

USE OF LEAF EXTRACT OF NONI, GUAVA AND MANGO AS A PHYTOIMMUNOSTIMULANT FOR IMMUNITY OF CARP (*CYPRINUS CARPIO*) AGAINST *AEROMONAS HYDROPHILA*

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Abstract. Carp (*Cyprinus carpio* L) is one of the most widely cultivated freshwater fish in Asia, with the main problem in farming being bacterial infections caused by *Aeromonas hydrophila*. Immunostimulants are the materials that boost the immune system by using certain immunostimulants, such as vaccines or any specific antigenic material. This study determined the effect of mango, noni, and guava leaf extract on carp immunity. The fish were fed with commercial fish (CF) only (a1), CF containing noni leaf extract 1.5 g/100 g (a2), CF containing guava leaf extract 1.5 g/100 g (a3), and CF containing mango leaf extract 1.5g/100 g (a4). The diet increased the total of carp leukocytes from 29.28 to 56.78 x 10⁴ (a3), 32.89 x 10⁴ (a2), and 25.68 x 10⁴ cells/mm³ (a3) on day 45 after the fish were challenged with *A. hydrophila*. The highest lymphocyte percentage was recorded in a3 (84.00%), followed by a2 (82.33%), and a4 (80.33%). The highest percentage of monocytes was in a1(13.67%), followed by a4 (11.33%), a2 (9.67%), and a3 (8.67 %). The highest percentage of neutrophils was in a1 (9.33%), followed by a4 (8.33%), a2 (8.00%), and a3 (7.33%). The highest percentage of pagocytosis index was in a3 (21.00%), followed by a4 (15.67%), a2(13.00 %), and a1 (12.67 %). The highest survival rate was recorded in a3 (94.67%), followed by a4 (90.67%), a1 (80.00%), and a2 (78.67%). It is concluded that dietary phytoimmunostimulant improved the immun status of carp, where the best results were obtained from the diet of guava leaf extract, followed by the noni and mango diet.

Keywords: *Aeromonas hydrophila*, carp, haematological characters, physiological parameters, phytoimmunostimulant.

INTRODUCTION

Carp (*Cyprinus carpio* L) is one of the most widely cultivated freshwater fish in yard ponds, flowing water, and floating net cage systems. The main problem in carp farming is the occurrence of bacterial infections, which result in decreased fish production. To overcome this problem, various antimicrobial compounds such as antibiotics and other chemicals are generally used. However, the application of antibiotics during fish production can cause other problems, namely antibiotic-resistant bacteria and food safety, because many antibiotics are already stored in the fish's body (Effendi et al, 2022^a). Therefore, alternative treatments are needed which are more environmentally friendly and do not cause a resistant effect on bacteria (Song et al, 2014; Igiebor et al, 2025).

One type of disease that is often found in carp cultivation is a bacterial disease caused by the bacterium *Aeromonas hydrophila*. This bacterium causes hemorrhagic septicemia, which is characterised by swelling of the stomach, intestines, lesions, skin abscesses, exophthalmia, and bleeding, especially in the gills and operculum. (Pathania et al, 2002).

Immunostimulants, often referred to as immunostimulators, are substances (drugs and foods) that boost the activity of any component of the immune system, hence stimulating the immune system. Immune responses to certain immunostimulants, such as vaccinations or any antigen, are antigen-specific in

nature. Non-specific immunostimulants, such as adjuvants and non-specific immunostimulators, work independently of antigenic specificity to enhance immune response to other antigens or stimulate immune system components (Secombes et al., 1992). Immunostimulants have been utilised as dietary supplements to increase weight gain, feed effectiveness, and/or disease resistance in farmed fish in order to preserve fish health and improve performance (Vallejos-Vidal et al, 2016; Mureșan et al., 2025).

The use of immunostimulants is an alternative to the use of antibiotics and chemicals. According to Awad et al. (2017), immunostimulants are materials that can improve the function of the immune system by using substances that stimulate the immune system. Plant-derived compounds have many benefits, such as stimulation of appetite and growth, stimulation of the immune system, antimicrobial and antifungal activity, as well as anti-stress effects (Shoemaker et al, 2001; Baghizadeh et al, 2015). Phytopharmaca that can be utilised to enhance the immune system of fish include mango leaves (*Mangifera indica*), noni leaves (*Morinda citrifolia*) and guava leaves (*Psidium guajava*) (Amulejoye et al, 2020; Tawwab et al., 2020).

Red blood cell (RBC) count is one of the immune cell measures that is widely used to assess potential unintended side effects (anaemia) caused by immunostimulants provided in supplemented feed. Interestingly, RBCs have drawn more attention recently

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because it has been claimed that these cells are involved in the immunological response of rainbow trout (Morera et al, 2011). Lysozyme and the complement system are examples of humoral components involved in the innate immune response in addition to the cellular response (Magnadóttir, 2006; Secombes et al., 1992).

The content of secondary metabolites such as alkaloids, terpenoids, phenolics, polyphenols, quinones, lectins, and polypeptide compounds is mostly alternatives to antibiotics, chemicals, vaccines, and other synthetic compounds (Harikhrisnan et al. 2011; Hai et al, 2015). The compounds contained in these plants are expected to improve the immune system in carps, as evaluated from the total leukocytes, leukocyte differentiation, and phagocytosis activity. This study aims to determine the effect of phytoimmunostimulants (mango, noni and guava leaf extract) on the differentiation of carp immunity challenged by the fish pathogenic bacteria *A. hydrophila*.

MATERIAL AND METHOD

Research methods

This research was carried out from January to April 2025 at the Marine Microbiology Laboratory, Faculty of Fisheries and Marine Sciences, University of Riau, Indonesia. The method used was an experimental method with a one-factor completely randomised design (CRD), namely dietary phytoimmunostimulant species, with 4 treatment levels and 3 repetitions. The treatment levels were a1 (experimental control unit or feeding the fish with commercial fish feed Hi Provit 781-2 only), a2 (feeding the fish with commercial fish feed Hi Provit 781-2 containing noni leaf extract of 1.5 g / 100 g), a3 (feeding the fish with commercial fish feed Hi Provit 781-2 containing guava leaf extract of 1.5 g /100 g), and a4 (feeding the fish with commercial fish feed Hi Provit 781-2 containing mango leaf extract of 1.5 g /100 g).

Preparation of phytoimmunostimulant fish feed

The main ingredient of the feed was the commercial fish feed Hi-Pro-Vite 781-2, a commercial fish feed produced by PT. Central Pangan Pertiwi Animal Feedmill Co. Ltd., Karawang, West Java, Indonesia. The composition of this fish pellet is 31-33% protein, 4-6% fat, 3-5% and 9-10% water content. The medicinal plant, namely noni leaves (*Morinda citrifolia*), guava leaves (*Psidium guajava*), and mango leaves (*Mangifera indica*) were collected from community plants around the University of Riau campus, Pekanbaru, Indonesia. The leaves were washed, air dried, and dried in the sun. After drying, the leaves were chopped into small pieces and blended, and finely ground. The powder was filtered through a sieve with a size of 100-200 mesh and then mixed with finely ground Hi-Pro-Vite 781-2 fish feed pellets (1.5 g/ 100 g) and re-milled to form new pellets according to the size of the mouth openings of the test fish (Effendi et al. 2022^b).

Acclimatisation and maintenance of fish

Carp, measuring 8-12 cm, were obtained from the owner of a fish hatchery in Rao, Pasaman, West Sumatra, Indonesia. In the laboratory, the carps were acclimatised for 7 days in fresh water, and then to

brackish water (salinity 5 ppt) for another 7 days. On day 15, fish began to be fed with phytoimmunostimulant feed. The containers were a plastic drum with a capacity of 100 L, which was filled with 80 L of brackish water. The stocking density of each drum was 25 individuals. The fish were reared for the next 30 days.

Preparation of *A. hydrophila* isolates

The *A. hydrophila* isolates used were obtained from a collection of virulent isolates at the Marine Microbiology Laboratory, University of Riau. The isolates were then cultured in nutrient agar media (Oxoid) and incubated in an incubator for 24 hours. For the inoculation of fish with *A. hydrophila*, the pathogen was then sub-cultured in the nutrient medium (Oxoid).

Experimental infection of fish

After being reared for 30 days (15 days of acclimatization and 15 days of being fed a diet containing phytoimmunostimulants), the fish were challenged with *A. hydrophila*. The bacterial solution density was 10⁸ CFU/ml and injected intramuscularly by using a 1 ml syringe. The fish were returned to the drum and reared for 15 days and fed with phytoimmunostimulant fish feed.

Measurement of haematological and physiologic parameters

The haematological and physiological parameters measured were total leukocytes, leukocyte differentiation, phagocyte activity and survival rate. Measurements were carried out at the beginning of the experiment, on day 30, day 45 or 15 days after the challenge with *A. hydrophila*.

Total leukocytes

The procedure for calculating total leukocytes refers to Blaxhall et al. (2006). The total number of leukocytes was counted under a binocular microscope in 4 large haemocytometer boxes with the following formula:

$$\sum \text{Leukosit} = \sum n \times 50 \text{ cel/mm}^3$$

where:

$\sum n$ = the total number of leukocytes in the 4 large squares

50 = dilution factor

Leukocyte differentiation

Calculation of leukocyte types based on the method of Blaxhall et al (2006). The types of leukocytes observed were lymphocytes, monocytes, and neutrophils. Then it was counted up to 100 cells and calculated by the following formula:

Cell percentage of lymphocytes, monocytes, and neutrophils = $\sum n \times 100\%$

where:

$\sum n$ = number of cells counted (lymphocytes, monocytes, and neutrophils)

Phagocytosis index

The procedure for determining the phagocytosis index refers to Anderson et al. (1993). The percentage of phagocytic cells can be calculated by observing the

number of cells that phagocytise bacteria. The method of calculation is as follows:

$$\text{Phagocytosis index} = \frac{\text{Phagocytosis cells}}{100} \times 100\%$$

Survival rate

Carp survival rates of fish were calculated using the following formula:

$$\text{SR} = \frac{N_t}{N_o} \times 100\%$$

Dimana:

SR = Survival rate (%)

Nt = The number of live fish at the end of the study

No = The number of live fish at the beginning of the study

Water quality

The water quality parameters measured were: temperature, dissolved oxygen (DO), salinity and pH. Measured on days 1, 30 and 45.

Data analysis

All data (total leukocytes, leukocyte differentiation, phagocytic activity, and survival rates were tabulated

and then analysed by using analysis of variance (ANOVA). If the treatment shows a significant difference where $P < 0.05$, then the Students Newman-Keuls test is carried out. Water quality parameters were analysed descriptively.

RESULTS

Total of leukocytes

The average number of carp leukocytes at the beginning of the study was 29.28×10^4 cells/mm³. After 14 days fed with dietary phytoimmunostimulant (day 30), the total of leukocytes ranged from 15.98 to 48.66×10^4 cells/mm³. The highest average leukocyte levels were recorded in a3 (48.66×10^4 cells/mm³), followed by a2 (29.16×10^4 cells/mm³), a4 (24.80×10^4 cells/mm³), and a1 (15.99×10^4 cells/mm³). On day 45 or 15 days after the challenge with *A. hydrophila*, the average pattern of leukocyte levels was also the same as the results of the examination on day 30, where the highest average leukocyte levels were recorded in a3 (56.78×10^4 cells/mm³), then followed by a2 (32.89×10^4 cells/mm³), a4 (25.68×10^4 cells/mm³), and a1 (15.99×10^4 cells/mm³) (Table 1).

Table 1.

Average total of leukocytes (10^4 cells/mm³) of carp fed with commercial fish feed Hi Provit 781-2 containing phytoimmunostimulant.

Treatments	Day 1	Day 30	Day 45
a1 (control unit)	29,28 ± 0,70	15,99 ± 1,29	11,91 ± 1,56
a2	29,28 ± 0,70	29,16 ± 2,24	32,89 ± 2,83
a3	29,28 ± 0,70	48,66 ± 2,29	56,78 ± 6,29
a4	29,28 ± 0,70	24,80 ± 3,39	25,68 ± 3,72

a1: experimental control unit or feeding fish with commercial fish feed Hi Provit 781-2 only.

a2: feeding the fish with commercial fish feed Hi Provit 781-2 containing noni leaf extract 1.5 g / 100 g.

a3: feeding fish with commercial fish feed Hi Provit 781-2 containing guava leaf extract 1.5 g /100 g.

a4: feeding fish with commercial fish feed Hi Provit 781-2 containing mango leaf extract 1.5 g /100 g.

The results of the statistical analysis of variance (ANOVA) showed that feeding fish phytoimmunostimulants containing feed (noni leaves, guava leaves, and mango leaves) affected the total leukocytes in carp after 30 and 45 days of rearing ($P < 0.05$). The results of the Student Newman-Keuls analysis showed that the control treatment was significantly different from the others, and there were significant differences between the treatments.

Lymphocyte percentage

After 14 days of feeding with dietary phytoimmunostimulant, the average percentage of carp

lymphocytes (day 30) ranged from 77.67-83.67%. The highest average lymphocyte percentage was recorded in a3 (83.67%), followed by a2 (82.33%), a1 (81.67%), and a4 (77.67%). On day 45 or 15 days after the challenge, the highest average lymphocyte percentage was recorded in a3 (84.00%), followed by a2 (82.33%), a4 (80.33%), and a1 (77.00%) (Table 2). The results of the ANOVA analysis showed that feeding the animals with commercial fish feed Hi Provit 781-2 containing phytoimmunostimulants had an effect on the percentage of carp lymphocytes on the 30th day of rearing ($P < 0.05$).

Table 2.

Percentage of lymphocytes (%) of carp fed with commercial fish feed Hi Provit 781-2 containing phytoimmunostimulant.

No.	Treatments	Day 30	Day 45
1.	a1 (control unit)	81,67 ± 3,79	77,00 ± 3,00
2.	a2	82,33 ± 1,16	82,67 ± 1,53
3.	a3	83,67 ± 2,51	84,00 ± 3,00
4.	a4	77,67 ± 4,04	80,33 ± 1,52

a1: experimental control unit or feeding fish with commercial fish feed Hi Provit 781-2 only.

a2: feeding the fish with commercial fish feed Hi Provit 781-2 containing noni leaf extract 1.5 g / 100 g.

a3: feeding fish with commercial fish feed Hi Provit 781-2 containing guava leaf extract 1.5 g /100 g.

a4: feeding fish with commercial fish feed Hi Provit 781-2 containing mango leaf extract 1.5 g /100 g.

Monocyte percentage

The average percentage of carp monocytes at day 30 ranged from 7.33-13.00%. The highest average monocyte percentage was recorded in a4 (13.00%), followed by a1 (10.00%), a3 (9.33%), and a2 (7.33%). On day 45 or 15 days after the challenge with *A. hydrophila*, the highest average percentage of monocytes was recorded in a1 (13.67%), followed by a4

(11.33%), a2 (9.67%), and a3 (8.67 %) (Table 3). The statistical analysis of variance (ANOVA) showed that feeding the fish with commercial fish feed Hi Provit 781-2 containing phytoimmunostimulants had an effect on the percentage of monocytes and carps on day 45 (P<0.05). However, further test results on the percentage of monocytes showed that there was no significant difference among the treatments.

Table 3.

The percentage of monocytes (%) of carp fed with commercial fish feed Hi Provit 781-2 containing phytoimmunostimulant.

No.	Treatments	Day 30	Day 45
1.	a1 (control unit)	10,00 ± 1,73	13,67 ± 1,53
2.	a2	7,33 ± 1,52	9,67 ± 1,53
3.	a3	9,33 ± 1,52	8,67 ± 1,53
4.	a4	13,00 ± 2,00	11,33 ± 1,16

a1: experimental control unit or feeding fish with commercial fish feed Hi Provit 781-2 only.

a2: feeding the fish with commercial fish feed Hi Provit 781-2 containing noni leaf extract 1.5 g / 100 g.

a3: feeding fish with commercial fish feed Hi Provit 781-2 containing guava leaf extract 1.5 g /100 g.

a4: feeding fish with commercial fish feed Hi Provit 781-2 containing mango leaf extract 1.5 g /100 g.

Neutrophil percentage

The average percentage of carp neutrophils on day 30 ranged from 7.00 to 9.33%. The highest average percentage of neutrophils was recorded in a4 (9.33%), followed by a1 (7.67%), a2 (7.33%), and a3 (7.00%). On day 45 or 15 days after the challenge test with *A.*

hydrophila, the highest average percentage of neutrophils was recorded in a1 (9.33%), followed by a4 (8.33%), a2 (8.00%), and a3 (7.33%) (Table 4). The statistical analysis of variance showed that the dietary phytoimmunostimulant had an effect on the percentage of carp neutrophils on the 45th day (P<0.05).

Table 4.

The percentage of neutrophils of carp (%) fed with commercial fish feed Hi Provit 781-2 containing dietary phytoimmunostimulant.

No.	Treatments	Day 30	Day 45
1.	a1 (control unit)	7,67 ± 2,30	9,33 ± 1,53
2.	a2	7,33 ± 1,52	8,00 ± 1,00
3.	a3	7,00 ± 1,73	7,33 ± 1,53
4.	a4	9,33 ± 2,08	8,33 ± 0,58

a1: experimental control unit or feeding fish with commercial fish feed Hi Provit 781-2 only.

a2: feeding the fish with commercial fish feed Hi Provit 781-2 containing noni leaf extract 1.5 g / 100 g.

a3: feeding fish with commercial fish feed Hi Provit 781-2 containing guava leaf extract 1.5 g /100 g.

a4: feeding fish with commercial fish feed Hi Provit 781-2 containing mango leaf extract 1.5 g /100 g.

Phagocytosis index

The phagocytosis index was determined only on day 45. The highest average percentage of phagocytosis index was recorded in a3 (21.00%), followed by a4 (15.67%), a2 (13.00 %), and a1 (12.67 %) (Table 5). The analysis of variance (ANOVA) showed that feeding the fish with Hi Provit 781-2 containing phytoimmunostimulants and challenge with *A. hydrophila* bacteria had an effect on the carp

phagocytosis index (P<0.05). The results of the ANOVA revealed that the administration of dietary phytoimmunostimulants and a challenge test with *A. hydrophila* bacteria affected the carp phagocytosis index (P<0.05). Further test results showed that the treatment was not significantly different between treatments a1, a2, a3 and a4. However, the best phagocytosis index was recorded in a3 (21,00 %), followed by a4 (15.67 %), a2 (13.00 %), and a1 (12.67 %).

Table 5.

Phagocytosis index of carp fed with commercial fish feed Hi Provit 781-2 containing phytoimmunostimulant.

No.	Treatments	Average phagocytosis index (%)
1.	a1 (control unit)	12,67 ± 2,51
2.	a2	13,00 ± 2,00
3.	a3	21,00 ± 1,16
4.	a4	15,67 ± 4,42

a1: experimental control unit or feeding fish with commercial fish feed Hi Provit 781-2 only.

a2: feeding the fish with commercial fish feed Hi Provit 781-2 containing noni leaf extract 1.5 g / 100 g.

a3: feeding fish with commercial fish feed Hi Provit 781-2 containing guava leaf extract 1.5 g /100 g.

a4: feeding fish with commercial fish feed Hi Provit 781-2 containing mango leaf extract 1.5 g /100 g.

Survival rate

The percentage of survival rates was calculated at the end of the experiment (day 45), which ranged from 78.67 - 94.67%. The highest survival rate was recorded in a3 (94.67%), followed by a4 (90.67%), a1 (80.00%), and a2 (78.67%) (Table 6). The results of the ANOVA

showed that feeding the carp with Hi Provit 781-2 containing phytoimmunostimulants and challenged with *A. hydrophila* bacteria had a significant effect on the survival rate of the tested fish at the end of the study ($P < 0.05$).

Table 6.

Survival rates of carp fed with commercial fish feed Hi Provit 781-2 containing dietary phytoimmunostimulants.

No.	Treatments	Survival Rate (%)
1.	a1 (control unit)	80,00 ± 4,00
2.	a2	78,67 ± 4,62
3.	a3	94,67 ± 2,31
4.	a4	90,67 ± 2,31

a1: experimental control unit or feeding fish with commercial fish feed Hi Provit 781-2 only.

a2: feeding the fish with commercial fish feed Hi Provit 781-2 containing noni leaf extract 1.5 g / 100 g.

a3: feeding fish with commercial fish feed Hi Provit 781-2 containing guava leaf extract 1.5 g /100 g.

a4: feeding fish with commercial fish feed Hi Provit 781-2 containing mango leaf extract 1.5 g /100 g.

Water quality parameters

Water quality measurements during the study showed that the water temperature ranged from 28-30°C, pH ranged from 6.6-6.8, DO ranged from 5.1-5.4 mg/l, and salinity remained at 5‰. The quality of the water during the study was considered good and not polluted according to the quality standards for carp maintenance. Salinity during the study was controlled in brackish water conditions (5‰).

DISCUSSION

Dietary stimulant with guava leaf, noni and mango extract increased total leukocytes. The compounds are cells in the blood that are made in the bone marrow and are found in the blood and lymph tissue. Serves to help the body fight infection and some diseases. High leukocyte levels usually occur because the body is sick, but sometimes this is also a symptom that the body is stressed. Leukocytes help the body fight infections and other diseases. Leukocytes form antibodies when a foreign object enters the body. Antibodies will be used by the immune system to provide stimulation, identify, and neutralise incoming foreign objects such as bacteria. The greater the antigen stimulation, the greater the antibodies that will be produced (Wang et al, 2016).

Lymphocytes are a type of white blood cell. T cells, natural killer cells, and B cells make up lymphocytes, which protect against viral infections and create proteins that aid the host in battling infection (antibodies). To combat bacteria, viruses, and other potentially hazardous invaders, they produce antibodies. There are two primary categories of lymphocytes, including lymphocytes (T cells), which are responsible for directing the immune response of the host body and actively pursuing and eliminating cancerous and contaminated cells. The purpose of B lymphocytes (B cells) is to produce antibodies, a protein that fights bacteria, viruses, and other foreign invaders (Alberts et al, 2002).

In this study, the highest lymphocyte percentage was recorded as 84.00% (guava), followed by 82.33%

(noni), 80.33% (mango), and experimental control (77.00%). The increase in lymphocytes is suspected because these lymphocytes are active and form antibodies to fight foreign bodies that enter the carp's body after the challenge test. Lymphocytes will spread throughout the body to fight foreign objects that enter to prevent infection. Kumar et al. (2022) stated that an increase in the percentage of lymphocytes is a reflection of the success of the fish immune system in developing a cellular (non-specific) immune response as a trigger for an immune response.

The number of lymphocytes was lower in the a4 treatment; lymphocytes experienced a significant decrease because the antibodies that worked against foreign bodies were only non-specific and were not affected by stimulants derived from herbal plants. This is supported by the opinion (Effendi et al, 2022^a), which stated that the decrease in lymphocytes occurs because most of the lymphocytes move to other circulations, entering body tissues where there is inflammation.

The monocyte performs the phagocytosis job as neutrophils, but they have an additional task: they deliver pathogen fragments to T cells so that the pathogens can be reidentified and eliminated. A mounting antibody reaction results from this. Eventually, monocytes leave the bloodstream and develop into tissue macrophages, which eliminate cellular debris and fight pathogens. The neutrophils are ineffective in removing both attacking bacteria and dead cell debris. Monocytes are believed to have a substantially longer active life than neutrophils because they can refill the contents of their lysosomes (Song et al, 2014).

The finding of this study the percentage of monocytes was decreased. The decrease is thought to be due to the presence of foreign objects that enter, so that monocytes play a role in fighting these foreign objects by eating dead cells and attacking microorganisms, so that the immune response will be stronger against foreign objects, and the number of monocytes will be reduced. According to Wheater et al. (2002), the

proportion of monocyte cells decreased due to a blood balance response to an increase in the proportion of other types of leukocyte cells, namely lymphocytes.

In the control treatment (a1), there was an increase in neutrophils, which was thought to be due to a natural response to infection with *A. hydrophila*, which was injected into the fish's body. Fish have an innate immune system that has the ability to maintain and increase the body's immunity against incoming foreign substances. This is in accordance with the statement of some previous work that reported an increase in the number of neutrophils caused by a bacterial infection; neutrophils are produced by the lymph to be sent to the site of infection. Neutrophils play a role in fighting infection and providing protection against various disease threats. The decrease in neutrophils occurs due to undergoing autolysis after successfully suppressing infection from microbes or foreign bodies that enter the host's body. (Hai, 2015). A low percentage of neutrophils indicates the absence of invading microorganisms.

The highest percentage of neutrophils was recorded in the experimental control, followed by the mango, noni, and guava diets. The assessment of non-specific immunological parameters and, consequently, the effects of these treatments on the innate immune system have received the majority of attention when examining the immunostimulant effect of dietary supplements in fish. The innate immune system performs its protective role using both cellular and humoral components. Leukocytes, particularly monocytes, macrophages, and granulocytes, are the primary cellular components of the innate immune system (Magnadóttir, 2006). Neutrophils are the most prevalent type of granulocyte and have been found in Cypriniformes, Salmoniformes, and Perciformes (Flerova et al., 2013). Neutrophils and macrophages produce bioactive substances that are used for pathogen detection and eradication, cellular communication and activation, the start of an adaptive immune response, and eventually, the resolution of an inflammatory response and tissue healing (Silva et al., 2012).

Neutrophils make up around 60–70% of the circulating leukocytes, making them the most prevalent white blood cell. They protect themselves against bacterial or fungal infection (Albert et al, 2002). Usually, they are the first to react when a microbial infection occurs. Neutrophils are abundant in the pus from wounds and actively phagocytose germs. After phagocytosing a few infections, these cells are unable to replenish the lysosomes that are utilised to digest microorganisms and eventually perish. The most prevalent type of cell in the initial phases of acute inflammation is a neutrophil (Wheater et al., 2002).

In this study the highest phagocytosis index and percentage of monocytes were recorded on the guava diet, followed by mango, noni and experimental control. Phagocytosis is the process by which cells use the cell membrane to engulf large particles, creating internal compartments called phagosomes. A cell that performs phagocytosis is called a phagocyte. Calculation of phagocytosis activity was carried out to see the ability of leukocyte cells to eat foreign objects, especially bacterial attacks. Based on the results of this study,

dietary phyto-immunostimulants affected leukocyte cells so they were able to phagocytose foreign bodies that entered the body of the fish.

Most phagocytosis, one of the primary mediators of innate immunity to eliminate pathogens such as bacteria, viruses, and parasites, is also carried out by neutrophils and macrophages. These immune cell subtypes are also known as phagocytes. This microbe-killing mechanism sets off a series of antimicrobial activities that, among other things, activate cells, produce oxidative radicals, and produce cytokines that cause an inflammatory response (Silva et al., 2012).

The fish survival rate at the end of the study was quite remarkable compared to the experimental control (a1). This shows that the immune response generated through a mixture of Hi Provit 781-2 with noni leaves, guava leaves, and mango leaves can suppress fish mortality. Nie (2015) investigated the effects of *Rehmannia glutinosa* on growth performance, immunological parameters, and disease resistance to *A. hydrophila* in common carp and reported the same thing.

Ferdous et al. (2017) also explained that guava leaf extract can increase nonspecific immunity against foreign bodies in the body. It is suspected that the increase in total leukocytes that occurred on day 14 after the challenge test using *A. hydrophila* was due to the carp's body response to the entry of foreign objects into their body. This opinion is supported by the report of Amelia et al (2021), which states that guava leaf extract, as an immunostimulant of common carp, infected with motile aeromonas septicemia.

The increase in total leukocytes in the treatment of guava leaves is thought to be because guava leaves contain active compounds in the form of flavonoids, alkaloids, tannins, and saponins, which can improve growth performance and the carp immune system (Effendi et al, 2022^b; Rosenhech et al., 2025). The haematological and immunological responses of common carp (*C. carpio*) fed diets supplemented with olive leaf extract were improved (Rajabiesterabadi et al., 2019). Ferdous et al. (2017) also reported that guava leaf extract can increase nonspecific immunity against foreign bodies in the body of tilapia. Guava leaves contain the highest flavonoids compared to noni leaves and mango leaves (Ayodele et al., 2015; Zhang et al, 2020).

CONCLUSION

In general, dietary phytoimmunostimulants improved the immune status of carp, where the best results were obtained from dietary guava leaf extract, followed by noni and mango leaf extract. Dietary stimulant with guava leaf, noni and mango extract increased total leukocytes from 29.28 to 56.78, 32.89, and 25.68 x 10⁴ cells/mm³, respectively, on day 45 after the fish were challenged with *A. hydrophila*. The highest lymphocyte percentage was recorded as 84.00% (guava), followed by 82.33% (noni), 80.33% (mango), and experimental control (77.00%). There was a different pattern of increasing the percentage of monocytes and neutrophils, where the highest average was recorded in the experimental control, followed by the mango, noni, and guava diets. The highest

phagocytosis index and percentage of monocytes were recorded on the guava diet, followed by mango, noni and experimental control. The highest survival rate was recorded in the guava dietary (94.67%), followed by mango (90.67%), experimental (80.00%), and noni dietary (78.67%).

AUTHORS CONTRIBUTIONS

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The authors have not any conflict of interest to the result of this study.

REFERENCES

Alberts B, Johnson A, Lewis M, Raff M, Roberts K, Walter P. Leukocyte also known as macrophages functions and percentage breakdown. *Molecular Biology of the Cell* (4th ed.). 2002. New York: Garland Science.

Amelia R, E. Fidyandini HP, Harpeni E, The application of guajava leaf extract (*Psidium guajava* Linnaeus) as immunostimulant of common carp (*Cyprinus carpio* Linnaeus) infected by motile aeromonas septicemia. *Journal of Aquatropica Asia*, 6(2): 48-59. 2021. <https://www.journal.ubb.ac.id/index.php/aquatropica/article/view/2564>.

Amulejoye FD, Adebayo OT, Dada AA, Reproductive impact of aqueous extract of *Mangifera indica* leaves on female African catfish *Clarias gariepinus* Broodstock. *International Journal of Fisheries and Aquatic Studies*. 8(3):657-662. 2020.

Anderson DP, Siwicki AK, Basic hematology and serology for fish health programs. Paper presented in Second Symposium on Diseases in Asia Aquaculture “Aquatic Animal Health and the Environment” 25-29 October 1993, Phuket, Thailand. pp. 185-202. 1993.

Awad E, Awad A, Role of medicinal plants on growth performance and immune status in fish. *Fish*

Shellfish Immunol. 67:40-54. doi: 10.1016/j.fsi.2017.05.034. 2017.

Ayodele, OD, Dolapo EO, Total Antioxidant Activity, Total Phenolic and Total Flavonoid Content of Some Plant Leaves in South-West Nigeria. *International Journal of Scientific & Engineering Research*. 6(1):418-427. 2015.

Baghizadeh E, Khara H, Variability in hematology and plasma indices of common carp *Cyprinus carpio*, associated with age, sex and hormonal treatment. *Iranian Journal of Fisheries Sciences*, 14(1): 99-111. 2015. URL: <http://jifro.ir/article-1-1827-en.html>

Blaxhall PC, Daisley KW, Routine methods for use with fish blood. *Journal of Fish Biology* 5(6):771 – 781. (2006). doi:10.1111/j.1095-8649.1973.tb04510.x.

Effendi I, Yoswaty D, Syawal H, Austin B, Lyndon AR, Kurniawan R, Wahyuni S, Al-Harbi A, The use of medicinal herbs in aquaculture industry: a review. *current aspects in pharmaceutical research and development* 7, 7–20. 2022^a. <https://doi.org/10.9734/bpi/caprd/v7/2190C>.

Effendi I, Yoswaty D, Syawal H, Austin B, Lyndon A. R, Kurniawan R, Wahyuni S, Effects of herbal growth promoters on common carp (*Cyprinus carpio*). *AACL Bioflux* 15(3):1096-1103. 2022^b.

Ferdous J, Hossain MMM, Jaman MHU, Rupom AH, Tonny NI, Jaman A. *Psidium guajava* leaf extracts fed to mono-sex Nile tilapia *Oreochromis niloticus* enhance immune response against *Pseudomonas fluorescens*. *European Journal of Clinical and Biomedical Sciences*. 3(1):34-42. 2017. doi: 10.11648/j.ejcs.20170301.16.

Flerova EA, Balabanova LV. Ultrastructure of granulocytes of teleost fish (Salmoniformes, Cypriniformes, Perciformes), *Journal of Evolutionary Biochemistry and Physiology*, 49 (2013) 223–233. 2013. doi:10.1134/S0022093013020126.

Hai NV, The use of medicinal plants as immunostimulants in aquaculture: a review. *Aquaculture*. 446(1):88-96. 2015. <https://doi.org/10.1016/j.aquaculture.2015.03.014>

Harikrishnan R, Balasundaram C, Heo M-S, Review: Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. *Aquaculture* 317, 1-15. 2011.

Igiebor FA, Simon PN, Phytochemical profiling and green synthesis of silver and copper nanoparticles using *Hibiscus sabdariffa* and *Xylopiia aethiopicia*. *Studia Universitatis “Vasile Goldiș”, Seria Științele Vietii*. 35(1): 34 – 42. 2025.

Kumar S, Choubey AK, Srivastava PK, The effects of dietary immunostimulants on the innate immune response of Indian major carp: A review. *Fish Shellfish Immunol*. Apr;123:36-49. 2022. doi: 10.1016/j.fsi.2022.02.039.

- Magnadóttir B. Innate immunity of fish (overview), *Fish Shellfish Immunol.* 20 (2006): 137–51. 2006. doi:10.1016/j.fsi.2004.09.006.
- Morera, D, Roher, N, Ribas, L, Balasch, J.C, Donate, C, Callol, A, Boltana, S, Roberts, S, Goetz, G, Goetz, F.W, MacKenzie, S.A. 2011. RNA-Seq reveals an integrated immune response in nucleated erythrocytes. *PLoS One.* 6 (2011) e26998.
- Mureşan ML, Virchea L.-I, Cormoş G, Muntean AC, Crăciunaş M-T, Gligor F-G, Chiş E, Combination therapy: herbal products with antibiotics in combating bacterial resistance – a mini-review of the literature. *Studia Universitatis “Vasile Goldiş”, Seria Ştiinţele Vietii.* 35(1): 23 – 33. 2025.
- Nie G-X, Effects of *Rehmannia glutinosa* on growth performance, immunological parameters and disease resistance to *Aeromonas hydrophila* in common carp (*Cyprinus carpio* L.). *Aquaculture* 435, 293-300. 2015.
- Pathania D, Katoch R, Mahajan A, Sharma M, Paul R, Etiopathological investigations on clinical dropsy in carps (*Cyprinus carpio*) due to *Aeromonas hydrophila* in Himachal Pradesh. *Indian Journal of Animal Sciences* (2002) 72(2) 197-19. 2002.
- Rajabiesterabadi H, Yousefi M, Hoseini SM, Enhanced haematological and immune responses in common carp *Cyprinus carpio* fed with olive leaf extract-supplemented diets and subjected to ambient ammonia. *Aquaculture Nutrition*, 2020:00:1-9. 2019. doi:10.1111/anu.13035.
- Rosenhech E, Lobiuc A, Zamfirache M-M, Analysis of phytochemical parameters with potential pharmaceutical applications in three *Viola* species, subgenus *viola*. *Studia Universitatis “Vasile Goldiş”, Seria Ştiinţele Vietii.* 35(1): 14-22. 2025.
- Secombes CJ, Fletcher TC, The role of phagocytes in the protective mechanisms of fish, *Annu. Rev. Fish Dis.* 2 (1992) 53–71. 1992. doi:10.1016/0959-8030(92)90056-4.
- Shoemaker CA, Klesius PH, Lim C, Immunity and disease resistance in fish. *Nutrition and Fish Health* (ed. by Lim C, Webster CD), pp.149-162. 2001.
- Silva MT, Correia-Neves M, Neutrophils and macrophages: the main partners of phagocyte cell systems. *Front Immunol.* 2012;3:174. 2012. doi:10.3389/fimmu.2012.00174.
- Song SK, Beck BR, Kim D, Park J, Kim J, Kim HD, Ringø E. Prebiotics as immunostimulants in aquaculture: a review. *Fish Shellfish Immunol.* 40(1):40-8. 2014. doi: 10.1016/j.fsi.2014.06.016.
- Tawwab MA, Hamed HS, Antagonistic effects of dietary guava (*Psidium guajava*) leaves extract on growth, hemato-biochemical, and immunity response of cypermethrin-intoxicated Nile tilapia, *Oreochromis niloticus*, finger lings. *Aquaculture.* 529(2):668-735. 2020. https://doi.org/10.1016/j.aquaculture.2020.735668
- Vallejos-Vidal E, Reyes-López F, Teles M, MacKenzie S, The response of fish to immunostimulant diets. *Fish & shellfish immunology*, 56, 34–69. 2016. https://doi.org/10.1016/j.fsi.2016.06.028.
- Wang W, Sun J, Liu C, Xue Z, Application of immunostimulants in aquaculture: current knowledge and future perspectives. *Aquaculture Research*, 2016, 1–23. 2016. doi:10.1111/are.13161.
- Wheater PR, Stevens A, Wheater's basic histopathology: a colour atlas and text (PDF). Edinburgh: Churchill Livingstone. 2002.
- Zhang W, Wang J, Chen Y, Zheng H, Xie B, Sun Z, Flavonoid compounds and antibacterial mechanisms of different parts of white guava (*Psidium guajava* L. cv. Pearl). *Natural Product Research*, 34(11), 1621–1625. 2020. https://doi.org/10.1080/14786419.2018.1522313