Therapeutic potential of *Allium sativum* in hemato-immunological responses and disease resistance in *Labeo rohita* against *Aeromonas hydrophila*

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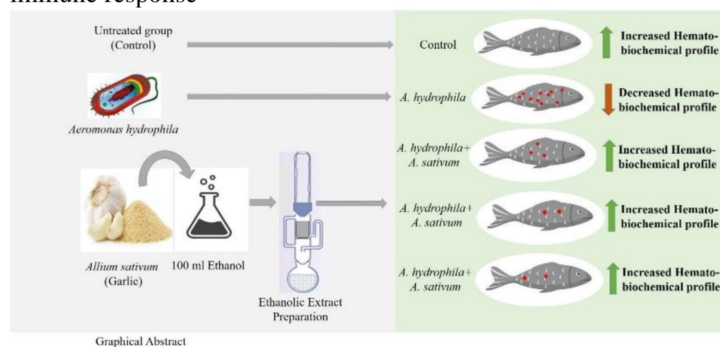
Abstract- Medicinal plants offer effective substitute to chemotherapeutic agents, particularly antibiotics. The study was conducted to evaluate the antibacterial efficacy of ethanolic extract of *Allium sativum (A. sativum)* against *Aeromonas hydrophila* (*A. hydrophila*) infection in *Labeo rohita* (*L. rohita*). Three different concentrations of *A. sativum*, 4 g/kg, 8 g/kg, and 12 g/kg were employed against *A. hydrophila* challenge. Fish (30±5g) were divided into six groups; Control, *A. hydrophila* challenged, *A. hydrophila* + *A. sativum* (4 g/kg), *A. hydrophila* + *A. sativum* (8 g/kg), *A. hydrophila* + *A. sativum* (12 g/kg) and *A. hydrophila* + Erythromycin. Herbal supplements were given for 14 days and sampling was performed 7 and 14 dpi (days post infection). Significant variations among hematological, biochemical and immune activities were observed between control and treated groups. When compared within the challenged groups, pathological symptoms were lessened in the *A. hydrophila* challenged+extract treated groups than the untreated infected fish. Interestingly, medium and high concentrations (8g/kg and 12g/kg) were effective to combat bacterial challenge. Moreover, after infection better fish survival was noted in extract treated groups as compared to untreated groups. Our study presents that herbal supplementation boosts-up fish immune system for better disease resistance against infection.

Index Terms- *Allium sativum*, antibacterial activity, characterizations, ethanolic extract, herbal supplementation, immune response



Graphical Abstract

I. Introduction

The intensive culture of food fish has led to outbreaks of various bacterial diseases, resulting in annual economic losses to the aquaculture industry. Carp culture faces an alarming situation due to pathogenic challenges resulting in increased infection rate and mass mortalities owing to loss of immune function. Aeromonas Septicaemia caused by *A. hydrophila* cause around 90% mortality during an outbreak [1]. The most common symptoms associated with *A. hydrophila* are loss of appetite, sores around the mouth, skin haemorrhages and lesions [2].

Antibiotics pose a serious global problem in aquafarming as they result in the production of antibiotic-resistant bacterial pathogens in fish species. In view of these undesirable effects caused by the antibiotics, there is an urgent need for eco-friendly, disease-preventive measures to promote sustainable aquaculture. During the last decade, attention has been increasingly focused on the use of herbal-based immunostimulants for fish disease control, particularly for those that elevate a nonspecific defence mechanism activating early protection against infections [3].

Eco-friendly alternatives for therapeutic and prophylactic purposes in health management of aquatic animals are therefore of high priority, as they meet both environmental and societal demands. The active molecules contained in plants, such as alkaloids, terpenoids, saponins and flavonoids may provide inhibitory activity against pathogenic microorganisms [4]. *A. sativum* (garlic) is an aromatic herbaceous plant with a long history of use in traditional medicines. Its medicinal properties are attributed to various bioactive compounds, particularly organosulfur compounds, such as thiosulfanates (R-S-S (O)-R), which play a significant role in health benefits. *A. sativum* possesses antimicrobial, hypolipidemic, antihypertensive, hepatoprotective, insecticidal, antibacterial and antifungal properties [5]. In addition to potential antibacterial activity, plant extracts may improve the non-specific immunity of several cultured fish, as well as the digestibility and availability of nutrients, thereby resulting in an increase in hemato-biochemical and enzymatic activity in plant supplemented fish [6].

Extensive research has been conducted on herbal supplementation to combat various fish infections, enhancing their health and resilience. However, there are fewer studies specifically addressing the optimal dosage, identification of bioactive compounds, and the long-term effects of *A. sativum* extract on fish health and performance, particularly in maintaining consistent antibacterial efficacy. The purpose of this investigation was to design the antibacterial potential of *A. sativum* in an *in-vivo* assay. The principal active compounds were identified by HPLC, FTIR and UV-Vis analysis. Hemato-biochemical and immune activities of treated groups were analyzed.

II. MATERIALS AND METHODS

2.1. Preparation of *A. sativum* extract

Fresh *A. sativum* weighed 700g collected from Kasur, Pakistan were washed with distilled water, peeled off and dried under shade for 4 weeks. Dried *A. sativum* were crushed to make fine powder using electric grinder. Ethanolic extract was prepared by soxhlet apparatus using *A. sativum* powder and ethanol for 24 hours. Liquid extract was filtered using whatmann filter paper. The extract obtained was air-dried and stored until required (Figure 1).

2.2. Experimental Design

L. rohita juveniles (30±5g) were obtained from the Fisheries Training and Research Institute Manawa, Lahore, and were acclimatized to the laboratory conditions in 60L glass tanks for 2 weeks and transferred into well aerated glass aquariums (VENUSAQUA, AP-308A). Fish were fed with a commercial feed at the rate of 3% of body weight for 2 times daily. The composition of commercial feed was Crude protein 30%, Fat 3.5%, Ash 20.8%, Crude fat 7.5% and Moisture 10% (Hi-tech Aqua Feeds, Pvt. Ltd, Gujranwala, Pakistan). Almost 20% of the tank water was exchanged on daily basis. Basic physiochemical parameters of the water were monitored during the study as follows; Temperature ranged between 27-30 °C, Dissolved oxygen 5.7- 7.7 mg/L, pH 6.8-7.4 and Ammonia concentration 0.1- 0.29 mg/L.

2.3. Treatment groups

A. sativum extract was supplemented in commercial feed at three different concentrations such as; low (4g/kg), medium (8g/kg) and high (12g/kg). Fish were divided randomly (*n*=15 fish per aquarium) into six groups; Control, *A. hydrophila* challenged, *A. hydrophila* + *A. sativum* (4g/kg), *A. hydrophila* + *A. sativum* (8g/kg), *A. hydrophila* + *A. sativum* (12g/kg) and *A. hydrophila* + Erythromycin. A 14 days trial was conducted (triplicates) and fish were intramuscularly injected with *A. hydrophila* infection.

2.4. Pathogen challenge

Bacterial (*A. hydrophila*) culture was prepared in nutrient broth (Himedia, Ltd, Lahore, Pakistan) containing essential ingredients necessary for optimum bacterial growth and sterilized by autoclaving at 121°C for 15-20 minutes. *A. hydrophila* strain inoculated in the culture and then incubated overnight in shacking incubator at 200 rpm for growth. For enumeration, serial dilution was prepared in 1ml sterile phosphate buffer saline (PBS) to an optical density (OD) of approximately 0.5. The OD was measured at wavelength of 365 nm by using spectrophotometer. Bacterial dose was prepared in PBS for intramuscular injections at a rate of 1×10^7 CFU/ml [7].

2.5. Characterizations

2.5.1. High Performance Liquid Chromatography (HPLC) Analysis

Phenolic standard of interest e.g., gallic acid was used for quantification of phenolic compound in *A. sativum* extract. The standard solution was prepared in methanol (1mg/ml). Chromatography was performed with a 4.6 mm × 250 mm i.d., 5 µm Agilent plus C18 column. The injection volume was 20 µL and temperature was kept at 25°C. Mobile phase A was water and B was methanol. The procedure was as follows: 0–30 minutes, B was 5–100%; 30–40 minutes, B was 100%; 40–50 minutes, B was 5% at a flow rate of 0.8 mL/min, 254nm. The flow rate of the drying gas was 10.0 L/min and nitrogen was used as the collision gas.

2.5.2. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed for the identification of functional groups using FTIR spectrophotometer (IRTracer-100, Shimadzu, Japan) at a resolution of 4 cm^{-1} and scan range of 500-4000 cm^{-1}.

2.5.3. Ultraviolet visible spectrophotometry (UV-Vis)

The optical properties of *A. sativum* were characterized by UV-Vis spectrophotometry (Shimadzu, UV-1800, Kyoto, Japan).

2.6. Blood Collection

To collect blood samples, fish were anesthetized using clove oil (100 µg/L) (Qarshi limted, Pakistan) for 40-60 sec. Fish samples were obtained randomly from each group. Samples were collected on 7 and 14 dpi. Blood was collected from caudal vein and centrifuged at 4000 rpm (PRO-ANALYTICAL.C1015) for ten minutes. The obtained serum (supernatant) was used for further analysis.

2.7. Hematological Assays

To collect blood samples, fish were anesthetized using clove oil (100 µg/L) for 40-60 sec (Qarshi limted, Pakistan) and samples were collected randomly from each group (*n*=5). Samples were obtained at 7 and 14 dpi and stored in EDTA (ethylenediaminetetraacetic acid) coated tubes. Level of red blood cells count (RBCs), white blood cells count (WBCs), hemoglobin (Hb), hematocrit (Hct%), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were measured using an automated blood analyzer (Sysmex KX-21, Kobe, Japan).

2.8. Serum Biochemical Analysis

Blood was allowed to coagulate and afterward it was centrifuged at 4000 rpm (C1015 Micro Prime Centrifuge, Pocklington, UK) for 10 min at 4 °C. The obtained serum (supernatant) was used for the analysis of enzymes and serum proteins. ALP level was measured by LAB Kit (REF 30133, Canovelles, Spain), AST and ALT activities were determined by using Spectrum Kits (REF 291005, Ismailia, Egypt) and (REF 292005, Ismailia, Egypt), respectively. Total protein was analyzed by using Crescent Diagnostics Kit, Cat. No. CS.610. (Jeddah, Saudi Arabia). Albumin was estimated by ARENA Bio Scien Kit (BS.1AL02.10.0200, Ismailia, Egypt), Globulin was checked by deducting the albumin from total protein and A/G ratio was obtained by dividing albumin from globulin.

2.9. Immune Analysis

Myeloperoxidase (MPO) assay was performed by using, MPO ELISA BT-LAB kit (Cat. No. E0880Hu, Birmingham, England). The Respiratory Burst Activity (RBA) was assessed by reduction of nitroblue tetrazolium (NBT) to formazan as described by Biller-Takahashi et al. [8]. Serum Lysozyme Activity (SLA) was determined by the turbidimetric assay [9].

2.10. Statistical Analysis

Data was statistically evaluated by using Graph Pad Prism Software (Version: 9.4.1, San Diego, USA). The results were expressed as mean±SEM and analyzed using two-way analysis of variance (ANOVA), followed by Tukey's comparison analysis. Results with $p < 0.05$ or lower were considered statistically significant.

III. RESULTS

3.1. Characterizations

3.1.1. HPLC analysis

HPLC analysis verified the presence of gallic acid peaks in the pure *A. sativum* extract sample, with a retention time of 3.34 min that was closely aligned with the standard peak observed at 3.12 minutes (Figure 2A,B).

3.1.2. FTIR Spectroscopy

The presence of IR band and their corresponding functional groups in *A. sativum* extract were detected. Functional groups associated to phytochemical compounds (alkaloid, flavonoid, terpenoid, amines, carboxylic acids and phenolic compounds) corresponds to –C–O stretch, C–O bend, C–N stretch, C–H stretch and O–H stretch (Figure 2C).

3.1.3. UV-visible Spectroscopy

The optical behavior of the ethanolic extract was studied by observing the UV-Vis spectrum. The spectra of *A. sativum* extract showed the absorption peak at 358nm which indicated the characteristic absorption of phenolic compounds (Figure 2D).

3.2. Hematological analysis

Hematological analysis was performed at 7dpi and 14dpi (Table 1). At 7 dpi, RBCs, Hb, HCT, MCV, MCH, and MCHC level significantly decreased in *A. hydrophila* challenged group while WBCs level increased significantly in *A. hydrophila* challenge fish as compared to the control. *A. sativum* treatment showed recovery in challenged groups, high concentration was seen to be more effective in WBCs, RBCs and Hb. Hb content recovered at medium and high concentration. MCH level showed improvement at low and high, while MCHC level recovered at low and medium concentrations comparable to the control (Table 1).

At 14 dpi, a significant decreased hematological contents were observed in *A. hydrophila* challenged group while WBCs level increased significantly in challenged group as compared to the control. When challenged fish was treated *A. sativum* extract, WBCs and Hb showed recovery effect at medium and high concentrations, while RBCs was only recovered at low concentration. HCT and MCH showed improved level at low and high concentrations comparable to the control group (Table 1).

3.3. Enzyme analysis

On day 7 and 14, ALT and AST levels were significantly increased upon *A. hydrophila* challenged as compared to the control group. The improved ALT level was observed on day 14 at low and medium concentrations of *A. sativum* treated groups comparable to the control group (Figure 3A). At 7 dpi, AST level recovered at medium and high concentrations of *A. sativum* while at 14 dpi, recovery effect was noted at high concentration (12g/kg) of *A. sativum* treated group (Figure 3B). At 7dpi, improved ALP level was noted at medium, high and erythromycin treated groups while at 14dpi, restored ALP level was observed in all *A. sativum* treated groups (Figure 3C).

3.4. Biochemical analysis

Total protein, albumin and globulin level significantly decreased in *A. hydrophila* challenged group as compared to the control group at 7 and 14 dpi, when challenged fish treated with *A. sativum* extract improved total protein level was achieved comparable to the control group. On day 7 and 14, total protein level showed recovery trend at medium and high concentrations of *A. sativum* treated groups in comparison to control (Figure 4A). At 7 and 14 dpi, albumin level restored to normal and low and medium *A. sativum* concentrations were more effective (Figure 4B). Maximum globulin level recovered at high concentration at both time points in comparison to control (Figure 4C). A/G ratio significantly increased at 14 dpi while *A. sativum* treatment to challenged fish showed recovery effect at all concentrations compared to the control group (Figure 4D).

3.5. Immune analysis

RBA significantly increased in *A. hydrophila* challenged group as compared to the control group. *A. sativum* extract treatment to challenged fish exhibited recovery at 7 dpi, in medium and high concentrations of plants comparative to the control. At 14 dpi, all plant treated and erythromycin treated groups showed noticeable improvement comparable to the control group (Figure 5A). MPO activity was significantly high in *A. hydrophila* challenged group as compared to the control group at 14 dpi. A noticeable recovery was obtained at all three concentrations comparable to the control (Figure 5B). SLA significantly decreased in *A. hydrophila* challenged group as compared to the control group at both time points. Treatment to challenged fish displayed improved fish health and better SLA at 7 dpi in medium concentration of *A. sativum.* After 14 dpi, medium and high *A. sativum* treated groups showed considerable recovery in comparison to the control (Figure 5C).

IV. DISCUSSION

Fish raised in intensive cultures live in extremely stressful environments, which further suppresses their immune systems and leads to illness outbreaks. Immunostimulants have the potential to be beneficial in fish culture as well as in the management of fish illnesses. *A. sativum* is indeed a valuable medicinal herb with broad applications in aquaculture due to its antimicrobial and immune-boosting properties. Its active compounds play a key role in enhancing disease resistance in fish. Studies have demonstrated that *A. sativum* as a feed additive strengthens the immune response, leading to improved resistance to pathogens like *A. hydrophila* in both *Oncorhynchus mykiss* and *Labeo rohita* [10].

HPLC peaks revealed the presence of phenolic compound such as; gallic acid in *A. sativum* extract. FT-IR is a valuable analytical technique used to identify organic and inorganic compounds in plants by detecting specific molecular vibrations. FT-IR analysis has shown the presence of various phytochemicals in *A. sativum* extract. A range of bioactive compounds in *A. sativum* extract were identified including: alkaloids, proteins, lipids, oils, flavanoids, gums, phenols, saponins, steroids, tannins, and terpenoids [11,12].

The use of hematological procedures has proven valuable in assessing fish health. Incorporating *A. sativum* into fish diets enhances immunological activity and positively influences the hematological parameters in various fish species [13]. In the current study, a decrease in red blood cell (RBC) count and an increase in white blood cell (WBC) count were observed at post-challenge. The changes in WBC count reflect the fish's response to infection, with the increase indicating a state of stress that may arise from the activation of defense mechanisms against the pathogen. Additionally, there was a decline in hematocrit (Hct) and hemoglobin (Hb) levels following the challenge, likely due to severe bacterial infection disrupting hematopoiesis, impairing osmoregulation, and causing tissue damage from the pathogen and other stressors [10]. However, the application of *A. sativum*-enriched feed resulted in an increase in RBC count, Hct and Hb content compared to the control suggesting a potential immunostimulatory effect [14,10].

Globulin levels are crucial for maintaining a robust immune system and supporting immune functions in the blood. Albumin plays an essential role in sustaining osmotic pressure, which is necessary for the proper distribution of body fluids, and serves as a carrier in plasma. The inclusion of *A. sativum* in the feed resulted in significant changes in serum total protein, albumin, globulin, and the albumin:globulin ratio. The increase in serum total protein, albumin, and globulin levels are indicative of enhanced innate immunity [15].

Pathogenic infections affect the liver and its functions, including the release of liver enzymes (ALT and AST) into the blood. A substantial increase in ALT and AST activity is a chief indicator of hepatic dysfunction [16]. Elevated level of liver AST an ALT were observed upon *A. hydrophila* challenge [17]. The current study had showed improved AST and ALT activity in *A. sativum* extract treatment groups which showed stabilized health status of fish. In the present study, ALP activity was significantly decreased in *A. hydrophila* challenged group, while improved level was recorded in the treatment group compared to the control. These results are concordance with Gobi et al. [18] who reported that the ethanolic leaf extract supplemented diet at 10 mg/g has increased the ALP activity in *O. mossambicus* after 30 days post challenge infection. However, higher ALP level suggests improved liver cell accumulation, improved absorption of the extract in the fish's body, and normal cell activity [18].

A. sativum enhances non-specific immunity by stimulating phagocytosis and respiratory burst [19]. It accelerates macrophage activity, a process where microorganisms and cellular debris are engulfed and eliminated, thereby boosting respiratory burst and strengthening the immune response. Current studies indicated improved immune activities (RBA, SLA and MPO) of fish supplemented with different concentration of *A. sativum* after 14 days post challenged in *L. rohita*. Fish fed *A. sativum*-enriched diets at concentrations of 10 g and 15 g/kg feed exhibited a significant increase in respiratory burst and serum lysozyme activity, enhancing the immune response of sea bass. *A. sativum* contains therapeutic components, such as hydroxyl radicals and superoxide anions [20], which act as natural defense mechanisms against infections [10]. After 20 days of feeding, leucocyte myeloperoxidase activity was increased by the administration of *Catla catla* with *C. dactylon* ethanolic extract following an *A. hydrophila* challenge [21].

V. CONCLUSION

The findings of this study demonstrate that *A. sativum* possesses immunostimulant properties and is effective in enhancing protection in *L. rohita* against *A. hydrophila* infection. Ethanolic *A. sativum* extract improve the hemato-biochemical profile and immune responses of the fish, as well as increase the survival rate in the *A. sativum*-treated groups. The highly appreciable responses were observed at medium and high concentrations (8g/kg and 12g/kg) of *A. sativum* dose is likely due to the bioactive components present in *A. sativum*. Overall, we conclude that herbal extract of *A. sativum* can be used as a safe and potent alternative to antibiotics for controlling bacterial infections in aquaculture.

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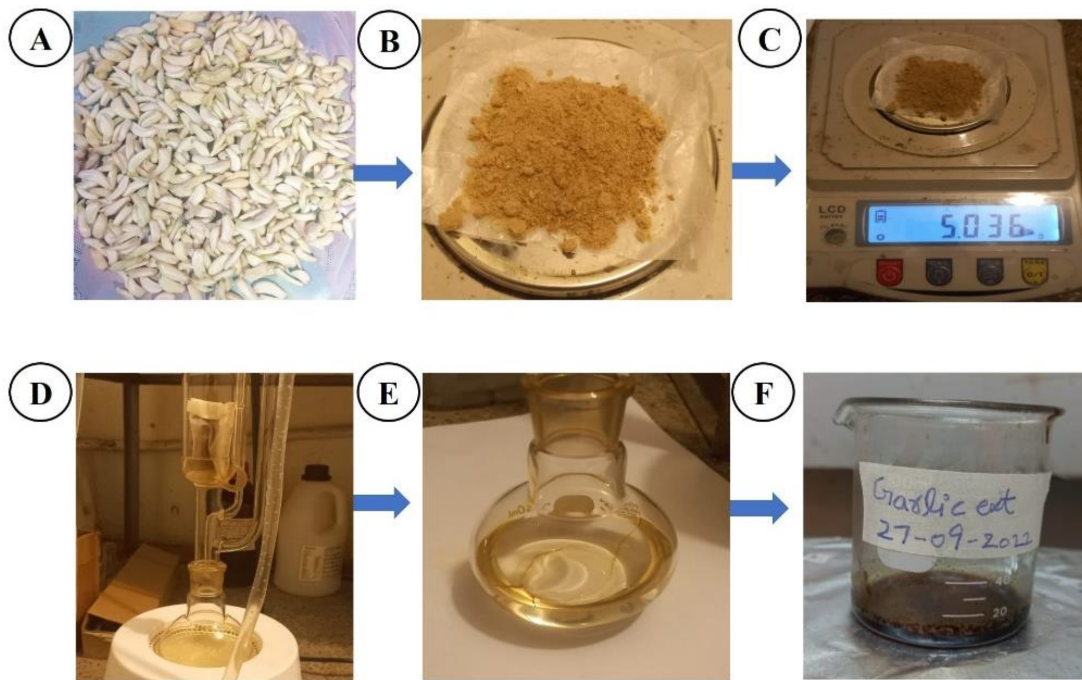

Figure 1. (A-F) Preparation of ethanolic extract of *A. sativum*. (A) Fresh bulbs of *A. sativum* washed and shade dried (B) Leaves grinded into fine powder (C) Weighed (D) Soxhlet extraction of *A. sativum* (E) Filtered extract (F) *A. sativum* extract dried and stored.

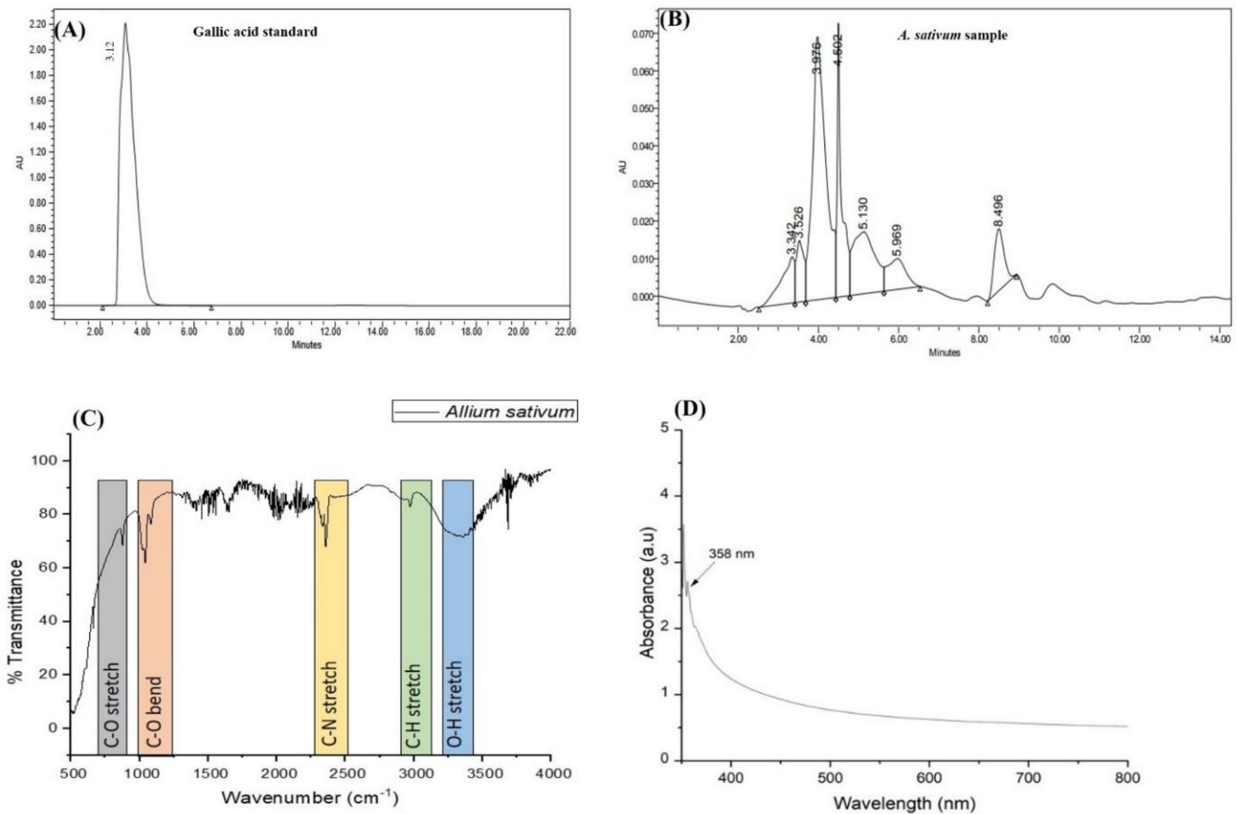

Figure 2. (A) The HPLC spectra of gallic acid standard (B) The HPLC spectra of *A. sativum* extract (C) FT-IR spectra of *A. sativum* extract (D) UV-Vis spectra of *A. sativum* extract.

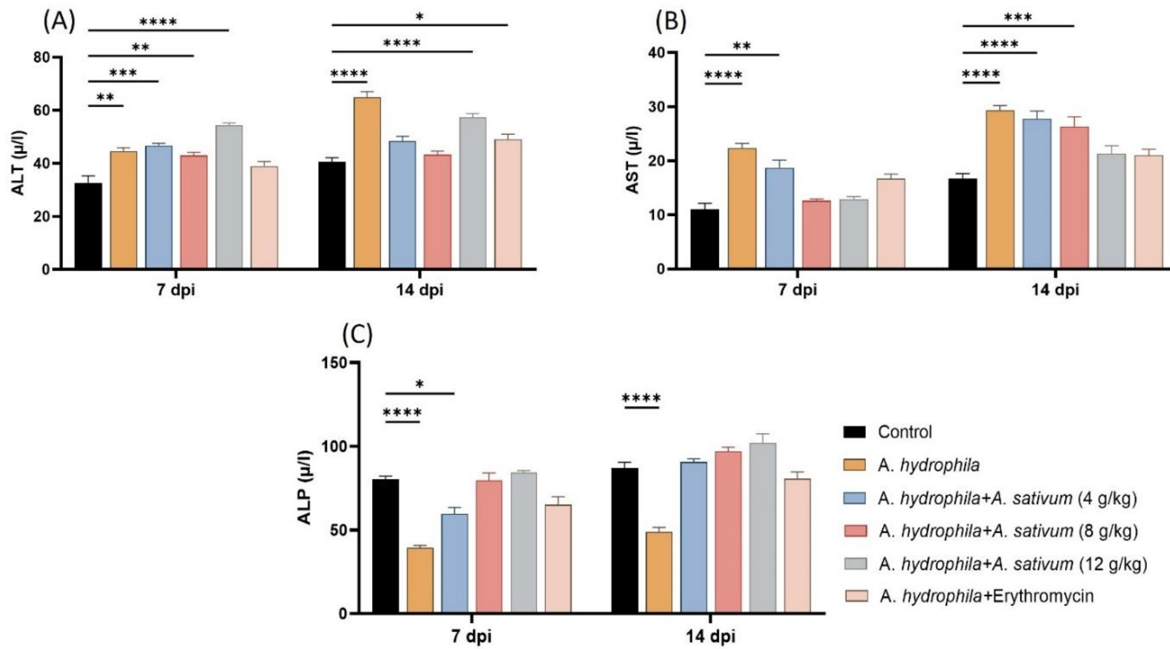

Figure 3. Enzyme analysis of *A. sativum* treated groups against *A. hydrophila* challenge on 7 and 14 days: (A) Alanine transaminase (ALT), (B) Aspartate transaminase (AST) and (C) Alkaline phosphatase (ALP). Two-way ANOVA and Tukey's test ($p<0.05$) were performed to analyze the variability between two weeks. Data are presented as mean±SEM (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$).

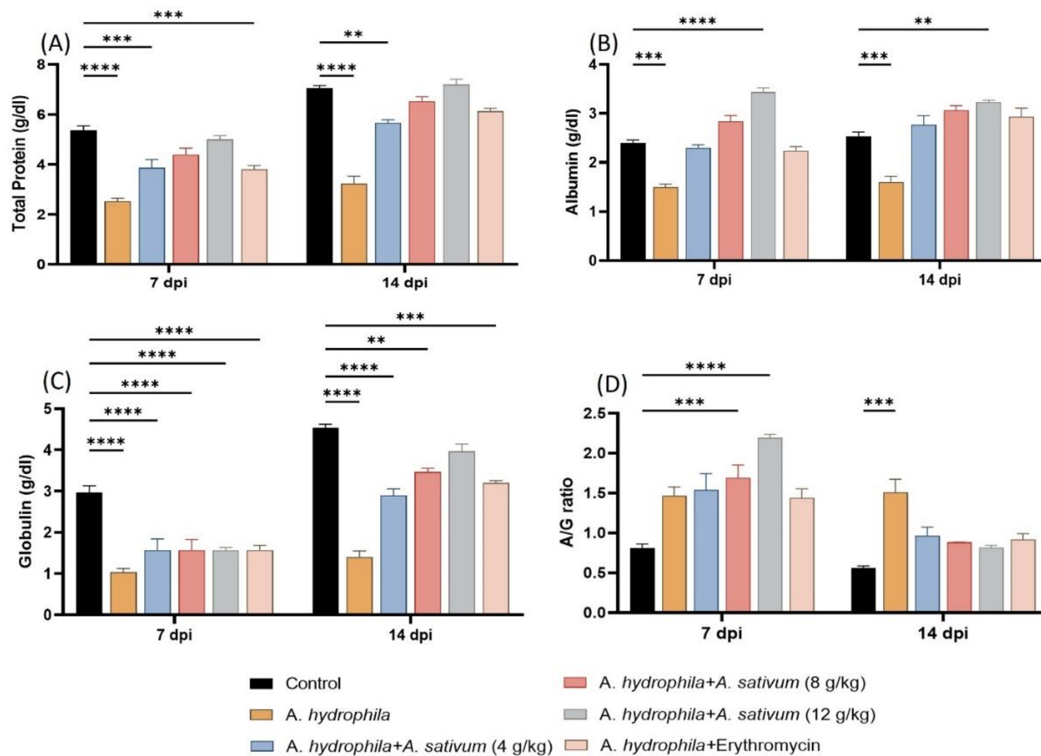

Figure 4. Biochemical analysis of *A. sativum* treated groups after 7 and 14 dpi against *A. hydrophila* challenge: (A) Total protein, (B) Albumin, (C) Globulin and (D) A/G ratio. Two-way ANOVA and Tukey's test ($p<0.05$) were performed to analyze the variability between two weeks. Data are presented as mean±SEM (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$).

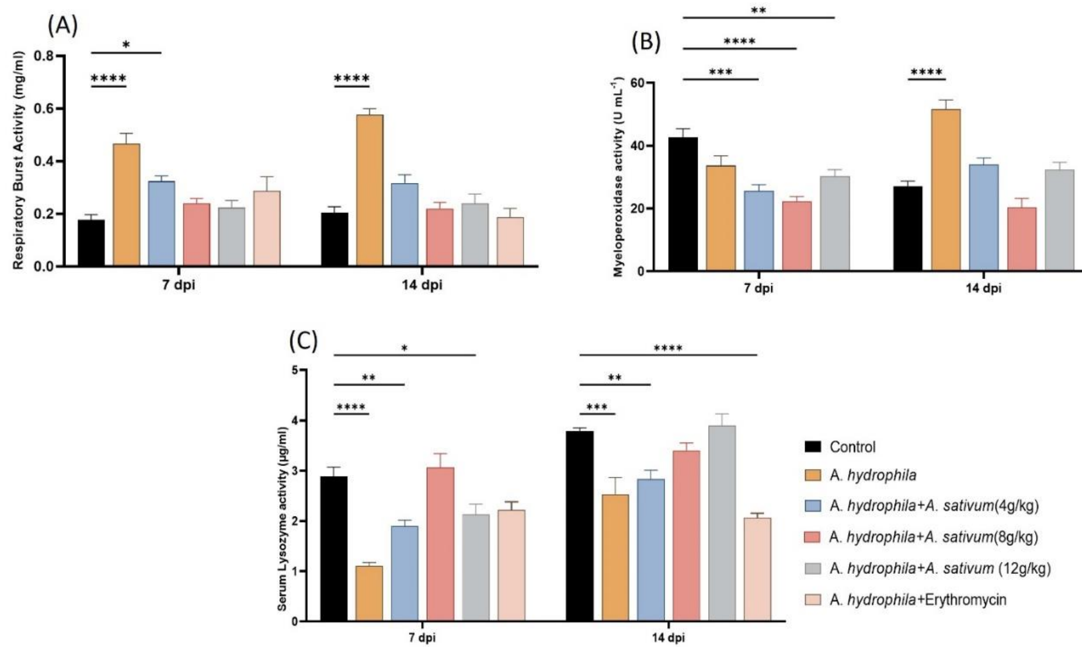

Figure 5. (A) Respiratory Burst (RB) Activity, (B) Myeloperoxidase (MPO) Activity and (C) Serum Lysozyme activity (SLA) in *A. sativum* treated groups on 7 and 14 dpi against *A. hydrophila* challenged assay. Data are presented as mean±SEM (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

Table 1. Effect of *A. sativum* extract supplementation on hematological parameters of *L. rohita* followed by *A. hydrophila* challenge.

	Parameters	Control	*A. hydrophila*	*A. hydrophila*+ *A. sativum* (4g/kg)	*A. hydrophila*+ *A. sativum* (8g/kg)	*A. hydrophila*+ *A. sativum* (12g/kg)	*A. hydrophila*+ Erythromycin
7 dpi *A. hydrophila*	RBCs x10^6 /uL	1.70±0.10^a	0.433±0.12^b	1.06±0.08^a	0.733±0.20^b	1.09±0.06^a	0.49±0.15^b
	WBCsx10^3 /uL	3.46±0.12^a	4.96±0.29^b	4.76±0.37^b	4.80±0.28^b	4.20±0.32^a	4.06±0.14^a
	Hb g/dl	3.96±0.12^a	1.96±0.08^b	2.80±0.23^b	3.03±0.16^a	3.03±0.34^a	2.50±0.23^b
	HCT (PVC) %	10.36±0.32^a	4.36±0.60^b	6.26±0.50^b	7.43±0.49^b	7.00±0.29^b	4.53±0.49^b
	MCV fL	139.00±6.30^a	70.00±4.04^b	95.33±4.70^b	107.00±7.55^b	82.33±3.75^b	132.33±4.72^a
	MCH %	42.00±1.52^a	25.33±2.33^b	30.00±1.73^a	38.66±2.60^b	30.33±1.45^a	36.33±1.20^b
	MCHC %	54.66±3.38^a	37.66±3.75^b	39.00±2.30^a	50.66±4.09^a	48.66±5.17^b	45.66±1.76^b
14 dpi *A. hydrophila*	RBCs x10^6 /uL	2.46±0.14^a	0.86±0.14^b	2.50±0.20^a	0.80±0.23^b	0.93±0.08^b	2.03±0.42^a
	WBCs x10^3 /uL	3.40±0.25^a	5.83±0.44^b	5.10±0.43^b	3.23±0.08^a	3.63±0.24^a	3.46±0.17^a
	Hb g/dl	4.93±0.35^a	2.43±0.24^b	6.46±0.12^b	3.93±0.26^a	3.93±0.27^a	3.00±0.49^b
	HCT (PVC) %	11.96±0.52^a	7.30±0.56^b	11.96±1.56^a	9.90±0.153^b	10.10±1.02^a	7.20±0.43^b
	MCV fL	152.33±3.84^a	113.33±7.75^b	163.00±3.60^a	141.00±4.72^a	124.33±2.02^b	131.66±4.48^b
	MCH %	44.00±2.33^a	27.66±2.40^b	37.66±2.90^a	43.33±0.88^b	37.00±1.52^a	44.33±1.76^b
	MCHC %	79.66±4.41^a	53.66±1.45^b	56.3±2.186^b	85.00±1.73^a	69.33±3.33^b	63.33±0.08^b

* Data are expressed as mean±SEM. Different superscripts in each parameter show significant differences ($P<0.05$) between control and treated groups.