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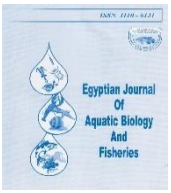

Immunological Response Assessment Through Hematology, Phagocytosis, and Gill Histology in Hybrid Giant Grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) Vaccinated Against *Vibrio harveyi* Using Integrated Whole Cell-Extracellular Product-Nano-Chitosan-*Caulerpa lentillifera* Formulation

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ABSTRACT

Vibriosis is a significant pathogenic issue leading to high mortality rates in giant grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*), mostly due to infections by *Vibrio harveyi*. This study examined the effectiveness of an oral vaccine formulation that includes extracellular product (ECP) extracts and whole cell (WC) preparations of *Vibrio harveyi*, in conjunction with nano-chitosan and *Caulerpa lentillifera* extract, to improve vibriosis resistance in giant grouper. The experimental protocol entailed the cultivation of *Epinephelus* spp. juveniles (10– 12cm total length) over a 30-day duration across five distinct treatments: Control; *Caulerpa lentillifera* extract treatment; ECP in conjunction with nano-chitosan; WC in conjunction with nano-chitosan; and a combination of ECP, WC, nano-chitosan, and *Caulerpa lentillifera* extract. The primary response parameters evaluated comprised hematological profiles, phagocytic activity, and histological investigation of the gills. The results indicated that the combination of nano-chitosan and WC derived from *Vibrio harveyi*, supplemented with *Caulerpa lentillifera* extract, greatly improved immune system activation relative to alternative therapies. This formulation significantly enhanced hematological indices, phagocytic responses, and gill histoarchitecture in giant grouper after exposure to *Vibrio harveyi*. The data indicate the efficacy of nano-chitosan, WC, and *Caulerpa lentillifera* -based oral immunization as a prophylactic approach against *Vibrio harveyi* infections in aquaculture systems, necessitating additional research for commercial application.

INTRODUCTION

The pearl gentian grouper, a hybrid marine species *Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*), is a significant aquaculture species that is widely cultivated in Indonesia. Recent years have seen a rise in cultivation challenges due to unfavorable environmental conditions and a variety of infectious agents, including viral, bacterial, and parasitic pathogens. Vibriosis, primarily attributed to *Vibrio harveyi*, has emerged as a prevalent and serious disease, significantly affecting fish health and growth performance, and resulting in considerable economic losses **(Liu *et al.*, 1997; Gudding & Van, 2013; Yu *et al.*, 2013; Mohamad *et al.*, 2019)**.

Antibiotic therapy has historically been the main management strategy for controlling vibriosis in aquaculture systems. Widespread antibiotic use has led to the emergence of antibiotic-resistant bacterial strains and environmental contamination due to the accumulation of drug residues **(Wei *et al.*, 2020)**. The efficacy of vaccination strategies has led to reduced pathogen prevalence and decreased antibiotic dependency, underscoring the importance of advancing vaccine development for vibriosis prevention and improving aquatic animal health in aquaculture systems. Formalin-inactivated vaccines have been widely utilized in aquaculture in recent decades, showing effective protection against multiple pathogens **(Sommerset *et al.*, 2005; Huang *et al.*, 2014; Liu *et al.*, 2015; Nguyen *et al.*, 2017)**. Moreover, various studies have demonstrated that vaccine-adjuvant combinations can improve protective efficacy against bacterial infections relative to single-component vaccine administration **(Nguyen *et al.*, 2017; Xu *et al.*, 2019)**. Thus, the identification of suitable immunostimulants as adjuvants is crucial for enhancing vaccine-induced immune responses **(Soltani *et al.*, 2014; Labarca *et al.*, 2015)**. Numerous studies indicate that vaccines containing bacterial ECP in conjunction with other bioactive compounds offer enhanced protection against bacterial diseases relative to single-component vaccine formulations **(Arijo *et al.*, 2005; Medina *et al.*, 2015)**. Although many vaccines have been developed for various *Vibrio* species in aquaculture, *Vibrio harveyi* has not been thoroughly addressed among the targeted diseases **(Toranzo, Santos & Barja, 1997)**. The pathogenic mechanisms of *Vibrio harveyi* are significantly influenced by ECP **(Zorrilla *et al.*, 2003)**, highlighting the potential benefits of integrating inactivated ECP as immunogenic elements in vaccine formulations. Previous investigations have shown that ECP-enriched whole-cell vaccines can provide effective protection against various pathogenic vibrios, including *Photobacterium damselae* subsp. *piscicida* **(Magariños *et al.*, 1994a, b; Romalde & Magariños, 1997)**, *V. vulnificus* **(Fouz *et al.*, 2001)**, and *V. alginolyticus* **(Moriñigo *et al.*, 2002)**.

Recent research has identified *Caulerpa lentillifera* as a promising component for vaccine formulations due to its immunostimulant properties and growth-promoting effects in both fish and shrimp species **(Hertika *et al.*, 2023; Hertika *et al.*, 2024)**. This marine macroalga shows considerable potential for improving immune system function via mechanisms triggered by vaccine components, thus facilitating the development of oral

vaccines for grouper species. Chitosan nanoparticles have been widely investigated for vaccine development, serving as adjuvants and antigen carriers owing to their biodegradability, biocompatibility, ability to penetrate intercellular barriers, and strong binding affinity with vaccine components. Chitosan-based delivery systems in aquaculture have been effectively utilized in fish vaccines, showing significant improvements in adaptive immune responses and mucosal immunity enhancement **(Vinay *et al.*, 2018; Gong *et al.*, 2022)**.

The present study investigated the administration of a combined oral vaccine that includes WC and ECP of *Vibrio harveyi*, nano-chitosan, and *Caulerpa lentillifera* extract to improve disease resistance in hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) against *Vibrio harveyi* infection. This study investigated the immunological responses triggered by the vaccine components, utilizing thorough immune profiling and histological examination of gill tissues. This integrated vaccination strategy presents a viable alternative for managing *Vibrio harveyi* outbreaks in hybrid grouper aquaculture. Assessing the effectiveness of the vaccine is essential for tackling significant health issues in the aquaculture sector, with implications for improving bacterial resistance and ultimately increasing productivity and sustainability in aquaculture.

MATERIALS AND METHODS

1. Fish preparation

The cantang giant grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*), measuring 10– 12cm in length, were kept in well-aerated containers under seawater conditions with a salinity of 30– 33ppt and a temperature range of 29– 31°C. The fish underwent a one-week acclimatization period before the experiment started. The fish received a daily allocation of commercial feed, administered twice per day.

2. *Caulerpa lentillifera* extraction

Caulerpa lentillifera extraction was performed following the protocol described by **Hertika *et al.* (2024)**. *Caulerpa lentillifera* was dehydrated in an oven at 60°C, ground into a fine powder, and then sieved using an 80-mesh screen. The powdered *C. lentillifera* was subjected to maceration in ethanol for four days, after which filtration was performed. The ethanol extraction residue underwent aqueous re-extraction in three sessions, each lasting six hours at a temperature of 60°C.

3. Extracellular product (ECP) of *Vibrio harveyi* culture

The culture supernatant of *Vibrio harveyi* was subjected to filtration and heat treatment at 70°C for 30 minutes to isolate the ECP antigen. Sterility verification involved inoculating 100µl of ECP onto nutrient agar (NA) plates, followed by incubation at 25°C for a duration of 24 to 48 hours. Following the confirmation of sterility, indicated by the absence of bacterial growth, ECP preparations were stored at 4°C for subsequent use.

4. Whole cell (WC) of *Vibrio harveyi*

Bacterial suspensions of *Vibrio harveyi* (20mL) from tryptic soy broth (TSB) medium were transferred into 100mL Erlenmeyer flasks. Formalin (2%, 0.4mL) was incorporated and homogenized, then stored at 4°C for 24 hours. Bacterial pellets were harvested and transferred to a total of 10 microcentrifuge tubes after 24 hours. The suspensions underwent centrifugation at 12,000rpm for 5 minutes at 4°C, after which the supernatants were discarded. Bacterial pellets were washed with 300µl of PBS, gently resuspended, and subsequently pooled into the final tube. The final suspension was subjected to centrifugation at 12,000rpm for 5 minutes at 4°C, after which the supernatant was removed and the pellet was resuspended in 400µl of PBS. Preparations were maintained at 4°C prior to utilization.

5. Vaccination and challenge

The giant grouper hybrid (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) utilized in this study measured approximately 12cm and was housed in container boxes with a water capacity of 130 liters. Each container housed 10–12 fish, distributed across five treatment groups over a period of 30 days: (A) *Vibrio harveyi* infection (control), (B) *Caulerpa lentillifera* extract, (C) ECP + nano-chitosan, (D) WC + nano-chitosan, and (E) ECP + WC + nano-chitosan + *Caulerpa lentillifera* extract. The acclimatized fish received vaccination following a 15-minute adaptation period. The vaccine was administered orally, consisting of 0.1ml of antigen mixed with chitosan at a 1:10 antigen-to-chitosan ratio **(Suprapto *et al.*, 1996)**. Infection was induced through intramuscular injection of 0.1ml of *Vibrio harveyi* solution, which contained 10^6 cells/ml **(Bere *et al.*, 2023)**. The procedure is detailed in Fig. (1).

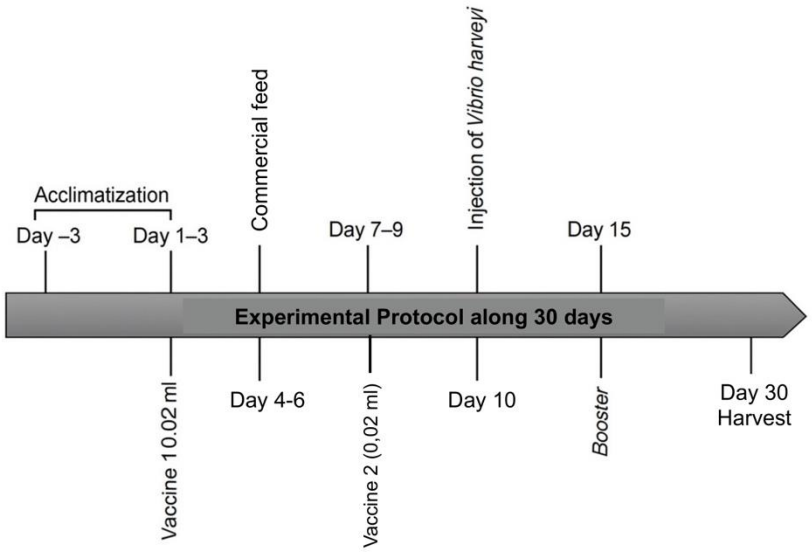

Fig. 1. Vaccination and challenge

6. Hematology analysis

a. Erythrocyte

Erythrocytes were analyzed according to the methodology established by **Blaxhall and Daisley (1973)**. Blood samples from grouper fish were collected using a toma pipette to the 0.5 scale, subsequently mixed with Hayem's solution to the 101 scale, and homogenized. The initial three drops of the blood sample are discarded, after which the sample is placed onto the hemocytometer and covered with a cover glass. Hayem's solution is employed for the enumeration of erythrocytes, which results in the lysis of white blood cells. The calculation for converting the number of erythrocytes is expressed by the formula: Total Erythrocytes = Counted Erythrocytes × 10^6 (cells/mm³).

b. Leukocyte

Leukocytes were analyzed according to **Utami *et al.* (2013)**. Blood samples were collected using a 0.5 leukocyte pipette and subsequently diluted with Turk's solution. Blood is examined under a microscope at 1000× magnification utilizing a hemocytometer. Turk's solution effectively lyses red blood cells, inhibits leukocyte aggregation, and maintains leukocyte integrity for accurate counting. The calculation for converting the number of leukocytes is as follows: Total leukocytes equal Total leukocyte count multiplied by 50 (cells/mm^3).

c. Hemoglobin

The Sahlinometer method determines hemoglobin levels as outlined by **Fangidae *et al.* (2018)**. This procedure involves measuring hemoglobin concentration using a Sahli tube filled with 0.1 N HCl to scale 2, followed by the collection of a blood sample with a Sahli pipette to scale 0.02ml. The blood sample is introduced into the Sahli tube, which contains 0.1 HCl, and is homogenized until a dark brown color is achieved. The blood sample was diluted with distilled water until its color corresponded with the color indicator on the Sahli hemometer, resulting in the measurement of hemoglobin concentration.

d. Differential leukocyte

The method for preparing a differential leukocyte smear, as described by **Davis *et al.* (2008)**, involves obtaining a fish blood sample and creating the smear on a glass slide. The preparation was fixed by immersing it in methanol for 5 minutes, followed by drying after the fixation process. The preparation was incupated in Giemsa solution for 30 minutes, followed by washing and drying post-staining. A microscope with a magnification of 40×10 was utilized for observation purposes. The leukocyte differential is determined by quantifying the numbers of lymphocytes, monocytes, and neutrophils. The calculation for converting the leukocyte differential is performed using the formula: %Cell = (Counted cells / 100) × 100%

7. Phagocytosis analysis

The phagocytosis method described by **Yuniastutik (2019)** involves the collection of 100µL of blood, which is then transferred into an Eppendorf tube. The *V. harveyi* bacteria were prepared at a density of 10^8 and combined with PBS (Phosphate Buffered Saline) in a 1:1 ratio of bacteria to PBS. One hundred microliters of bacteria mixed with PBS were combined with one hundred microliters of blood in the Eppendorf tube. Incubation was conducted based on the specified treatments, at room temperature of 25°C and an incubator temperature of 30°C, for durations of 40 minutes and 50 minutes, respectively, followed by the preparation of a blood smear. The preparation of blood smear specimens requires two clean, grease-free glass slides. The initial object glass serves as a platform for examining the blood drop, while the subsequent object glass is utilized to spread the blood drop, facilitating the formation of a thin layer. The object glass for blood spreading is positioned at a 24-degree angle, with an approximate angle of 45 degrees, and is moved rapidly to create a thin layer. The blood sample is dried at room temperature and fixed with absolute methanol by applying methanol over the entire blood smear, which is then allowed to dry at room temperature. Following drying, the blood smear is stained using Giemsa stain in accordance with the Giemsa and Wright method, a modification of the Romanowsky technique. The phagocytosis activity test during the staining process may utilize a 7% Giemsa stain. Giemsa staining involves applying several drops of Giemsa to the blood smear and allowing it to incubate for 20 minutes. Phagocytosis activity, as described by **Huang *et al.* (2024)**, can be calculated using the formula: Phagocytosis activity (%) = (Number of phagocytic cells performing phagocytosis / Number of observed phagocytic cells) × 100.

8. Histology (gills and stomach)

Histopathological examination was performed as described by **Digambiro and Purwanto (2024)**. The gills of the giant grouper hybrid (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) from each treatment were collected and promptly fixed in 10% neutral buffered formalin; dehydration was performed using ascending grades of ethanol. The specimens were subsequently cleared using two changes of xylene. Following the application of soft paraffin for blocking, serial sections with a thickness of 4µm were prepared. The percentage of tissue damage was calculated by dividing the number of damaged tissue areas by the total number of analyzed tissue areas, and this value was expressed as a percentage. The calculation was derived from observations conducted with OlyVIA 2.9.1 software (Olympus, USA), employing a single field of view instead of direct microscopic examination. The field of view was divided into multiple grid squares, with squares showing damage identified and quantified as damaged tissue. The total number of analyzed tissue squares encompassed both damaged and undamaged regions within the

field of view. The sections were stained with hematoxylin-eosin, and the calculation of damage was performed using the following formula:

$$\text{Histology damage (\%)} = \frac{\text{The total (square) damage of tissue}}{\text{The total (square) of tissue}}$$

9. Data analysis

Data were expressed as mean ± standard deviation (SD). The differences among three or more groups were assessed for statistical significance using one-way analysis of variance (ANOVA), followed by least significant difference (LSD) post-hoc testing. Statistical significance was established at $P < 0.05$. Analyses were conducted using SPSS for Windows (Version 20.0, SPSS Inc., Chicago, USA), while graphical representations were created with GraphPad Prism 7 (GraphPad Software, Inc., USA).

RESULTS

1. Hematology analysis

The results of the hematological profile analysis (erythrocyte, leukocyte, and hemoglobin) for the hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) treated with extracellular product (ECP), whole cell (WC) from *Vibrio harveyi*, and *Caulerpa lentillifera* extract based on nano-chitosan as distinct oral vaccine formulations are illustrated in Fig. (2). Variations in the hematological parameters of the cantang grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) were observed after infection with *Vibrio harveyi*, indicating differing immunostimulatory responses among the treatment groups. Significant enhancements across all parameters were observed in fish treated with *Caulerpa lentillifera* extract combined with nano-chitosan under the whole cell (WC) challenge condition. The erythrocyte counts in this group reached a significantly elevated mean of approximately 290 cells/mm³, suggesting an improved capacity for oxygen transport. Simultaneously, leukocyte levels increased significantly to approximately 280 cells/mm³, indicating an enhanced innate immune response. Hemoglobin concentrations demonstrated a significant increase, with the peak value approaching 11%, reflecting enhanced blood quality and overall physiological condition. Compared to the control and other treatment groups, which included those receiving the extract alone, bacterial challenge (either ECP or WC) alone, or combinations excluding nano-chitosan, only the WC + extract + nano-chitosan group consistently showed statistically significant ($P< 0.05$) improvements across all hematological indicators. The findings highlight the synergistic potential of *C. lentillifera* extract and nano-chitosan as an effective immunostimulatory agent, promoting hematopoietic function and enhancing disease resistance in *V. harveyi*-infected grouper.

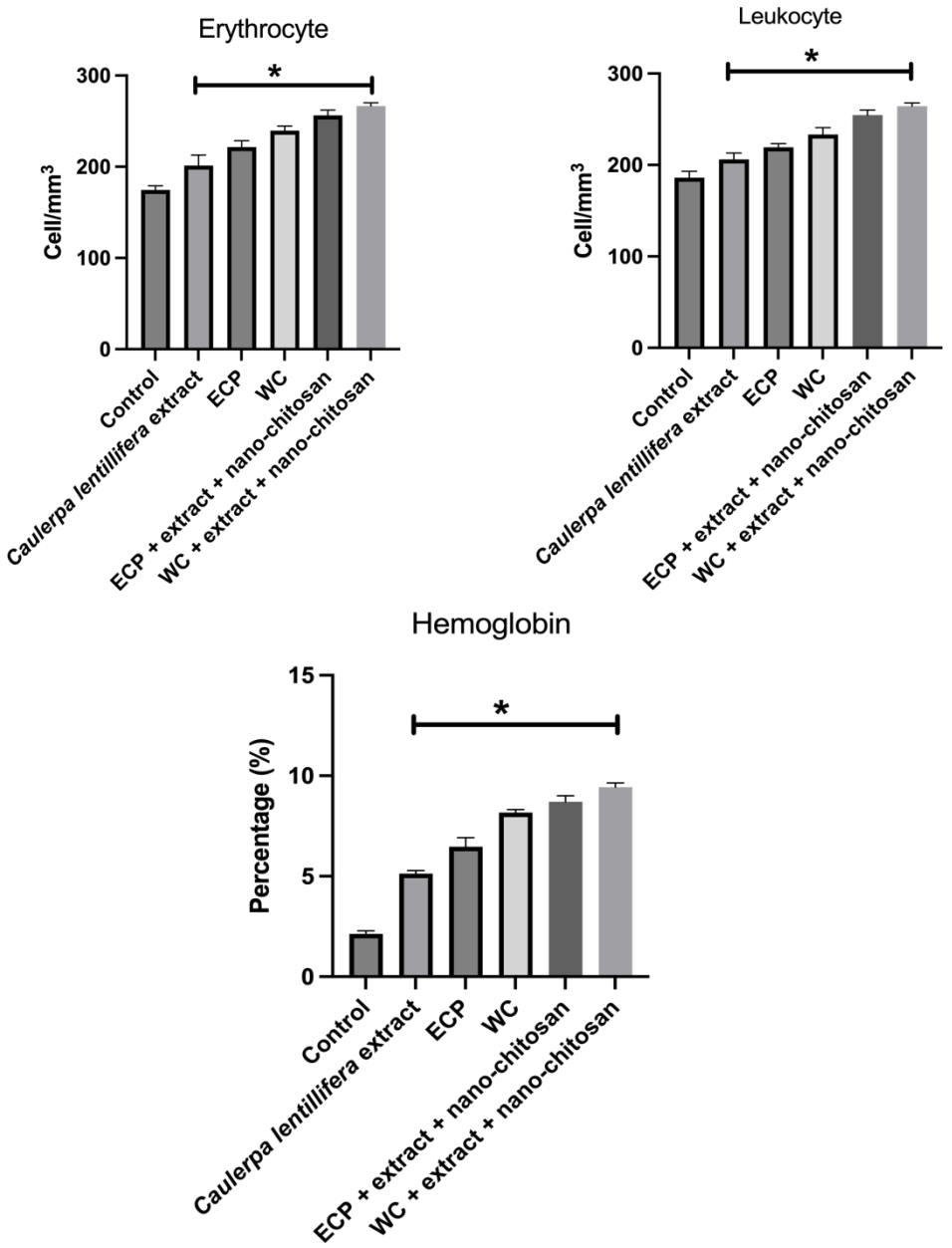

Fig. 2. Hematology analysis (erythrocyte, leukocyte, and hemoglobin) of hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) against *Vibrio harveyi* induced by various oral vaccine formulations—comprising nano-chitosan-based extracellular products (ECP), whole cells (WC) of *Vibrio harveyi*, and *Caulerpa lentillifera* extract. The data are expressed as mean±SD, with **P*<0.05 statistically significant

Leukocyte profiling in hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) after oral vaccination and subsequent challenge with *Vibrio harveyi* demonstrated significant immunomodulatory effects in all leukocyte subsets (Fig. 3). The administration of vaccine formulations containing nano-chitosan-encapsulated extracellular products (ECP), whole cells (WC) of *V. harveyi*, and *Caulerpa lentillifera* extract led to notable

increases in leukocyte proportions. Neutrophil counts rose significantly, from 11.6% in the control group to 45.6% in fish treated with the combined WC + extract + nano-chitosan formulation, suggesting enhanced innate immune activation. A similar trend was noted in basophils, increasing from 2.8 to 13.2%, indicating the activation of pro-inflammatory or hypersensitivity-related pathways. Eosinophil levels increased from 6.5% in controls to 20.4% in the treatment group, indicating an enhanced capacity to respond to extracellular pathogens. The percentage of monocytes, associated with phagocytic and antigen-presenting functions, rose from 8.2% in control subjects to 31.8%, highlighting a significant cellular immune response. Lymphocyte levels, indicative of adaptive immunity, increased significantly from 4.2% to 15.6%. Moderate increases were observed in groups receiving ECP alone, WC alone, or combinations that included *C. lentillifera* extract or nano-chitosan, indicating a synergistic immunostimulatory effect resulting from the integration of bacterial antigens, algal bioactives, and nanocarrier systems. The findings indicate that the WC + extract + nano-chitosan formulation is the most effective oral vaccine candidate, capable of inducing a comprehensive leukocytic response that includes both innate and adaptive immune pathways in hybrid grouper challenged with *V. harveyi*.

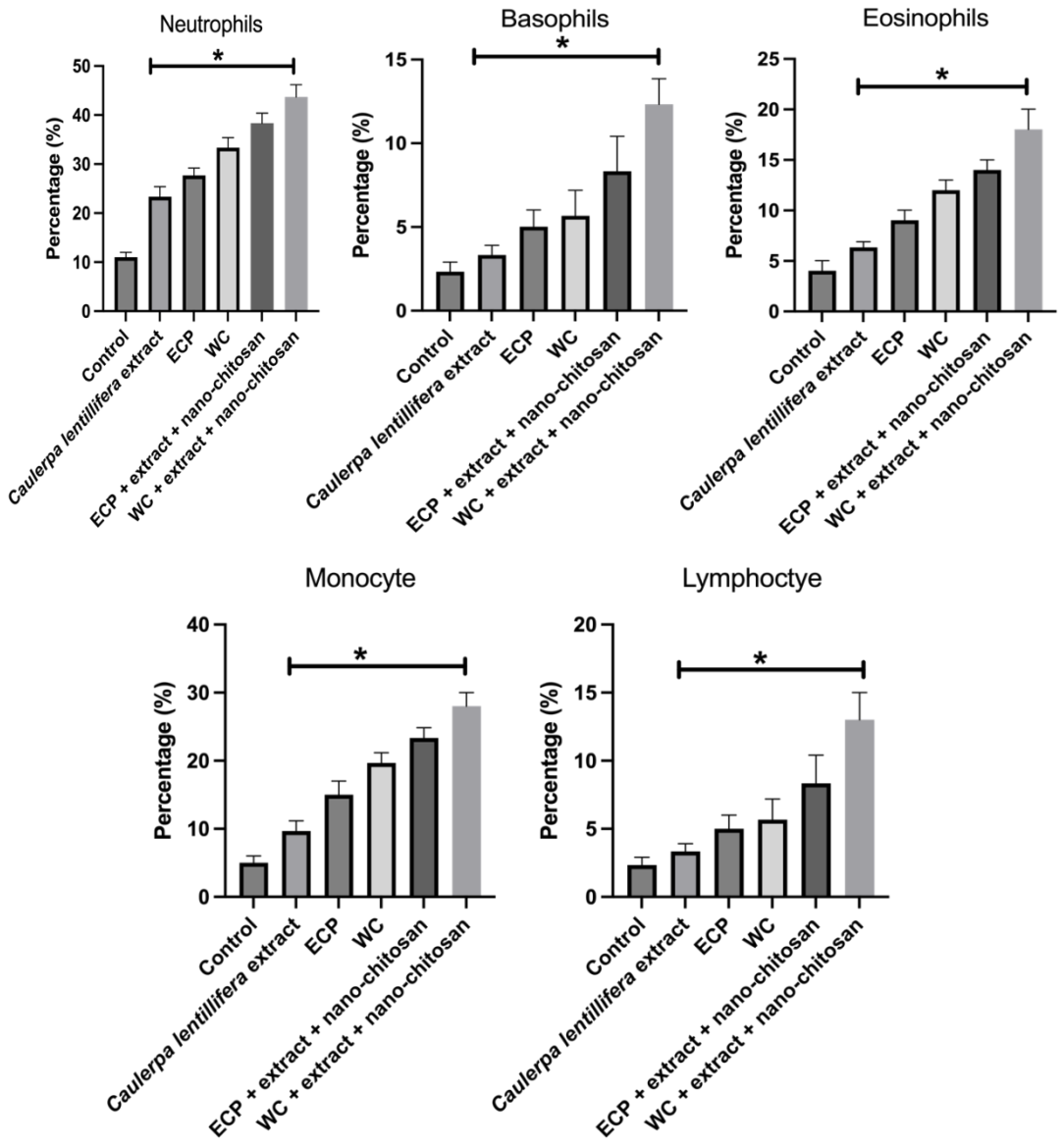

Fig. 3. Differential leukocyte analysis of hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) against *Vibrio harveyi* induced by various oral vaccine formulations—comprising nano-chitosan-based extracellular products (ECP), whole cells (WC) of *Vibrio harveyi*, and *Caulerpa lentillifera* extract. The data are expressed as mean±SD, with **P*<0.05 statistically significant.

2. Phagocytosis analysis

The phagocytic activity of hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) showed a progressive increase across treatments with different oral vaccine formulations, including nano-chitosan-based extracellular products (ECP), whole cells (WC) of *Vibrio harveyi*, and *Caulerpa lentillifera* extract, as illustrated in Fig. (4). The control group exhibited the lowest phagocytic percentage (23.4 ± 2.1%), indicating

baseline innate immune function. The administration of *C. lentillifera* extract alone resulted in an increase in phagocytic activity to 32.8 ± 2.7%, indicating a modest immunostimulatory effect due to algal bioactives. Fish treated with ECP alone demonstrated an increase to 39.6 ± 3.4%, suggesting the activation of innate immune responses by bacterial extracellular antigens. The administration of WC resulted in a significant increase (48.7 ± 2.9%), likely attributable to the presence of intact pathogen-associated molecular patterns that effectively engage phagocytic receptors. The ECP + extract + nano-chitosan group exhibited a phagocytic rate of 61.2 ± 3.1%, indicating additive effects from bacterial antigens, algal-derived immunomodulators, and the delivery of nano-chitosan. The WC + extract + nano-chitosan group exhibited the highest activity at 68.4 ± 2.5%, significantly surpassing all other treatments ($P< 0.05$), as denoted by the asterisk, highlighting a synergistic enhancement of innate immune function. The findings indicate that the WC + extract + nano-chitosan formulation significantly enhances phagocytic capacity in hybrid grouper, suggesting its potential as an effective oral vaccine candidate for protection against *V. harveyi* in aquaculture

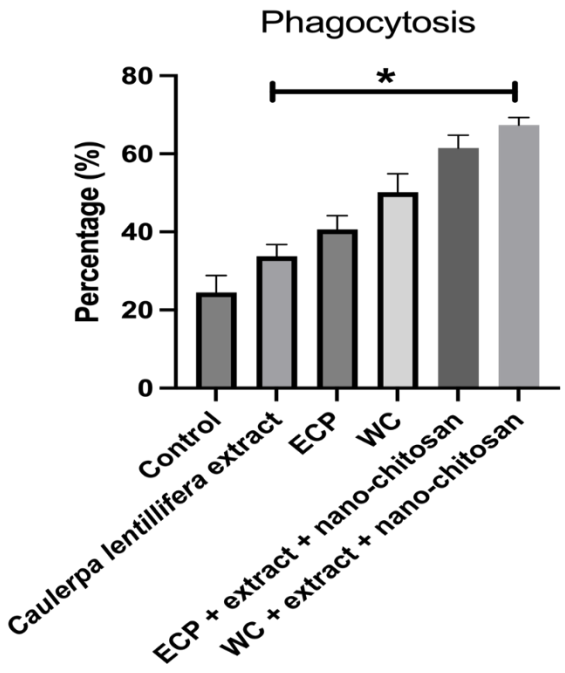

Fig. 4. Differential leukocyte analysis of hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) against *Vibrio harveyi* induced by various oral vaccine formulations—comprising nano-chitosan-based extracellular products (ECP), whole cells (WC) of *Vibrio harveyi*, and *Caulerpa lentillifera* extract. The data are expressed as mean±SD, with *P<0.05 statistically significant.

3. Histology of gill of hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*)

Based on the results of the Fig. (5), histopathology gills are detected from the combination treatment of ECP + WC + nano-chitosan + *Caulerpa lentillifera* extract on grouper fish infected with *V. harveyi* bacteria. The highest number of histopathology gills was significantly observed in treatment (E). The cohort of *Caulerpa lentillifera* extract exhibited a modest enhancement in histopathology gills, but the ECP + nano-chitosan therapy indicated further improvement. The greatest treatment (E), was recorded in the ECP + WC + nano-chitosan + *Caulerpa lentillifera* extract group, which exhibited a significant difference from the control group (P<0.05).

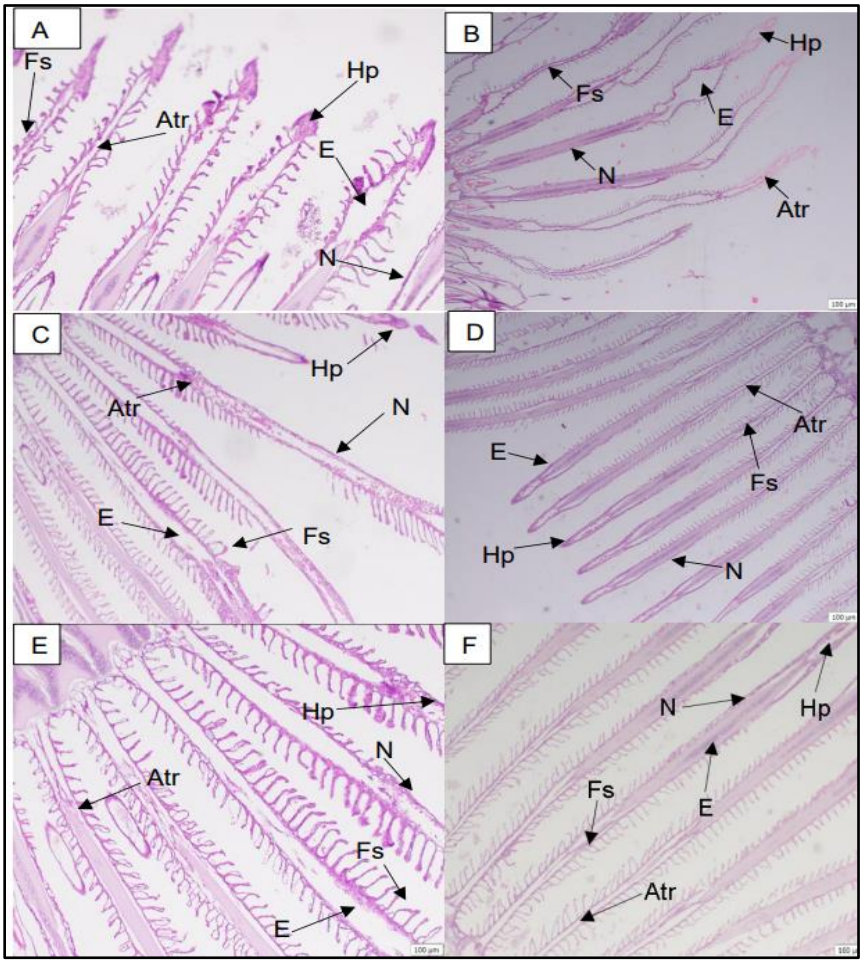

Fig. 5. Analysis of histology in the structure of gill tissue in of hybrid grouper induced by oral vaccine and *Caulerpa lentillifera* extract againts *Vibrio harveyi*. a) *Vibrio harveyi* infection (control); b) *Caulerpa lentillifera* extract; c) ECP + nano-chitosan; d) WC + nano-chitosan; e) WC + nano chitosan + Caulerpa lentillifera extract. (N) Necrosis, (HP) Hypertropy, (FS) Fussion, (Atr) Atrophy, (E) Edema. (400x magnification, Microscope Olympus TL4).

The bacterial infection caused by *Vibrio harveyi* poses a significant threat to the cultivation of hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*), leading to considerable physiological disturbances, particularly in respiratory organs like the gills. Histopathological examinations of gills are essential for evaluating tissue damage resulting from bacterial infections and for assessing the efficacy of an oral vaccine derived from *Caulerpa lentillifera* extract, augmented with nanochitosan and combined with extracellular product (ECP) and whole cell *Vibrio harveyi*. The image below illustrates the histological structure of the gills of hybrid grouper fish exposed to various treatments. Several histopathological parameters, including secondary fusion (Fs), necrosis (N), hyperplasia (Hp), edema (E), and lamellar atrophy (Atr), were assessed to evaluate the tissue response to vaccination.

Table 1. Scoring evaluation of gill tissue damage in hybrid grouper induced by oral vaccine and *Caulerpa lentillifera* extract against *Vibrio harveyi* infection

Treatment	Test	Edema	Artropi	Hiperplasia	Nekrosis	Fusi	Total (%)	Average
A	1	49	3,2	2,7	5,51	0,64	61,05%	12,21
	2	37,43	4,01	2,67	13,63	1,2	58,94%	11,79
	3	30,66	2,7	3,38	16,90	1,14	54,78%	11
B	1	13,80	2,38	4,60	18,39	0,79	39,96%	8
	2	15,80	1,88	5,53	16,20	1,25	40,66%	8,1
	3	12,96	2,05	3,73	18,52	0,95	38,21%	7,64
C	1	14,71	2,1	3,23	15,21	1,2	36,45%	7,29
	2	13,26	1,7	4,71	13,26	0,73	33,66%	6,73
	3	12,01	1,25	3,43	13,35	1,18	31,22%	6,24
D	1	14,95	2	6,43	4,09	1,75	29,22%	5,8
	2	17,80	1,05	3,58	3,26	1,21	27,24%	5,4
	3	15,81	3,46	4,17	2,40	1,50	27,27%	5,45
E	1	12,95	2	4,43	2,09	1,75	23,22%	4,64
	2	14,80	1,05	3,58	3,26	1,21	23,90%	4,78
	3	13,81	1,46	2,17	2,40	1,50	21,34%	4,26
F	1	10,95	2	4,43	2,09	1,75	21,22%	4,24
	2	12,80	1,05	3,58	3,26	1,21	21,59%	4,38
	3	10,81	1,46	2,17	2,40	1,50	18,34%	3,66

This analysis aims to elucidate the effectiveness of the oral vaccine in augmenting fish resistance to *Vibrio harveyi* infection and enhancing the histological condition of the gills. Histopathological analysis of the gills was performed to assess the efficacy of the oral vaccine in improving the immune response of grouper fish against *Vibrio harveyi* infection. This table outlines histological parameters, including edema, atrophy, hyperplasia, necrosis, and lamellar fusion, utilized to evaluate the extent of gill tissue damage resulting from infection. The results indicate variations in damage levels across treatments, reflecting the physiological response of the fish to infection and the efficacy of the administered vaccine. Certain treatments demonstrated reduced damage levels, suggesting the vaccine's potential to effectively bolster the immune response against *Vibrio harveyi* infection. The scoring results are displayed in Table (1).

Histopathological scoring of gill tissue in hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) challenged with *Vibrio harveyi* demonstrated significant variations in lesion severity across treatments utilizing different oral vaccine formulations, including nano-chitosan-based extracellular products (ECP), whole cells (WC) of *V. harveyi*, and *Caulerpa lentillifera* extract (Table 1). The control group (Treatment A) displayed the most significant pathological alterations, recording an average cumulative damage score of 12.21 and a total lesion percentages of 61.05, 58.94, and 54.78% across three replicates, respectively. The lesions exhibited significant edema (up to 49%), pronounced necrosis (up to 16.90%), moderate hyperplasia (2.67–3.38%), and lamellar fusion (0.64–1.20%), indicating acute and extensive tissue degeneration in the absence of immunoprophylaxis. The administration of *C. lentillifera* extract alone (Treatment B) resulted in an average total damage reduction to 8.10, with lesion percentages recorded at 40.66, 38.21, and 36.28%, respectively. The observed attenuation was mainly linked to decrease edema (13.80–15.80%) and necrosis (16.20–18.53%), indicating that the bioactive compounds in the extract provide partial structural protection to the gills. The administration of ECP (Treatment C) resulted in a further reduction of average lesion severity to 6.73, with total damage percentages recorded at 33.66, 31.22, and 29.22%. Additionally, there was a decrease in necrosis, ranging from 13.53 to 14.93%, and slight reductions in edema were observed. The administration of WC (Treatment D) resulted in a lower average damage score of 5.40, with lesion percentages of 27.24, 27.27, and 23.90%. Necrosis significantly decreased to 2.26–3.26%, suggesting robust protective effects facilitated by intact bacterial antigens that activate innate immunity. The ECP + extract + nano-chitosan formulation (Treatment E) demonstrated enhanced efficacy, yielding an average score of 4.26 and lesion percentages of 21.32, 21.29, and 21.34%. This formulation consistently suppressed necrosis (2.09–2.80%) and hyperplasia. The most significant protection was noted in the WC + extract + nano-chitosan group (Treatment F), which exhibited the lowest average damage score of 3.66 and lesion percentages of 21.59, 20.54, and 18.34%. This group exhibited consistently low levels of necrosis (2.26–2.80%), reduced edema (10.81–12.80%), and the lowest hyperplasia scores (2.17–3.58%). These findings highlight the synergistic protective effects of bacterial antigens, algal bioactives, and nano-chitosan-mediated antigen delivery in maintaining gill structural integrity. The findings collectively indicate that the WC + extract + nano-chitosan formulation is the most effective in mitigating *V. harveyi*-induced gill pathology in hybrid grouper, thereby supporting its potential as an oral vaccine strategy in aquaculture.

DISCUSSION

At present, there are no commercially viable vaccines for fish that specifically target *V. harveyi* infections. Multiple investigations have demonstrated that inactivated *V. harveyi* may confer immunological protection to fish **(Zheng *et al.*, 2012; Nguyen *et al.*, 2017; Xu *et al.*, 2019)**. Additionally, numerous new vaccines for aquaculture are under development, focusing on safety, long-term protection, and cost-effectiveness **(Zhang *et***

***al.*, 2020)**. The development of oral vaccines for grouper aimed at *Vibrio harveyi* represents a key focus in current aquaculture research. Innovative approaches utilizing nano-chitosan technology, a biocompatible polymer, have emerged as a viable method for oral vaccine delivery, owing to its biodegradability and low toxicity. **Li *et al.* (2013)** and **Wu *et al.* (2020)** demonstrated the effectiveness of chitosan nanoparticles in oral vaccine delivery for fish, indicating an enhanced immune response to *Vibrio harveyi*. **Zhang *et al.* (2020)** demonstrated the effectiveness of this method in protecting fish species, such as the orange-spotted grouper. **Jazayeri *et al.* (2020)** examined the use of *Vibrio harveyi* extracellular products (ECPs) in nanoparticle-based oral vaccines, demonstrating increased specific antibodies and immunological markers in fish. In this study showed the absolute profile hemotology of fish was the highest in the ECP +WC + nano-chitosan + *Caulerpa lentillifera* extract group, showing a significant difference (P<0.05) compared to other groups. The control group (infected with *Vibrio harveyi* without treatment) exhibited the lowest profile hematology, indicating that the infection negatively impacted fish development. Other treatments, including *Caulerpa lentillifera* extract alone and ECP +WC + nano-chitosan, resulted in moderate erythrocytes, leukocytes, and hemoglobin improvements. A similar trend was observed in the differential leukocyte and phagocytosis. The highest differential leukocyte and phagocytosis was recorded in the ECP + WC + nano-chitosan + *Caulerpa lentillifera* extract group, significantly differing from the control (P<0.05). Other treatments improved differential leukocyte and phagocytosis compared to the control, but the highest enhancement was seen upon combining all three components (ECP, WC, nano-chitosan, and *Caulerpa lentillifera* extract). This suggests that this combination provides the best differential leukocyte and phagocytosis promoting effects in infected fish. Many studies show that whole cell vaccines play a significant role in inducing an immune response and increasing the resistance to diseases in the host´s system **(Nguyen *et al.*, 2017; Huang *et al*., 2019; Wei *et al.*, 2020; Mohamad *et al.*, 2021; Strem *et al.*, 2023)**, as it was demonstrated in this research. The significant involvement of extracellular products (ECP) in the pathogenesis of this microbe has been shown **(Zorrilla *et al.* 2003)**, making it pertinent to consider including inactivated ECPs of this species into a prospective vaccine. Prior researchers have documented the efficacy of ECP-enriched whole-cell vaccines against *P. damselae* subsp. *piscicida* **(Magariños *et al.* 1994a, b; Romalde & Magariños 1997)**, *V. vulnificus* **(Fouz *et al.*, 2001)**, and *V. alginolyticus* **(Morin˜igo *et al.*, 2002)**. **Rajan *et al.* (2023)** investigated the immunostimulatory effects of oligosaccharides, demonstrating enhanced specific and non-specific immune responses in pearl grouper. **Lin *et al.* (2023)** examined the efficacy of Astragalus and Ganoderma lucidum polysaccharides in augmenting immunity associated with the *Vibrio harveyi* DNA vaccine. **Liu *et al.* (2020)** created polymer microparticles for the encapsulation of the pleurocidin peptide and recombinant protein, demonstrating the ability to elicit protective immunity against *Vibrio harveyi* in grouper fish. **Huang *et al.* (2019)** investigated anti-idiotype vaccines derived from antibodies that replicate the epitopes of *Vibrio harveyi*, demonstrating potential in

conferring protective immunity in grouper fish. **Strem *et al.* (2023)** and **Wan *et al.* (2023)** examined the amalgamation of formalin-killed *Vibrio harveyi* cells with chitosan oligosaccharides as a potent approach to augment vaccination efficacy. Subsequent study has disclosed advancements in vaccine creation. **Nguyen *et al.* (2018)** created a vaccine including recombinant outer membrane proteins of *Vibrio harveyi*, exhibiting an augmented immune response in orange-spotted grouper. **Chuang *et al.* (2014)** discovered that outer membrane proteins, including glyceraldehyde-3-phosphate dehydrogenase, induced protective immunity against *Vibrio harveyi*. **Yanuhar *et al.* (2012)** found immunogenic proteins of *Vibrio harveyi* that stimulate interleukin-4 synthesis in grouper, hence eliciting an immunological response. **Suprapto and Sukmawati (2010)** demonstrated that whole cell *Vibrio alginolyticus* serves as an oral vaccine to mitigate high mortality rates in the grouper larvae. **Arijo *et al.* (2008)** established that the extracellular product from *Vibrio harveyi* can elicit an immunological response in fish, underscoring its potential as a vaccine candidate. The toxicity of extracellular compounds derived from *Vibrio harveyi* has been evaluated on hybrid grouper fish larvae. The vaccination combination presents a viable approach to augment the immune response and confer robust protection against this virus in grouper fish. Studies have investigated the use of immunostimulants to enhance vaccine efficacy in aquaculture. In additional, *Caulerpa lentillifera* has been demonstrated to enhance the immune system, including hematological parameters and phagocytosis, in both fish and shrimp **(Hertika *et al.*, 2023; Hertika *et al.*, 2024)**. It provides a comparable outcome to another investigation. Numerous studies have examined the diverse immunological responses in organisms from *Caulerpa* sp. **Yuniarti *et al.* (2015)** observed an elevation in total leukocytes, monocytes, lymphocytes, neutrophils, macrophages, and phagocytic activity following induction by *Caulerpa racemosa* in gourami. Moreover, *Caulerpa lentillifera* enhanced growth in shrimp **(Putra *et al.*, 2019)** and milkfish **(Putra *et al.*, 2021)** via the incorporation of extracts into their meal.

The combination of ECP, WC, nano-chitosan, and *Caulerpa lentillifera* extract demonstrates the highest efficacy in mitigating histopathological damage to the gills of the cantang grouper. This part delineates the findings of the research and engages in a comprehensive debate. Concerning vaccine safety for fish, a prior histological investigation conducted within 0–3 months post-vaccination of trout with 0.1µg pIHNw-G indicated only temporary damage to muscle tissue linked to needle injection, and by 90 days, no significant difference was observed between vaccinated and mock-vaccinated fish. Elevated dosages of 50µg pIHNwG **(Garver *et al.*, 2005)** or 20µg of the VHS-G DNA vaccine **(Lorenzen *et al.*, 2005)** induced a significant infiltration of inflammatory cells at the injection site, peaking two to four weeks post-vaccination and subsequently diminishing. Based on these findings, histological examinations were conducted solely on tissues from fish at the most recent time point to evaluate any long-term effects resulting from pIHNw-G vaccination. Analysis of fish two years post-vaccination indicated that

intramuscular administration of 0.1µg IHNV DNA vaccine did not seem to cause enduring harm to muscle tissue. This is unsurprising, given the significant regenerating capacity of fish skeletal muscle documented by various authors **(Mitial *et al.*, 1974; Anderson & Roberts, 1975; Dutta & RAJ, 1994; Unguez *et al.*, 1998)**. The lack of systemic histopathological damage in the various tissues analyzed, along with the consistent protection against viral challenge, indicates that the expression of the IHNV G protein from the low dose of IHNV DNA vaccination utilized in these investigations is both safe and efficient for fish.

CONCLUSION

In conclusion, this study presents compelling evidence that the ECP + WC + nano-chitosan + *Caulerpa lentillifera* extract formulation improves hematological parameters (erythrocytes, leukocytes, and hemoglobin), differential leukocyte counts, phagocytosis, and gill histopathology, thereby establishing it as a promising oral vaccine candidate for the management of *Vibrio harveyi* infection in hybrid grouper. These findings possess potential uses for enhancing aquaculture sustainability and fish health management.

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