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In Silico and *In Vivo* Potential of Fraction Red Betel Leaf as an Immunostimulant Agent in White-leg Shrimp

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ABSTRACT

Production of white-leg shrimp (*Litopenaeus vannamei)* in aquaculture with advanced technology ultimately results in high mortality rates in cultivation. Infectious diseases, including *Vibrio* spp., can cause mortality with necrosis symptoms in the white-leg shrimp hepatopancreas. Disease prevention through enhancing immunity is highly effective in controlling diseases in shrimps. The current study aimed to obtain information on the compounds present in the fraction of *Piper (P.) crocatum* as an immunostimulant agent used *in silico.* The current study investigated the absorption, distribution, metabolism, excretion, and toxicity (ADME/T), and determined the optimal fraction dosage of *P. crocatum* when injected as an immunostimulant substance. In this study, *in silico* analysis was conducted by accessing several servers, while the shrimp's immune response was evaluated using a completely randomized design experiment with four treatments (10 individuals/container) and three replications, including 0 (control), 0.5 µg/g, 1 µg/g, and 1.5 µg/g. The shrimp's immunity was examined by injecting the *P. crocatum* fraction initially, followed by a second injection 24 hours later. Shrimp hemolymph was collected before the injection of the *P. crocatum* fraction and 24 hours after the injection. Hemolymph was collected at both time intervals to assess total hemocyte count (THC), differential hemocyte count (DHC), and phenoloxidase (PO) as the immune response of shrimp before and after administration of *P. crocatum* fraction. Two compounds were confirmed immunostimulant agents in a fraction of *P. crocatum*, 2-Amino-1,3,4-octadecanetriol, and erucamide. The immune response values for THC (14.17 ± 2.45 × 10^6 cells mL^{-1}), DHC hyaline (53 ± 4.5%), semi-granular cells (52 ± 4.0%), and granular cells (43 ± 40%), and PO (0.112 ± 0.016 units/λ=490) at a concentration of 1.5 µg/g showed a significant increase in number and percentage compared to the control. These results indicate the presence of two compounds in fraction one *P. crocatum*, as candidates for immunostimulant agents. Administration of 1.5 µg/g of a fraction of *P. crocatum* is the appropriate dose as an immunostimulant agent when administered via injection method for white-leg shrimp.

Keywords: Immunostimulant, *In silico*, *Litopenaeus vannamei*, *Piper crocatum*

INTRODUCTION

White-leg shrimp (*Litopenaeus vannamei*) cultivation in Indonesia has been practiced by shrimp farmers for a long time due to its significance as an important commodity in the fisheries sector (Amelia et al., 2021). White-leg shrimp (*L. vannamei*) is a commodity that can be cultured at high densities, with a density range of 500-1000 individuals/M^3 (Suantika et al., 2018). Indonesia's crustacean commodity production is more than 10% of the world's total, with 15% of the total value derived from aquaculture. It is reported that 75% of Indonesia's total shrimp production comprises the white-leg shrimp commodity (FAO, 2016). As an economically important commodity, white-leg shrimp in Indonesia has experienced an annual production increase of 16%. Producing white-leg shrimp in the aquaculture industry with advanced technology eventually leads to a high mortality rate in cultivation (FAO, 2013). The occurrence of infectious diseases, caused by bacteria such as *Vibrio alginolyticus* (Li et al., 2008), *Vibrio parahaemolyticus* (Pena-Navarro et al., 2020; Saputra et al., 2023), and *Vibrio harveyi* (Rungrassamee et al., 2014) were reported before. Vibriosis can cause mortality rates of up to 100% (Soto-Rodriguez et al., 2015). Infection of *Vibrio* spp. in white-leg shrimp has been reported by Saputra et al. (2023). The results showed hepatopancreas damage, such as necrosis and hemocyte infiltration, leading to melanization.

Several studies have researched using herbal plants as bioactive immunostimulant agents to address this condition. Applying bioactive immunostimulants is safe from chemical residues, serves as an alternative to antibiotics, is environmentally friendly, and significantly enhances the immune system of aquatic animals (Van Hai, 2015; Vijayaram et al., 2022). Herbal plants containing phenolic and flavonoid compounds have been reported to enhance innate immune in shrimp, such as the herbal plant zingerone, *Scutellaria baicalensis*, and *Galla chinensis* (Chang et al., 2012; Pan and Yan, 2020), *Procambarus clarkia* (Zhang et al., 2021) and *Ocimum basilicum* (Abdel-Tawwab et al., 2022). The immunity parameters with normal range in shrimp are total hemocyte count (THC) 4.10 - 5.01 x 10^6 cell mL^{-1}, and

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differential hemocyte count (DHC), Hyaline (29.67-32.92%), Granular Cells (6.93-11.55%), and semi granular cells (45.03-50.85%) (Fadjar et al., 2020).

Red betel leaf (*Piper crocatum*) is an herbal plant with flavonoid content and a high total phenolic value (Saputra et al., 2016). Azhar et al. (2021) reported that the application of red betel leaf extract (*Piper crocatum*) to tiger shrimp at a dose of 0.5% resulted in a higher THC value of 7.70×10^6 cells mL-1, compared to controls with a value of 3.89×10^6 cells mL^{-1}. Moroever, DHC indicated a significant increase in hyaline cells, reaching 82.94%, compared to controls with a value of 45.24%. The compound content of *Piper (P.) crocatum* is used as medicine in fisheries aquaculture. Further studies are needed to optimize the use of herbal plants as medications in aquaculture (Reverter et al., 2014). The effectiveness of herbal plants should be studied *in silico* before *in vivo* testing. *In silico* studies involve computerized prediction methods for the biological activity of a compound, which can efficiently optimize the use of laboratory resources (Frimayanti et al., 2018). The *in silico* study method is a computerized method that accesses the PubChem web database. The website provides information on millions of descriptions of chemical compounds, chemical structures, and biological activities (Kim et al., 2016). *In silico* method predicting antimycobacterial, antifungal, and antioxidant properties (Jamkhande et al., 2016; Biswal et al., 2019).

Several studies have reported the benefits of *P. crocatum* extract in shrimp as potential immunostimulant agents (Emrizal et al., 2014; Azhar et al., 2021). However, there is a lack of *in silico* and *in vivo* data on the application of *P. crocatum* fractions as immunostimulants in white-leg shrimp. Therefore, based on this background, this study aims to determine compounds from a fraction of *P. crocatum* as immunostimulants through *in silico* prediction and to evaluate changes in the amount of innate immune in white-leg shrimp, including THC, DHC, and phenoloxidase (PO) parameters after injection of the fractions of *P. crocatum*.

MATERIALS AND METHODS

Ethical approval

The research was conducted in May - December 2022 at the Microbiology and Chemistry Laboratory of the Ahli Usaha Perikanan (AUP) Polytechnic, Serang, Banten-Indonesia. This study was approved by the Institutional Research Ethics Commission at the University of Malahayati, Indonesia, with ethical clearance 3490a/EC/KEP-UNMAL/V/2022.

Red betel leaf fractionation

Red betel leaves were collected from herbal plant farmers in Jogjakarta-Central Java, Indonesia. The average size of the *P. crocatum* leaves was 12 cm. Extraction was performed based on the method Saputra et al. (2016). Red betel leaf extraction was carried out by dissolving 100 grams of red betel leaf powder (dry) in methanol (250 ml) and then homogenizing with a magnetic stirrer for 3 hours. The filtrate obtained was concentrated in a rotary evaporator at a temperature of 40-45^{o}C. After the extraction, fractionation was carried out to obtain the fraction of *P. crocatum* using the column chromatography method with methanol: ethyl acetate (1:8, v/v) as the eluent (Nursyam et al., 2017). Before profiling using liquid chromatography high-resolution mass spectrometry (LC-HRMS) on the *P. crocatum* fraction, identifying flavonoid content was performed in each fraction of *P. crocatum* using 0.1 mg quercetin as the standard. The quercetin compound group was found in fractions of *P. crocatum* (Saputra et al., 2016). Furthermore, the fraction of *P. crocatum* was profiled using LC-HRMS, referring to the method of Carvalho and Ribeiro (2019). The analysis uses the Thermo Scientific Dionex Ultimate 3000 RSLCnano LC-HRMS model.

In Silico absorption, distribution, metabolism, excretion, and toxicity

Fractions of *P. crocatum* were analyzed for candidate drug prediction using the method of Amin et al. (2018). The LC-HRMS screening resulted in a fraction of *P. crocatum*, indicating various compounds. The highest abundance of compounds in the fraction of *P. crocatum* was filtered to obtain two target compounds. To obtain *in silico* absorption, distribution, metabolism, excretion, and toxicity (ADME/T) analysis information on the first fraction of *P. crocatum* in terms of the canonical simplified molecular input line entry system (SMILES), molecular weight (MW), hydrogen bond acceptor (HBA), hydrogen bonded donor (HBD), polar surface topology (TPSA), partition coefficient (iLOG P) were accessed on Swissadme, while for toxicity class and LD50 value accessed on ProTox II, and probability activity (Pa) were accessed on PASSonline (Banerjee et al., 2018; Supandi and Merdekawati, 2018). To obtain the LD50 value, the toxicity class was accessed using ProTox-II. By entering the SMILES of the target compound, Pro Tox II could predict the median lethal dose (LD50) in mg/kg weight and toxicity class (Banerjee et al., 2018). According to Filimonov et al. (2014), this compound could potentially have high experimental activity with a Pa value > 0.7. The compound is likely to be close to known pharmaceutical compounds within the range of 0.5 < Pa < 0.7. Meanwhile, the value of Pa < 0.5 indicates that the activity of the compound in the experiment is low.

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Immunostimulant activity

After predicting the probability of candidate compounds using the *in-silico* method, the experiment was followed by the *in vivo* method on test animals (white-leg shrimp). White-leg shrimp (*L. vannamei*) with an average weight of 11 ± 0.5 g were obtained from the teaching factory (TEFA) shrimp pond at the AUP Polytechnic in Serang, Banten, Indonesia. The white-leg shrimp were adapted in the container for three days before being injected with the red betel leaf fraction. Commercial feed (33% protein) was given at a dose of 10% of body weight/individual during treatment (24 hours). The application test of the *P. crocatum* fraction was carried out using a glass aquarium container (60 x 40 x 40 cm) with a volume of 60 L and a density of 10 individuals/container. After completing the LC50 test, the dose of the fraction of *P. crocatum* on white-leg shrimp (*L. vannamei*) was determined based on a study by Wang and Chen (2005). The shrimp's immune response was evaluated using a completely randomized design experiment with four treatments (10 individuals/container) and three replications (30 individuals/replications), including 0 (control), 0.5 µg/g, 1 µg/g, and 1.5 µg/g. The study of shrimp immunity was based on the method introduced by Fadjar et al. (2020) with a modification. In this modified approach, the shrimp were subjected to injections of the *P. crocatum* fraction at the outset and then again after a 24-hour interval. Following this procedure, the measurement of shrimp immunity was carried out. Shrimp hemolymph was collected before injection of the *P. crocatum* fraction and 24 hours after injection of the *P. crocatum* fraction. Hemolymph was collected at both times to assess Total Hemocyte Count (THC), Differential Hemocyte Count (DHC), and Phenoloxidase (PO) as the immune response of shrimp before and after administration of *P. crocatum* fraction. Each treatment and control was performed in three replicates using a completely randomized design. Water quality conditions include water salinity of 25-29 ppt, dissolved oxygen above 5 ppm, pH of 7, and a temperature of 29°C (Xu et al., 2016).

The THC and DHC observations were carried out according to the method of Liu and Chen (2004) and Wu et al. (2017) by homogenizing 0.1 ml of hemolymph with 0.900 ml of anticoagulant. After the hemolymph was homogeneous, THC and DHC were measured using a hemocytometer and light microscope (Olympus IX 71).

$$\text{THC} = \text{Average} \sum \text{Counted cell x} \frac{1}{\text{Volume large box}} \text{ x Dilution factor}$$

The PO activity was measured following Tenriulo et al. (2014) and Zhou et al. (2021). To do so, 100 µL of hemolymph was added to 900 µL of anticoagulant and centrifuged at 700 x rotary temperature setting of 4°C for 20 minutes. The pellets were separated, and then 1000 µL of cacodylate-citrate buffer was added and centrifuged again. The centrifuged pellet was dissolved in 0.2 mL of cacodylate buffer. The solution was then separated into two parts. The first solution was as an elicitor by incubating 0.1 mL of the solution with 0.05 mL trypsin (10 minutes at 25°C). Then, 0.05 mL of L-DOPA was added, and 0.8 mL of cacodylate buffer was added after 5 minutes. The second solution was used as a control, where 0.1 mL of the cell suspension was added to 0.05 mL of cacodylate buffer and 0.050 mL of L-DOPA.

Data analysis

Statistical analysis of the THC, DHC, and PO data was performed using one-way ANOVA in SPSS Version 21 (USA) with a confidence level of 95% ($p < 0.05$). Subsequently, Tukey's test was employed to determine significant differences among the results. The descriptive data analysis includes ADME/T analysis on the phytochemical fraction of *P. crocatum*, toxicity (ProTox-II, 2021), canonical SMILES, (SwissADME, 2023), and prediction of pharmacokinetic biological activity (PassOnline, 2023), which were carried out by accessing several servers. The analysis of obtained data for THC, DHC, and PO were presented in tabular form.

RESULTS AND DISCUSSION

Liquid chromatography high-resolution mass spectrometry analysis

Column chromatography was conducted, employing the quercetin standard as a reference, in line with the methodology proposed by Saputra et al. (2016). This procedure led to the examination of fractions from *P. crocatum* through liquid chromatography high-resolution mass spectrometry (LC-HRMS) instrument analysis. The LC-HRMS profiling results indicated 85 chromatograms (data not published), and 10 compounds with an abundance of > 1% (Figure 1). Then, a filter was applied by considering the probability activity (Pa) values of ≥ 0.5 and <0.7. The filter results concluded that two compounds were candidates for immunostimulant agents (Table 1).

The two compounds each have a very important role in medicine. The highest abundance value is found in the compound 2-Amino-1,3,4-octadecanetriol, which is 4.41%. This compound is an amino alcohol in which the molecular entity can accept a hydron from a donor (Bronsted acid) through an organic amino compound (Matsumoto et al., 1995). The total synthesis of 2-amino-l,3,4-octadecanetriol is an anti-tumor glycosphingolipid and immunostimulant derived from agelasphins. Meanwhile, the next compound is erucamide, with an abundance value of 4.09%. Erucamide is a primary fatty amide produced by condensing erucic acid carboxyl group with ammonia. This compound acts as a metabolite in both mammals and plants (Kenar et al., 2017). Is based on research by Gong et al. (2022), was reported that

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the extract of *Ficus tikoua Bur*, which contains erucamide, showed significant immunomodulatory effects by increasing cytokine release and inducible nitric oxide synthase (iNOS) and celecoxib (COX-2) expression in RAW264.7 cells.

Table 1. Liquid chromatography of high-resolution mass spectrometry screening the fraction of *Piper crocatum*

No	Name	Formulas	RT [minimum]	Area (Maximum)	mzCloud Best Match	Abundance (%)
1	2-Amino-1,3,4-octadecanetriol	C18 H39 N O3	13,02	560.866.154	78.8	4.41
2	Erucamide	C22 H43 N O	22,37	520.442.819	77.1	4.09

RT: Retention time, mzCloud: Mass Spectral

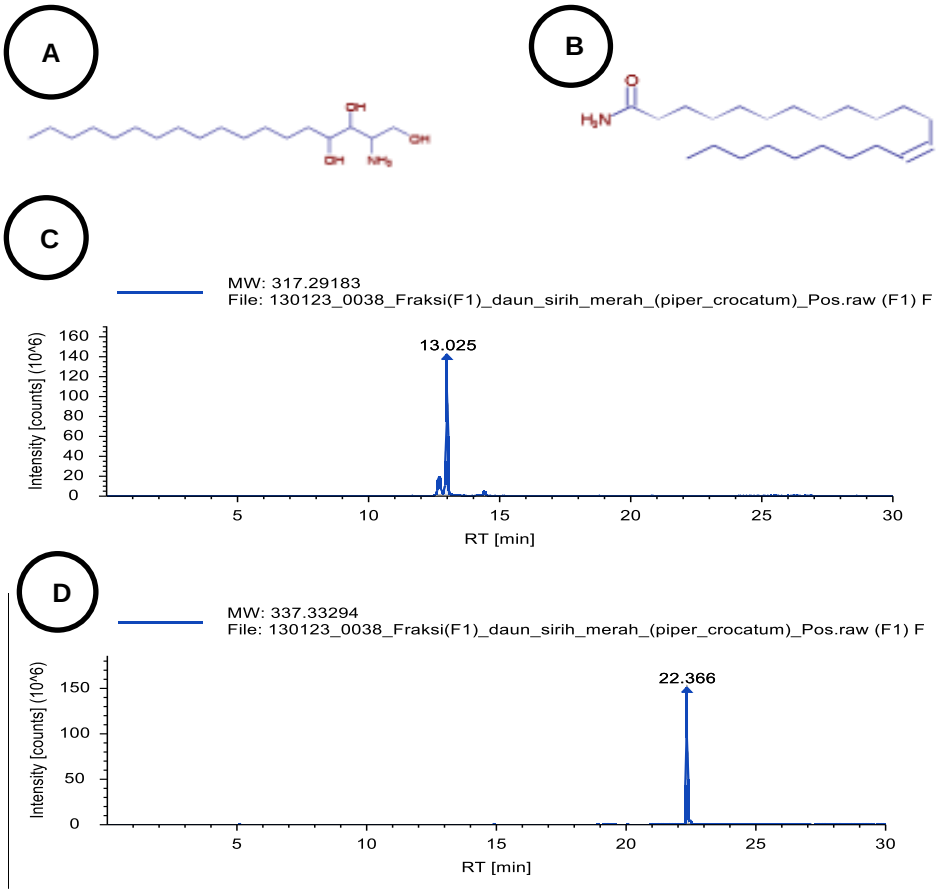

Figure 1. Structure and retention time-minimum screening of liquid chromatography high-resolution mass spectrometry (LC-HRMS) fractions of *Piper crocatum*. **A:** Structure of the compound 2-Amino-1,3,4-octadecanetriol. **B:** Structure of the compound erucamide. **C:** Retention time-minimum of the compound 2-Amino-1,3,4-octadecanetriol. **D:** Retention time-minimum of the compound erucamide. RT: Retention time, MW: Molecular weight.

***In silico* analysis absorption, distribution, metabolism, excretion, and toxicity**

Candidate compounds for drugs were analyzed *in silico*, encompassing Molecule, Canonical SMILES, MW, HBA, HBD, TPSA, iLOG P (Supandi and Merdekawati, 2018), and Lethal doses and toxicity class (Banerjee et al., 2018). The ADME/T analysis is presented in Table 2.

Table 2 shows two potential drug-candidate compounds that can be utilized as immunostimulants. Drug usage was determined at 90% based on the physicochemical properties, considering the five rules (RO5) provided by Lipinski method (Singh, 2016). The RO5 considers four important physicochemical properties, including MW < 500 Da, partition coefficient (iLog P) < 5, HBD < 5, and hydrogen bond acceptor HBA < 10 (Lipinski et al., 2012). According to Jemal et al. (2010), TPSA value ≤ 140 A^0 has ideal bioavailability. Two active compounds from the first fraction of *P. crocatum* had MW values within the range of 317.50-337.60 g/mol. As sated by Lipinski et al. (2012), MW value < 500 indicates that the compound can diffuse across cell membranes. The HBA, HBD, and iLOG P values for both compounds were < 10, < 5, and < 5, respectively, while the TPSA value was ≤ 140 A^0. Considering Lipinski's RO5 rule, it could be concluded that both compounds were ideal drug candidates.

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The toxicity values (LD50) analyzed by ProTox II showed a toxicity range of 750 mg/kg to 3,500 mg/kg. The toxicity classification indicated that erucamide belonged to class 4, while 2-Amino-1,3,4-octadecanetriol was for class 5. According to Supandi and Merdekawati (2018), the classification of this class means that the higher the LD50 value, the lower the toxicity value of the compound to the test animal.

Table 2. Absorption, distribution, metabolism, excretion, and toxicity analysis of the active compounds in a fraction of *Piper crocatum*

No	Molecule	Canonical SMILES	MW (g/mol)	HBA	HBD	TPSA (A^0)	iLOG P(o/w)	LD50 (mg/kg)	Toxicity Class
1	2-Amino-1,3,4-octadecanetriol	CCCCCCCCCCCCCCCC(C(C(CO)N)O)O	317,50	4	4	86,71	4,23	3.500	5
2	Erucamide	CCCCCCCC/C=C\CCCCCCCCCCCC(=O)N	337,60	1	1	43,09	5,02	750	4

SMILES: Simplified molecular input line entry system, MW: Molecular weight, HBA: Hydrogen bond acceptor, HBD: Hydrogen bound donor, TPSA: Topology Polar Surface Area, iLOGP: Partition coefficient, LD50: Lethal doses 50

The biological activity of *Piper crocatum* fraction

Biological activity showed that the two compounds were predicted to act as leukopoiesis stimulants, immunostimulants, immunomodulators, and macrophage stimulants, with Pa values ranging from 0.449 to 0.940 based on the Prediction of Activity Spectra for Substances (PASSonline method, Table 3). These prediction results demonstrated that active compounds from the fraction of *P. crocatum* possessed ideal biological activity in the immune system.

An important aspect in interpreting the prediction results is considering the highest Pa value as an indication of probability. As shown in Table 3, compounds 2-amino-1,3,4-octadecanetriol and erucamide had Pa values ≥ 0.5 for almost all biological activities except for immunomodulator activity (0.449). According to Filimonov et al. (2014), a Pa value > 0.7 (blue box) indicated a higher likelihood of finding experimental activity. The 0.5 < Pa < 0.7 (green box) range suggested that experimental activity was more likely to be found, but the compounds might resemble known pharmaceutical agents. Pa < 0.5 (gray box) indicated that the experimental activity would be lower because it had a Pa value of 0.499. As stated by Filimonov et al. (2014), Pa value below 0.5 indicates that the anticipated biological activity is likely to exhibit characteristics somewhat akin to an immunomodulator agent. Based on the Pa values of the two candidate compounds from the fraction of *P. crocatum*, it could be concluded that these two compounds had a relatively high potential to be considered immunostimulants for disease prevention in white-leg shrimp.

Table 3. Analysis of biological activities using prediction of activity spectra for substances to fraction from *Piper crocatum*

No	Molecule	Probability activity (Pa)			
		Leukopoiesis stimulant	Immunostimulant	Immunomodulator	Macrophage stimulant
1	2-Amino-1,3,4-octadecanetriol	0.805	0.670	0.547	0.555
2	Erucamide	0.713	0.559	0.449	0.940

Pa value > 0.7 (blue box), Value 0.5 < Pa < 0.7 (green box), Pa < 0.5 (grey box)

Immunostimulant activity

Shrimp immune response tests were observed to determine the treatment used in the subsequent stage of the research. Fraction of *P. crocatum* serves as the active ingredient of the immunostimulant compound used in the study. The innate immune defense system of white-leg shrimp against the application of an immunostimulant from the *P. crocatum* fraction was shown by the hemocyte profile on THC, DHC, and PO.

Total hemocyte count and differential hemocyte count

Hemocytes and differential hemocytes are innate immune responses in shrimp. Pathogen attacks or the presence of chemical compounds, such as octopamine, can enhance the immune response in shrimp (Hauton, 2012; Liu et al., 2019). Phagocytosis and encapsulation of foreign substances are highly important in the innate immunity (hemosit) of crustaceans (Cerenius et al., 2010). The hemocyte cell count is one of the parameters that indicate the activity of the immune response in shrimp. Table 4 shows the immune response of white-leg shrimp (*L. vannamei*) given an injection of *P. crocatum* fraction, with the variables of total hemocyte count and differential hemocyte count.

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Table 4. Total hemocyte count and differential hemocyte count levels in white-leg shrimp (*Litopenaeus vannamei*) after injection of *Piper crocatum* fraction

Treatment	Total hemocytes count/THC (10^6 cell mL^{-1})		Differential hemocyte count					
			Hyaline (%)		Granular cells (%)		Semi-granular cells (%)	
	Outset	24 hours post injection	Outset	24 hours post injection	Outset	24 hours post injection	Outset	24 hours post injection
0.5 µg/g	5.83±1.41	7.50±0.95 [a]	28±2.1	27±2.5 [a]	27±3.2	40±3.0 [b]	30±1.2	31±1.5 [a]
1 µg/g	7.70±1.25	9.83±1.27 [b]	26±2.6	26±3.1 [a]	26±3.1	40±4.2 [b]	28±1.5	31±3.5 [a]
1.5 µg/g	6.33±1.26	14.17±2.45 [c]	27±2.1	53±4.5 [b]	27±3.2	43±4.0 [c]	30±0.6	52±4.0 [b]
control	7.47±1.48	7.73±1.96 [a]	26±1.5	27±2.0 [a]	21±2.5	24±3.8 [a]	29±2.0	30±1.0 [a]

[abc] show a significant difference in a variable in the column ($p < 0.05$); Data presented as mean ± standard error

In treating immunostimulant administration at a dose of 1.5 µg/g, significant results (notations b and c) were observed after injection of *P. crocatum* fraction ($p < 0.05$). The THC value increased from 6.33 x 10^6 cells mL^{-1} to 14.17 x 10^6 cells mL^{-1} after injection (Table 4). Similar findings were reported by Azhar et al. (2021), indicating that post-feeding with 5% enrichment of *P. crocatum* significantly increased THC to 7.7 x 10^6 cells mL^{-1}, compared to the control group (3.1 x 10^6 cells mL^{-1}). Based on the statistical analysis of DHC, a dose of 1.5 µg/g showed significant results, compared to the control and doses of 0.5 µg/g and 1 µg/g ($p < 0.05$). In hyaline and semi-granular cells, there was a significant difference (HSD) increase from 27 % to 53 % and from 30 % to 52 % after injection ($p < 0.05$). There was no significant difference between the control group and the treatments with doses of 0.5 µg/g and 1 µg/g ($p > 0.05$). However, doses of 1.5 µg/g, 0.5 µg/g, and 1 µg/g showed significant differences in granular cells, compared to the control group ($p < 0.05$).

The increase in THC and DHC (hyaline, semi-granular cells, and granular cells) is an immune response to the fraction of *P. crocatum.* The compounds in the fraction of *P. crocatum* that had the highest likelihood as immunostimulant candidates were 2-Amino-1,3,4-octadecanetriol and erucamide, with a Pa value > 0.5 (Table 3). Both compounds had a MW value < 500, allowing diffusion into the shrimp cell membrane under such conditions. This follows RO5 of Lipinski. According to Biswal et al. (2019), a candidate with a MW value < 500 can easily diffuse into the cell membrane. When compounds enter the cell membrane, hemocyte receptor cells recognize them and respond by increasing the production of hemocytes. Hemocytes are integrated by a pair of epigastric tissues located precisely on the dorsal part of the anterior stomach. These tissues serve as the site of hemocyanin synthesis, so an increase in hemocyanin levels is directly proportional to an increase in hemocytes (Effendy et al., 2004). Hemocyte count activity plays an important role in pathogen attack through several stages, starting from the recognition stage to the cytotoxicity stage against pathogens (Cerenius et al., 2010). Innate immunity in hyaline cells performs phagocytosis (Johansson et al., 2000). The next stage is granular cells; semigranular cells produce melanin in a cytotoxic process against pathogens (Hauton, 2012).

The immune response at a dose of 1.5 µg/g showed a significant difference in results, compared to doses of 0.5 µg/g, 1 µg/g, and the control. The PO value in Table 5 indicated an increase from 0.081 to 0.112 units after injection. Chang et al. (2012) reported that applying zingerone enrichment at 1, 2.5, and 5 mg zingerone (kg diet)$^{-1}$ can significantly increase THC and PO levels in shrimp. In line with the significant increase of semi-granular and granular cells in Table 4, it indicated a series of synergistic immune responses in white-leg shrimp following the injection of the fractions of *P. crocatum* in the recognition, phagocytosis, melanization, and cytotoxicity systems. Cerenius et al. (2010) and Lee et al. (2020) stated that the pathogen-associated molecular pattern would degranulate and release the proPO system β-1,3-glucan binding protein.

Phenoloxidase

Phenoloxidase is the proPO system's terminal enzyme and is a primary immune indicator in crustaceans (Liu et al., 2019). The PO response after injection of the fraction of *P. crocatum* is presented in Table 5.

Table 5. Phenoloxidase levels in white-leg shrimp (*Litopenaeus vannamei*) after fraction injection of *Piper crocatum*

Treatment	Activities phenoloxidase/optical density ƛ=490 Units	
	Onset	24 hours post injection
0.5 µg/g	0.082 ± 0.003	0.084 ± 0.009 [a]
1 µg/g	0.081 ± 0.004	0.076 ± 0.004 [a]
1.5 µg/g	0.081 ± 0.003	0.112 ± 0.016 [b]
control	0.079 ± 0.002	0.078 ± 0.002 [a]

[abc] show a significant difference in the variable in the column ($p < 0.05$), Data presented as mean ± standard error; h: Hours

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CONCLUSION

Present findings indicated that two compounds (2-Amino-1,3,4-octadecanetriol, and erucamide) are identified as potential immunostimulants based on *in silico* (ADME/T) method. Subsequently, administration of the fractions of *P. crocatum* via injection at a dose of 1.5 µg/g resulted in a significant increase in THC 6.33 to 14.17 x 10^6 cell mL^{-1}, DHC (hyaline 27 % to 53 %, semi-granular 30 % to 52 %, and granular cells 27 % to 43 %), and PO 0.081 to 0.112 units. Further resaerach is needed to evaluate the effect of *P. crocatum* fraction as an immunostimulant agent in preventing the pathogenicity of acute hepatopancreatic necrosis disease (AHPND) caused by the bacterium *Vibrio parahaemolyticus*.

DECLARATIONS

Availability of data and materials

All data generated during the research are relevant and included in this published article.

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Authors' contributions

Afandi Saputra conducted data collection and data analysis and wrote the original manuscript. Maftcuh did the conceptualization and supervision. Sri Andayani and Uun Yanuhar assisted in data analysis, manuscript preparation, and revision. All authors read and confirmed the final draft of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethical consideration

The authors declare and confirm that the manuscript is original, has no misconduct, has never been published in another journal, and is confirmed to be published in this journal.

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