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Indigofera zollingeriana as an Alternative Feed and Immunostimulant For Snakehead Fish Infected with Aeromonas hydrophila

Siti Lestari[1]*, Rizki Eka Puteri[2], Rani Ria Rizki[2] , Guttifera[2], Donny Prariska[2], Raudhatus Sa'adah[3], Febby Hadi Setyawan[4], Ramanda Ahmad Rizal Rifa'i[5]

[1]Freshwater Aquaculture Study Program, Rejang Lebong State Community Academy, Jl Basuki Rahmat No27 Dwi Tunggal, Curup, Kabupaten Rejang Lebong, Bengkulu, Indonesia
[2]Fisheries Science Program, Faculty Of Agriculture, South Sumatra University, Jalan Letnan Murod Nomor 55, Talang Ratu, Palembang, Indonesia
[3]Food Agribusiness Study Program, Department of Agricultural Technology and Business Engineering, Sriwijaya State Polytechnic, Jl. Srijaya Negara Bukit Lama, Palembang City
[4]Aquaculture Study Program, Faculty of Agriculture, Samudra University, Jl. Prof.Dr.Syarief Thayeb, Meurandeh, Kec. langsa, Langsa City, Aceh, Indonesia
[5]Aquaculture Study Program, Faculty of Fisheries and Marine Science, Mulawarman University, Jl. Gn. Tabur, Gn. Kelua, Kec. Samarinda Ulu, Samarinda City, East Kalimantan

*Correspondence Author: sitilestari088@gmail.com

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ABSTRACT

The demand for snakehead fish in South Sumatra continues to increase, encouraging farmers to develop intensive cultivation. However, the main challenges of high feed costs and disease attacks need to be addressed to maintain optimal productivity. One alternative being studied is the use of Indigofera zollingeriana as a source of protein and immunostimulant in feed. This study aims to evaluate the effect of substituting fish meal with Indigofera flour on the immune response of snakehead fish (Channa striata) infected with Aeromonas hydrophila bacteria. The study used a Completely Randomized Design with four treatments of fish meal substitution with Indigofera zollingeriana flour, namely 0%, 15%, 30%, and 45%. The parameters observed included phytochemical tests, growth, blood profile, and water quality. The results of the phytochemical test showed the presence of flavonoids, alkaloids, tannins, and saponins. In the blood profile, the 45% treatment produced the highest erythrocyte value (4.54×10^4 cells) and hematocrit of 38%. The best growth was shown in the 30% treatment with an SGR value of 2.56±0.23d and an FCR of 1.61±0.11a, while the highest survival rate (84.44%) was obtained in the 45% treatment. These findings indicate that Indigofera has the potential as a functional feed to improve the immunity and productivity of snakehead fish.

INTRODUCTION

The high demand for snakehead fish fish (Channa striata), especially in South Sumatra, has encouraged fish farmers to start intensive snakehead fish farming. The increasing demand cannot be met solely by catching snakehead fish directly from their natural habitat. Continuous fishing of snakehead fish in the wild can also have a negative impact on the population of these fish in their natural habitat (KKP, 2020). However, intensive snakehead fish farming is not an easy task for fish farmers. There are many factors that need to be considered in the catfish farming process, such as seed quality, feed, and the environment (Muslim et al., 2021; Mutmainnah, 2013). The issues of feed prices and high disease rates in catfish need to be addressed so that catfish production can continue to increase.

One of the diseases that often attacks farmed snakehead fish is Motile Aeromonas Septicemia (MAS), caused by Aeromonas hydrophila. This bacterial infection causes mortality in farmed fish, resulting in significant losses for farmers due to high mortality rates (Kong et al., 2021; Li et al., 2022). One of the efforts that can be made to overcome Aeromonas hydrophila infection is by boosting the fish's immunity through feeding them high-quality nutrition and adding immunostimulants (Abdullah, 2014). One of the feed ingredients currently being developed for use as an alternative protein source in fish feed with good nutritional quality is Indigofera zollingeriana.

Indigofera zollingeriana is a type of leguminous tree that grows well in Indonesia (Abdullah, 2014). In addition to being high in protein, this plant also contains several bioactive chemicals or phytochemicals (tannins, saponins, phenols, flavonoids, terpenoids, alkaloids), minerals, vitamins, and fatty acids. These compounds can be used as antibacterial, neuroprotective, and antioxidant agents (Alaghe, 2020; Gerometta et al., 2020; Karakousi et al., 2018; Talha et al., 2022). The use of Indigofera zollingeriana in feed formulation, apart from being a source of protein, can also be used as an antimicrobial and immunomodulator.

Several studies have shown the use of Indigofera zollingerina flour on fish growth and its effect on fish color pigments (Mukti et al., 2019; Jefry et al., 2021; Pratama et al., 2019). Research on the use of Indigofera zollingeriana as an antibacterial, immunostimulant, anti-inflammatory, and antioxidant has also been widely conducted in animals and humans (Palupi et al., 2022; Javeda et al., 2020; Santi, 2017; Herawati et al., 2021). Given the potential of Indigofera zollingeriana, the researchers wanted to examine the potential use of Indigofera zollingeriana as an alternative protein source and also as an immunostimulant in snakehead fish (Channa striata) infected with the bacterium Aeromonas hydrophila.

METHOD

This research was conducted at the Fish Hatchery Laboratory in Sungai Dua Village, Banyuasin, South Sumatra, Department of Fisheries, Faculty of Agriculture, University of South Sumatra, from July to September 2023.

The research design used was a completely randomized design (CRD). The treatments in this study involved the addition of Indigofera zollingerina flour substituted with fish meal at different levels: 0%, 15%, 30%, and 45% (Shulikin *et al.*, 2021). The purpose of this treatment was to determine the most effective immunostimulant for snakehead fish (*Channa striata*). Each treatment was repeated three times and placed randomly. The tools and materials used during the study were: a 100 cm x 100 cm x 40 cm aquarium, a blower, aeration stones, a DO meter, a thermometer, a pH meter, a fish scoop, a bucket, digital scales, a syringe, EDTA, *Aeromonas hydrophila* bacteria, 3-5 cm long snakehead fish fry with a stocking density of 2 fish/liter, and *Indigofera zollingeriana* powder obtained from the agricultural office.

Research Procedure

Feed Formulation

The feed formulation was calculated using Microsoft Excel. The feed formula to be used consisted of fish meal, soybean meal, *Indigofera zollingeriana* flour, MBM, rice bran meal, wheat flour, vitamin-mineral premix, and tapioca. The ingredients were then sifted and weighed using digital scales according to the composition of each treatment. The ingredients are mixed, starting with the ingredients with the least composition, followed by those with more. Each treatment is made in 10 kg batches with a composition according to the formulation. Then it is printed using a pellet machine.

Table 1. Treatment Feed Formulation

Ingredient Composition (%)	**Treatment**			
	A (0%)	**B (15%)**	**C (30%)**	**D 45%)**
Fish Meal	22	18,70	15,40	12,10
Soy Bean Meal	23	23	23	23
Meat Bone Meal	28	28	28	28

Ingredient Composition (%)	Treatment			
	A (0%)	B (15%)	C (30%)	D 45%)
Indigofera zollingeriana flour	0	5,28	10,55	15,83
Rice Bran Meal	5	5	5	5
Corn Flour	5	5	5	5
Tapioca	14	12,02	10,05	8,07
vitamin-mineral premix	3	3	3	3
Total	**100**	**100**	**100**	**100**
Protein (%)	35,37	35,37	35,37	35,36
Fat (%)	7,38	7,50	7,62	7,75
Crude Fiber (%)	7,54	8,36	9,19	10,02
Ash (%)	11,39	8,53	8,66	8,79
Nitrogen-Free Extract (NFE) (%)	38,32	37,24	36,12	35,51
Energy (Kkal/Kg)	3.970	3.930	3900	3.890
Essential Amino Acids:				
- Arginine	1,66	1,58	1,50	1,42
- Histidine	0,62	0,69	0,76	0,83
- Isoleucine	1,02	1,04	1,06	1,09
- Leucine	1,76	1,74	1,71	1,69
- Lysin	1,61	1,54	1,46	1,38
- Methionine	0,57	0,59	0,61	0,63
- Cysteine	0,41	0,43	0,45	0,47
- Phenylalanine	1,16	1,20	1,25	1,29
- Tyrosine	0,81	0,79	0,77	0,76
- Threonine	0,98	1,04	1,10	1,16
- Tryptophan	0,27	0,27	0,28	0,29
- Valine	1,28	1,32	1,35	1,39

Research Method

Fish seeds were placed in each aquarium at 2 fish/L and maintained for 30 days. Feeding was 5% of the total biomass per day with a frequency of 3 times a day at 08.00, 12.00 and 16.00 WIB. Measurements of temperature, pH and DO of the maintenance water were carried out every day before siphoning in the morning and afternoon at 06.00 and 17.00 WIB. Meanwhile, measurements of ammonia levels were carried out every 10 days at 17.00 WIB. Snakehead fish (*Channa striata*) were infected with *Aeromonas hydrophila* bacteria on the 21st day. Blood profile observations were carried out at the beginning and end of the study.

The research parameters taken were phytochemical tests of *Indigofera zollingerina* flour, survival rate (SR), and absolute growth. The blood profile parameters observed were total leukocytes (cells/mm^3), total erythrocytes (cells/mm3), hemoglobin levels (G%), hematocrit levels (%) and glucose levels (Nursatia et al., 2017). The supporting parameters observed were water quality which included temperature, pH, DO, and ammonia.

Data Analysis

The research data were analyzed statistically using analysis of variance (ANOVA) with the F test at a 95% confidence level using SPSS 20 software. If the analysis showed a significant effect, a further Duncan test was carried out to determine the real differences between the treatments given.

RESULTS AND DISCUSSION

Phytochemical analysis

To determine the active compounds contained in *Indigofera zollingeriana* flour, phytochemical testing was conducted. Prior to phytochemical testing, *Indigofera zollingeriana*

flour was extracted using methanol as a solvent. The test results showed that crude *Indigofera zollingerina* extracts using methanol as a solvent contained active compounds, as shown in Table 2.

Table 2. Phytochemical Test Result

No	Identification of compounds	Parameter	Result	Description
1	Flavonoid	Orange, Red		The liquid turns orange (Positive (+))
2	Alkaloid	Brown Sediment		A brown sediment has formed (Positive (+))
3	Tannin	Brown		the liquid turns brown (Positive (+))
4	Saponin	Stable Foam		stable foam is formed (Positive (+))

In phytochemical tests, crude extracts of *Indigofera zollingeriana* showed the presence of active compounds in the form of flavonoids, alkaloids, tannins, and saponins. This indicates the antioxidant activity of these four compounds. The flavonoids, saponins, and tannins have antioxidant and antibiotic activities that can inhibit the growth of bacteria, viruses, and fungi. Phytochemical testing of crude extracts of *Indigofera zollingeriana* revealed the presence of important bioactive compounds such as flavonoids, alkaloids, tannins, and saponins. These compounds function as natural antioxidants that can ward off free radicals and support the immune system of fish. In snakehead fish (*Channa striata*), flavonoids, saponins, and tannins not only act as antioxidants, but also as natural antibiotics that effectively inhibit the growth of bacteria, viruses, and fungi, including disease-causing pathogens such as *Aeromonas hydrophila*. Thus, the use of *Indigofera zollingeriana* flour in snakehead fish (*Channa striata*) feed has the potential to boost the immune system, improve physiological conditions, and support better growth. This potential makes Indigofera a functional feed ingredient that not only replaces conventional protein sources but also provides added value in disease prevention efforts in snakehead fish farming (Ondho, 2020).

Blood Profile of Snakehead Fish

Snakehead fish (*Channa striata*) were raised for 28 days and fed artificial feed with a substitution treatment of *Indigofera zollingeriana* flour and fish meal. After 28 days of raising the snakehead fish, they were infected *with Aeromonas hydrophyla* bacteria and then tested. Blood profile measurements of the snakehead fish (*Channa striata*) were taken 24 hours after infection with *Aeromonas hydrophyla* bacteria. The results of the blood profile measurements are presented in Table 3.

Table 3. Blood Profile of Snake Head Fish

Parameter	Substitution treatment of *Indigofera zollingeriana* flour protein with fish flour protein				
	*Control	A (0%)	B (15%)	C (30%)	D (45%)
Leukocyte	4,51 X 10^4 ± 0,46^a	7,42 X 10^4 ± 0,23^c	7,13 X 10^4 ± 1,02^c	6,68 X 10^4 ± 0,82^{ab}	6,02 X 10^4 ± 0,49^b
Erythrocyte	5,47 X 10^4 ± 0,81^d	2,42 X 10^4 ± 1,32^a	3,34 X 10^4 ± 1,62^b	3,64 X 10^4 ± 2,13^{ab}	4,54 X 10^4 ± 0,54^c
Hemoglobin	8,21 ± 0,51^c	6,62 ± 0,44^a	7,24 ± 0,42^b	7,98 ± 0,56^b	8,02 ±1,02^c
Hematocrit	38 ± 1,42^d	35 ± 1,60^a	36 ± 0,83^b	37 ± 1,22^c	38 ± 0,42^d
Glucose	57 ± 1,41^a	82 ± 1,41^e	76 ±1,51^d	67 ± 1,15^c	61 ±2,82^b

*Control = without treatment and bacterial infection
A = fish meal substituted with Indigofera zollingerina flour (0%)
B = fish meal substituted with Indigofera zollingerina flour (15%)
C = fish meal substituted with Indigofera zollingerina flour (30%)
D= fish meal substituted with Indigofera zollingerina flour (45%)

The blood profile of snakehead fish (*Channa striata*) was obtained after 28 days of fish cultivation and then tested with *Aeromonas hydropilla* bacteria. The highest leukocyte value was found in the 0% treatment with a leukocyte value of 7. 42 × 10^4, while the lowest leukocyte count was observed in untreated snakehead fish (*Channa striata*) that were not challenged with *Aeromonas hydropilla* bacteria, at 4.51 × 10^4. The fish experienced an increase in leukocyte count due to infection or attack by *Aeromonas hydrophila* bacteria, and the feed in the 0% treatment did not contain antioxidant compounds capable of boosting the immune system in snakehead fish (*Channa striata*). The erythrocyte blood profile in snakehead fish (*Channa striata*) showed the highest value in the 45% feed treatment with an erythrocyte count of 4.54 × 10^4 and the lowest in the 0% feed treatment at 2.42 × 10^4. This is directly proportional to the hematocrit value, where the highest value was in the 45% feed treatment at 38% and the lowest in the 0% treatment at 35%.

Hemoglobin levels in snakehead fish (*Channa striata*) that had been raised for 28 days and then infected with *Aeromonas hydrophila* bacteria for 24 hours also decreased in the 0% feed treatment, while in the 45% treatment, hemoglobin levels were higher than in the other treatments, at 8.02. Blood hemoglobin levels are closely related to the number of erythrocytes in the fish's body. The lower the hemoglobin level in the fish's blood, the more likely the fish is to experience anemia after infection with *Aeromonas hydrophila* bacteria. Observations of blood glucose levels in snakehead fish (*Channa striata*) in all treatments fluctuated. The treatment with the best glucose value was the 45% treatment with a value of 61, which is within the normal range. High glucose levels were found in the 0% treatment with a value of 82. High glucose levels in fish blood indicate that the fish are experiencing stress.

The results showed that the blood profile of snakehead fish (*Channa striata*) was influenced by the level of *Indigofera zollingeriana* flour substitution in the feed. The highest leukocyte count was found in the 0% treatment (7.42 × 10^4 cells) due to Aeromonas hydrophila infection without the protection of bioactive compounds, while the lowest count was found in the control fish (4.51 × 10^4 cells). Conversely, the highest erythrocyte, hematocrit, and hemoglobin counts were obtained in the 45% treatment (4.54 × 10^4 cells; 38%; 8.02 g/dL), indicating better physiological conditions due to the flavonoid, saponin, and tannin content of *Indigofera zollingeriana*, which act as antioxidants and antibacterials. The decrease in erythrocytes and hemoglobin in the 0% treatment was thought to be caused by *Aeromonas hydrophila* toxin, which lyses red blood cells, potentially causing anemia in fish. Blood glucose levels also fluctuated, with the highest value in the 0% treatment (82 mg/dL) indicating stress, while the 45% treatment (61 mg/dL) was within the normal range. This condition shows that Indigofera can boost the immune response and suppress physiological stress in snakehead fish. This is in line with the opinion of Harikrishnan et al. (2011), who stated that bioactive

compounds such as flavonoids and saponins function as natural immunostimulants, increasing fish resistance to bacterial infections and supporting physiological blood balance. *Indigofera zollingeriana* is known to have good nutritional content, making it a potential alternative protein source in fish feed formulations. Based on proximate analysis results, Indigofera shoot flour contains 27.97% crude protein, 15.25% crude fiber, 0.22% calcium, 0.18% phosphorus, and bioactive pigments in the form of 507.6 mg/kg xanthophyll and carotenoids (Alagbe et al., 2020; Suharlina et al., 2019). This high protein content is reinforced by a complete amino acid profile, including histidine (0.67%), threonine (1.14%), arginine (1.67%), tyrosine (1.05%), methionine (0.43%), valine (1.56%), phenylalanine (1.60%), isoleucine (1.35%), leucine (2.26%), and lysine (1.57%) (Palupi et al., 2014). This essential amino acid profile not only supports growth but also indirectly enhances the immune system when consumed by animals (Huaa et al., 2019; Hastuti et al., 2020; Santi et al., 2018).

Indigofera zollingeriana also contains protein and amino acids. *Indigofera zollingeriana* is also rich in natural pigments such as xanthophyll, β-carotene, and carotenoids, which play an important role as antioxidants, thereby reducing oxidative stress in organisms (Hutapea et al., 2018). Other bioactive compounds include tannins, saponins, phenols, flavonoids, terpenoids, alkaloids, and steroids. The presence of these phytochemical compounds provides various physiological effects, including antibacterial, antifungal, antiviral, anti-inflammatory, hypolipidemic, neuroprotective, anti-allergic, hepatoprotective, antispasmodic, antioxidant, and immunostimulatory (Li et al., 2020; Geromettaa et al., 2020; Karakousi et al., 2018; Stalha et al., 2022; Javeda et al., 2020). This makes Indigofera not only a source of nutrition, but also a functional feed that can boost fish immunity.

Growth of Snakehead Fish

This study also examined the growth parameters of snakehead fish over a period of 28 days. The results of the growth parameters (SR, SGR, FCR, and PER) are presented in Table 4.

Tabel 4. Growth parameter results for snakehead fish.

Parameter	Substitution treatment of *Indigofera zollingerina* flour protein with fish flour protein			
	A (0%)	B (15%)	C (30%)	D (45%)
SR (%)	75,56±4,15[a]	80 ± 3,33[b]	82,22 ± 2,19[c]	84,44 ± 1,68[d]
SGR (%BW/day)	1,83±011[a]	2,16±0,02[b]	2,56±0,23[d]	2,23±0,41[c]
FCR	3,01±0,10[d]	2,22±0,04[c]	1,61±0,11[a]	1,95±0,03[b]
PER	1,04±0,05[a]	1,34±0,16[b]	1,76±0,24[d]	1,42±0,12[c]

The survival rate (SR) of snakehead fish was observed to average between 75.56 and 84.44%. The fluctuating SR values were due to the fish being infected with Aeromonas hydropilla bacteria, which caused the fish to become ill and die. The best specific growth rate, feed conversion ratio (FCR), and protein efficiency ratio (PER) in this treatment were found in the treatment with 30% indigofera flour substitution, with values of 2.56, 1.61, and 1.76, respectively. The use of indigofera flour in snakehead fish feed can be utilized at 30% because the fish are able to absorb the protein in the feed. However, in the 45% treatment, the fish experienced a decline in growth due to the fiber content in indigofera, which made it difficult for the fish to digest the feed. The best growth of snakehead fish was observed in the 30% treatment, while the best immunostimulant effect on snakehead fish was observed in the 45% treatment because the growth of the fish was inhibited by coarse fiber, while the best immune system of the fish was observed in the 45% treatment due to the antioxidant, active compound, and antibacterial content in indigofera flour.

Previous research reported that the addition of *Indigofera zollingeriana* leaf meal with a substitution rate of more than 15% in feed has been proven to provide good performance on the growth performance of various types of farmed fish. In catfish, the use of Indigofera can increase the daily growth rate while improving feed utilization efficiency. Similarly, in gourami, the addition of Indigofera has a positive effect on absolute length and absolute weight growth,

and supports fish survival. Meanwhile, in Sumatran fish, the nutritional content and bioactive compounds of Indigofera not only increase growth but also strengthen the immune system so that fish are more resistant to disease attacks (Mukti et al., 2019; Jefry et al., 2021; Pratama et al., 2019). This research shows that *Indigofera zollingeriana* not only functions as an alternative protein source, but also as a functional feed that plays a role in improving growth performance, maintaining health, and increasing the body's resistance to various environmental stresses and pathogen infections. Thus, the use of Indigofera in fish feed formulations has promising prospects to support sustainable fish farming.

CONCLUSION

Based on the results of the study, it can be concluded that Indigofera zollingeriana has great potential as a functional feed ingredient for snakehead fish (Channa striata). Phytochemical testing showed that it contains bioactive compounds such as flavonoids, alkaloids, tannins, and saponins, which play an important role in supporting the fish's immune system. In blood profiles, the 45% Indigofera flour substitution treatment improved the physiological health of the fish, with the highest erythrocyte value reaching 4.54×10^4 cells and a hematocrit of 38%. In terms of growth performance, the best results were obtained in the 30% treatment with an SGR value of 2.56±0.23d and an FCR of 1.61±0.11a, indicating more optimal feed efficiency. In addition, the highest survival rate (84.44%) was also found in the 45% treatment. This study indicates that Indigofera not only functions as an alternative protein source but also provides physiological benefits through enhanced immunity, growth, and survival rates in catfish, making it highly promising for use in the development of healthy and sustainable aquaculture feed.

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