International Journal on Advanced Science Engineering Information Technology

Vol.13 (2023) No. 6
ISSN: 2088-5334

Activity of Natural Compound *Pothos tener* Wall on *Aeromonas hydrophila* Infection to Prevent of Antibiotics

Media Fitri Isma Nugraha [a,*], Dwi Asih Kurniati [b], Sri Rukmini [c], Imam Taukhid [d], Hessy Novita [e], Bejo Slamet [d], Ikhsan Khasani [d], Dwi Budiyanto Trisnoharjono [f], Muh. Alias L Rajamuddin [g], Berna Elya [b]

[a] *Research Centre for Pharmaceutical Ingredients and Traditional Medicine-National Research and Innovation Agency of Indonesia, Cibinong Science Centre Area, Cibinong, Bogor, West Java, Indonesia*
[b] *Faculty of Pharmacy, University of Indonesia, Depok, West Java, Indonesia*
[c] *Department of Aquaculture, Faculty of Fisheries and Marine Science, IPB University, Bogor, West Java, Indonesia*
[d] *Research Center for Fisheries- National Research and Innovation Agency of Indonesia*
[e] *Research Center for Veterinary - National Research and Innovation Agency of Indonesia*
[f] *Research Center for Marine and Land Bioindustry- National Research and Innovation Agency of Indonesia*
[g] *Pangkep State Polytechnic of Agriculture South Sulawesi, Makassar, South Sulawesi, Indonesia*
Corresponding author: [*]medi005@brin.go.id/nugrahamediafitriisma@gmail.com

***Abstract*—This study aims to discover how influential *Photos tener* Wall is as an anti-bacterial treatment for *Aeromonas hydrophila*. Methods: *Cyprinus Carpio* were reared for 28 days, and on the 29th and 30th days before *Aeromonas hydrophila* infection, the fish were adequately fasted. On the 31st day, they were intramuscularly challenged with *A. hydrophila* (105 CFU/mL) (the first day in *A. hydrophila* infection). The treatments given were (I) immersed trial with fresh (live) *P tener* Wall: (H1) Immersed with 15 g of *P. tener* Wall plant, (H2) Immersed with 30 g of *P. tener* Wall plant, and (H3) Immersed with 60 g of *P. tener*; (II) feeding trials in which the treatments given were (P1) 2% of *P. tener* Wall powder mixed with 1 kg commercial diet and (P2) 4% of *P. tener* Wall powder mixed with 1 kg commercial diet; Experiment III combined the best results from experiment I (H2) and experiment II (P2) and Oxytetracycline 5 g/kg feed as a control antibiotic. The result obtained was that the treatment of 30 g of fresh *P. tener* Wall or adding 4% simplicial *P. tener* Wall in the diet could increase koi fish's immune response and resistance to *A. Hydrophila* has a survival rate that reaches 100%. This treatment has the same effect as using antibiotic Oxytetracycline 5 g/kg of feed. An important aspect for further research is that *P. tener* wall can be tested on other fish diseases caused by bacteria or fungi.**

***Keywords*—*Pothos tener* wall; *Cyprinus carpio*; GC-MS; LC-MS; immune response.**

Manuscript received 19 Jun. 2023; revised 5 Oct. 2023; accepted 29 Nov. 2023. Date of publication 31 Dec. 2023.

I. INTRODUCTION

Pothos tener Wall is an aquatic plant from Makassar, South Sulawesi, Indonesia. This plant can be found in the Bantimurung Bulusaraung waterfall area and is known as an endemic flora. A previous study by [1] described that *Pothos tener* Wall plant contains several secondary metabolite compounds, such as flavonoid, phenol, and glycoside, with a total phenol content of 8,519 mg GAE/gram and a total flavonoid content of 7.16 mg QE/gram. *Pothos tener* Wall is also part of the Araceae family with the same genus, namely *Pothos scandens,* which is known to have various pharmacological activities such as anti-microbial, antioxidant, antipyretic, antidiabetic, bronchodilator, and others [2], [3]. The Araceae family is one of the families used in traditional medicine in Asia [4]–[6]. One of the Araceae species that has been researched is *Pothos scandens*, which is known to have various pharmacological activities, such as anti-microbial, antioxidant, antipyretic, antidiabetic activity, and anti-microbial activity against *Escherichia coli* and *Candida albicans* [7], [8].

Koi carp is one of the most well-known ornamental freshwater fish species. The high potential in cultivating Koi carp has attracted many ornamental fish hobbyists worldwide [9], [10]. However, the market needs have not been fulfilled because of some problems in cultivating koi carp. The main problem is the infection of *Aeromonas hydrophila* bacteria, which causes 80-100% mortality in koi carp, known as Motile *Aeromonas Septicemia* (MAS) [11]–[14]. These pathogenic bacteria produce toxigenic substances that cause tissue

damage in fish, like sores around the mouth, skin hemorrhages, and lesions [15]–[19].

The latest disease control in fish cultivation is through antibiotics, such as penicillin, oxytetracycline, or ciprofloxacin. These materials often cause bacterial resistance and aquatic ecological damage. Over the last decades, many studies have shown that alternative phytopharmaceutical properties or immunostimulants are required to modulate the fish immune system more safely, in addition to anti-microbial properties, feed attractants, and growth promoters [20], [21]. Aquatic herbs are one of the safe phytopharmaceuticals for the aquatic environment and unchallenged human needs [22].

However, there has been no research on preventing carp from being infected with *A. hydrophila* using *P. tener* Wall. This condition urges a further study regarding the effectiveness of *P. tener* Wall in preventing the *A. hydrophila* infection in koi carp (*C. Carpio*), applied either as fresh materials in a fish maintenance environment or as supplemented materials in a fish diet. This study aims to find out how influential *P. tener* Wall is as an anti-bacterial treatment for *Aeromonas hydrophila* infection in koi fish (*Cyprinus Carpio*).

II. Materials and Methods

A. Plant Material

Fresh plant material was collected in April 2020 from Bantimurung Bulusaraung National Park waterfall, Makassar, Indonesia. The plant was authenticated at the Indonesian Institute of Sciences (LIPI), and the voucher specimen was deposited at the department for future reference (KU/AB/KSV/315). The fresh plant material was washed under running tap water to remove surface pollutants.

B. Fish and Bacteria Materials

The koi carp fish were obtained from the Ministry of Marine Affairs and Fisheries - Research Centre for Ornamental Fish Cultivation, Depok, West Java, Indonesia. Samples with an average length of 9 ± 0.5 cm and an average weight of 10 ± 2.1 g were stocked for ten fish/aquarium. *A. hydrophila* bacterial isolates were obtained from the collection of the Aquatic Organism Health Laboratory, Department of Aquaculture, the Faculty of Fisheries and Marine Sciences, IPB, West Java, Indonesia.

C. Ethics Statement

This experiment passed and met the animal ethics and animal welfare test requirements by the Animal Ethics Commission, Bogor Agricultural University, Indonesia, with registration number 219-2021.

D. Characterization and Culture Preparation of Aeromonas Hydrophila

Bacteria used were characterized following the Cowan's method (1974), and the KIT API 20NE (bio Mérieux, Inc., North Carolina, USA) test was performed by reading the results through the API WEB software (http://apiweb.biomeriux.com/strip/3). *A. hydrophila* bacteria were a collection of the Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, IPB West Java, Indonesia. They were tested for virulence level after being re-isolated on TSA (tryptic soy agar) and RS (Rimler-Shotts) media incubated at 30ºC for 24 hours. The characterization was performed based on the method [21].

E. Virulence Test

The virulence test aimed to evoke the bacterial virulence level. *A. hydrophila* used for the virulence test was cultured in TSB (tryptic soy broth) on a 140-rpm shaker at 29°C for 24 hours. Harvesting was performed through centrifugation for 10 minutes at 3000 rpm, then rinsed with sterile phosphate-buffered saline (PBS) and homogenized in a vortex. The bacteria were injected into the koi carp intramuscularly at 0.1 ml per fish. The liver, spleen, and kidney of fish with clinical symptoms were taken for bacterial isolation and identification. The bacteria obtained from those organs were isolated on RS medium and incubated at 25-37˚C for 24 hours to prove if the bacteria were indeed *A. hydrophila*. Bacterial characterization was also performed using the [21]method, API 20E kit, and API 20 Strep kit (bio Mérieux, Inc., North Carolina, USA). The isolate was used for further experiments after being proven to be an A. hydrophila bacteria.

F. Pothos Tener Wall GC-MS Analysis

DKI Jakarta Regional Health Laboratory carried out the GC-MS test using the Agilent Technologies 7890 gas chromatography instrument with an autosampler and a mass selective detector of the 5975 Chem Station data system. The capillary column separated compounds with a length of 30 m, a diameter of 0.20 mm, and a layer thickness of 0.11 µm. Samples were injected with a split ratio of 8:1 and a 1.2 mL/minute flow rate. Overtemperature conditions were carried out at an initial temperature of 80ºC, which was then increased to 3ºC/minute to 150ºC, held for 1 minute, increased to 20ºC/minute to 280ºC, and then held for 26 minutes. The injection temperature was maintained at 250ºC with carrier gas helium. Constituents were identified by comparing the library database to the tool.

G. Pothos Tener Wall LC-MS Analysis

The analysis was carried out by DKI Jakarta Regional Health Laboratory using Waters Alliance 2695 HPLC Pump Gradient Timetable with Mass Lynx 4.1 Software. HPLC was used with the MS Scan type with the ES + ion mode. A full-scan model from m/z 50-1200 was performed with a scan time of 5 seconds and an interscan of 0.1 seconds. The column used HPLC X Bridge® C18 3.5 µm (2.1 x 100 mm) for analysis. The solvent used was methanol with 90% water. The flow rate was 0.2 ml/min.

H. Experimental Fish, Feeding Trials, and Bacterial Challenges

The fish were kept for 28 days in a uniformly conditioned aquarium. On the 29th and 30th days, the fish were fasted. On the 31st day, the fish were given the treatment with *A. hydrophilla* infection. Post-infection observation began on the 32nd day (this was the first day of the post-infection of *A. hydrophila*). Feeding was performed thrice daily at 08.00, 12.00, and 16.00 (GMT +7) until apparent satiation.

I. Fish were Split into Three Experimental Groups

1) Immersion experiment with fresh (live) P. tener Wall: These experimental fish were fed with a commercial diet (Breeder Pro – CP Petindo). The treatments were:

- (H1) Koi immersed with 15 g of *P. tener* Wall plant during rearing.
- (H2) Koi immersed with 30 g of *P. tener* Wall plant during the rearing period.
- (H3) Koi immersed with 60 g of *P. tener* Wall plant during the rearing period.
- (K-) Fish injected with PBS without *P. tener* Wall treatment (negative control)
- (K+) Fish injected with *A. hydrophila* without *P.tener* Wall treatment (positive control).

2) Feeding experiment: One kg commercial feed was ground and mixed with *P. tener* Wall powder based on dose treatment, and an addition of 1%Carboxymethyl Cellulose (brand: Koepoe-koepoe) was used as a binder in 300 mL of water to mix this formula, which was then repelled. Fish in the treatment were fed commercially (Breeder Pro – CP Petindo). The treatments were as follows:

- (P1) 2% of P. tener Wall powder was mixed into the commercial diet during rearing.
- (P2) 4% of P. tener Wall powder was mixed into the commercial diet during rearing.
- (K-) Fish were injected with PBS without P. tener Wall treatment (Negative control).
- (K+) Fish were injected with A. hydrophila without P. tener Wall treatment (Positive control).

3) Experiment III combined the best results from Experiments I and II and was compared with an antibiotic. Fish in the treatment were given commercial feed (Breeder Pro – CP Petindo). The treatments were:

- (C) The best result in experiment I was mixed with the best result in experiment II (H2 + P2).
- (KA) Oxytetracycline 5 g/ kg feed.
- (K-) Fish were injected with PBS without *P. tener* Wall treatment (negative control).
- (K+) Fish were injected with *A. hydrophila* without *P. tener* Wall treatment (positive control).

J. Parameters

The parameters observed in this study included the survival rate [21], immune response: total erythrocytes, hemoglobin, hematocrit, total leucocytes, phagocytic activity, respiratory burst [23], and water quality. Microscopic Observations by Scanning Electron Microscope (SEM), using the SEM Model: JSM – IT 200, were conducted by the Zoology Department of the Biology Research Centre – National Research and Innovation Agency of Indonesia.

K. Data Analysis

All data were tabulated in Microsoft Excel 2010. Data analysis was performed using analysis of variance (ANOVA) with SPSS 16.0 software. The data were continuously analyzed using Duncan's test when a significant difference was found.

III. Results and Discussion

A. Results of Gas Chromatography-Mass Spectrometry (GC-MS) of P. tener Wall

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis is a technique used for analyzing mixed compounds in a sample where gas chromatography can separate the components of the mixture while mass spectroscopy will characterize each of the components that have been separated. GC-MS also provides specificity and sensitivity for simultaneous multi-component analysis (Shimadzu, 2020). In the results of the analysis of ethanol extract of leaves and stem of *P. tener* Wall, GC-MS showed 16 compounds in the leaf extract; the most important content was phytol compounds (33.47%), while seven compounds in the stem extracts had the most extensive content of 31.72% for hexadecenoic acid, methyl ester and followed by 9.12-octadecadienoic acid which amounted to 29.54%. The analysis results can be seen in Tables 1 and 2, with the chromatogram results shown in Figures 1 and 2.

TABLE I
COMPOUNDS CONTENT OF P. TENER WALL LEAF BY GC-MS

RT	Compound name	Molecular formula	Molecular weight	Content (%)	Compound group	Biological activity
4,332	Butyric Acid-2-D1	C4H8O2	89,0	4,02	Fatty acid	[24], [25]
26,259	(2E)-3,7,11,15-Tetramethyl-2-Hexadecene	C20H40	280.0	2,05	Alkene	[26], [27]
26,362	Neophytadiene	C20H38	278.0	3,61	Sesquiterpenoid	[28], [29]
26,431	Phytene	C20H40	280.0	3,56	Carotenoid	-
26,528	2-Pentadecanone, 6,10,14-Trimethyl	C18H36O	268.0	13,45	Diterpenoid	[30]
26,672	-Loliolide	C11H16O3	196.0	2,42	Carotenoid	[31]–[33]
26,879	3,5-Dimethyl-1,6-Heptadiene	C9H16	124.0	1,39	Fatty acid	-
27,396	Hexadecanoic Acid, Methyl Ester	C17H34O2	270.0	6,87	Fatty acid ester	[34]–[36]
27,948	Hexadecanoic acid, ethyl ester	C18H36O2	284.0	1,01	Fatty acid ester	[37]
28,637	Methyl (9Z, 12Z)-9,12-Octadecadienoate	C19H34	294.0	2,95	Fatty acid	[38]
28,672	Trans-13-Octadecenoic acid, methyl ester	C19H36O2	296.0	12,10	Fatty acid ester	[39], [40]
28,803	Phytol	C20H40O	297.0	33,47	Diterpene alcohol	[41]–[43]
29,024	Methyl (9Z,12Z)-9,12-Octadecadienoate	C19H34	294.0	2,21	Fatty acid	[44], [45]
29,072	Z, Z-10,12-Hexadecadien-1-ol acetate	C18H32O2	280.0	2,85	Fatty acid	-
29,361	Oxirane, tridecyl-	C15H30O	226.0	2,22	Polymer	[46]
30,079	1-Propadienylcyclohexanol	C9H14O	123.0	3,81	Alcohol	-

TABLE II
COMPOUNDS CONTENT ANALYSIS OF P. TENER WALL STEM BY GC-MS

RT	Compound name	Molecular formula	Molecular weight	Content (%)	Compound group	Biological activity
26,541	2-Pentadecanone, 6,10,14-trimethyl	C18H36O	268.0	2,63	Ketone	[47]
27,410	Hexadecanoic Acid, Methyl Ester	C17H34O2	270.0	31,72	Fatty acid ester	[34]
27,955	Hexadecanoic acid, ethyl ester	C18H36O2	284.0	2,37	Fatty acid ester	[37]
28,196	Hexadecanoic Acid, 14-Methyl-, Methyl Ester	C18H36O2	284.0	3,36	Fatty acid ester	-
28,658	9,12-Octadecadienoic acid, methyl ester	C19H34O2	294.0	13,64	Fatty acid ester	[48], [49]
28,679	9-12, Octadecadienoic acid, methyl ester	C19H34O2	294.0	29,54	Fatty acid ester	[48]
28,831	Heptadecanoic acid, 16-methyl-, methyl ester	C19H38O2	298.0	16,46	Fatty acid ester	-

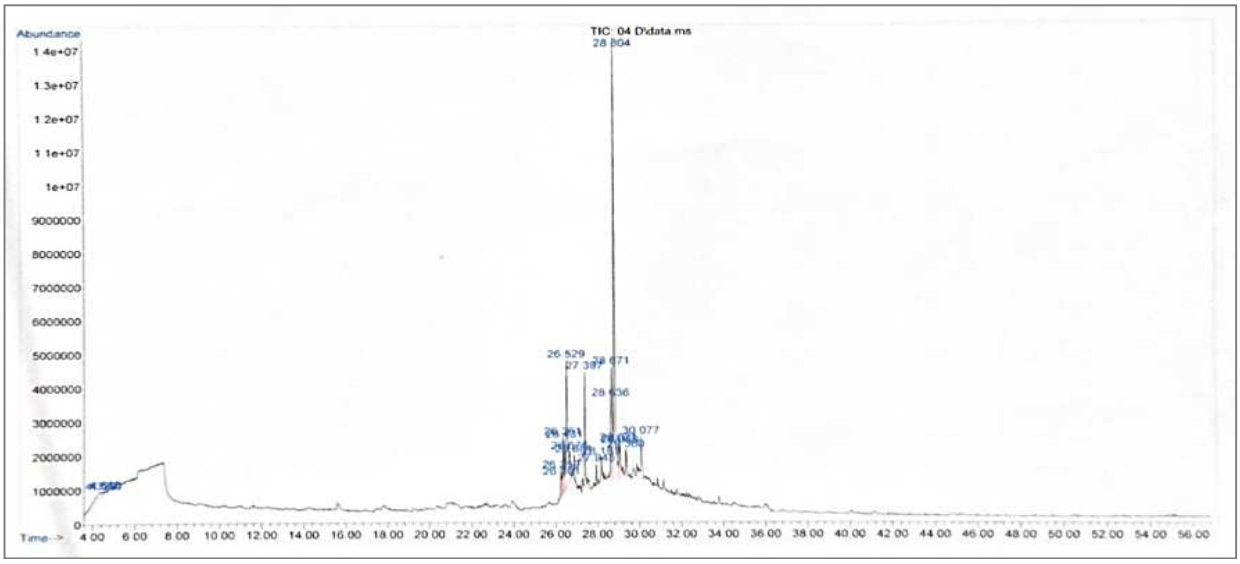

Fig. 1 Chromatogram analysis of compound content of *P. tener* Wall leaf by GC-MS

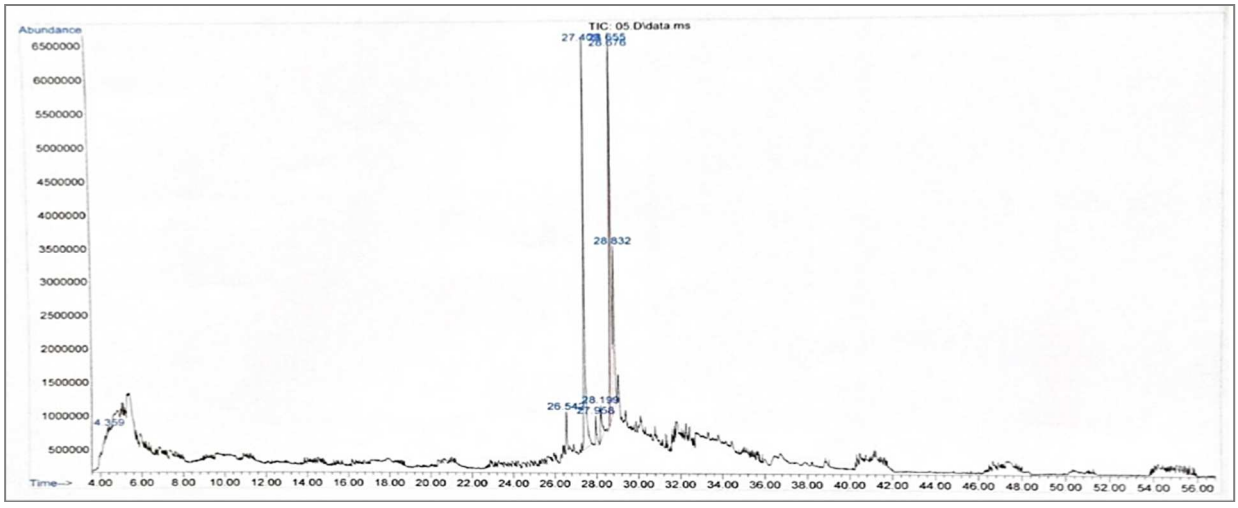

Fig. 2 Chromatogram analysis of compound content of *P. tener* Wall stem by GC-MS

LC-MS analysis was carried out on ethanol extract of *P. tener* Wall leaves. Five peaks were detected with retention times of 1.28, 2.13, 16.27, 18.91, and 20.70 minutes (Figure 3). Meanwhile, four peaks were detected in the ethanol extract of P. tener Wall stems, with retention times of 1.28, 15.24, 17.12, and 19.84 minutes (Figure 4).

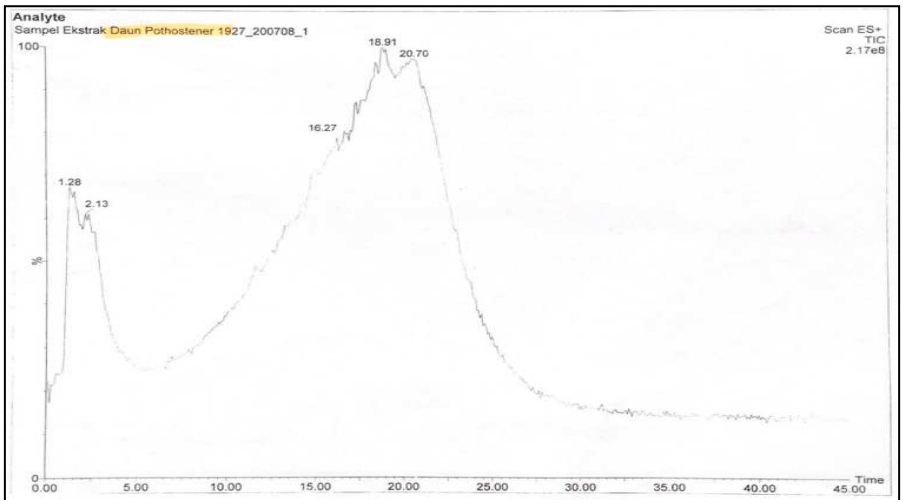

Fig. 3 Chromatogram analysis of compounds content of *P. tener* Wall leaf with LC-MS

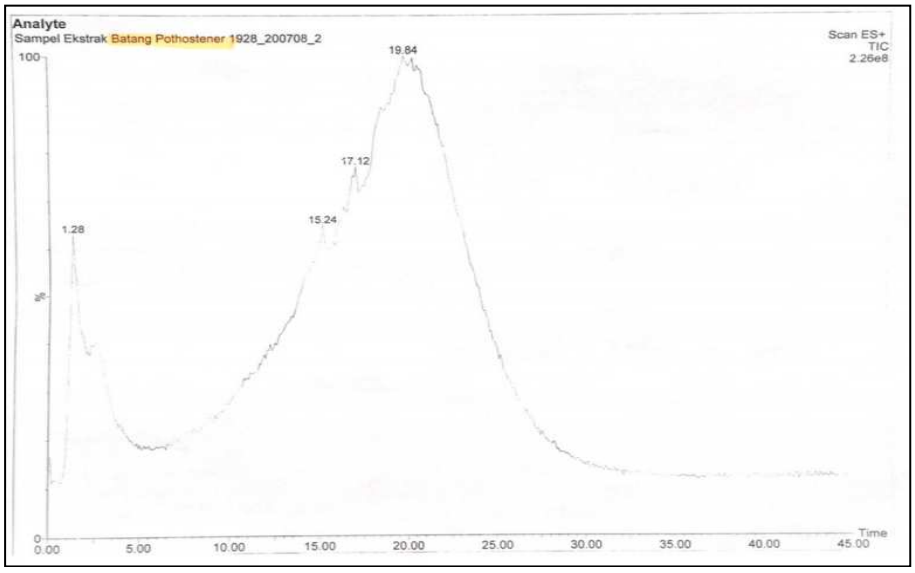

Fig. 4 Chromatogram analysis of compound content of *P. tener* Wall stem with LC-MS

B. Survival Rate

Survival rate is the relative percentage of organisms that survive from the initial to the end of the rearing period. The survival rate data of koi carp before and after the challenge test are presented in Table 3.

TABLE III
THE SURVIVAL RATE OF KOI CARP PRE AND POST-INFECTION

Treatment	Parameter	
	Pre infection (D1-D28)	Post-infection (D2-D6)
Fresh plant treatment		
K+	100±0.0%[a]	10±0.0%[d]
K-	100±0.0%[a]	100±0.0%[a]
H1	100±0.0%[a]	47±0.6%[b]
H2	100±0.0%[a]	43±0.6%[bc]
H3	100±0.0%[a]	33±1.2%[c]
Simplicial plant dietary supplementation treatment		
K+	100±0.0%[a]	20±0.0%[a]
K-	100±0.0%[a]	100±0.0%[c]
P1	100±0.0%[a]	43±0.6%[b]
P2	100±0.0%[a]	53±1.5%[b]
Fresh plant application and plant dietary supplementation		
K+	100±0.0%[a]	43±2.0%[a]
K-	100±0.0%[a]	100±0.0%[b]
KA	100±0.0%[a]	100±0.0%[b]
C	100±0.0%[a]	100±0.0%[b]

C. Water Quality Parameters and Experimental Fish, Feeding Trials, and Bacterial Challenges

Table 4. Water quality parameters during the rearing period. The pH and DO temperatures in the three treatments were almost identical on average. The highest ammonia was found in the aquarium during experiment II, while the highest nitrite was found in the aquarium during experiment I, and the highest nitrate was found in the aquarium during experiment III.

TABLE IV
THE SURVIVAL RATE OF KOI CARP PRE AND POST INFECTION

Parameter	Experimental Treatment		
	I	II	III
Temperature	26–30.5ºC	26.3–30.5ºC	25–30ºC
pH	6.3–7.4	6.0–7.5	6.8–7.4
DO	6.3–6.6 mg/L	6.4–6.6 mg/L	6.3–6.6 mg/L
Ammonia	0.00–10.88 mg/L	7.50–10.76 mg/L	0.048–0.222 mg/L
Nitrite	0.00–5.46 mg/L	0.00–0.31 mg/L	0.045–0.080 mg/L
Nitrate	0.107–0.489 mg/L	0.00–2.29 mg/L	1.616–3.212 mg/L

TABLE V

KOI CARP IMMUNE RESPONSE BEFORE *AEROMONAS HYDROPHILA* INFECTION

Treatment	The Day Before Infection (D1-D28)											
	Hb (g%)		Ht (%)		AF (%)		RB (OD = 630 nm)		TE (× 10^6 sel mm^{-3})		TL (× 10^4 sel mm^{-3})	
	Day		Day		Day		Day		Day		Day	
	1	28	1	28	1	28	1	28	1	28	1	28
Fresh plant treatment (I)												
K+	6,1±0,3^a	9,2±1,6^a	9,7±0,1^a	23,8±0,55^a	37±3,3^a	38±6,4^a	0,28±0,03^a	0,27±0,05^a	0,62±0,04^a	0,68±0,10^a	3,85±0,68^a	4,73±0,26^b
K-	6,1±0,3^a	9,3±1,1^a	9,7±0,1^a	23,1±1,19^a	37±3,3^a	38±6,4^a	0,28±0,03^a	0,27±0,05^a	0,62±0,04^a	0,66±0,11^a	3,85±0,68^a	4,73±0,26^b
H1	6,1±0,3^a	12,7±1,6^a	9,7±0,1^a	26,6±1,28^a	37±3,3^a	42±4,9^a	0,28±0,03^a	0,30±0,05^a	0,62±0,04^a	0,76±0,06^a	3,85±0,68^a	6,06±0,29^d
H2	6,1±0,3^a	10,3±1,1^a	9,7±0,1^a	26,1±1,16^a	37±3,3^a	48±4,4^a	0,28±0,03^a	0,27±0,03^a	0,62±0,04^a	0,81±0,17^b	3,85±0,68^a	5,37±0,29^c
H3	6,1±0,3^a	10,1±1,9^a	9,7±0,1^a	23,1±1,30^a	37±3,3^a	41±3,6^a	0,28±0,03^a	0,27±0,05^a	0,62±0,04^a	0,78±0,04^a	3,85±0,68^a	3,39±0,20^a
Simplicial plant dietary supplementation treatment (II)												
K+	6,5±1,3^a	8,9±1,2^a	9,7±0,6^a	25,0±5,0^a	28±2,6^a	31±3,21^a	0,24±0,06^a	0,30±0,02^a	0,54±0,03^a	0,86±0,10^a	2,80±0,25^a	5,29±0,77^a
K-	6,5±1,3^a	8,1±2,6^a	9,7±0,6^a	22,0±6,1^a	28±2,6^a	31±3,21^a	0,24±0,06^a	0,30±0,02^a	0,54±0,03^a	0,89±0,13^a	2,80±0,25^a	5,29±0,77^a
P1	6,5±1,3^a	9,7±2,4^a	9,7±0,6^a	20,0±6,6^a	28±2,6^a	42±2,71^b	0,24±0,06^a	0,28±0,04^a	0,54±0,03^a	0,92±0,30^a	2,80±0,25^a	6,80±0,87^a
P2	6,5±1,3^a	9,7±2,4^a	9,7±0,6^a	28,9±6,2^a	28±2,6^a	46±3,51^b	0,24±0,06^a	0,31±0,04^a	0,54±0,03^a	0,98±0,30^a	2,80±0,25^a	7,81±0,95^b
Fresh plant application and plant dietary supplementation (III)												
K+	6,5±0,2^a	10,9±0,7^a	21,7±7,5^a	36,72±1,23^a	31±5,03^a	39±3,1^a	0,28±0,07^a	0,34±0,08^a	0,35±0,07^a	0,44±0,03^a	6,25±0,91^a	6,74±0,25^a
K-	6,5±0,2^a	10,6±0,2^a	21,7±7,5^a	35,97±2,68^a	31±5,03^a	39±3,1^a	0,28±0,07^a	0,34±0,08^a	0,35±0,07^a	0,46±0,07^a	6,25±0,91^a	6,74±0,25^a
KA	6,5±0,2^a	12,9±1,3^a	21,7±7,5^a	39,44±3,06^a	31±5,03^a	61±6,1^b	0,28±0,07^a	0,34±0,08^a	0,35±0,07^a	0,48±0,07^a	6,25±0,91^a	7,51±0,59^a
C	6,5±0,2^a	12,0±1,4^a	21,7±7,5^a	47,00±2,95^a	31±5,03^a	65±3,1^b	0,28±0,07^a	0,34±0,08^a	0,35±0,07^a	0,65±0,10^b	6,25±0,91^a	8,19±0,40^b

Note: Different letters in each bar (mean value standard deviation) show statistical differences (Duncan multiple distance test; $p<0.05$). Description: (HB) hemoglobin levels, (HT) hematocrit levels, (AF) phagocytic activity, (RB)respiratory burst, (TE) total erythrocytes, (TL) total leukocytes, (H1) Koi immersed with 15 g of *P. tener* Wall plant during rearing. (H2) Koi immersed with 30 g of *P. tener* Wall plant during the rearing period. (H3) Koi immersed with 60 g of *P. tener* Wall plant during the rearing period. (K-) fish injected with PBS without *P. tener* Wall treatment, (K+) Fish injected with *A. hydrophila* without *P. tener* Wall treatment. (P1) 2% of *P. tener* Wall powder was mixed into the commercial diet during the rearing period. (P2) 4% of *P. tener* Wall powder was mixed into the commercial diet during the rearing period. (KA) Oxytetracycline 5 g/ kg feed. (C) the best result in experiment I was mixed with the best result in experiment II (H2 + P2).

TABLE VI

KOI CARP IMMUNE RESPONSE AFTER *AEROMONAS HYDROPHILA* INFECTION

Treatment	The Day After Infection											
	Hb (g%)		Ht (%)		AF (%)		RB (OD = 630 nm)		TE (× 10^6 sel mm^{-3})		TL (× 10^4 sel mm^{-3})	
	Day		Day		Day		Day		Day		Day	
	2	6	2	6	2	6	2	6	2	6	2	6
Fresh plant treatment (Treatment A)												
K+	4,8±0,4^a	5,0±0,2^a	14,6±5,3^a	20,0±2,0^a	46±5,1^b	40±3,3^b	1,01±0,08^b	0,56±0,11^b	0,66±0,03^a	0,48±0,09^a	10,23±0,79^c	3,59±0,99^a
K-	8,8±1,1^b	9,5±0,9^b	23,1±3,8^a	28,9±4,3^b	39±5,1^a	33±3,3^a	0,56±0,11^a	0,32±0,10^a	0,78±0,14^a	0,96±0,16^b	6,79±0,62^a	4,72±0,36^b
H1	7,6±1,4^b	9,7±1,4^b	25,0±1,0^a	38,1±0,8^c	50±3,3^b	47±1,5^c	1,06±0,17^b	0,82±0,11^c	0,71±0,13^a	1,15±0,06^b	9,14±0,96^b	6,49±0,67^c
H2	7,1±0,8^b	8,5±1,4^b	24,0±1,0^a	28,0±0,7^b	53±6,7^b	42±1,2^b	1,06±0,12^b	0,66±0,06^b	0,97±0,11^a	1,21±0,12^b	11,61±0,61^c	5,89±0,82^b
H3	5,4±0,9^a	5,5±0,4^a	18,2±5,9^a	25,6±3,3^b	50±4,7^b	44±1,5^b	1,02±0,13^b	0,73±0,10^c	0,71±0,13^a	1,03±0,05^b	9,91±0,78^b	5,47±0,56^b
Simplicial plant dietary supplementation treatment (Treatment B)												
K+	5,1±0,7^a	5,5±0,3^a	23,0±1,0^{bc}	22,0±1,0^a	44±2,5^b	31±2,5^a	0,81±0,06^b	0,80±0,18^b	0,63±0,11^a	1,08±0,19^a	8,70±0,78^{ab}	6,99±0,37^b
K-	6,9±1,0^{ab}	6,0±0,8^{ab}	13,4±1,3^a	33,6±1,8^b	31±2,0^a	30±1,0^a	0,42±0,14^a	0,27±0,06^a	0,84±0,18^b	1,44±0,33^b	7,64±0,24^a	5,77±0,24^a
P1	6,5±0,8^a	8,8±1,8^b	18,8±2,3^b	35,0±1,4^b	48±6,0^c	33±3,6^a	0,89±0,18^b	0,78±0,06^b	0,76±0,11^b	1,03±0,20^a	9,55±0,74^{bc}	6,25±0,27^b
P2	9,3±2,2^b	11,9±2,4^c	22,1±2,6^c	48,4±1,5^c	55±6,4^c	44±1,5^b	1,24±0,13^c	1,18±0,06^c	0,95±0,12^b	1,74±0,28^b	9,97±0,34^c	7,03±0,77^b
Fresh plant application and plant dietary supplementation (Treatment C)												
K+	7,0±0,9^a	7,6±0,5^a	20,79±3,2^a	32,05±5,7^a	61±3,1^b	41±3,1^a	0,40±0,04^b	0,29±0,06^a	0,31±0,05^a	0,69±0,02^a	6,79±0,14^b	6,70±0,36^a
K-	9,7±0,3^b	10,9±0,7^b	24,05±6,6^b	32,74±9,2^a	53±2,0^a	39±5,0^a	0,30±0,01^a	0,26±0,04^a	0,41±0,03^b	0,71±0,03^a	6,00±0,23^a	6,94±0,21^b
KA	9,5±1,1^b	11,8±0,5^c	29,06±5,8^c	34,26±9,8^a	63±6,1^b	57±7,0^b	0,45±0,08^c	0,43±0,09^b	0,47±0,06^c	0,87±0,10^b	8,99±0,22^c	7,11±0,24^b
C	10,9±1,0^b	12,6±0,7^c	33,06±4,3^c	35,19±1,3^a	67±4,2^b	61±7,0^b	0,54±0,07^c	0,49±0,07^b	0,54±0,03^c	1,02±0,03^c	9,93±0,25^d	9,02±0,22^c

Note: Different letters in each bar (mean value ± standard deviation) show statistical differences (Duncan multiple distance test; $p<0.05$). Description: (HB) hemoglobin levels, (HT) hematocrit levels, (AF) phagocytic activity, (RB) respiratory burst, (TE) total erythrocytes, (TL) total leukocytes, (H1) Koi immersed with 15 g of *P. tener* Wall plant during rearing. (H2) Koi immersed with 30 g of *P. tener* Wall plant during the rearing period. (H3) Koi immersed with 60 g of *P. tener* Wall plant during the rearing period. (K-) fish injected with PBS without *P. tener* Wall treatment, (K+) Fish injected with *A. hydrophila* without *P. tener* Wall treatment. (P1) 2% of *P. tener* Wall powder was mixed into the commercial diet during the rearing period. (P2) 4% of *P. tener* Wall powder was mixed into the commercial diet during rearing. (KA) Oxytetracycline 5 g/ kg feed. (C) the best result in experiment I was mixed with the best result in experiment II (H2 + P2).

D. Scanning Electron Microscope (SEM) of Aeromonas hydrophila

We observed Aeromonas hydrophila bacteria with SEM to see whether the bacterial cells were damaged by treating *P. tener* wall. The performance of *A. hydrophila* cells, with and without *P. tener* Wall treatment, was observed with the Scanning Electron Microscope. *A. hydrophila* cells without *P. tener* Wall treatment showed healthy cell conditions, no cell damage, and active cell self-dividing (Fig5). *A. hydrophila* bacterial cells were treated with *P. tener* wall, and all the cells were broken and suffered some damages (Fig 6).

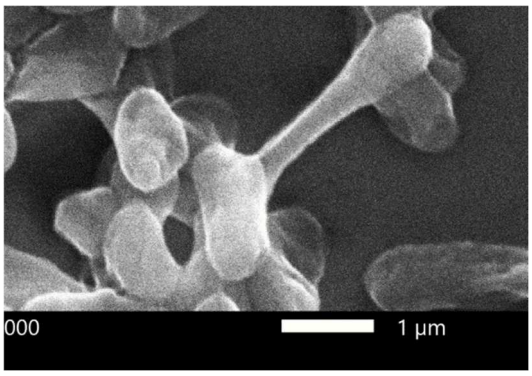

Fig. 5 The results of SEM observations on cell *A. Hydrophila*, *without P. tener* Wall Treatment. (healthy cell)

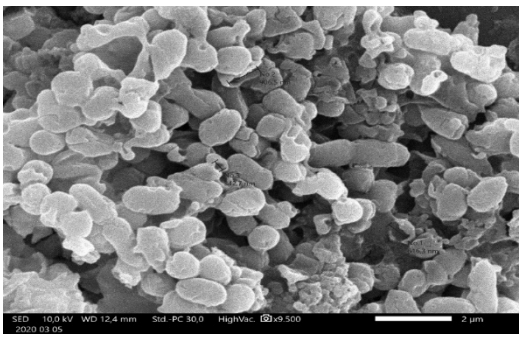

Fig. 6 Result of SEM of cell damage of *A. Hydrophila*, with *P. tener* wall treatment

E. Discussion

The highest hemoglobin levels were obtained from fresh plant treatment (treatment I), while the lowest values were obtained from favorable control treatment. The highest hematocrit level on the 6th day of the post-challenge test was found in the H2 treatment and significantly different from other treatments. A significant difference in the phagocytic activity level was found on the 6th day of the post-challenge test between the P2 treatment and the K+, K-, and P1 treatment. The highest phagocytic activity level was obtained from the P2 treatment.

The respiratory burst activity level on the 6th day of the post-challenge test was significantly different from the other treatments, as the highest value was found in the H2 treatment. In the experiment, I obtained an insignificant difference ($p>0.05$) in total erythrocytes on the 0th and 28th days of the pre-challenge test. In experiment II, the number of erythrocytes on the 0th and 28th days of the pre-challenge test had significant differences among the treatments. Experiment III showed that the total erythrocyte value was significantly different on the 28th day before the challenge test. The highest value of total erythrocytes on the 2nd and 6th days after the challenge test was obtained in the *P. tener* Wall application treatment, and it was significantly different among the treatments. The highest value of total leucocytes in H2 treatment. On the 2nd day of the post-challenge, a test was obtained in the P. *tener* Wall application treatment, which was significantly different from antibiotic and positive and negative control treatments.

Leaves of *P. tener* Wall contain Diterpene alcohol and diterpenoid, which function as anti-inflammatory, anti-microbial, anti-microbial, anti-inflammatory, antifungal, Antioxidant, Larvicidal, acaricidal. The stem contains the highest Fatty acid ester which has functions as, nematicide, hemolytic anti-bacterial, antioxidant, anti-microbial, hypercholesterolemia, nematicide, anti-inflammatory, antifungal, antiviral, anticancer, antifungal.

The results showed that the new *P. tener* Wall application could increase the immune response and the survival rate of koi carp after the challenge test with *A. hydrophila*. The survival rate in the fresh *P. tener* Wall application was significantly different ($p<0.05$) from the other treatments and better than the favorable control treatment. The survival rate in dietary *P. tener* Wall simplicial supplementation was higher than that in the favorable control treatment (Table 3). Simplicial *P. tener* Wall supplemented diet treatments provided a better health condition than non-supplemented diet treatments. Several studies reported that herbal plants are highly influential in inducing the fish body's immune system against disease in the form of either fresh, extracted, or isolated active components. This study is like that of [50], which mentioned that dietary supplementation of herbal plants in fish could promote growth, minimize stress, and increase health status. Moreover, previous studies reveal that natural herbs can be used as potential alternative properties to synthetic herbs in aquaculture due to the active biological metabolite contents utilized to modulate the fish body immune system [51]–[54].

The high survival rate in the *P. tener* Wall application treatments (Table 3) was like that of the K- and KA treatments. This condition showed that the non-specific immune system in koi carp had been formed and could attack bacteria, which might be due to the secondary metabolite compounds of *P. tener* Wall modulating the immune system of koi carp. This condition followed the results of a study [55], which stated that the survival rate of rohu carp (Labeo rohita) fed with Andrographis paniculate-supplemented diets after *A. hydrophila* infection was higher than those fed with a non-supplemented diet.

The application of *P. tener* Wall is proposed to improve the immune response of cultivated fish for disease resistance. The results showed that the application of *P. tener* Wall could improve the non-specific immune response of koi carp. In addition, [56] stated that the metabolites in herbs, such as polysaccharides, flavonoids, saponins, alkaloids, essential oils, and organic acids, had a beneficial impact on the fish immune system and several parameters of non-specific immune responses.

Total erythrocytes, hemoglobin, and hematocrit increased after *P. tener* Wall treatment was applied. This is like the results of the study conducted by [57]–[60]. Leucocytes play

an essential role in the non-specific immune system of fish against pathogenic infections. This study showed a significant increase in the total leucocytes after the challenge test with *P. tener* Wall application, which was higher than the control and *P. tener* Wall antibiotic treatments. This can increase the total leucocytes and immunity level against pathogenic attacks. The increase occurring in this study was caused by the fish body's immune response to disease infection. The number of Total leucocytes indicates an increase in immunity level, followed by level of phagocytic activity. Several studies have shown that herbal plant feeding contains metabolic compounds that can induce leukocyte production in rainbow trout [61].

The leucocyte system works in two ways to prevent disease: phagocytosis and antibody formation. This study also showed a significant increase in the phagocytic activity in *P. tener* Wall compared to the control treatment. The increasing phagocytic activity indicates that *P. tener* Wall immune stimulant in fresh ingredients and dietary-supplemented material could spread the immune system in koi carp fish. In addition, herbal plant treatment has shown a significant increase in the phagocytic activity in fish [55], [56]. Water quality is one of the essential factors in fish cultivation. During the rearing period, the water quality parameters provide values that still meet the requirements for koi carp fish. This was thought to be due to adding aquatic plants to the rearing media [62], [63].

IV. Conclusion

This study concludes that each administration of 30 g of fresh *P. tener* Wall or the addition of P. *tener* Wall simplicial in the diet can increase koi fish's immune response and resistance to *A. hydrophila*. The application of a mixed supplementation treatment of 30 g fresh *P. tener* Wall and 4% *P. tener* Wall in the diet gave the best results, with a survival rate reaching 100%.

Acknowledgment

The authors are very grateful to Ministry of Education, Culture, Research, and Technology for funding Media Fitri Isma Nugraha through INSINAS Grand Research Program in 2020 and 2021 under the contract number 6/E/ KPT/ 2020) and 15/E1/ KPT/ 2021. Also, we thank the Indonesian Research Institute for Ornamental Fish Culture–the Ministry Marine and Fisheries. Bogor University Faculty of Fisheries and Marine Science, IPB University, Bogor, West Java, Indonesia.

References

[1] M. F. I. Nugraha *et al.*, "Identification of aquatic plant species from Bantimurung waterfall and their phytochemical compounds analysis," *Ann. Bogor.*, vol. 23, no. 1, pp. 41–48, 2019, doi: 10.14203/ann.bogor.2019.v22.n2.41-48.

[2] M. Phumthum and H. Balslev, "Anti-infectious plants of the thai karen: A meta-analysis," *Antibiotics*, vol. 9, no. 6, pp. 1–15, 2020, doi: 10.3390/antibiotics9060298.

[3] C. A. Ukwubile *et al.*, "Antioxidant, anti-inflammatory, analgesic and in vitro-in vivo cytotoxicity effects of Spondias Venulosa (Engl.) Engl. leaf extracts on MCF-7/S0.5 and OV7 cancer cell lines," *Sci. African*, vol. 13, p. e00917, 2021, doi: 10.1016/j.sciaf.2021.e00917.

[4] M. R. Ramli, S. Malek, P. Milow, and N. J. Aziz, "Traditional knowledge of medicinal plants in the kampung orang asli donglai baru, hulu langat, malaysia," *Biodiversitas*, vol. 22, no. 3, pp. 1304–1309, 2021, doi: 10.13057/biodiv/d220329.

[5] H. Zhou *et al.*, "Ethnobotanical study on medicinal plants used by Bulang people in Yunnan, China," *J. Ethnobiol. Ethnomed.*, vol. 19, no. 1, 2023, doi: 10.1186/s13002-023-00609-0.

[6] S. Y. Hsiung *et al.*, "Structures of the xyloglucans in the monocotyledon family Araceae (aroids)," *Planta*, vol. 257, no. 2, pp. 1–16, 2023, doi: 10.1007/s00425-023-04071-w.

[7] D. Dutta, M. J. Bordoloi, and N. K. Bhattacharyya, "Genus Pothos: A Review of Chemical Constituents and Biological Activities," *Rasayan J. Chem.*, vol. 14, no. 04, pp. 2161–2170, 2021, doi: 10.31788/RJC.2021.1446353.

[8] D. Dutta, M. Bordoloi, and N. Bhattacharyya, "Antimicrobial Compound Isolated From The Plant Pothos scandens L.," *Egypt. J. Chem.*, vol. 64, no. 9, pp. 0–0, Apr. 2021, doi: 10.21608/ejchem.2021.56540.3216.

[9] G. Napolitano *et al.*, "Towards sustainable aquaculture systems: Biological and environmental impact of replacing fishmeal with Arthrospira platensis (Nordstedt) (spirulina)," *J. Clean. Prod.*, vol. 374, p. 133978, 2022, doi: 10.1016/j.jclepro.2022.133978.

[10] J. Sridharan, A. Samraj, S. S. K. John, and C. Antony, "Effect of replacement of dietary fish meal with silkworm pupae meal and black soldier fly larvae meal as a combination diet on the growth and digestive performance of koi carp Cyprinus carpio var. koi in the nursery phase," *Indian J. Fish.*, vol. 70, no. 1, pp. 103–110, 2023, doi: 10.21077/ijf.2023.70.1.121903-15.

[11] K. Honein, S. S. S. D. S. Jagoda, A. Arulkanthan, H. Ushio, and S. Asakawa, "Draft Genome Sequence of *Aeromonas hydrophila* Strain Ae25, Isolated from a Septicemic Moribund Koi Carp (*Cyprinus carpio*) in Sri Lanka," *Genome Announc.*, vol. 6, no. 5, pp. 11–12, Feb. 2018, doi: 10.1128/genomeA.01523-17.

[12] F. Fikri, D. K. Wardhana, A. Purnomo, S. Khairani, S. Chhetri, and M. T. E. Purnama, "Aerolysin gene characterization and anti-microbial resistance profile of Aeromonas hydrophila isolated from milkfish (Chanos chanos) in Gresik, Indonesia," *Vet. World*, vol. 15, no. 7, pp. 1759–1764, 2022, doi: 10.14202/vetworld.2022.1759-1764.

[13] M. S. Monir *et al.*, "Feed-based bivalent vaccine upregulates expressions of immune-related genes in systemic and mucosal tissues of red hybrid tilapia (Oreochromis niloticus × O. mossambicus) against Streptococcus iniae and Aeromonas hydrophila," *Aquac. Int.*, vol. 30, no. 5, pp. 2641–2659, 2022, doi: 10.1007/s10499-022-00925-4.

[14] V. Kumar *et al.*, "Outbreak of Ichthyophthirius multifiliis associated with Aeromonas hydrophila in Pangasianodon hypophthalmus: The role of turmeric oil in enhancing immunity and inducing resistance against co-infection," *Front. Immunol.*, vol. 13, no. September, pp. 1–19, 2022, doi: 10.3389/fimmu.2022.956478.

[15] A. Fernández-Bravo and M. J. Figueras, *An update on the genus Aeromonas: Taxonomy, epidemiology, and pathogenicity*, vol. 8, no. 1. 2020.

[16] Y. J. Yuan, F. Guan, C. Yu, Z. Chen, and J. Zhang, "Isolation and toxigenic characteristics of a tetrodotoxin producing bacteria," *Turkish J. Fish. Aquat. Sci.*, vol. 22, no. 1, 2022, doi: 10.4194/TRJFAS19531.

[17] R. Hazarika *et al.*, "Clostridioides difficile in food and food products of animal origin in Assam, India," *Anaerobe*, vol. 81, p. 102723, 2023, doi: 10.1016/j.anaerobe.2023.102723.

[18] K. Kamala, S. Rajeshkumar, and P. Sivaperumal, "The predominance of Shiga toxin-producing E. coli in the Southeast Coast of India," *Mar. Pollut. Bull.*, vol. 174, no. November 2021, p. 113188, 2022, doi: 10.1016/j.marpolbul.2021.113188.

[19] C. A. Lydon, J. Mejia-Fava, L. Collado-Vides, H. Eskelinen, and J. P. Berry, "Identification of apparently neurotoxic metabolites from assemblages of marine filamentous cyanobacteria associated with the intoxication of captive bottlenose dolphins (Tursiops truncatus) in the Florida Keys," *Chemosphere*, vol. 288, no. P1, p. 132423, 2022, doi: 10.1016/j.chemosphere.2021.132423.

[20] G. Arslan, "Cytokine gene expression, immune responses and disease resistance of oncorhynchus mykiss after raphanus sativus by-products supplementation," *Turkish J. Fish. Aquat. Sci.*, vol. 21, no. 10, pp. 521–535, 2021, doi: 10.4194/1303-2712-v21_10_05.

[21] D. Wahjuningrum, I. D. N. Fitrianingrum, W. Widanarni, and D. A. S. Utami, "Banana (Musa paradisiaca) midrib extract as a curative agent of motile aeromonad septicemia in giant gourami (osphronemus gouramy)," *Turkish J. Fish. Aquat. Sci.*, vol. 21, no. 12, pp. 589–602, 2021, doi: 10.4194/1303-2712-v21_12_02.

[22] B. Priscilia, M. F. I. Nugraha, H. Novita, and B. Elya, "Antioxidant and anti-bacterial assay against fish pathogen bacteria of

Kjellbergiodendron celebicum (Koord.) merr. leaf extract," *Pharmacogn. J.*, vol. 12, no. 1, pp. 173–179, 2020, doi: 10.5530/pj.2020.12.26.

[23] Y. Yakubu, A. M. Talba, C. M. Chong, I. S. Ismail, and K. Shaari, "Effect of Terminalia catappa methanol leaf extract on nonspecific innate immune responses and disease resistance of red hybrid tilapia against Streptococcus agalactiae," *Fish Shellfish Immunol.*, vol. 18, no. November, pp. 140–145, Nov. 2020, doi: 10.1016/j.aqrep.2020.100555.

[24] N. K. K *et al.*, "Is butyrate a natural alternative to dexamethasone in the management of CoVID-19?," *F1000Research*, vol. 10, no. May, 2021, doi: 10.12688/f1000research.51786.1.

[25] S. Thongyim, S. Chiangchin, H. Pandith, Y. Tragoolpua, S. Jangsutthivorawat, and A. Panya, "Anti-Inflammatory Activity of Glyceryl 1,3-Distearate Identified from Clinacanthus nutans Extract against Bovine Mastitis Pathogens," *Antibiotics*, vol. 12, no. 3, 2023, doi: 10.3390/antibiotics12030549.

[26] Z. Liu *et al.*, "Responses of Aroma Related Metabolic Attributes of Opisthopappus longilobus Flowers to Environmental Changes," *Plants*, vol. 12, no. 8, 2023, doi: 10.3390/plants12081592.

[27] Y. J. Lee, L. K. Putri, B. J. Ng, L. L. Tan, T. Y. Wu, and S. P. Chai, "Blue TiO2 with tunable oxygen-vacancy defects for enhanced photocatalytic diesel oil degradation," *Appl. Surf. Sci.*, vol. 611, no. PB, p. 155716, 2023, doi: 10.1016/j.apsusc.2022.155716.

[28] CHEBI, "neophytadiene (CHEBI:145817)," *CHEBI*, 2020. https://www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI:145817 (accessed May 30, 2023).

[29] S. J. Thomas, H. Li, A. P. Praplan, H. Hellén, and F. Bianchi, "Complexity of downy birch emissions revealed by Vocus proton transfer reaction time-of-flight mass spectrometer," *Front. For. Glob. Chang.*, vol. 5, no. November, pp. 1–15, 2022, doi: 10.3389/ffgc.2022.1030348.

[30] L. S. Herbeck, D. Unger, Y. Wu, and T. C. Jennerjahn, "Effluent, nutrient and organic matter export from shrimp and fish ponds causing eutrophication in coastal and back-reef waters of NE Hainan, tropical China," *Cont. Shelf Res.*, vol. 57, pp. 92–104, Apr. 2013, doi: 10.1016/j.csr.2012.05.006.

[31] S. Radman *et al.*, "Bioprospecting of Less-Polar Fractions of Ericaria crinita and Ericaria amentacea: Developmental Toxicity and Antioxidant Activity," *Mar. Drugs*, vol. 20, no. 1, 2022, doi: 10.3390/md20010057.

[32] M. H. Liang, Y. J. He, D. M. Liu, and J. G. Jiang, "Regulation of carotenoid degradation and production of apocarotenoids in natural and engineered organisms," *Crit. Rev. Biotechnol.*, vol. 41, no. 4, pp. 513–534, 2021, doi: 10.1080/07388551.2021.1873242.

[33] L. Wang *et al.*, "In vitro and in vivo photoprotective effects of (-)-loliode isolated from the brown seaweed, sargassum horneri," *Molecules*, vol. 26, no. 22, 2021, doi: 10.3390/molecules26226898.

[34] I. Bano and G. S. Deora, "Preliminary Phytochemical Screening and Gc-Ms Analysis for Identification of Bioactive Compounds From Abutilon Fruticosum Guill and Perr. a Rare and Endemic Plant of Indian Thar Desert," *Int. J. Pharm. Sci. Res.*, vol. 11, no. 6, pp. 2671–2679, 2020, doi: 10.13040/IJPSR.0975-8232.11(6).2671-79.

[35] U. T. Ferdous, A. Nurdin, S. Ismail, and Z. N. Balia Yusof, "Evaluation of the antioxidant and cytotoxic activities of crude extracts from marine Chlorella sp.," *Biocatal. Agric. Biotechnol.*, vol. 47, no. July 2022, p. 102551, 2023, doi: 10.1016/j.bcab.2022.102551.

[36] G. M. Miya, A. O. Oriola, B. Payne, M. Cuyler, N. Lall, and A. O. Oyedeji, "Steroids and Fatty Acid Esters from Cyperus sexangularis Leaf and Their Antioxidant, Anti-Inflammatory and Anti-Elastase Properties," *Molecules*, vol. 28, no. 8, pp. 1–14, 2023, doi: 10.3390/molecules28083434.

[37] P. E. Joshua, C. J. Anosike, R. O. Asomadu, D. E. Ekpo, E. N. Uhuo, and O. F. C. Nwodo, "Bioassay-guided fractionation, phospholipase A2-inhibitory activity and structure elucidation of compounds from leaves of Schumanniophyton magnificum," *Pharm. Biol.*, vol. 58, no. 1, pp. 1069–1076, 2020, doi: 10.1080/13880209.2020.1839510.

[38] P. von der Ohe and R. Aalizadeh, "S13 | EUCOSMETICS | Combined Inventory of Ingredients Employed in Cosmetic Products (2000) and Revised Inventory (2006)," May 28, 2020. https://zenodo.org/record/3959386 (accessed May 30, 2023).

[39] B. Ali, Y. K. Rawal, Sidharth, and C. Thayes, "Nutritional composition, phytochemical screening, antioxidant, GC-MS, and FTIR analysis of methanolic extract of Prunus armeniaca," *Arab. J. Med. Aromat. Plants*, vol. 8, no. 3, pp. 149–169, 2022, doi: 10.48347/IMIST.PRSM/ajmap-v8i3.33827.

[40] M. Hassan *et al.*, "LC-MS and GC-MS Profiling of Different Fractions of Ficus platyphylla Stem Bark Ethanolic Extract," *J. Anal. Methods Chem.*, vol. 2022, 2022, doi: 10.1155/2022/6349332.

[41] M. T. Islam *et al.*, "Phytol anti-inflammatory activity: Pre-clinical assessment and possible mechanism of action elucidation," *Inflammopharmacology*, vol. 27, no. 4, pp. 857–862, 2020, [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/32583784/.

[42] A. Shukla, S. Tyagi, V. Gupta, P. Jain, T. Kanai, and R. Tripathi, "FT-IR, GC-MS, and HPLC Profiling of the Bioactive Constituents of Ethyl Acetate Fraction of Eichhornia crassipes as a Hepatoprotectant," *Lett. Appl. NanoBioScience*, vol. 12, no. 4, 2023, doi: 10.33263/LIANBS124.096.

[43] J. Baan, M. Holloway-Phillips, D. B. Nelson, and A. Kahmen, "The metabolic sensitivity of hydrogen isotope fractionation differs between plant compounds," *Phytochemistry*, vol. 207, no. April 2022, p. 113563, 2023, doi: 10.1016/j.phytochem.2022.113563.

[44] Z. Yang *et al.*, "Biostimulation of sulfate-reducing bacteria used for treatment of hydrometallurgical waste by secondary metabolites of urea decomposition by Ochrobactrum sp. POC9: From genome to microbiome analysis," *Chemosphere*, vol. 282, p. 131064, 2021, doi: 10.1016/j.chemosphere.2021.131064.

[45] A. Swargiary, M. K. Roy, and H. Boro, "Persicaria strigosa (R.Br.) Nakai: a natural anthelmintic?," *Parasitol. Res.*, vol. 120, no. 9, pp. 3215–3227, 2021, doi: 10.1007/s00436-021-07249-x.

[46] H. Yin, W. Li, M. Xu, D. Xu, and P. Wan, "The role of tetradecane in the identification of host plants by the mirid bugs Apolygus lucorum and Adelphocoris suturalis and potential application in pest management," *Front. Physiol.*, vol. 13, no. December, pp. 1–13, 2022, doi: 10.3389/fphys.2022.1061817.

[47] M. Duraisamy and R. Selvaraju, "Analysis of Chemical Compounds By Using Gas Chromatography and Mass Spectrum Analysis, in Vitro Antioxidant and Anti-bacterial Activity of Methanolic Extracts of Seaweed Ulva Flexuosa Wulfen (Green Algae)," no. January, 2021, doi: 10.13140/RG.2.2.21866.75209.

[48] A. S. M. A. Reza *et al.*, "Antiproliferative and antioxidant potentials of bioactive edible vegetable fraction of Achyranthes ferruginea Roxb. in cancer cell line," *Food Sci. Nutr.*, vol. 9, no. 7, pp. 3777–3805, 2021, doi: 10.1002/fsn3.2343.

[49] A. Balasubramanian, R. Ganesan, Y. K. Mohanta, J. Arokiaraj, and M. Saravanan, "Characterization of bioactive fatty acid metabolites produced by the halophilic Idiomarina sp. OM679414.1 for their anti-microbial and anticancer activity," *Biomass Convers. Biorefinery*, no. 0123456789, 2023, doi: 10.1007/s13399-023-04687-8.

[50] T. Hou, H. Liu, and C. Li, "Traditional Chinese herb formulas in diet enhance the non-specific immune responses of yellow catfish (Pelteobagrus fulvidraco) and resistance against Aeromonas hydrophila," *Fish Shellfish Immunol.*, vol. 131, no. August, pp. 631–636, Dec. 2022, doi: 10.1016/j.fsi.2022.10.050.

[51] M. Zare, N. Esmaeili, S. Paolacci, and V. Stejskal, "Nettle (Urtica dioica) Additive as a Growth Promoter and Immune Stimulator in Fish," *Aquac. Nutr.*, vol. 2023, 2023, doi: 10.1155/2023/8261473.

[52] A. Panase, M. Thirabunyanon, J. Promya, and C. Chitmanat, "Influences of Bacillus subtilis and fructooligosaccharide on growth performances, immune responses, and disease resistance of Nile tilapia, Oreochromis niloticus," *Front. Vet. Sci.*, vol. 9, 2023, doi: 10.3389/fvets.2022.1094681.

[53] E. Nikiphorou and E. Philippou, "Nutrition and its role in prevention and management of rheumatoid arthritis," *Autoimmun. Rev.*, vol. 22, no. 7, p. 103333, 2023, doi: 10.1016/j.autrev.2023.103333.

[54] E. E. Hussein *et al.*, "Effect of dietary sage (Salvia officinalis L.) on the growth performance, feed efficacy, blood indices, non-specific immunity, and intestinal microbiota of European sea bass (Dicentrarchus labrax)," *Aquac. Reports*, vol. 28, no. December 2022, p. 101460, 2023, doi: 10.1016/j.aqrep.2022.101460.

[55] R. Palanikani *et al.*, "Efficacy of Andrographis paniculata supplements induce a non-specific immune system against the pathogenicity of Aeromonas hydrophila infection in Indian major carp (Labeo rohita)," *Environ. Sci. Pollut. Res.*, vol. 27, pp. 23420–23436, 2020.

[56] P. Elumalai, A. Kurian, S. Lakshmi, C. Faggio, M. A. Esteban, and E. Ringø, "Herbal Immunomodulators in Aquaculture," *Rev. Fish. Sci. Aquac.*, vol. 29, no. 1, pp. 1–25, 2020, doi: 10.1080/23308249.2020.1779651.

[57] S. Duman and A. Şahan, "Effects of β-1,3/1,6 glucan dietary supplements on some immunological and hematological health markers in Siberian sturgeon (Acipenser baerii) infected with Aeromonas hydrophila," *Pol. J. Vet. Sci.*, vol. 26, no. 1, pp. 109–118, 2023, doi: 10.24425/pjvs.2023.145012.

[58] S. M. Aly, A. E. Eissa, N. Abdel-Razek, and A. O. El-Ramlawy, "The anti-bacterial activity and immunomodulatory effect of naturally synthesized chitosan and silver nanoparticles against Pseudomonas fluorescence infection in Nile tilapia (Oreochromis niloticus): An in vivo study," *Fish Shellfish Immunol.*, vol. 135, no. December 2022, p. 108628, 2023, doi: 10.1016/j.fsi.2023.108628.

[59] S. M. I. Khalil *et al.*, "Immune profiling of rainbow trout (Oncorhynchus mykiss) exposed to Lactococcus garvieae: Evidence in asymptomatic versus symptomatic or vaccinated fish," *J. Fish Dis.*, vol. 46, no. 7, pp. 731–741, 2023, doi: 10.1111/jfd.13782.

[60] S. H. Almarri, A. A. Khalil, and A. T. Mansour, "Oreochromis niloticus , and Its Tolerance to Thermal Stress and Aeromonas sobria Infection," 2023.

[61] A. Gholamhosseini *et al.*, "Effect of dietary supplements of Artemisia dracunculus extract on the haemato-immunological and biochemical response, and growth performance of the rainbow trout (Oncorhynchus mykiss)," *Aquac. Res.*, vol. 52, no. 5, pp. 2097–2109, May 2021, doi: 10.1111/are.15062.

[62] J. Xu *et al.*, "Nitrogen and phosphorus removal in simulated wastewater by two aquatic plants," *Environ. Sci. Pollut. Res.*, vol. 28, no. 44, pp. 63237–63249, Nov. 2021, doi: 10.1007/s11356-021-15206-5.

[63] M. M. Kamdem and P. V. Otomo, "Developmental performance of Eristalis tenax larvae (Diptera: Syrphidae): Influence of growth media and yeast addition during captive rearing," *J. Exp. Zool. Part A Ecol. Integr. Physiol.*, vol. 339, no. 5, pp. 503–513, 2023, doi: 10.1002/jez.2696.