C_{14}H_{28}O_2$		Antibacterial, catalytic

12	15.965	15.975	4h-1,4-Epoxy-4a,7-Methanonaphthalene, 1,5,6,7,8,8a-Hexahydro-, 1.Alpha.,4.Alpha	$C_{11}H_{14}O$		Antibacterial, Antioxidant activity
13	16.09	16.1	8-Amino-4-(3-Hexoxy)-6-Methoxy-3-Methylquinoline	$C_{11}H_{12}N_2O$		Antibacterial, Anticancer activity
14	16.3	16.31	Neophytadiene	$C_{20}H_{38}$		Antiarrhythmic, Antibacterial, Antimalarial activity
15	16.36	16.37	3,7,11,15-Tetramethylhexadecyl Acetate	$C_{22}H_{44}O_2$		Antibacterial, Antioxidant
16	16.475	16.485	2-Naphthalenol, 1-Amino-7-Methoxy-	$C_{11}H_{10}O_2$		Antiviral, Anticancer
17	16.55	16.56	3,7,11,15-Tetramethyl-2-Hexadecen-1-Ol	$C_{20}H_{40}O$		Antiseizure, Anti-inflammatory
18	16.745	16.755	2-Hexadecen-1-Ol, 3,7,11,15-Tetramethyl-, (R-(R*,R*-(E)))-	$C_{20}H_{40}O$		Anti-diabetic, ameliorative, Antimalarial activity
19	17.07	17.08	Octadecane, 1-Chloro-	$C_{18}H_{37}Cl$		Anti-diabetic, Anti-inflammatory
20	17.19	17.2	Hexadecanoic Acid, Methyl Ester	$C_{18}H_{37}Cl$		Antibacterial, Antioxidant
21	17.485	17.495	2-(1,3-Benzothiazol-2-Ylsulfanyl)Ethanol	$C_9H_9NO_2S_2$		Antiviral, Anticancer
22	17.635	17.645	N-Hexadecanoic Acid	$C_{16}H_{32}O_2$		Anti-inflammatory, Antiviral activity
23	17.74	17.75	N-Hexadecanoic Acid	$C_{16}H_{32}O_2$		Anticancer activity

24	17.865	17.875	Hexadecanoic Acid, Ethyl Ester	$C_{18}H_{36}O_2$		Anticancer, Antidiuretic activity
25	18.53	18.54	Heptadecanoic Acid	$C_{17}H_{34}O_2$		Antioxidant, Anticancer activity
26	18.76	18.77	Nonadecyl Pentafluoropropionate	$C_{22}H_{39}F_5O2$		Antibacterial, Antioxidant
27	18.81	18.82	1,6,10,14,18,22-Tetracosahexaen-3-Ol, 2,6,10,15,19,23-Hexamethyl	$C_{30}H_{50}O$		Antiviral, Anticancer activity
28	18.89	18.895	9,12,15-Octadecatrienoic Acid, Methyl Ester	$C_{19}H_{32}O_2$		Ant seizure, Anti-inflammatory, Antidiuretic activity
29	19.005	19.005	2-Hexadecen-1-Ol, 3,7,11,15-Tetramethyl-, (R-(R*,R*-(E)))-	$C_{20}H_{40}O$		Anti-diabetic, Antioxidant activity
30	19.105	19.115	Tetracosane	$C_{24}H_{50}$		Antibacterial, catalytic
31	19.325	19.335	Dichloroacetic Acid, Tridec-2-Ynyl Ester	$C_{15}H_{24}Cl_2O_2$		Antibacterial, Antioxidant activity
32	19.505	19.515	2-Aminoethanethiol Hydrogen Sulfate (Ester)	$C_{13}H_{29}NO_3S_2$		Antibacterial, Antioxidant activity
33	19.735	19.745	Octadecanoic Acid, Ethyl Ester	$C_{20}H_{40}O_2$		Antibacterial activity
34	20.695	20.705	4,9,13,17-Tetramethyl-4,8,12,16-Octadecatetraenal	$C_{22}H_{36}O$		Antiviral, Anticancer

35	20.695	21.135	4,8,12,16-Tetramethylheptadecan-4-Olide	$C_{21}H_{40}O_2$		Anti-inflammatory, Antiviral activity
36	21.125	21.325	(2e,6e,10e)-3,7,11,15-Tetramethyl-2,6,10,14-Hexadecatetraenyl Acetate	$C_{22}H_{36}O$		Anticancer, Antibacterial activity
37	22.27	22.28	Behenic Alcohol	$C_{22}H_{46}O$		Antiviral activity
38	22.315	22.325	Nerolidyl Acetate	$C_{17}H_{28}O_2$		Antioxidant, Anticancer
38	22.44	22.45	Hexadecanoic Acid, 2-Hydroxy-1-(Hydroxymethyl)Ethyl Ester	$C_{19}H_{38}O$		Antibacterial, Atidiabetic
40	22.49	22.5	Hexadecanoic Acid, 2-Hydroxy-1-(Hydroxymethyl)Ethyl Ester	$C_{19}H_{38}O$		Antiarrhythmic, Antibacterial, Anticancer activity
41	22.585	22.595	1,2-Benzenedicarboxylic Acid	$C_6H_4(COOH)_2$		Antibacterial, Antioxidant
42	22.7	22.71	2,4,6-Tris-(1-Phenylethyl)-Phenol	$C_{30}H_{30}O$		Antiviral, Anticancer activity
43	23.005	23.015	2,4,6-Tris-(1-Phenylethyl)-Phenol	$C_{30}H_{30}O$		Ant seizure, Anti-inflammatory, Antidiuretic activity
44	23.86	23.87	9,12-Octadecadienoic Acid (Z,Z)-, 2,3-Dihydroxypropyl Ester	$C_{21}H_{40}O_5$		Antiarrhythmic, Antibacterial, Anticancer activity
45	23.905	23.915	9-Octadecenoic Acid (Z)-, Tetradecyl Ester	$C_{32}H_{62}O_2$		Anti-inflammatory, Antidiuretic activity

46	24.12	24.13	Octadecanoic Acid, 2,3-Dihydroxypropyl Ester	$C_{21}H_{42}O$		Antioxidant activity
47	24.19	24.2	Octadecanoic Acid, 2,3-Dihydroxypropyl Ester	$C_{21}H_{42}O$		Antibacterial, catalytic
48	24.335	24.345	.Gamma.-Sitosterol	$C_{29}H_{50}O$		Antioxidant activity

Lysozyme activity

Lysozyme activity, a crucial immune enzyme, was significantly ($p < 0.05$) higher in the serum of fish in the triherbal extract groups at all time points, including post challenge, compared to the control group showed in Figure 3. The highest lysozyme activity was observed in the T5 group (1 g) on day 40, with a value of 11.66 ± 0.57 µg/ml. This indicates that the triherbal extract effectively enhanced the bactericidal properties of fish, contributing to a robust immune defence (30).

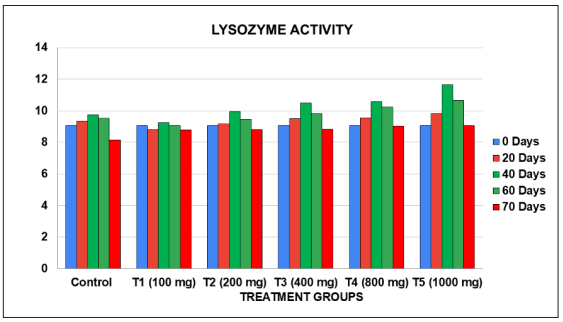


Figure 3. Lysozyme assay of ethanolic leaf extract

Myeloperoxidase activity

Myeloperoxidase activity an indicator of neutrophil activation increased significantly ($p < 0.05$) in the experimental groups following 20 days of supplementation, while no significant change was observed in the control group showed in Figure 4. The highest myeloperoxidase activity was recorded in the T5 group (49.33 ± 0.57) after 60 days of feeding. After pathogen challenge, the highest myeloperoxidase activity was again observed in the T5 group (31.66±0.57), suggesting a stronger immune response in fish fed higher concentrations of the triherbal extract (31).

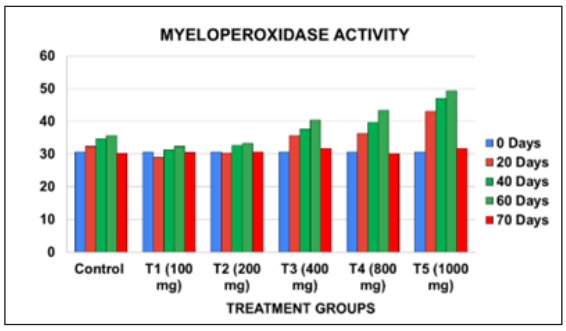


Figure 4. Myeloperoxidase assay of ethanolic leaf extract

Serum antiprotease activity

Serum antiprotease activity, which plays a crucial role in modulating immune responses, was significantly higher ($p < 0.05$) in the T3 (70.0 ± 1.73) and T4 (74.33 ± 1.15) groups compared to T1 (60.33 ± 1.52), which was close to the control group (62.66 ± 0.57) after 20 days of supplementation showed in Figure 5. Over time, all experimental groups showed a gradual increase in antiprotease activity, with T5 exhibit-

ing the highest activity (59.66 ± 0.57) post challenge. This increase was influenced not only by the concentration of the herbal extract but also by the duration of supplementation (32).

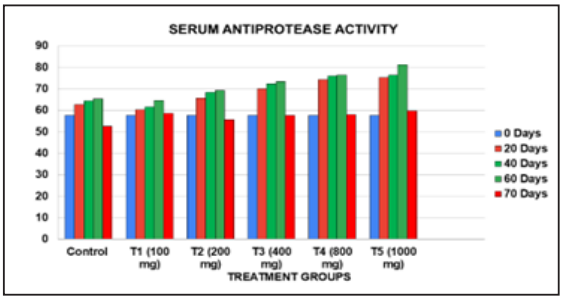


Figure 5. Serum Antiprotease assay of ethanolic leaf extract

Acid and alkaline phosphatase activity

Acid phosphatase activity in the experimental groups was significantly ($p < 0.05$) higher on days 20, 40 and 60 compared to the control. The most notable increase occurred on day 20 with the T4 and T5 groups showing the highest acid phosphatase activity showed in showed in Figure 6. Post challenge, the lowest acid phosphatase activity was recorded in the control group and T1-T3, while the highest was observed in T5 (0.46 ± 0.02 IU/l). Similarly, alkaline phosphatase activity gradually increased as the concentration of the triherbal extract increased. The highest activity was recorded in the T5 group (0.266 ± 0.003 IU/l) by day 60.

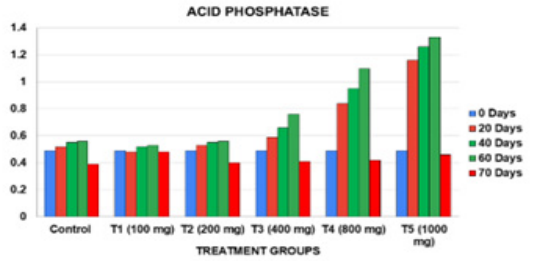
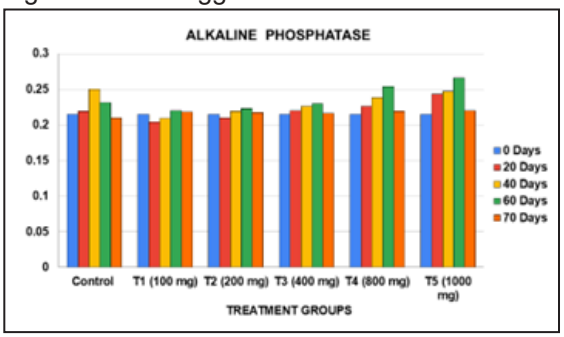


Figure 6. Acid Phosphatase assay of ethanolic leaf extract

Post challenge, the lowest levels of alkaline phosphatase activity were observed in the control group and T1-T2, while the highest levels were found in T5 (0.220 ± 0.004 IU/l) showed in Figure 7. This suggests that the triherbal extract



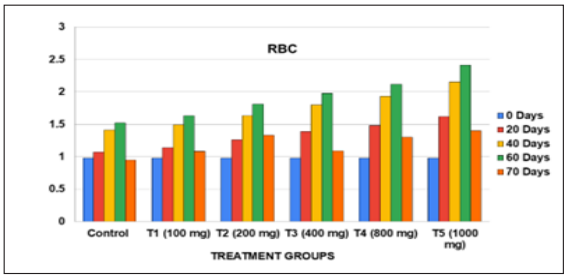
supports enzymatic activities that are vital for immune responses and metabolic health (33).

Figure 7. Alkaline Phosphatase assay of ethanolic leaf extract

Hematological parameters

A significant ($p < 0.05$) increase in red blood cell (RBC) counts was observed in all treatment groups on days 40 and 60, with the T5 group showing the highest increase on day 60 and 10 days post challenge showed in Figure 8. Similarly, white blood cell (WBC) counts were significantly higher in T4 and T5 groups compared to the control group throughout the feeding period with T5 showing the greatest increase at day 60. After challenge with *P. fluorescens*, significant ($p < 0.05$) differences in WBC counts were observed across all treatment groups compared to the control. Hemoglobin content was significantly higher ($p < 0.05$) in T3, T4 and T5 groups compared to the control, indicating a better oxygen carrying capacity in these fish, which is crucial for recovery after infection (34). Serum protein content was significantly different between treatment groups and the control showed in Figure 9. At day 20, fish in T2 and T4 showed significantly higher serum protein levels compared to T1. By the end of the 60 days feeding period fish in T2 and T3 groups had significantly higher serum protein levels compared to the T1 group. Post challenge,

serum protein content was significantly higher in the T3, T4 and T5 groups compared to the control, highlighting the nutritional and immune



enhancing benefits of the triherbal supplementation (35).\

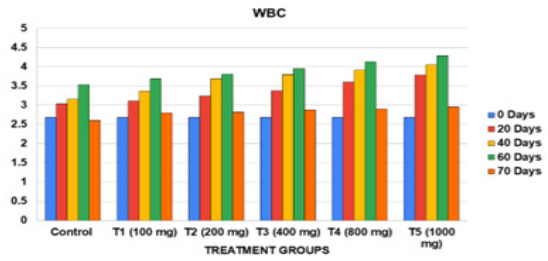


Figure 8. RBC assay of ethanolic leaf extract

Figure 9. WBC assay of ethanolic leaf extract

Relative percentage survival (RPS)

The relative percentage survival (RPS) of *L. rohita* after the challenge with *P. fluorescens* demonstrated significantly ($p < 0.05$) higher disease resistance in the triherbal supplemented groups compared to the control showed in Figure 10. The highest survival rate (85%) was recorded in T2 and T3, followed by T4 (83%) and T1 (80%). The lowest survival rate was observed in T5, which suggests that higher concentrations of the herbal extract may sometimes result in a less optimal immune response (36).

Molecular docking analysis

To identify potential lead compounds for antibacterial drug development, molecular docking was performed on 13 compounds derived from the medicinal plants used in the study showed in Figure 11 (a, b and c) (37). Three

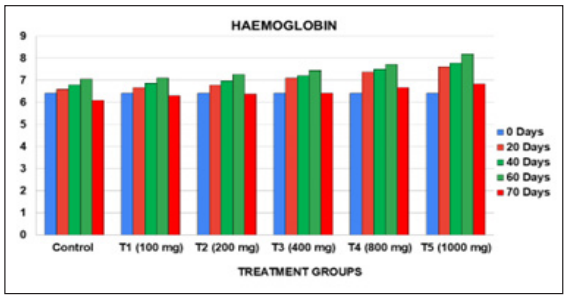
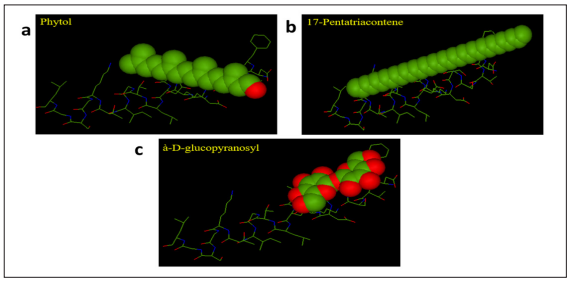


Figure 10. Haemoglobin assay of ethanolic leaf extract

compounds showed the highest molecular binding energies such as *Phytol* from *Avicennia marina* (-231.39 kcal/mol), *17-Pentatriacontene* from *Eichhornia crassipes* (-267.35 kcal/mol) and *β-D-glucopyranosyl* from *Azadirachta indica* (-223.37 kcal/mol) (38). These compounds exhibited strong binding to the target proteins involved in bacterial virulence. Their identification as potential lead molecules suggests that further exploration of their mechanisms of action in both *in vivo* and *in vitro* models could lead to the development of antibacterial treatments for managing *Pseudomonas sps.,* infections in aquaculture (39,40). In conclusion, the results from this study demonstrate that dietary supplementation with triherbal extracts enhances the immune responses, biochemical parameters and disease resistance of *Labeo rohita*. The findings suggest that the herbal extracts not only improve the fish innate immune functions but also hold potential for developing new strategies to combat bacterial pathogens in aquaculture. Further studies, including detailed molecular investigations are required to fully understand the mechanisms behind these effects and to validate the therapeutic potential of these natural compounds in aquaculture.



Conclusion

The present study provides compelling evidence for the potential of bioactive compounds derived from *Avicennia marina*, *Eichhornia crassipes* and *Azadirachta indica* in combating bacterial pathogens in aquaculture, specifically targeting *Pseudomonas sps*. Through molecular docking analysis, the study establishes that the bioactive compounds Phytol**,** 17-Pentatriacontene and β-D-glucopyranosyl form stable intermolecular hydrogen bonds with the active site of the AprX protein, a critical virulence factor in *Pseudomonas sps*. The successful docking between these compounds and the AprX protein is supported by favourable binding energies and Glide scores, indicating that these bioactive molecules have a strong potential to act as inhibitors of the AprX protein. The observed binding affinities suggest that these compounds could serve as promising candidates for the development of antimicrobial drugs targeting *Pseudomonas sps.,* pathogens in aquaculture. This is particularly important given the increasing challenge of bacterial infections in fish farming, where *Pseudomonas sps*. contribute to high mortality rates in various species. The inhibition of the AprX protein, which plays a significant role in bacterial virulence, could help reduce the pathogenicity of *Pseudomonas sps*. and improve the health and survival rates of farmed fish. However, while these findings provide a strong foundation for the development of antibacterial treatments, further experimental studies are essential to fully elucidate the mechanisms through which these bioactive compounds interact with the AprX protein and exert their antimicrobial effects. *In vivo* and *in vitro* studies will be crucial to validate the efficacy, safety and long term impact of these compounds as potential drugs in aquaculture. Additionally, exploring their pharmacokinetic properties and potential for resistance development will be critical in ensuring the sustainability and effectiveness of these natural compounds as a part of integrated disease management strategies in aquaculture systems.

Declaration of Conflict of Interest

The authors declared that there is no conflict of interest

Data Availability

Human and Animal Rights Declaration

All authors hereby declare that there are no ethical concerns related to human or animal rights in this study.

Ethical approval

Not applicable.

Consent for publication

All the authors agreed to publish the data in this journal.

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