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Dietary Chamomile Nanoparticles Enhance Growth, immunity and Disease Resistance in the Nile Tilapia (*Oreochromis niloticus*) challenged with *Aeromonas hydrophila*

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ABSTRACT

Aeromonas hydrophila is a significant foodborne pathogen with economic consequences in aquaculture. This study investigated its prevalence in the Nile tilapia from fish farms in Kafr El-Shaikh Governorate, revealing a 39% infection rate. All isolates carried the *aerA* gene, and 50% had the *hlyA* gene. An *in vivo* trial was conducted to assess the effects of dietary Nano Chamomile on the Nile tilapia over 60 days. Fish were divided into five groups receiving diets with 0.0% **(**control**)**, 0.5%, 1.0%, 1.5%, and 2.0% Nano Chamomile. Results showed significant improvements in growth performance, feed utilization, immune responses **(**white blood cell and lymphocyte counts, lysozyme activity, phagocytic activity**)**, and antioxidant enzymes **(**catalase, superoxide dismutase**)**. Proinflammatory gene expression **(***IL6, IL-1β, TNF***)** was significantly downregulated in the 1.0 and 1.5% groups. These groups also exhibited reduced clinical symptoms associated with infection. The optimal effects were observed at 1.5% **(**15 g/kg**)** Nano Chamomile supplementation.

INTRODUCTION

One of the final avenues for enhancing contributions to food security in developing nations seems to be aquaculture. In some countries, it is now the fastest-growing sector in agriculture, with freshwater aquaculture accounting for the majority of total aquaculture production. Africa exemplifies this global trend, where aquaculture provides millions of people with low-cost, high-quality food, generates income for farming and fishing households, and plays a central role in numerous local and national economies **(Kitessa *et al*., 2014)**.

In recent years, the use of plant-based dietary supplements has become a prominent focus in aquaculture research. These natural extracts are gaining attention due to their positive effects on the immune system and overall health of both animals and humans. Studies have recently revisited their potential as antimicrobial agents and as alternatives to conventional chemotherapy in aquaculture settings **(Olusola *et al*., 2013; Reverter *et al*., 2014)**. Owing to their antioxidant, antibacterial, and immune-boosting properties, herbal plants are increasingly recognized for their role in managing and preventing fish diseases **(Galina *et al*., 2009; Chakraborty *et al*., 2011)**. More than 60 different plant species are currently being utilized in aquaculture to promote fish health and to improve resistance to disease **(Bulfon *et al*., 2013)**. Nevertheless, further research is essential to evaluate their efficacy and safety, considering the wide range of bioactive compounds in plants and the potential environmental consequences of their use **(Neelavathi *et al*., 2013)**.

Chamomile (*Matricaria chamomilla L*.), a well-known medicinal plant from the Asteraceae family, is native to southern and eastern Europe **(Srivastava *et al*., 2010)**. It has become a widely used therapeutic herb in both folk and traditional medicine. Chamomile is rich in a variety of natural compounds, including coumarins, flavonoids, polyacetylenes, sesquiterpenes, and terpenoids **(Haghi *et al*., 2014; Qasem *et al*., 2022)**. Chamomile exhibits various pharmacological activities, including antioxidative, antibacterial, anti-inflammatory, antifungal, analgesic, anticancer, anti-hypoglycemic, anti-stress, antihypertensive, and hepatoprotective properties **(Miraj & Alesaeidi, 2016)**.

Nanoscale drug delivery systems for herbal medicines offer significant potential to enhance their biological activity and to overcome the limitations associated with chemical or synthetic drugs. Integrating herbal remedies into nanodrug delivery systems could expand the use of herbal treatments and improve the management of various diseases **(Chatterjee *et al*., 2023)**.

One of the most significant challenges faced by fish farming is disease. Fish diseases result from interactions between the pathogen, the fish, and the environment, rather than a single factor. Motile aeromonad septicemia (MAS), caused by *A. hydrophila*, leads to tissue swelling, dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia in fish. MAS has a high mortality rate in both wild-caught and farmed freshwater and marine fish species, which significantly impacts the global fish industry **(Beaz-Hidalgo & Figueras, 2013)**.

Microbes can acquire antibiotic resistance determinants without direct exposure to antibiotics, or they can develop resistance under selective pressure. The widespread use of antibiotics, both in medicine and outside of it, has significantly contributed to the proliferation of resistant bacteria **(Goossens *et al*., 2005)**.

Aeromonas hydrophila is associated with its pathogenicity (virulence) through the production of several extracellular products, including proteases, haemolysins, aerolysin, and cytolytic enterotoxins **(Hu *et al*., 2012)**.

This study uniquely focuses on the use of Nano Chamomile as a feed additive in aquaculture, a plant that has not been extensively explored in the context of nanotechnology applications. While much of the existing research on nano-herbal feed additives has centered around well-established herbs such as garlic, turmeric, or mint, our study highlights the potential of chamomile, a widely known herbal remedy, in its nanoparticle form. The incorporation of Nano Chamomile in aquafeeds offers a novel approach, particularly due to its proven antioxidant, anti-inflammatory, and immunomodulatory properties, which are crucial for enhancing fish health and disease resistance. Furthermore, unlike many studies that only explore single-dose applications, this research investigates varying doses of Nano Chamomile, providing a comprehensive assessment of its effectiveness at different concentrations. The unique combination of chamomile's bioactive compounds with nanotechnology presents a promising alternative to traditional feed additives, and our findings offer valuable insights into its potential to support sustainable aquaculture practices. This study therefore not only adds to the growing body of nano-herbal research but also introduces a new dimension by exploring a less-explored herb with significant promise for fish health and growth promotion.

MATERIALS AND METHODS

Collection of samples

In this study, a total of 100 apparently healthy and diseased Nile tilapia (*Oreochromis niloticus*) were randomly selected from various farms in Kafr El-Shaikh Governorate. The fish were collected alive. For bacteriological analysis, the fish were transported from the farms to the Animal Health Research Institute, Kafr El-Shaikh branch, in sterile polythene bags filled with chlorine-free, aerated tap water. Upon arrival, the fish were clinically examined, with particular attention given to external lesions, following the guidelines outlined by **Noga (2010)**.

Bacteriological examinations

Isolation and identification of *Aeromonas hydrophila* isolates

Under aseptic conditions, tissue samples were taken from the gills, heart, liver, spleen, and kidneys, and inoculated into Tryptic Soy Broth (TSB). The broth cultures were incubated aerobically at 37°C for 24 hours. Following incubation, loops of the broth culture were streaked onto *Aeromonas* Isolation Agar Base, m-*Aeromonas* Selective Agar Base, and Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar. These media were incubated at 37°C for another 24 hours. Colonies with characteristic appearances were selected, subjected to morphological assessment, and biochemically confirmed based on the guidelines in **Bergey**'s Manual **(2005)**.

Antibiotic susceptibility testing

The disk diffusion method was applied to evaluate the antibiotic susceptibility of ten *Aeromonas hydrophila* isolates against nine commercially available antibiotics, following the protocol described by **Jorgensen and Turnidge (2007)**. The antimicrobial agents tested included Amoxicillin–Clavulanic acid (AMC, 30µg), Trimethoprim–Sulfamethoxazole (SXT, 25µg), Cefotaxime (CTX, 30µg), Doxycycline (DO, 30µg), Streptomycin (S, 10µg), Tylosin (TL, 15µg), Enrofloxacin (ENR, 5µg), Ciprofloxacin (CIP, 5µg), and Gentamycin (GEN, 10µg). The diameter of the inhibition zones around each disc was measured in millimeters to assess bacterial sensitivity. The results were interpreted as sensitive, intermediate, or resistant, in accordance with the Clinical and Laboratory Standards Institute **(CLSI, 2018)** criteria.

Amplification of *Aeromonas hydrophila* 16S rRNA and selected virulence genes via PCR technique

DNA extraction

The QIAamp DNA Mini Kit from Qiagen, Germany, GmbH, was used to extract DNA from the samples, with some modifications to the manufacturer's instructions. Briefly, 200µl of the sample suspension was incubated for 10 minutes at 56°C with 10µl of proteinase K and 200µl of lysis buffer. After incubation, 200µl of absolute ethanol was added to the lysate. The mixture was then washed and centrifuged as per the instructions provided by the manufacturer. In the final step, 100µl of elution buffer was applied to extract the nucleic acids.

Oligonucleotide Primer

Primers used were supplied from Metabion (Germany), they are listed in Table (1).

PCR amplification.

A 25µl reaction was prepared using 12.5µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1µl of each primer (20 pmol concentration), 5.5µl of water, and 5µl of DNA template. The reaction was conducted in an Applied Biosystems thermal cycler, model 2720.

Analysis of PCR products

The PCR products were separated by electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature, with a gradient of 5V/cm. Each gel slot contained 20µl of PCR products for analysis. Fragment sizes were determined using a Generuler 100 bp ladder (Fermentas, Germany). The gel image was captured using a gel documentation system (Alpha Innotech, Biometra), and the data were analyzed using PC software.

Table 1. Primer sequences, target genes, amplicon sizes, and cycling conditions

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
***A. hydrophila* 16S rRNA**	F-GAAAGGTTGATGCCTAATACGTA R-CGTGCTGGCAACAAAGGACAG	685	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Gordon *et al.*, 2007**
Aerolysin	F-CACAGCCAATATGTCGGTGAAG R-GTCACCTTCTCGCTCAGGC	326	94˚C 5 min.	94˚C 30 sec.	52˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	**Singh *et al.*, 2008**
Hemolysin	F-GGCCGGTGGCCCGAAGATACGGG R-GGCGGCGCCGGACGAGACGGGG	592	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Rozi *et al.*, 2017**

Preparation of nanoparticles

Chamomile was purchased from a local market in Kafr El-Shaikh City, Egypt. It was thoroughly cleaned with double the amount of distilled water, filtered, and allowed to air dry.

To create Nano Chamomile powder, the dried chamomile was ground using a home mill. The grinding process was carried out with a Braun mixer grinder at the third speed for 10 minutes, followed by sieving. If larger particles were observed, the material was re-crushed and sieved under the same conditions. The resulting fine powder was stored in closed glass bottles until use **(Elgendy *et al.*, 2023)**.

Characterization of nanoparticles

The size, shape, and grain boundaries of the synthesized Nano Chamomile powder were examined using Scanning Electron Microscopy (SEM). For elemental analysis, SEM imaging was performed with an Energy Dispersive X-ray (EDX) system (Quattro S, Thermo Scientific). The material was freeze-dried, mixed with potassium bromide (KBr) at a 1:100 (w/w) ratio, compressed into 2mm discs, and analyzed using an FTIR spectrophotometer (JASCO) in the 4000-400 cm^-1 range to determine the structural information and functional groups present in the sample.

Fish, diet preparation, and experimental design

A total of three hundred Nile tilapia (*O. niloticus*) fingerlings, with an initial weight of 19.5 ± 0.2g per fish, were supplied from a private farm in Kafr El-Shaikh, Egypt. Before the trial, the fish were housed in three indoor circular fiberglass tanks (1 m^3) and were acclimatized to experimental conditions for 15 days. During this acclimatization period, the fish were fed a control diet containing 30% crude protein and

7% crude fat. After acclimatization, the fish were randomly assigned to 15 glass tanks (30 x 40 x 60cm), with five experimental groups represented in triplicate. Each tank housed 20 fish. The fish were fed diets supplemented with varying concentrations of Nano Chamomile: 0.0% (G1, control), 0.5% (5g Nano Chamomile/kg diet; G2), 1% Nano Chamomile (10g Nano Chamomile/kg diet; G3), 1.5% Nano Chamomile (15g Nano Chamomile/kg diet; G4), and 2% Nano Chamomile (20g Nano Chamomile/kg diet; G5). All tanks were provided with continuous aeration, and half of the water was replaced daily with fresh, dechlorinated water.

During the 60-day feeding trial, the fish were fed the test diets to satiation (3% of body weight). Water quality parameters, including temperature, pH, oxygen, salinity, and total ammonia nitrogen, were recorded throughout the experiment as follows: 26 ± 0.2°C, pH 6.9 ± 0.1, oxygen 5.5 ± 0.1mg/ L, salinity 10.5ppt, and total ammonia nitrogen 0.1 ± 0.02mg/ L.

Table (2) outlines the components and proximate analysis of the formulated diets. All feed ingredients were initially ground to a fine powder, weighed precisely, and hand-mixed for 5 minutes before being transferred to a mechanical mixer for an additional 15 minutes to ensure uniformity. The blended mixture was then processed into pellets using a laboratory pelletizer with a diameter range of 1.6– 2.1mm. The resulting pellets were left to air-dry at ambient temperature and were subsequently stored in a freezer until use, following the guidelines of **NRC (1994)**.

Table 2. Composition and nutritional profile of the test diets (Percentage on Dry Matter Basis)

Physical composition	**Ingredient g/kg**				
	G1	**G2**	**G3**	**G4**	**G5**
Dehulled-SBM **(**46%**)**[1]	440	440	440	440	440
Yellow corn **(**8%**)**	364	364	364	364	364
Fishmeal **(**67%**)**	60	60	60	60	60
Gluten **(**62%**)**	50	50	50	50	50
Wheat bran **(**12%**)**	50	50	50	50	50
Fish oil	10	10	10	10	10
Coated vitamin C	1	1	1	1	1
Vitamins mixture[2]	1	1	1	1	1
Minerals mixture[3]	1	1	1	1	1
Na Cl	5	5	5	5	5
Lime	7	7	7	7	7
Mono ca phosphate	8	8	8	8	8

Sodium bicarbonate	1	1	1	1	1
Carboxymethyl cellulose	2	2	2	2	2
Nano Chamomile*	0	5	10	15	20
Chemical composition	**Nutrient contents**				
Dry matter	90	90	90	90	90
Crude protein	30	30	30	30	30
Ether extract	7	7	7	7	7
Crude fiber	8	8	8	8	8
Ash	7.5	7.5	7.5	7.5	7.5
Energy **(**KJg^{-1}**)** **	18	18	18	18	18

[1] Dehulled Soybean Meal **(**46%**)**: Contains 46% crude protein, sourced from soybean meal with the hulls removed.

[2] Vitamin Premix **(**g/kg of feed**)**: Includes β-carotene (0.10), vitamin D_3 (0.01), menadione sodium bisulfite (K_3) (0.05), DL-α-tocopherol acetate (E) (0.38), thiamine nitrate (B_1) (0.06), riboflavin (B_2) (0.19), pyridoxine hydrochloride (B_6) (0.05), cyanocobalamin (B_{12}) (0.0001), biotin (0.01), inositol (3.85), niacin (nicotinic acid) (0.77), calcium pantothenate (0.27), folic acid (0.01), choline chloride (7.87), para-aminobenzoic acid (0.38), and cellulose (1.92).

[3]Mineral Premix (g/kg of feed): Comprised of magnesium sulfate ($MgSO_4$) (5.07), disodium hydrogen phosphate (Na_2HPO_4) (3.23), dipotassium hydrogen phosphate (K_2HPO_4) (8.87), iron citrate (1.10), calcium lactate (12.09), aluminum hydroxide ($Al(OH)_3$) (0.01), zinc sulfate ($ZnSO_4$) (0.13), copper sulfate ($CuSO_4$) (0.004), manganese sulfate ($MnSO_4$) (0.03), calcium iodate ($Ca(IO_3)_2$) (0.01), and cobalt sulfate ($CoSO_4$) (0.04).

* The dose of 0.5% and 1% Nano Chamomile was added according to **Chatterjee and Ramamurthy (2024)**.

** Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 kJ g^{-1}, respectively and carbohydrate was calculated by the difference: 100- **(**protein +lipid + ash**)**.

Collection of samples

At the end of the 60-day feeding period, fish were subjected to a 24-hour fasting period. Subsequently, growth performance metrics were calculated using specific formulas, taking into account the body weight and length of individual fish from each tank.

- Weight gain (%) = (final weight-initial weight) x 100/initial weight.

- Specific growth rate (SGR, $\%day^{-1}$) = [Ln (final weight)-Ln (initial weight)/duration] x 100.
- Feed conversion ratio (FCR) = total dry feed intake (g)/(Final body weight (g) – Initial body weight (g)).

- Feed Conversion Efficiency (FCE): FCE was calculated as the ratio of live weight gain (g) to the amount of dry feed consumed (g).
- Protein Efficiency Ratio (PER): PER was determined by dividing the live weight gain (g) by the total dry protein intake (g).
- Survival Rate (%): Survival was expressed as: 100 × (number of fish at the end / number of fish at the beginning).

Hematobiochemical and immune parameters

At the end of the 60-day feeding trial, blood was collected from the caudal vein of six fish per treatment group (two fish from each replicate). One portion of the blood samples was collected using anticoagulant, as outlined by **Schalm (1986)**, to evaluate hematological indices along with phagocytic activity and index as described by **Kawahara *et al.* (1991)**. Another portion was drawn without anticoagulant for serum separation, which was then preserved at -20°C for further analysis. Lysozyme activity was assessed by measuring the lytic effect on *Micrococcus lysodeikticus* (Sigma M 3770), following the method of **Ellis (1990)**. Serum antioxidant status was analyzed by determining the activity of superoxide dismutase (SOD) and catalase (CAT) using commercial assay kits obtained from Bio-diagnostic Co., Egypt. Lipid peroxidation was estimated by quantifying malondialdehyde (MDA) levels, using reagent kits provided by Cusabio Biotech Co., Ltd. (China), in accordance with the manufacturer's protocol.

Real-time PCR procedure for IL-1β, IL-6, and TNF gene expression

Total RNA was extracted from tissue samples utilizing the RNeasy Plus Mini Kit (Qiagen), following the protocol provided by the manufacturer. The quantity and quality of the RNA were evaluated using a NanoDrop ND-1000 spectrophotometer **(**NanoDrop Technologies, Wilmington, DE, USA**)** by recording the absorbance at 260nm and calculating the 260/280 ratio. Following RNA assessment, complementary DNA **(**cDNA**)** was synthesized using the High-Capacity RNA-to-cDNA Master Mix Kit.

PCR amplification was carried out using gene-specific primers and the Thermo Scientific Maxima® SYBR Green/ROX qPCR Master Mix (2×), employing a Rotor-Gene Q real-time PCR system (Qiagen, USA). The primers (listed in Table (3)) were designed using Primer Express 3.0 software (Applied Biosystems, USA) and their specificity was confirmed through BLAST analysis against the NCBI database. Melting curve analysis was performed to verify the specificity and to identity each amplified product. The relative mRNA expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method based on the obtained threshold cycle (Ct) values.

Table 3. Primers of interleukin 1-β **(**IL-1β**)**, interleukin 6 **(**IL6**)** and tumor nicrosis factor (TNF) in the *O. niloticus*

	Primer sequence (5' →3')	Accession No.
TNF-α	F-GAAGCAGCTCCACTCTGATGA	JF957373.1
	R-ACAGCGTGTCTCCTTCGTTCA	
IL1β	F-AAGGATGACGACAAGCCAACC	XM_003460625.2
	R-GCGGACAGACATGAGAGTGC	
IL6	F-ACAGAGGAGGCGGAGATG	LOC102077017
	R-GCAGTGCTTCGGGATAGAG	
β-actin	F-CCACACAGTGCCCATCTACGA	EU887951.1
	R-CACGCTCTGTCAGGATCTTCA	

Whole body and physiological parameters

Three individual fish were randomly selected from each replicate and stored at -20°C for subsequent analysis. The proximate composition of all diets and whole fish specifically dry matter, crude protein, crude fat, and ash content was determined following the standard procedures outlined by **AOAC (2007)**. Additionally, the viscera and liver of the Nile tilapia were carefully dissected and individually weighed to calculate the viscerosomatic index **(**VSI**)** and hepatosomatic index (HSI) using the following equations:

- VSI (%) = (Viscera weight / Fish weight) × 100
- HSI (%) = (Liver weight / Fish weight) × 100

Pathogenicity test

After blood collection, the remaining fish from each treatment group (20 fish per group) were kept for an additional 10 days under the same rearing conditions and continued to receive their respective diets. Subsequently, a total of 60 fish (20 from each group) were subjected to an experimental challenge using a virulent strain of *Aeromonas hydrophila*, which had been previously isolated and confirmed through both biochemical and molecular methods. The bacterial suspension was adjusted to a concentration of 3×10^8 CFU/mL using McFarland Standard No. 1, as described by **Fadl *et al.* (2017)**. Each fish was intraperitoneally **(**IP**)** injected with 0.2 mL of the suspension. Throughout the 10-day observation period following the challenge, fish were carefully monitored for any visible clinical symptoms. Both morbidity and mortality rates were calculated, with

morbidity percentage determined using the formula:
• Morbidity **(%)** = [(Fish showing clinical signs) / (Total number of fish challenged)] × 100

Histopathological examination

Intestinal samples for histopathological evaluation were collected from the middle section of the intestines of fish across different treatment groups. Tissue samples were preserved in 10% neutral buffered formalin, then subjected to dehydration, clearing, and paraffin embedding. Thin sections, approximately 5µm thick, were prepared and stained using hematoxylin and eosin, following the procedure outlined by **Bancroft and Layton (2013)**. Histomorphometric parameters including villus height, width, and the space between villi were measured using ImageJ software (National Institutes of Health, MD, USA). All measurements were expressed in micrometers (µm). The ImageJ tool is openly accessible at: https://imagej.nih.gov/ij/download.html.

Statistical analysis

The obtained data were subjected to one-way analysis of variance using SPSS version 22 (SPSS Inc., IL, USA). The Shapiro-Wilk and Levene tests were used to asses the homogeneity and normality of variance. Differences between means were tested at the 5% probability level using Duncan ˈs test as a *post- hoc* test.

Biosafety measures

Throughout the study, all required biosafety protocols were strictly followed in accordance with the pathogen safety data sheet. Appropriate personal protective equipment (PPE) was utilized, effective disinfectants were applied, and proper waste disposal procedures were implemented. In addition, all experimental fish were disposed of via incineration to ensure safe and ethical handling.

RESULTS

1. **Incidence of *Aeromonas hydrophila* infection in tilapia fish samples**

Out of a total of 100 tilapia fish samples, comprising both healthy and diseased individuals, 39 samples tested positive for *Aeromonas hydrophila*.

2. Antimicrobial susceptibility test for some *Aeromonas hydrophila* isolates

Table 4. Antimicrobial susceptibility test for some *Aeromonas hydrophila* isolates (No.=10)

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Tylosine (TL)	1	10	-	-	9	90
Trimethoprim/ Sulphamethoxazole (SXT)	3	30	1	10	6	60
Doxycycline (DO)	5	50	3	30	2	20
Amoxicillin clavulanic acid (AMC)	6	60	2	20	2	20
Cefotaxime (CTX)	9	90	-	-	1	10
Streptomycin (S)	9	90	-	-	1	10
Enrofloxacin (ENR)	10	100	-	-	-	-
Gentamycin (GEN)	10	100	-	-	-	-
Ciprofloxacin (CIP)	10	100	-	-	-	-

3. Prevalence of *Aeromonas hydrophila* 16S rRNA, some virulence genes in some isolates

Table 5. Prevalence of *Aeromonas hydrophila* 16S rRNA, some virulence genes in some isolates

Sample	*Aeromonas hydrophila* 16S rRNA	*Aerolysin*	*Hemolysin*
1	+	+	+
2	+	+	+
3	+	+	-
4	+	+	-
5	+	+	-
6	+	+	-
7	+	+	+
8	+	+	+
Total %	8(100%)	8(100%)	4(50%)

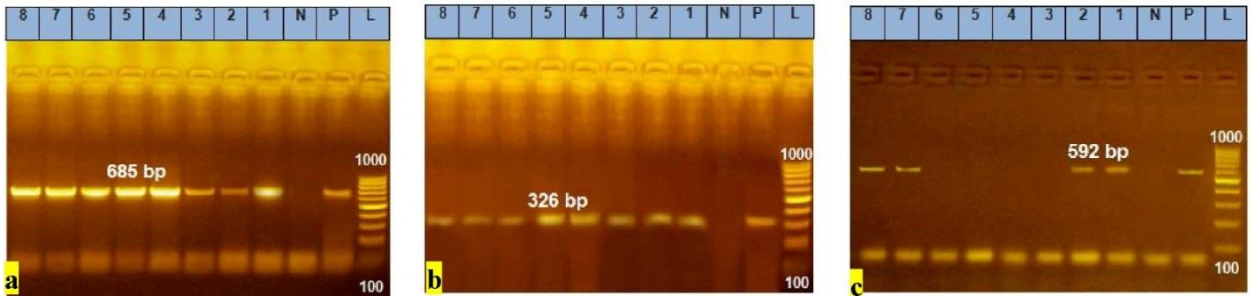

Fig. 1 Gel electrophoresis image showing PCR results for *Aeromonas hydrophila.* **(a)** 16S rRNA, Lane L: DNA size marker **(**100–1000 bp**)**. Lane Pos: Positive control showing a 685 bp band for the 16S rRNA gene. Lanes 1 to 8: Samples confirmed positive for *A. hydrophila* 16S rRNA amplification. **(b)** *Aerolysin* gene. Lane L: DNA ladder ranging from 100 to 1000 bp. Lane Pos: Positive control exhibiting a band at 326 bp for the *Aerolysin* gene. Lanes 1 to 8: *A. hydrophila* isolates testing positive for the *Aerolysin* virulence gene. **(c)** *Hemolysin* gene. Lane L: DNA ladder ranging from 100 to 1000 bp. Lane Pos: Positive control displaying a 592 bp band corresponding to the *Hemolysin* gene. Lanes 1, 2, 7, and 8: *A. hydrophila* isolates showing positive amplification for the *Hemolysin* virulence gene.

4. Characterization analysis of the nano chamomile

4.1. Scanning electron microscope imaging

The surface morphology of Nano Chamomile powder was examined using Field Emission Scanning Electron Microscopy (FE-SEM) (Fig. 2a). The micrographs revealed the presence of well-defined, individual particles exhibiting two distinct morphological forms: rod shaped structures with widths ranging from 490 to 560nm, and uniformly distributed semi-spherical particles with an average diameter of approximately 100nm.

4.2. Energy-dispersive x-ray spectroscopy (EDX) analysis

The elemental composition of Nano Chamomile powder was analyzed using Energy Dispersive X-ray (EDX) spectroscopy. The EDX spectrum (Fig. 2b) revealed the presence of carbon (52%), oxygen (43.98%), potassium (2.1%), chlorine (1.06%), and cerium (0.70%).

4.3. FTIR spectroscopy

The presence of various functional groups in the synthesized Nano Chamomile was detected using FTIR analysis. The FTIR spectrum obtained for Nano Chamomile (Fig. 2c) revealed the presence of multiple functional groups in the nano powder.

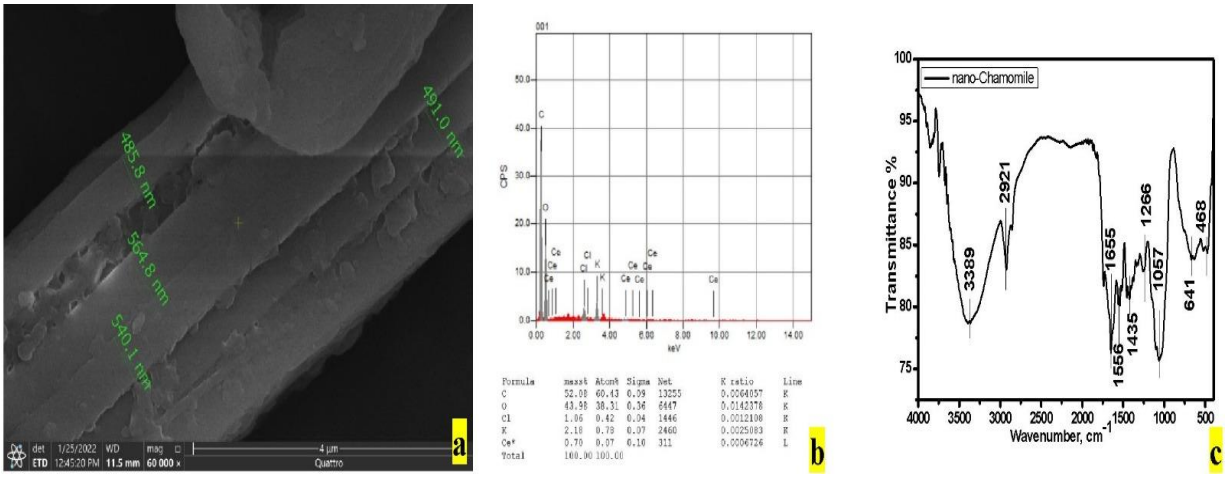

Fig. 2 (a). The SEM image of the prepared Nano Chamomile; **(b)**: The EDX pattern of Nano Chamomile; **(c)**: the FTIR spectrum of Nano Chamomile

5. Growth performance

As shown in Table (6), the growth performance of *Oreochromis niloticus* was notably influenced by dietary Nano Chamomile supplementation. Fish fed Nano Chamomile-supplemented diets (G3 and G4) exhibited significantly higher ($P< 0.05$) final body weight (FBW), weight gain (WG), specific growth rate (SGR), feed conversion efficiency (FCE), and protein efficiency ratio (PER) compared to the control group. Additionally, the feed conversion ratio (FCR) was significantly reduced ($P< 0.05$) in these groups. However, no significant differences ($P> 0.05$) were observed in feed intake or survival rate among the tested groups.

Table 6. Growth metrics following a 60-day feeding period

Parameter	G1	G2	G3	G4	G5
Initial weight	19.7±0.3	19.5±0.7	19.5±1.5	19.4±0.8	19.5±1.2
Final weight	47.4±0.4^{ab}	48.52±0.1^{b}	48.11±1.7^{ab}	47.25±0.7^{ab}	47±0.3 ^{a}
Weight gain	140.7±0.7^{a}	148.6±1.2^{b}	147.8±0.3^{b}	143.35±0.2^{ab}	141.8±3.8^{ab}
SGR	1.36±0.02^{a}	1.41±0.002^{b}	1.39±0.01^{ab}	1.37±0.01^{ab}	1.36±0.005^{a}
Survival	97.5±2.5	97.5±2.5	95±5	97.5±2.5	97.5±2.5
Feed intake	38.05±0.25	37.05±0.55	36.25±1.45	37.55±0.95	38.15±0.55
FCR	1.38±0.005^{b}	1.28±0.007^{ab}	1.27±0.01^{a}	1.35±0.01^{ab}	1.35±0.06 ^{ab}
FCE	0.73±0.007^{a}	0.78±0.012^{b}	0.79±0.017^{b}	0.74±0.006^{a}	0.72±0.001^{a}
PER	2.75±0.03^{a}	2.95±0.02^{b}	2.98±0.04^{b}	2.8±0.06^{a}	2.72±0.01^{a}

*Values are expressed as the mean ± standard error (SE) based on three replicates (n=6). Means with different superscript letters indicate statistically significant differences among treatments ($P<0.05$). Abbreviations: SGR – specific growth rate; FCR – feed conversion ratio; FCE – feed conversion efficiency; PER – protein efficiency ratio.

6. Hematological analysis

Hematological analysis revealed that diets supplemented with Nano Chamomile (Groups G4 and G3) significantly ($P< 0.05$) enhanced several blood parameters, including red blood cell (RBC) count, hemoglobin concentration (Hb%), and the percentages of heterophils, lymphocytes, monocytes, and eosinophils, compared to the control group. Nevertheless, the dietary addition of Nano Chamomile had no significant impact ($P> 0.05$) on packed cell volume (PCV) or total white blood cell (WBC) count.

Table 7. Hematological parameters in the Nile tilapia fed test diets

Parameter	G1	G2	G3	G4	G5
HB	4.36 ± 0.05^{a}	4.89 ± 0.11^{ab}	5.5 ± 0.1^{b}	5.57 ± 0.35^{b}	4.25 ± 0.34^{a}
RBCs	2.05 ± 0.05^{ab}	2.3 ± 0.03^{ab}	2.4 ± 0.1^{ab}	2.55 ± 0.2^{b}	1.93 ± 0.13^{a}
PCV	22.32±0.4	22.66±0.5	23.25±0.5	25.35±1.2	21.63±1.05
WBCs	38±1.3	43.45±0.4	46.14±1.3	46.07±1	41.7±3.5
heterophils	22.45 ± 0.96^{c}	20.7 ± 0.6^{bc}	15.8133 ± 0.9^{ab}	14.09 ± 0.9^{a}	19.9 ± 1.6^{bc}
Lymphocyte	67.38 ± 1.6^{a}	71.46 ± 0.6^{ab}	77.4 ± 1.2^{bc}	80.47 ± 0.85^{c}	72.6 ± 2.5^{ab}
Monocytes	6.67 ± 0.3^{b}	5 ± 0.6^{ab}	4.3 ± 0.3^{a}	3.67 ± 0.3^{a}	5 ± 0.6^{ab}
Esinophils	3.3 ± 0.3^{b}	2.67 ± 0.3^{ab}	2.3 ± 0.3^{ab}	1.67 ± 0.3^{a}	2.3 ± 0.3^{ab}
Basophils	0.17±0.03	0.13±0.03	0.1±0.00	0.1±0.00	0.13±0.03

*Results are presented as mean values ± standard error (SE) based on three replicates (n=6). Treatments with differing superscript letters indicate significant differences at the $P<0.05$ level.

7. Antioxidant activity and immune parameters

Antioxidant parameters in the Nile tilapia following 60 days of dietary treatment are summarized in Table (8). Groups G3 and G4 exhibited significantly increased ($P< 0.05$) superoxide dismutase (SOD) and catalase (CAT) activities relative to other treatments. Additionally, these groups showed a significant reduction ($P< 0.05$) in malondialdehyde (MDA) levels, indicating an improved oxidative status. Table (8) outlines the immune responses observed in the Nile tilapia across the experimental groups. Groups G3 and G4 demonstrated significantly increased ($P< 0.05$) phagocytic and lysozyme activities compared to the rest. Additionally, the phagocytic index was notably higher ($P< 0.05$) in the groups receiving Nano Chamomile supplementation (G4 and G5) than in the other groups.

8. Gene expression

Fig. (3) illustrates the fold changes in the expression of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor (TNF) genes in *Oreochromis niloticus*. Dietary supplementation with Nano Chamomile at 1% and 1.5% significantly ($P< 0.05$) downregulated the expression levels of IL-6, IL-1β, and TNF compared to the control groups.

Table 8. Impact of Nano Chamomile supplementation on serum antioxidant levels and serum immunity parameters in the Nile tilapia following a 60-day feeding period

Parameter	MDA	CAT	SOD	phagocyticactivity	phagocyticindex	lysozyme
G1	4.31±0.6^{b}	46.95±2.5^{a}	27.2±2.3^{a}	17.43±1.74^{a}	2.02±0.1^{a}	2.93±0.3^{a}
G2	3.74±0.5^{b}	58.43±1.7^{ab}	37.5±4.7^{ab}	18.58±0.8^{a}	2.66±0.27^{ab}	4.23±0.4^{ab}
G3	1.84±0.2^{a}	66.18±2.15^{bc}	43.48±2.3^{bc}	24.44±1.07^{ab}	3.83±0.3^{bc}	5.46±0.2^{b}
G4	1.58±0.1^{a}	79.2±5.8^{c}	52.5±1.2^{c}	29.84±0.9^{b}	5.07±0.1^{d}	7.94±0.7^{c}
G5	4±0.4^{b}	56.98±5.02^{ab}	40.27±3^{abc}	22.48±3.2^{ab}	4.25±0.5^{c}	5.39±0.4^{b}

*Values are presented as means ± standard error (SE) based on three replicates (n = 6). Different superscript letters denote statistically significant differences between treatments ($P < 0.05$). SOD: Superoxide dismutase; CAT: Catalase; MDA: Malondialdehyde.

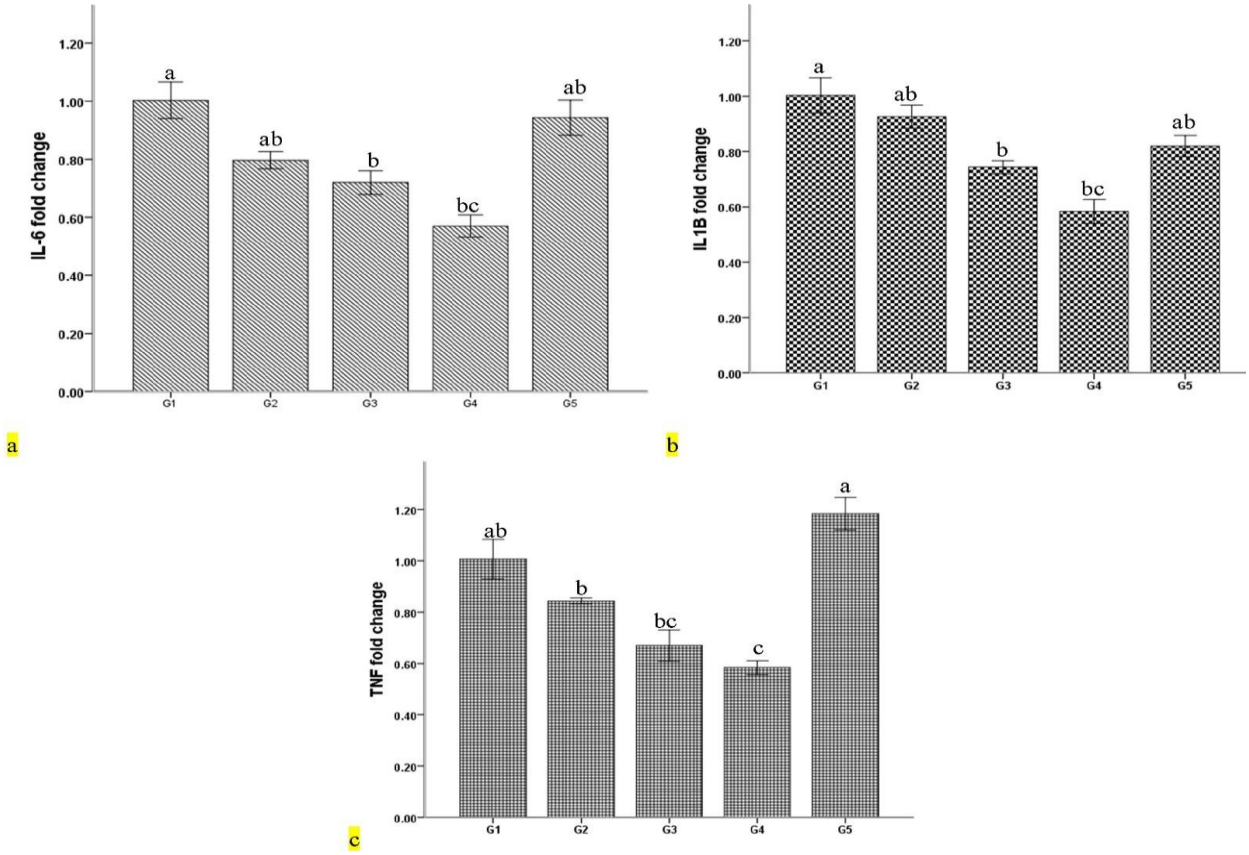

Fig. 3. Expression levels of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor (TNF) in *Oreochromis niloticus* fed diets supplemented with varying concentrations of Nano Chamomile

Relative expression was normalized to β-actin using the 2^−ΔΔCt method. Data are presented as means with standard error (SE) bars (n=6 per group). Different superscript letters above the bars indicate statistically significant differences between treatments ($P<0.05$).

9. Whole-body composition and somatic parameters in the Nile tilapia

Table (9) displays the data on body composition and somatic measurements of the Nile tilapia. No statistically significant differences (*P> 0.05*) were observed in the whole-body proximate composition or somatic indices among the groups fed the different experimental diets.

Table 9. Proximate composition and somatic indices of the Nile tilapia following 60 days of dietary treatments

Item	G1	G2	G3	G4	G5
Dry matter	30.9± 0.2	30.5± 0.2	30.4± 0.2	30.1± 0.2	30.1± 0.2
Moisture	69.1±0.5	69.5±0.2	69.6±0.5	69.9±0.3	69.9±0.2
Crude protein	16.2±0.3	16.4±0.3	16.4±0.3	16.2±0.3	16.25±0.3
Crude lipid	6.7±0.2	6.8±0.2	6.8±0.2	6.7±0.2	6.55±0.2
Crude ash	6.5± 0.4	6.3± 0.4	6.6± 0.4	6.5± 0.4	6.5± 0.4
[1]VSI (%)	8.4±0.6	8.2±0.6	8.5 ±0.6	8.3±0.6	8.3±0.6
[2]HSI (%)	2.4±0.1	2.5±0.18	2.8 ±0.15	2.7±0.12	2.7±0.12

*Data are presented as mean values ± standard error (SE) from three replicates. Means within the same row that have identical superscript letters do not differ significantly ($P<0.05$). [1]VSI: Viscerosomatic index = (weight of viscera / total fish weight) × 100. [2]HSI: Hepatosomatic index = (liver weight / total fish weight) × 100.

10. Clinical and postmortem observations

Following the *Aeromonas hydrophila* challenge, only one mortality was recorded throughout the entire experimental period. Moribund fish in Groups 1 and 2 exhibited severe clinical signs, whereas those in Groups 3 and 4 displayed milder symptoms. Observed external signs included exophthalmia, hemorrhages on the tail and skin, and fin rot. Internally, affected fish showed pale, enlarged livers, distended gall bladders filled with bile, and congested gills, as illustrated in Fig. (4). The percentage of moribund fish within the first seven days post-challenge is presented in Table (10).

Table 10. Moribund % of challenged fish

	1 st day	2nd day	5th day	7th day
G1	100^{a}	100^{a}	72^{a}	36^{a}
G2	50^{b}	75^{c}	31^{c}	12^{b}
G3	39^{c}	77^{c}	17^{e}	11^{b}
G4	50^{b}	75^{c}	25^{d}	12^{b}
G5	52^{b}	90^{b}	48^{b}	38^{a}

Different letters in the same column indicate significant differences (n=20).

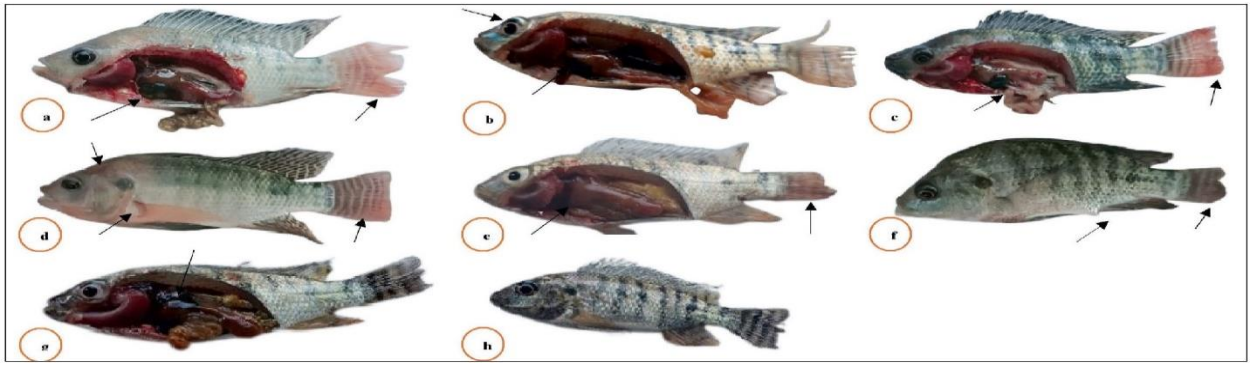

Fig. 4. Clinical and postmortem observations in all groups: (a) Control group showing distension of gall bladder, hemorrhagic liver, some skin hemorrhage, and hemorrhagic tail . (b) Group 2 showing eye protrusion and hemorrhagic liver. (c) Group 3 distension of gall bladder, and hemorrhagic tail. (d) Group 3 showing skin hemorrhage, hemorrhage at the base of the pectoral fin, and skin ulcer. (e) Group 4 showing enlarged and hemorrhagic liver and tail erosion. (f) Group 4 showing tail erosion, dark coloration, and protrusion of the anus. g) Group 5 showing green hemorrhagic liver (retention of bile in the liver). (h) Group 5 showing dark coloration and body stiffness

Re-isolation trials from internal organs of artificially infected fish with *Aeromonas hydrophila*

Bacteriological examination of homogenized gill and organ samples confirmed the presence of the inoculated *Aeromonas hydrophila*. Moreover, dietary supplementation with 1.5% Nano Chamomile (G4), followed by 1% Nano Chamomile (G3), resulted in a reduced frequency of bacterial re-isolation from internal organs compared to the positive control group (G1).

12.Intestinal morphology

As presented in Table (11) and Fig. (4), after 60 days of the experiment, dietary supplementation with Nano Chamomile in Groups G3 and G4 resulted in a significant ($P < 0.05$) increase in both intestinal villi length and goblet cell count compared to the other treatment groups.

Table 11. Micromorphology of the intestine of the Nile tilapia fed test diets

	Tested parameters		
Treatments	**Villi length**	**Intervilli-distance**	**Gobletcells**
G1	415.96±33.2^{a}	54.05±10.7	74.7±2.5^{a}
G2	453.73±26.6^{a}	37.52±7.04	85±2.9^{ab}
G3	633.34±22.3^{b}	51.74±4.5	116.34±3.02^{c}
G4	736.07±46.96^{b}	42.3±8.7	136.84±1.6^{d}
G5	451.91±14.3^{a}	43.58±14	88.4±1.04^{b}

*Data are presented as means ± standard error (SE) for three replicates (n = 6). Means with different superscript letters differ significantly among treatments ($P < 0.05$).

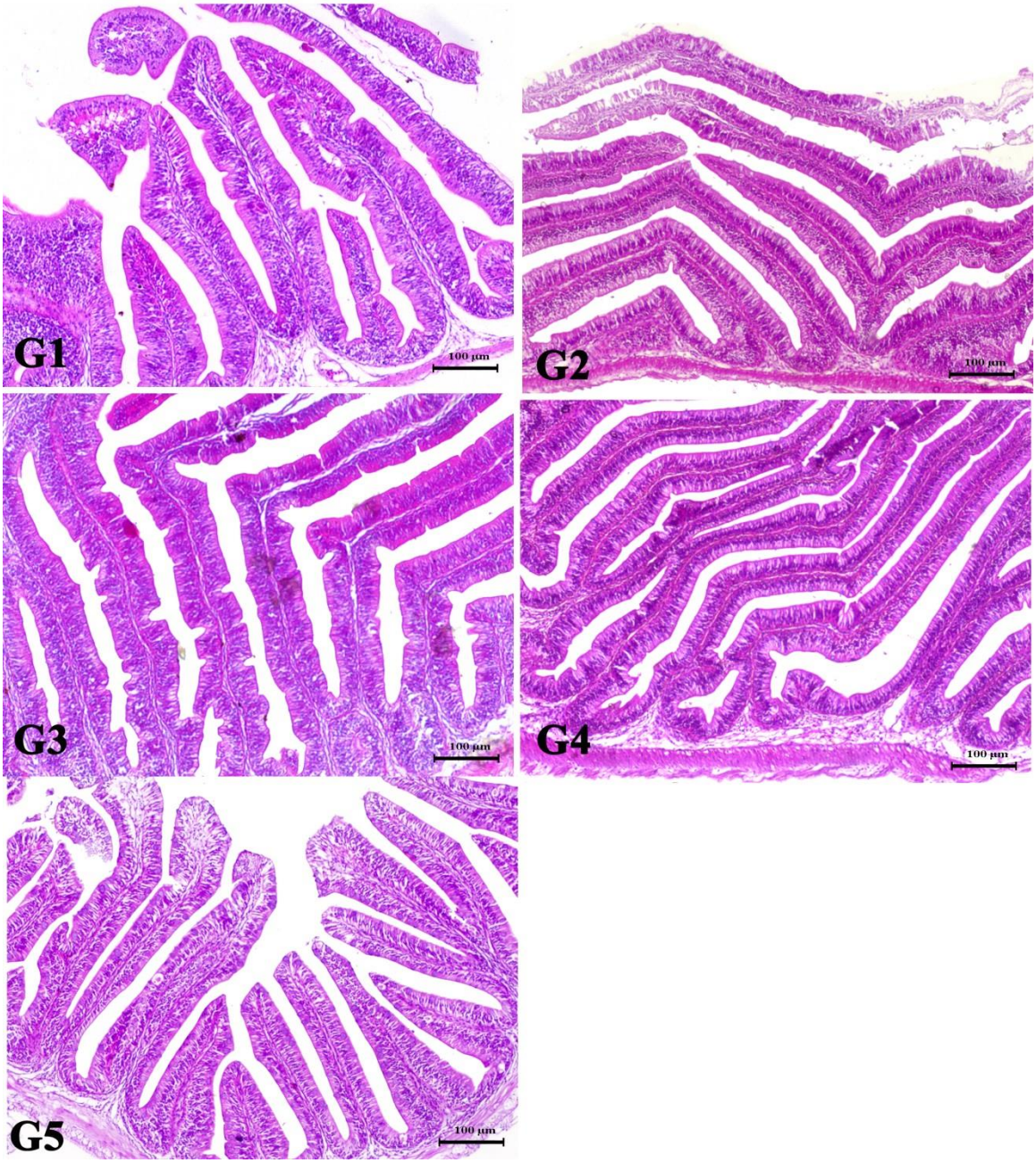

Fig. 5. Histological sections of the mid-intestine of Nile tilapia. Group 1 (G1) displays normal intestinal villi; Group 2 (G2) shows increased villi length; Group 3 (G3) exhibits a marked increase in villi length; Group 4 (G4) also shows a pronounced elongation of villi; and Group 5 (G5) reveals a moderate increase in villi length. Stained with H&E, scale bar = 200 µm

DISCUSSION

Our findings revealed that *Aeromonas hydrophila* was detected in 39% of the examined fish samples. This result is comparable to that reported by **El-Ashram (2002)**, who recorded a prevalence of 47.3% among diseased tilapia, and **El-Gohary *et al*.**

(2020), who found that 46.7% of fish samples from farms were positive for motile aeromonads. In contrast, lower prevalence rates were observed by **Kahraman *et al*. (2020)**, who detected *A. hydrophila* in only 15% of shrimp samples. In this context, **Nagar *et al*. (2011)** reported a 3.85% occurrence in 52 freshwater fish samples. On the other hand, a higher prevalence of 65.63% was documented by **Salem *et al*. (2020)**. Variations in prevalence across studies may be attributed to differences in sampling seasons, detection techniques, sample collection procedures, and hygiene practices applied at the sampling sites.

The antimicrobial susceptibility results indicated that all *Aeromonas hydrophila* isolates exhibited 100% sensitivity to Enrofloxacin, Gentamicin, and Ciprofloxacin. In contrast, 90% of the isolates were resistant to Tylosin, and 60% showed resistance to Trimethoprim/Sulfamethoxazole. These findings are in agreement with those of **Dahdouh *et al*. (2016)** observed that *Aeromonas hydrophila* strains recovered from freshwater fish exhibited susceptibility to antibiotics such as Enrofloxacin, Ofloxacin, and Gentamycin. Similarly, **Basri *et al*. (2020)** observed resistance of *A. hydrophila* to Sulfamethoxazole/Trimethoprim. Additionally, **Adah *et al*. (2021)** found that 66.7% of *A. hydrophila* isolates were highly sensitive to Gentamycin.

Molecular analysis based on 16S rRNA gene sequencing confirmed that all eight isolates belong to *Aeromonas hydrophila*. These findings are consistent with those of **Basri *et al*. (2020)**, who reported the presence of the 16S rRNA gene in all examined strains. Similarly, **Hamouda *et al*. (2019)** detected the gene in six out of seven isolates, confirming *A. hydrophila* as the species through this species-specific genetic marker.

Investigating virulence genes is essential for assessing the pathogenic potential of bacterial isolates and identifying potential targets for infection control strategies. In this study, the *aerA* **(***aerolysin***)** gene was detected in all eight randomly chosen *A. hydrophila* isolates, reflecting a complete (100%) occurrence among the tested samples. This finding aligns with the results of **Sonkol *et al*. (2020)**, who similarly identified the *aerA* gene in all of their examined isolates. However, it contrasts with the study by **Nagar *et al*. (2011)**, which failed to detect the *aerA* gene in the examined strains. Additionally, our results showed that the *hly* **(**hemolysin**)** gene was present in four out of the eight *A. hydrophila* isolates, corresponding to a 50% detection rate. This is slightly higher than the 30% reported by **Sonkol *et al*. (2020)**.

Field Emission Scanning Electron Microscopy (FE-SEM) was employed to analyze the surface structure of the Nano Chamomile powder, as illustrated in Fig. (2.a). The FE-SEM images displayed clearly defined, separate particles characterized by two distinct shapes: rod-like forms measuring between 490 and 560nm in width, and evenly distributed, nearly spherical particles with an average size of about 100nm.

The elemental composition of Nano Chamomile powder was analyzed using Energy Dispersive X-ray (EDX) spectroscopy. The EDX spectrum (Fig. 2b) revealed the presence of carbon (52%), oxygen (43.98%), potassium (2.1%), chlorine (1.06%), and

cerium (0.70%). These findings are consistent with the observations documented by **Parlinska-Wojtan *et al*. (2016)**.

The presence of various functional groups in the synthesized Nano Chamomile was detected using FTIR analysis. The FTIR spectrum obtained for Nano Chamomile (Fig. 2c) revealed the presence of multiple functional groups in the nano powder. The broad absorption peak centered at 3389 cm^{-1} is characteristic of the hydroxyl group (O-H) stretching mode of alcohols or phenols, as well as the N-H stretching vibration of amide groups present in the plant **(Fatima *et al*., 2016)**. The peak around 2921cm^{-1} is attributed to the C-H stretching vibrations of the alkane group. The sharp and intense peak at 1655cm^{-1} likely corresponds to the stretching vibration of the aldehyde carbonyl **(**C=O**)** groups.

The low-intensity absorption band observed at 1435cm^{-1} is attributed to the bending vibrations of C–OH groups present in alkanes and aromatic amines derived from the plant material. Several low-intensity peaks appearing between 1380 and 1315cm^{-1} are associated with C–N stretching vibrations of aromatic amines, as described by **Parlinska-Wojtan *et al*. (2016)**. The peak at 1266 cm^{-1} likely corresponds to O–H deformation and C–H stretching of carboxylic groups, in addition to the N–H bending of amide II. Moreover, the absorption band at 1057 cm^{-1} is indicative of C–O–C stretching vibrations related to aliphatic amines.

In general, the distinct absorption peaks detected within the 1600– 1000cm^{-1} range suggest the presence of aldehydes and cinnamaldehyde in cinnamon, consistent with the findings reported by **Ma *et al*. (2016)**. The peaks observed in the 900–600cm^{-1} range are characteristic of primary and secondary amines and amides, as previously noted by **Szymczycha-Madeja *et al*. (2013)**.

There is an increasing focus on developing practical and cost-effective strategies to incorporate medicinal products into aquafeeds, aiming to promote more sustainable aquaculture practices. Herbal additives have been traditionally used in aquaculture to improve fish health and immunity through various mechanisms, such as stimulating enzyme activity, enhancing growth, and exhibiting antimicrobial properties **(Van Hai, 2015)**. Nanotechnology offers a novel and promising approach in animal nutrition, with the potential to improve the health, immune response, nutrient bioavailability, and overall productivity of aquatic species. Due to their greater specific surface area, nanoscale feed additives possess enhanced absorption capabilities, leading to an improved efficacy.

This study aimed to assess the impact of Chamomile Nanoparticles (CNPs) on the growth metrics, gut health, and antioxidant enzyme activities in the Nile tilapia (*Oreochromis niloticus*). The findings revealed that incorporating Chamomile Nanoparticles (CNPs) into the diet markedly improved final body weight, weight gain, specific growth rate, and feed conversion ratio in comparison to the control group fed without CNPs. These findings are consistent with those of **Subasinghe *et al*. (2009)** and **El-Dakar *et al*. (2023)**, who reported that dietary supplementation with 1% chamomile

flower meal (CFM) improved all growth parameters in the Nile tilapia. Such improvements may be attributed to the high content of bioactive compounds in chamomile, including flavonoids and terpenoids, which enhance feed palatability, thereby promoting better growth performance and feed utilization **(Al-Dabbagh *et al*., 2017)**.

Previous studies have evaluated the effects of chamomile powder and extract on fish performance. The results indicated that *Matricaria chamomilla L.*, when administered at 1% as a powder and 5% as an extract, yielded the most favorable outcomes in terms of growth performance, hematological parameters, and resistance to *Aeromonas hydrophila* infection.

Moreover, **AbdelTawab *et al*. (2022)** reported that dietary supplementation with chamomile exerts growth-promoting and stimulatory effects, with the optimal dose being 3.0 g/kg of diet. The current findings could be attributed to chamomile's ability to enhance digestive enzyme activity and nutrient absorption, modify intestinal structure, and stimulate appetite in Nile tilapia.

Hematological parameters serve as vital indicators for diagnosing health disorders, evaluating nutritional status, and monitoring the overall hygiene and health of fish. In the present study, hematological analysis showed that diets supplemented with Nano Chamomile (groups G3 and G4) resulted in a significant **(***P*< 0.05**)** increase in several hematological parameters, including red blood cell (RBC) count, hemoglobin concentration **(**Hb%**)**, heterophils, lymphocytes, monocytes, and eosinophils, compared to the control group. These findings are consistent with those reported by **Esmaeili *et al*. (2021)** and **El-Dakar *et al*. (2023)**, who found that dietary inclusion of chamomile flower meal **(**CFM**)** at 1% led to the highest values of Hb, hematocrit **(**Hct**)**, and RBCs.

Meanwhile, although packed cell volume **(**PCV**)** and white blood cell **(**WBC**)** counts also increased in response to Nano Chamomile supplementation, the changes were not statistically significant (*P*> 0.05). WBC count is considered one of the key indicators of fish health, as it reflects the immune response to infections and is influenced by a range of physiological and pathological factors, including disease, inflammation, nutrition, stress, age, temperature, sex, and hormonal fluctuations **(Clauss *et al*., 2008)**.

Many plant-derived compounds are considered to function as natural antioxidants, helping to neutralize free radicals and to reduce the generation of oxygen-derived species **(Chakraborty & Hancz, 2010)**. Key components of the body's antioxidant defense include enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which work together to convert reactive oxygen species (ROS) into less toxic molecules, thereby minimizing oxidative damage **(Madeira *et al*., 2013; Hoseinifar *et al*., 2021)**. Additionally, malondialdehyde (MDA) is a well-known byproduct of lipid peroxidation and serves as a key biomarker for oxidative damage to lipids.

The antioxidant results of the current study align with earlier research demonstrating that dietary supplementation with Chamomile Nanoparticles significantly

enhances antioxidant activity in the Nile tilapia **(Mayada *et al.*, 2023)**. The antioxidant effects of chamomile are attributed to its volatile oils, total flavonoids, and polysaccharides, which have been shown to effectively scavenge free radicals. These findings further support the scientific basis for chamomile's antioxidant potential.

Similarly, **Abdel-Tawwab *et al.* (2023)** reported that Nano-Curcumin supplementation in the Nile tilapia diets led to significant increases in SOD and CAT activities, along with a marked reduction in MDA levels. In contrast, **Bao *et al.* (2022)** observed a significant decline in MDA activity in the group receiving 0.2% Nano-Curcumin; however, no significant effects were noted on SOD and CAT activities in serum **(***P*> 0.05**)**, suggesting that antioxidant responses may vary depending on the concentration and biological context.

Phagocytosis is one of the most crucial defense mechanisms against pathogenic bacteria. In this investigation, Nile tilapia administered diets containing 1% and 1.5% Chamomile Nanoparticles (CNPs) demonstrated marked improvements in both phagocytic function and lysozyme activity. Moreover, a notable elevation (*P< 0.05*) in the phagocytic index was observed in the groups supplemented with 1.5% and 2% chamomile nanoparticles (CNPs). These findings align with those reported by **Talpur (2014)**, who observed significantly elevated phagocytic activity in *Lates calcarifer* fed a medicinal plant **(**MP**)**-supplemented diet, indicating an improvement in the fish's nonspecific immune response.

Lysozyme, an important antibacterial enzyme in the innate immune system, plays a vital role by breaking down peptidoglycan bonds in bacterial cell walls. However, our results regarding lysozyme activity differ from the findings of **Talpur (2014)**, **Adel *et al.* (2015)** and **Paknejad *et al.* (2020)**, suggesting variability that could be attributed to differences in experimental design, fish species, or supplement formulations.

Interleukin-6 **(**IL-6**)** is chiefly produced by T lymphocytes and macrophages to activate immune mechanisms during inflammatory responses **(Hirano, 1998)**. Interleukin-1β (IL-1β) was one of the earliest cytokines discovered in fish using gene homology approaches **(Zou *et al.*, 1999)**. Tumor necrosis factor-alpha **(**TNF-α**)**, a major pro-inflammatory mediator in mammals, is secreted by immune cells like macrophages, monocytes, neutrophils, natural killer cells, and T cells in reaction to bacterial lipopolysaccharides. A comparable immunological response has been observed in fish species, such as the rainbow trout **(Laing *et al.*, 2001)**.

In this study, incorporating Nano Chamomile at 1% and 1.5% in the diet significantly (*P*< 0.05) suppressed the expression of IL-6, IL-1β, and TNF-α genes. This response is likely linked to the strong anti-inflammatory activity of chamomile essential oil (CEO), which is known to reduce the production of inflammatory cytokines such as TNF-α and IL-1β. These outcomes align with observations by **Mayada *et al.* (2023)**, who noted improved cytokine profiles when CEO was administered alongside aluminum

nanoparticles (ALNPs). The anti-inflammatory action of chamomile is largely attributed to its high flavonoid content.

Carcass composition serves as an important indicator of Tilapia physiology and can reflect variations in nutritional status. In the present study, the carcass composition of the Nile tilapia did not show significant differences among the groups fed different levels of Nano Chamomile, indicating that the proximate body composition of the Nile tilapia is not notably affected by varying Nano Chamomile concentrations in the diet. This contrasts with previous studies on chamomile supplementation in tilapia diets. For instance, **El-Dakar *et al.* (2023)** reported an improvement in carcass protein content in the Nile tilapia reared under a biofloc system with chamomile supplementation. Such discrepancies may be attributed to differences in the rearing system or the higher dosage of the additive used in previous studies.

Aeromonas hydrophila is a bacterium commonly found in freshwater environments. It is facultatively anaerobic and obtains its energy through chemo-organoheterotrophic metabolism. As reported by **Ali *et al*. (2014)**, *Aeromonas hydrophila* is linked to several pathological conditions in fish, such as gastroenteritis and septicemia, and is recognized as the primary pathogen responsible for Motile Aeromonas Septicemia (MAS).

Clinical observations and macroscopic examinations of the fish and their organs were conducted on the 1st, 2nd, 5th, and 7th days post-exposure. Clinically, signs such as lethargy, loss of appetite, surface swimming, body darkening, and fin erosion with lysis were observed, consistent with the findings of **Banu and Yilmaz (2011)**. Additionally, exophthalmia (eye protrusion), along with hemorrhages on the tail and body, were noted. Macroscopic examination revealed hepatomegaly with a pale appearance, and an enlarged gall bladder filled with dense emerald green bile, in agreement with the observations reported by **Banu and Yilmaz (2011)**.

The main location for nutrient absorption in fish is the gut. Nutrients enter and exit intestinal enterocytes through specific transporters at the brush border and basolateral membranes **(Adeshina *et al*., 2019; Abdel-Latif *et al*., 2020)**. The addition of CNPs, specifically 1 and 1.5%, to the diet of Nile tilapia resulted in a dose-dependent increase in the length/width of the villi and the area absorbed in the mid-intestine, according to intestinal histomorphometry. On the other hand, long villi are typically linked to improved gut health, increased performance, and high nutrient and absorption efficiency **(Sklan *et al*., 2004; Huerta-Aguirre *et al*., 2019)**. Comparability: The intestinal architecture, particularly the lamina, has significantly improved in *Oreochromis* spp. when photomicrographed with Nano Curcumin. A photomicrograph of the intestinal tissues in *Oreochromis* spp. treated with Nano Curcumin reveals marked enhancement in intestinal architecture, characterized by a clear lamina propria and smooth apical surfaces of the intestinal villi.

CONCLUSION

Our research indicates that *Aeromonas hydrophila* and its virulence- associated genes play a major role in causing fish diseases. Among these, the *aerA* (*aerolysin*) and *hylA* (*haemolysin*) genes have emerged as one of the most effective methods for identifying the virulence chracterisitics of *A. hydrophila.* The bacterial isolates demonstrated *in vitro* sensitivity to several antibiotics, including Ciprofloxacin, Gentamycin, and Enrofloxacin.

This study aimed to introduce a new perspective on the application of Nano herbiotics in aquaculture. The findings indicated that Chamomile Nanoparticles (CNP), a natural growth enhancer, can effectively replace conventional synthetic growth promoters in fish diets. Supplementation with CNP notably improved the growth performance, intestinal structure, overall health, and disease resistance of *Oreochromis niloticus*. The results suggest that incorporating CNP at a level of 15 g/kg in feed could contribute to advancing sustainability in aquaculture practices.

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