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Potential of *Eurycoma longifolia*, *Curcuma zedoaria*, and *Allium sativum* extracts as phytobiotics for shrimp health

Potensi ekstrak *Eurycoma longifolia*, *Curcuma zedoaria*, dan *Allium sativum* sebagai fitobiotik untuk meningkatkan kesehatan udang

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ABSTRACT

This study evaluated the efficacy of *Eurycoma longifolia, Curcuma zedoaria*, and *Allium sativum* in improving the immune response and resistance of whiteleg shrimp to prevent *Vibrio parahaemolyticus* infection. The study consisted of two phases, an in vitro phase to determine the compounds contained in three medicinal plants as antibacterials, followed by an in vivo phase to evaluate the effect of the medical plant extract on immune response and robustness against *V. parahaemolyticus*. The results from the first phase revealed that bioactive compounds present in *E. longifolia* were more varied and had higher concentrations with a lower bactericidal value when compared to those found in *C. zedoaria* or *A. sativum*. In the second phase of the experiment, the medicinal plant extract was added to the feed with a dose that was determined according to the first phase results. The treatments tested in the second phase were 1.6% *E. longifolia* extract dietary addition (EL16), 6.4% *C. zedoaria* extract dietary addition (CZ64), 6.4% *A. sativum* extract dietary addition (AS64) and phytobiotics mixture of 1:1:1 (C1) dietary addition, as well as no phytobiotic for negative control treatment and positive control. The results from the second stage demonstrated that dietary phytobiotic extract addition enhances the immunological responses and improves the shrimp survival against *V. parahaemolyticus* challenge compared to the control group. In conclusion, *E. longifolia*, *C. zedoaria*, and *A. sativum* showed different bioactive compound profiles, which affect their efficacy against *V. parahaemolyticus*, with EL16 showing higher efficacy.

Keywords: *Allium sativum*, *Curcuma zedoaria*, *Eurycoma longifolia*, *Penaeus vannamei*, phytobiotic

ABSTRAK

Penelitian ini mengevaluasi efikasi *Eurycoma longifolia*, *Curcuma zedoaria*, dan *Allium sativum* dalam meningkatkan respon imun dan resistensi udang vaname untuk mencegah infeksi *Vibrio parahaemolyticus*. Penelitian ini terdiri dari dua tahap: tahap *in vitro* untuk menentukan senyawa yang terkandung dalam tiga tanaman obat sebagai antibakteri, diikuti oleh tahap in vivo untuk mengevaluasi efek ekstrak tanaman obat terhadap respon imun dan ketahanan terhadap *V. parahaemolyticus*. Hasil dari tahap pertama mengungkapkan bahwa senyawa bioaktif yang ada dalam *E. longifolia* lebih bervariasi dan memiliki konsentrasi yang lebih tinggi dengan nilai bakterisida yang lebih rendah jika dibandingkan dengan yang ditemukan di *C. zedoaria* atau *A. sativum*. Pada percobaan tahap kedua, ekstrak tanaman obat ditambahkan ke pakan dengan dosis yang ditentukan sesuai dengan hasil tahap pertama. Perlakuan yang diuji pada tahap kedua adalah penambahan 1,6% ekstrak *E. longifolia* dalam pakan (EL16), penambahan 6,4% ekstrak *C. zedoaria* dalam pakan (CZ64), penambahan 6,4% ekstrak *A. sativum* dalam pakan (AS64) dan campuran fitobiotik 1:1:1 (C1) dalam pakan, serta tanpa fitobiotik untuk perlakuan kontrol negatif dan kontrol positif. Hasil dari tahap kedua menunjukkan bahwa penambahan ekstrak fitobiotik dalam pakan meningkatkan respons imunologi dan memperbaiki kelangsungan hidup udang terhadap tantangan *V. parahaemolyticus* dibandingkan dengan kelompok kontrol. Sebagai kesimpulan, *E. longifolia*, *C. zedoaria*, dan *A. sativum* menunjukkan profil senyawa bioaktif yang berbeda, yang mempengaruhi efikasinya terhadap *V. parahaemolyticus*, dengan EL16 menunjukkan efikasi yang lebih tinggi.

Kata kunci: *Allium sativum*, *Curcuma zedoaria*, *Eurycoma longifolia*, fitobiotik, *Penaeus vannamei*

INTRODUCTION

Disease outbreaks have massively hampered shrimp production. The most challenging issue for the cultivation of shrimp are the bacterial diseases caused by Vibrio species, collectively called vibriosis (Chandrakala & Priya, 2017). Various species of Vibrio, such as *V. harveyi, V. vulnificus, V. anguillarum*, *V. parahaemolyticus, V. alginolyticus, V. campbellii,* and *V. splendidus* have been identified and isolated from the infected shrimp (Abdel-Latif *et al*., 2022). Infected shrimp usually present clinical signs such as empty intestines, reduced feeding activity, and discoloration of gills, which ranges from red to brown, thereby leading to tissue damage and high mortalities (Elias *et al*., 2023). Diseases associated with Vibrio infections include luminescent disease, acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome (EMS), white feces disease (WFD) (de Souza & Wan, 2021), and the highly lethal vibrio disease (HLVD) caused by the *Vibrio parahaemolyticus* strain (VpHLVD) (Yang *et al*., 2023).

Shrimp health is one of the critical aspects in the successful completion of the aquaculture project. The effective adoption of good management practices during the hatchery and grow-out phases could prevent and control Vibrio infections. While chemicals and antibiotics have been used conventionally to control pathogenic bacteria, many countries restrict their use because of the possible risks of bioaccumulation in shrimp and the ambient ecosystem coupled with the appearance of antibiotic-resistant strains (Singer *et al*., 2019). On the other hand, medicinal plants represent sustainable alternatives, being easy to apply, biodegradable, and thereby minimizing their ecological footprint even when applied over a long period (Citarasu, 2010; Hai, 2015).

Empirical studies have shown that probiotic supplementation in shrimp feed can increase their appetite, activate digestive enzyme activity, stimulate immune responses, and raise resistance against pathogens in shrimp and other aquaculture species (Pu *et al*., 2017). Other studies on medicinal plants using leaf powder of *Syzygium cumini* in feed formulation have also shown improvement in the growth performance of whiteleg shrimp (*Litopenaeus vannamei*) (Prabu *et al*., 2018). The inclusion of medicinal plants in animal feed has also been reported to increase growth rates and enhance immune systems, resulting in higher resistance to diseases (Dadras *et al*., 2023). Other possible phytobiotics are *Eurycoma longifolia,* commonly known as pasak bumi (Rehman *et al*., 2016); *Curcuma zedoaria,* commonly known as white turmeric (Gharge *et al*., 2021); and *Allium sativum,* commonly known as garlic (Delgado *et al*., 2023).

The efficacy *of E. longifolia* might be related to its richness in bioactive constituents, such as quassinoids, alkaloids, glycosides, eurycomanol, and euricomanone (Rehman *et al*., 2016). Roots of this species have been reported to exert anticancer, chemopreventive, and immunomodulatory activities, which may be attributed to the presence of quassinoids, flavonoids, and alkaloids. Besides, *E. Longifolia* has been reported to exhibit numerous pharmacological activities, such as antimalarial, antitumor, anticancer, antidiabetic, aphrodisiac, anxiolytic, and antiparasitic activities (Khanam *et al*., 2015). *C. zedoaria* has been reported to have anti-inflammatory properties (Rahaman *et al*., 2021), in addition to antifungal and antimicrobial activities (Chachad *et al*., 2016; Gharge *et al*., 2021). *A. sativum* contains highly reactive organic sulfur compounds that exhibit strong antimicrobial activities against both gram-positive and gram-negative bacteria even at low concentrations (Salehi *et al*., 2019). The antimicrobial properties of garlic also include antifungal and antiviral activities, which can be attributed primarily to its sulfur derivatives, including allicin (Bhatwalkar *et al*., 2021).

This study aims to evaluate the efficacy of administering extracts of *E. longifolia, C. zedoaria,* and *A. sativum* as immunostimulants through feed to enhance the immune response and disease resistance of whiteleg shrimp as part of an effort to prevent infections caused by *V. parahaemolyticus*.

MATERIALS AND METHODS

Time and place

This study was carried out from April to June 2023 at the Marine Center, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University. Health analyses were performed at the Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB. Phytochemical analyses were conducted at the Biopharmaceutical Study Center Laboratory and the Spice and Medicinal Plants Research Center Laboratory.

Preparation of phytobiotic powders and extracts

Samples of the materials used were obtained from the Anyar traditional market in Bogor City, West Java. The garlic and white turmeric used were cleaned from dirt with water, then cut into small pieces and dried in an oven for 24 hours at 60°C. The dried test plants were then mashed and filtered with a 0.5–1 mm mesh size sieve. Pasak Bumi in the form of wood shavings was formed into a powder using a wood powder grinding machine before being dried in an oven for 24 hours at 60°C.

Extraction was done by weighing 50 grams of each simplicia powder, the powder was extracted using 96% ethanol as a solvent with a ratio of 1:10 (w/v). Furthermore, it was macerated for 24 hours with stirring using an orbital shaker at room temperature. The maceration results were filtered using Whatman filter paper no. 41. The remaining filter was re-maerated twice with the same method as the first maceration. The maceration results were concentrated with a vacuum evaporator at a temperature of 30-45°C then stored at a temperature of -20°C for use in the next stage (Adeshina *et al*., 2018).

Test feed preparation

The feed used in this study was commercial shrimp feed with a protein content of 41%, crude fibre of 3%, fat of 7%, water content of 12%, and ash content of 13%. Five types of treatment feeds were prepared by mixing phytobiotic extracts into the feed manually using 2% (v/v) egg white binder and 6% (v/b) water as a solvent. The feed consisted of feed for the primary treatment with the addition of 1.6% pasak bumi extract (EL16), 6.4% white turmeric extract (CZ64), 6.4% garlic extract (AS64) and a mixture of the three phytobiotics 1:1:1 (C1), and feed without the addition of phytobiotics for the negative control (KN) and positive control (KP) treatments. The dose of each extract was determined based on the minimum bactericidal concentration (MBC) value obtained. The mixed feed was air-dried and then stored in a refrigerator at a temperature of 4°C before use.

Shrimp rearing and *V. parahaemolyticus* challenge test

Two hundred shrimp weighing 2.53 ± 0.22 g were transferred into each cylindrical fibre tank with a water volume of 450 L (density of 445 shrimp/m3). Feed was given to the shrimp four times a day, namely at 07.00, 11.00, 15.00, and 19.00 WIB, with a feeding rate of 7%. The maintenance of vaname shrimp was carried out for 42 days. The challenge test of *V. parahaemolyticus* bacteria 106 CFU/mL intramuscular injection was carried out at the end of the 42nd day of maintenance in an aquarium container measuring 60×30×30 cm3 as many as 15 shrimp per aquarium and observed for seven days.

Observation parameters

Phytochemical screening

Phytochemical screening or qualitative identification of chemical compounds in samples includes alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, steroids, and glycosides. Furthermore, the quantitative value of the total phenolic and flavonoid content of each phytobiotic extract was measured using the spectrophotometric method (Sahreen *et al*., 2010; Mayur *et al*., 2010).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) was done following the procedures of Zhang *et al*. (2018), with some modifications. Stock solutions of each extract were prepared at 64 mg/mL and then serially diluted (1:1 dilution factor) in SWC liquid media in test tubes, giving a concentration of 64, 32, 16, 8, 4, 2, 1, 0.5, 0.125, and 0.0625 mg/mL. For negative control, PBS was used, while positive control was done using chloramphenicol at 30 µg/mL, all in replicates of three sets. Each treatment tube was infused with 100 µL of *Vibrio parahaemolyticus* RfR, which was of density 10^8 CFU/mL after washing twice with PBS. The number of bacterial colonies of the negative control, which was harmful, was counted prior to the incubation (data from the 0hour K* treatment).

The tubes were then placed in an incubator shaker at 28-29°C and maintained at the speed of 140 rpm for a duration of 24 hours. After the incubation, 100 µL of all treatment tubes were plated onto TCBS media and once more placed in the incubator for 24 hours. It was noted that MIC was the lowest concentration that inhibited the bacterial growth activity (bacteriostatic) when compared to the negative control K* before the incubation. It was, however, noted that the concentration used to kill 99% of the bacteria

was the MBC, which indicates the bactericidal concentration.

Total hemocyte count (THC)

Shrimp hemolymph was withdrawn from the ventral sinus by a sterile syringe containing 1 mL of anticoagulant (4°C) (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55), in a 1:3 ratio in order to determine the total hemocyte count. The mixture of hemolymph and anticoagulant was put in an Eppendorf tube and homogenized by shaking the hand in a figure-eight motion. The total number of hemocytes was counted by placing the hemolymph-anticoagulant mixture into the counting chamber of a hemocytometer, where hemocyte cells were observed at a 100× magnification under a light microscope. The hemocyte cells were observed under a light microscope at a 100-fold magnification. (Hamsah *et al*., 2019). Total hemocyte count was calculated using the formula below.

$$\text{Total hemocyte} = \text{Average} \sum \text{cell} \frac{1}{\text{volume of large box}} \times \text{dilution factor}$$

Phagocytosis activity (AF)

Measuring phagocytic activity: 100 µL of the shrimp hemolymph sample was mixed with 25 µL of *Staphylococcus aureus* suspension (10^7 CFU/mL) and incubated for 20 minutes. Smear preparation was then made and fixed with methanol for five minutes. After being air-dried again, it was stained for 20 minutes by Giemsa dye. Smear preparation was observed under 400× microscope and the phagocytic activity was assessed by counting the number of 100 phagocytic cells showing the process of phagocytosis (Chen *et al*., 2014).

Respiratory burst (RBs) (Cheng *et al*., 2004)

Respiratory burst activity was determined by measuring superoxide anion (O2-) production based on the indicator nitroblue tetrazolium (NBT). In brief, a 100 µL solution of hemolymph mixed with anticoagulant was incubated at ambient temperature for 30 minutes. Then the sample was centrifuged at 3,000 rpm for 20 minutes before the supernatant was discarded. The resulting cell pellet was suspended in 100 µL of 0.3% NBT solution and incubated at room temperature for two hours. After the incubation, the resulting suspension was centrifuged at 3,000 rpm for 10 minutes, the supernatant being removed. To the pellet were added 100 µL of absolute methanol, after which it underwent the same steps of centrifugation. After this, the pellet was washed twice using 70% methanol and dried, treated with 120 µL of potassium hydroxide (KOH) and 140 µL of dimethyl sulfoxide (DMSO), before being resuspended into the wells of a microplate. The staining intensity was measured with a spectrophotometer at a wavelength of 630 nm with KOH or DMSO as a control blank. Respiratory burst activity was expressed as the reduction of NBT per 10 µL of hemolymph.

Phenoloxidase (PO) activity

Activity of phenoloxidase was measured by quantification of dopachrome produced upon using L-DOPA as a substrate. One milliliter of a mixture of hemolymph-anticoagulant was spun at 3,000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was discarded, and the pellet of cells resuspended in 1 mL cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7). The suspension was centrifuged again under the same conditions, and the supernatant was removed. The cell pellet was resuspended in 200 µL of cacodylate buffer.

A 100 µL aliquot of the cell suspension was treated with 50 µL of trypsin solution (1 mg/mL in cacodylate buffer) and incubated at 25°C for 10 minutes. Then, 50 µL of L-DOPA (3 mg/mL in cacodylate buffer) was added to it and incubated for five minutes. Finally, the cell suspension was filled up with 800 µL of cacodylate buffer before being transferred to the wells in a microplate. The standard solution, in contrast to the test solution, contained 100 µL of hemocyte suspension, 50 µL of cacodylate buffer instead of trypsin, and 50 µL of L-DOPA. Optical density (OD) was read at 492 nm by spectrophotometer. Phenoloxidase activity was expressed as dopachrome formation per 50 µL of hemolymph, following the method of (Zhao *et al*., 2017).

Total vibrio count (TVC) and population *V. parahaemolyticus*

The total bacterial count (TBC) and total Vibrio count (TVC) were analyzed from the intestines of shrimp, while the total *Vibrio parahaemolyticus* RfR (TVp) was analyzed from both the intestines and hepatopancreas. In the preparation of the shrimp samples, the body surface was first disinfected with 95% ethanol and then washed three times with sterile distilled water. The

intestines and hepatopancreas (taken from the fourth abdominal segment) were subsequently cut into small pieces using scissors until complete homogenization occurred. Each organ sample (0.1 g) was further homogenized in 0.9 mL of PBS solution and plated onto appropriate agar media. TVC was quantified on thiosulfate citrate bile-salt sucrose (TCBS) agar, TVp was determined on TCBS agar supplemented with rifampicin, and TBC was measured on SWC agar. Plates were incubated at 28°C, and bacterial colonies were enumerated after 24 hours.

Survival rate

The survival rate (SR) was calculated before the challenge test and once more seven days after the challenge test using the formula described by Liu *et al*. (2018):

$$\text{Survival rate (\%)} = \frac{Nt}{N0} \times 100$$

Note:

Nt = Number of live shrimp at the end of the study (pcs)

No = Number of live shrimp at the beginning of the study (pcs)

Data analysis

The data obtained were organized and tabulated using Microsoft Excel 2019. Homogeneity and normality tests were performed using the Post Hoc test. One-way ANOVA was then done to analyze the data by SPSS version 24. Wherever significant differences were obtained, further analyses were carried out by the Duncan test at a 95% confidence interval. Descriptive analysis was also done for the parameters of phytochemical screening, MIC, MBC, and histopathological data.

RESULTS AND DISCUSSION

Result

Phytochemical screening

The results of phytochemical screening of ethanol extracts of *E. longifolia*, *C. Zedoaria*, and *A. sativum*, including alkaloid test, saponin test, tannin test, phenol test, flavonoid test, triterpenoid test and steroid test, are presented in Table 1. The test results show that each phytobiotic extract contains alkaloid, saponin, phenolic, flavonoid, and triterpenoid compounds, and no steroid content was found in each phytobiotic extract. Meanwhile, tannin compounds were only found in the pasak bumi extract and not in the white turmeric and garlic extracts. The results of the total phenol and total flavonoid measurements are shown in Table 2. The highest total phenol and flavonoid values were found in the pasak bumi extract, 9.61% (w/w) and 13.38 QE/g.

Minimum inhibitory concentration (MIC) and *minimum bactericidal concentration* (MBC)

Pasak bumi E. longifolia and garlic *A. sativum* showed lower MIC capability of 0.31 mg/mL

Table 1. Results of phytochemical screening of *E. longifolia, C. zedoaria*, and *A. sativum* extracts.

Phytochemical Compounds	Ethanol Extract		
	E. longifolia	*C. zedoaria*	*A. sativum*
Alkaloids	+	+	+
Saponins	+	+	+
Tannins	+	-	-
Phenolics	+	+	+
Flavonoids	+	+	+
Triterpenoids	+	+	+
Steroids	-	-	-

Table 2. Results of total phenol and total flavonoid analysis of *E. longifolia*, *C. zedoaria*, and *A. sativum* extracts.

Sample	Parameter	
	Total Phenol % (b/b)	Total Flavonoid mg QE/g
Pasak Bumi Extract	9.61	13.38
White Turmeric Extract	4.72	4.88
Garlic Extract	0.68	6.63

Note: Flavonoid content is expressed as quercetin equivalent (QE) in mg/g dry extract.

while white turmeric *C. zedoaria* produced MIC value of 64 mg/mL. Pasak bumi also had the lowest MBC value compared to white turmeric and garlic, which was 16 mg/mL. Determination of the dose of phytobiotic extract was determined based on the MBC value of each phytobiotic extract.

Total haemocyte count

THC of whiteleg shrimp after feeding with single and combination phytobiotic extracts and post-infection is shown in Figure 1. After 42 days of maintenance with feeding containing single and combination phytobiotic extracts, the THC of whiteleg shrimp increased significantly (P<0.05) compared to the control. During maintenance and post-infection, whiteleg shrimp in the pasak bumi extract treatment (EL16) had the highest THC value compared to other phytobiotic extract treatments.

Phagocytic activity

The results of measuring the phagocytosis activity (AF) parameters showed that after each treatment, each extract was higher and significantly different (p<0.05) compared to the control, with the best treatment being the pasak bumi extract (Figure 2).

Phenoloxidase activity

The single and combined phytobiotic extract administration results in higher PO activity than the control after 42 days of maintenance

Table 3. Minimum inhibitory concentration and minimum bactericidal concentration of *E. longifolia*, *C. zedoaria* and *A. sativum* against *V. parahaemolyticus*.

	E. longifolia	*C. zedoaria*	*A. sativum*
MIC (mg/mL)	0.31	0.125	0.31
MBC (mg/mL)	16	>64	>64

Note: MIC= minimum inhibitory concentration, MBC= minimum bactericidal concentration.

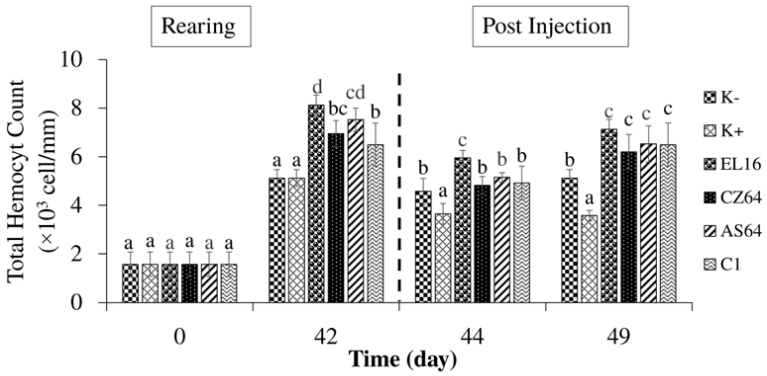

Figure 1. Total haemocyte count of white leg shrimp on days 0 and 42 during maintenance and days 44 and 49 after challenge with *V. parahaemolyticus*. Different uppercase letters in the same row indicate significantly different results between treatments (Duncan p<0.05).

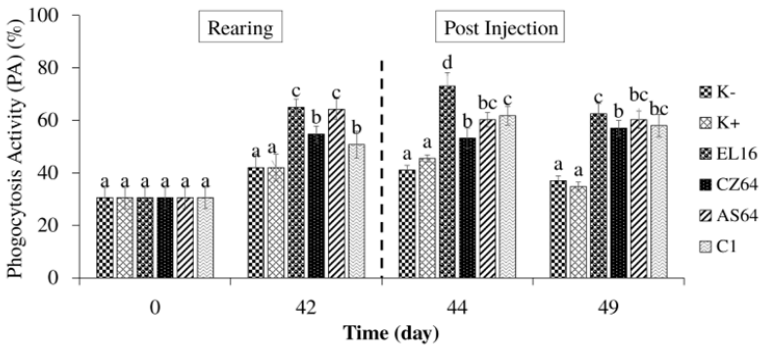

Figure 2. Phagocytic activity of whiteleg shrimp on days 0, 42, and 49 after challenge with *V. parahaemolyticus*. Different uppercase letters in the same row indicate significantly different results between treatments (Duncan p<0.05).

(P<0.05). The PO activity of whiteleg shrimp in the single and combined phytobiotic extract treatments post-infection was also significantly higher (P<0.05) than the KP and KN treatments. During maintenance and post-injection, whiteleg shrimp in the EL16 extract treatment had the highest THC value compared to the CZ64, AS64, and C1 treatments.

Respiratory burst activity

In line with the RB results, administration of single and combined phytobiotic extracts provided higher PO activity than the control after 42 days of maintenance (P<0.05). The RB activity of white leg shrimp in the single and combined phytobiotic extract treatments post-injection was also significantly higher (P<0.05) than the KP and KN treatments. During maintenance and post-injection, whiteleg shrimp in the EL16 extract treatment had higher THC values compared to the CZ64, AS64, and C1 treatments.

Vibrio bacteria population

Administration of phytobiotic extract reduced the total V. parahaemolyticus RfR bacteria and Vibrio count in the intestines of vaname shrimp (Table 4).

Resistance to Vibrio parahaemolyticus

The survival rate (SR) of whiteleg shrimp after being challenged with *V. parahaemolyticus* in the EL16, CZ64, AS64, and C1 treatments was higher (p<0.05) compared to the favourable control treatment (Figure 5). The highest survival rate was in the EL16 treatment at 82.5%, followed by AS64, C1, and CZ64 at 75%, 67.5%, and 57.5%.

Discussion

Medicinal plant contains phytochemical components that act as natural immunostimulants, which increase the host's immune response against pathogenic organisms (Chabib *et al.*,

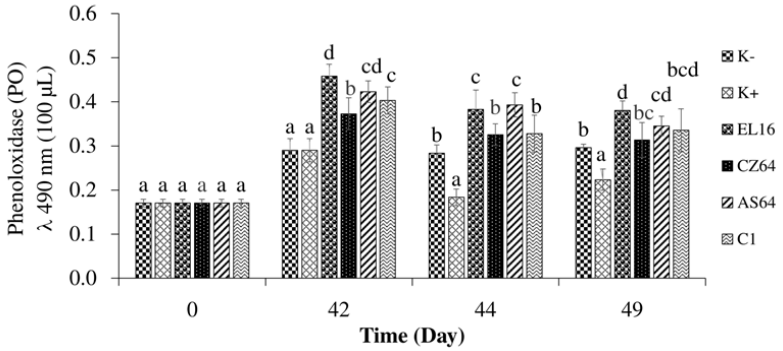

Figure 3. Phenoloxidase activity of white leg shrimp on days 0 and 42 during maintenance and on days 44 and 49 after challenge with *V. parahaemolyticus*. Different uppercase letters in the same row indicate significantly different results between treatments (Duncan p<0.05).

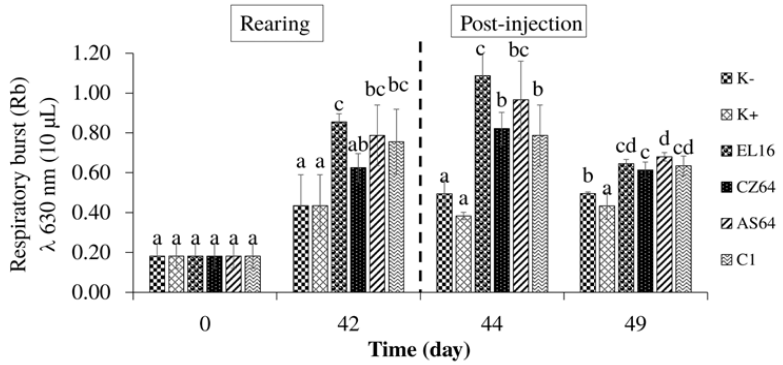

Figure 4. Respiratory burst of whiteleg shrimp on days 0 and 42 during maintenance and on days 44 and 49 after challenge with *V. parahaemolyticus*. Different uppercase letters in the same row indicate significantly different results between treatments (Duncan p<0.05).

2018). Some bioactive compounds have the capability to inhibit or prevent the growth of specific pathogens in the host. The antagonistic effect of *V. parahaemolyticus* by phytobiotic extracts is related to the phytochemical constituents present in each plant extract. Plant species with pharmacological activities are being related to phytocomponents such as glycosides, saponins, flavonoids, steroids, tannins, alkaloids, and terpenes (Batiha *et al*., 2020).

Alkaloids, saponins, phenolic compounds, flavonoids, and triterpenoids are found in all three phytobiotic extracts. On the other hand, tannin compounds were only found in the pasak bumi extract. *E. longifolia* extract has a higher phenol and flavonoid content, 9.61% (w/w) and 13.38 QU/g, respectively, compared to *C. zedoaria* and *A. sativum* extract. The content of active ingredients may be influenced by several factors, including plant type, extraction method, solvent type, and storage process.

Shrimp immunity is believed to increase in order to control diseases effectively. Numerous studies have shown that phytobiotics could improve immune function by increasing the total number of hemocytes, enhancing phagocytosis, stimulating antibacterial and bacteriolytic activities, increasing resistance to pathogens, and improving intestinal microbiota diversity in shrimp (Navin *et al*., 2016; Munaeni *et al*., 2020a). In this study, feeding with phytobiotic extracts led to increased immune responses, including total hemocyte counts (THC), phenoloxidase (PO), and respiratory bursts (RBs) in whiteleg shrimp, both before and after the challenge test. Phytobiotic extracts are posited to have immunostimulant capabilities that can activate immune responses in shrimp. Previous studies have shown that the use of herbal powder extracted from medicinal plants, which are rich in phytochemical components, can act as natural immunostimulants to enhance the host's immune system against pathogens (Chabib *et al*., 2018).

Table 4. Total Vibrio Count (TVC) and total *V. parahaemolyticus* RfR (VpR) in the intestines of whiteleg shrimp fed with additional phytobiotic extract.

Parameter (log 10 cfu/g)	Day	Treatment					
		KN	KP	EL16	CZ64	AS64	C1
TVC	0	4.39 ± 0.07^{a}	4.39 ± 0.07^{a}	4.39 ± 0.07^{a}	4.39 ± 0.07^{a}	4.39 ± 0.07^{a}	4.39 ± 0.07^{a}
	42	6.43 ± 0.42^{a}	6.43 ± 0.42^{a}	6.84 ± 0.47^{a}	6.68 ± 0.11^{a}	6.34 ± 0.08^{a}	6.69 ± 0.22^{a}
	44	6.62 ± 0.18^{a}	7.42 ± 0.43^{b}	7.63 ± 0.04^{b}	7.33 ± 0.34^{b}	7.38 ± 0.10^{b}	7.46 ± 0.14^{b}
	49	6.26 ± 0.12^{a}	6.44 ± 0.45^{a}	5.99 ± 0.26^{a}	6.38 ± 0.44^{a}	6.13 ± 0.25^{a}	6.45 ± 0.12^{a}
VpR	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	42	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	44	0.00 ± 0.00^{a}	5.93 ± 0.26^{c}	5.51 ± 0.09^{b}	5.83 ± 0.14^{c}	5.48 ± 0.11^{b}	5.74 ± 0.07^{bc}
	49	0.00 ± 0.00^{a}	4.84 ± 0.35^{d}	3.00 ± 0.38^{b}	3.72 ± 0.15^{c}	3.60 ± 0d.25^{c}	3.70 ± 0.14^{c}

Different superscript letters in the same row (mean value ± standard deviation) indicate differences (Duncan<0.05). Negative control (KN); positive control (KP); 1.6% pasak bumi extract treatment (EL16); 6.4% white turmeric extract treatment (CZ64); 6.4% garlic extract treatment (AS64), 1:1:1 combination of phytobiotic extracts (C1).

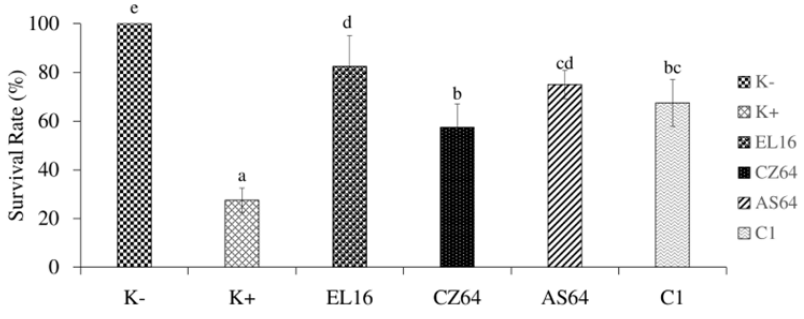

Figure 5. Survival rate (SVR) of whiteleg shrimp after challenge test (day 49) with *V. parahaemolyticus*.
Note: Different uppercase letters in the same row indicate significantly different results between treatments (Duncan p<0.05).

These bioactive compounds have the potential to inhibit or reduce the growth of specific pathogens. The inhibitory effect of *Vibrio parahaemolyticus* due to phytobiotic extracts is related to their respective phytochemical compositions. Phytochemical constituents like glycosides, saponins, flavonoids, steroids, tannins, alkaloids, and terpenes are responsible for the pharmacological activities of different plant species (Batiha *et al*., 2020). All three phytobiotic extracts contain alkaloids, saponins, phenols, flavonoids, and triterpenoids, but tannins are only found in the extract of *E. longifolia*. This extract also has a higher content of phenols (9.61% w/w) and flavonoids (13.38 QU/g) as compared to that of *C. zedoaria* and *A. sativum* extract.

The active compound content may change due to factors like species of the plant, method of extraction, type of solvent used, and storage conditions. Enhancing immunity in shrimp is one of the important approaches for effective control of diseases. The previous literature demonstrated that phytobiotics could enhance immune function, as evidenced by the increase of hemocyte count, phagocytosis, antibacterial activity, and bacteriolytic activity, together with improved resistance to pathogens and enhanced diversity of the gut microbiota in shrimp (Navin *et al*., 2016; Munaeni *et al*., 2020a). Dietary administration of phytobiotic extracts in the present study was noted to increase the immune parameters including THC, PO, and RB activity in whiteleg shrimp before and after the challenge test. The extracts showed immunostimulant activities that may enhance the immunity of shrimp.

Previous studies also reveal that phytobiotics and herbal powders can stimulate immune responses and improve the resistance against *V. parahaemolyticus* infection in shrimp (Munaeni *et al*., 2020b). Crustaceans, such as shrimp, depend on innate immune mechanisms involving both cellular and humoral responses (Tran *et al*., 2022). Shrimp hemocytes are classified into three categories: hyaline, semi-granular, and granular cells, which all contribute to different functions, including phagocytosis, encapsulation, and nodule formation, besides the production of immune components, such as agglutinins, prophenoloxidase (proPO), antimicrobial peptides, and protease inhibitors (Jiravanichpaisal *et al*., 2006). The cellular and humoral immune systems can operate independently or cooperatively to protect against pathogens (Zhang *et al*., 2021). Among the treatments tested, the use of *E. longifolia* extract (EL16) showed the best effect in improving the immune response of whiteleg shrimp, as indicated by the high values of THC, PO, and RB parameters before and after the challenge test.

Immunostimulant effects have been reported to be influenced by source, dose, route of administration, time, and the cultured species (Apines-Amar & Amar, 2015). The treatment of CZ64 and AS64 extracts in this study with 64 g/kg feed probably inhibited the immune system of shrimp, as it acts against the outcome. This observation was also noticed in the case of fish, where the authors of one study mentioned that medicinal plants can exhibit immunosuppressive actions at high concentrations (Jian & Wu, 2003).

Post-challenge test results showed that phytobiotic extracts reduced the population of *V. parahaemolyticus* in the intestine of shrimp. The TVC in the positive control group on day 44 showed a high correlation with the population of *V. parahaemolyticus* in the intestine. Similar studies have shown that Eleutherine bulbosa extracts could reduce *V. parahaemolyticus* populations in the intestine, hepatopancreas, and muscles of shrimp after the challenge test (Munaeni *et al*., 2020a). Phytobiotics effectively reduce *V. parahaemolyticus* density and stimulate the immune system, thus increasing the survival rate of shrimp. Among the treatments, the most effective extract to protect shrimp and increase survival rates was *E. longifolia* extract (EL16) compared to *C. zedoaria* (CZ64), *A. sativum* (AS64), or their combination (C1).

CONCLUSION

The phytobiotic extract that has been given can increase the immune response and resistance of whiteleg shrimp to *V. parahaemolyticus* RfR infection. EL16 extract treatment is the best treatment that can increase survival and immune response and reduce the population of *V. parahaemolyticus* RfR in the intestines of whiteleg shrimp after the challenge test.

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