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Utilization *Ecdysterone* Hormone of Krokot (Portulaca oleracea) Extract on Molting Activity in Mud Crabs (*Scylla serrate*)

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Abstract: The Scylla serrate mangrove crab is one of the leading fisheries commodities in terms of exports, only behind shrimp, tuna and shellfish. Crabs have a fairly high price in foreign markets and most of the mud crabs obtained from wild catches are soft shell crabs. In terms of price, cultivating soft shell crabs is quite profitable, but most cultivators have several problems such as long maintenance periods and inconsistent molting times so that they do not experience business continuity. Applying herbal extracts via injection is an effective method because all the substances that trigger molting directly enter the body. Physiological engineering is needed to speed up the molting phase and get the same molting time. This research aims to identify the ecdysterone content in purslane leaves and determine the optimum dose of purslane leaf extract which can encourage skin molting in mud crabs. This research used a Randomized Block Design (RBD). The in silicons test results show that the compound that has the smallest binding affinity is ecdysone, namely -6.80 kcal/mol. Based on analysis of variance, the best molting percentage occurred in treatment B at 55.50%. The fastest molting period occurred in treatment C, namely on days 4 to 29 after injection. Treatments B and C had the highest survival rate, namely 100%, while in terms of increasing body weight and carapace width, the effect of all treatments was not significantly different. The water quality results for all groups were still within the optimum value, temperature 26.40 – 33.80ºC; pH 6.83 – 7.20; salinity 20.70 – 26.70 ppt and dissolved oxygen 4 – 4.52 ppm

Keywords: *Ecdysterone;* Growth; Percentage Molting; *Portulaca oleracea*; Molting Period; Percentage Molting; *Scylla serrate*

Introduction

Mangrove crabs of the type Scylla serrate are one of the leading fishery commodities in terms of exports, just below shrimp, tuna and skipjack. The crab has a high price in foreign markets such as China, Hong Kong, Taiwan, Thailand, and Singapore (Bhuiyan et al., 2021). Most of the mangrove crabs obtained from natural catches are soka crabs. In terms of price between soft-shell and hard-shell crabs, soka crabs range from Rp55.000-Rp60.000/kg while hard-shell crabs range from Rp18.000/kg, each kilogram containing 10 crabs. In terms of price, soft-shell crab cultivation is quite profitable, however, most farmers have several obstacles in cultivation such as long rearing periods and molting times that are not simultaneous. The long rearing period causes high feed and operational costs, while the molting time that is not simultaneous results in stricter supervision during maintenance, making it less efficient in terms of time and energy (Silva, 2023; Sukardi et al., 2021; Mariani et al., 2022).

Various efforts have been made to overcome these obstacles such as stimulation through food manipulation the environment and leg organ autotomy

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techniques (Holleman et al., 2020; Sousa et al., 2024). The technology of mutilation or autotomy in crabs can cause faster molting, which is around 1 month, but the growth is low and the mortality rate is high (around 50%) because during the rearing period it experiences stress and infection (Fujaya et al., 2013). In addition to these two methods, there is another way, namely through injecting molting hormones such as 20- *Hydroxyecdysone*, but in terms of price it is very expensive around Rp 1.000.000/mg (Sarintang et al., 2020). 20-*Hydroxyecdysone* is a pure hormone resulting from the extraction of crustacean ecdysteroids, to overcome the high price of these hormones, a new breakthrough emerged using ecdisteroid hormones from plants also known as phytoecdisteroids (Arif et al., 2022).

The addition of phytoecdisteroids hormones has been carried out in previous studies, namely spinach extract (Amaranthus) (Rahma & Nurcahyanti, 2021; Lubis et al., 2023; Rao & Poonia, 2023). However, there are still disadvantages of using spinach extract and mulberry leaf extract, namely that it is difficult to do at the cultivator level because the extraction process is relatively difficult and also for spinach extract is very competitive with human needs. Therefore, for the purpose of diversifying the use of plant products, it is necessary to find and develop other sources of moltinginducing hormones, one of which is purslane leaves (*Portulaca oleracea*).

The application of herbal extracts by injection is an effective way because all moltingpromoting substances enter the body directly. The utilization of ecdisteroid hormones in crabs needs to be adjusted to their needs to carry out the molting process, due to the importance of the molting mechanism for crab growth and production, it is necessary to innovate in molting physiology engineering. This study aims to isolate and identify the ecdysterone content in purslane leaves and determine the optimum dose of purslane leaf extract that can spur molting in mangrove crabs.

Method

Research Location

Stage one research included maceration, liquid chromatography mass spectrometry test, High Performance Liquid Chromatography (HPLC) chromatography test and insilico analysis using Windows 10 laptop software. Maceration test was conducted at the Materia Medica Laboratory (Batu City), Liquid Chromatograph Mass Spectroscopy (LC-MS) test was conducted at the Chemical Engineering Laboratory the State Polytechnic of Malang and the HPLC test were carried out at the Laboratory of the Faculty of Pharmacy, State Islamic University of Malang, while the second phase of the research was carried out at the Community Pond in Curah Sawo Village, Gending District, Probolinggo Regency.

Research Design

The research design used was a randomized block design (RAK) (Shi et al., 2023). The groups in this study were divided into three parts, namely: inlet, outlet and ponds. Each group had 15 crab boxes (five treatments and three replications). The treatment in this study consisted of a control treatment, namely without being given the injection of the hormone ecdysteron and treatment with the injection of the hormone ecdysteron, including: A (12 µg/head), B (18 µg/head), C (24 µg/head), D (30 µg/head) and controls.

Research Tools and Materials

The tools used in this study included: crab box or plastic basket as a container for each test crab, 1 ml tube, DO meter, pH meter, glass beaker, rotary evaporator, blender, separating funnel, oven, filter polytetrafluoroethylene (PTFE), photodiode-array (PDA) detector, analytical balance, digital ruler, net and 1 mL volume syringe. The materials used in this study were divided into two parts, namely the main ingredients and additional materials. The main ingredients include: Mud crab (Scylla serrate) with a body weight of 70 grams with a total of 45 individuals (each treatment per head), 2400 grams of purslane (Portulaca oleracea) leaves obtained from Ngantang Village, Malang Regency.

Data Collection

Collected purslane leaves were dried at room temperature for approximately one week, the dried leaves were weighed as much as 2400 grams then put into a blender to be crushed, then macerated for 3x24 hours using 96% ethanol solvent to obtain crude extract. After three days of maceration, the crude extract obtained was concentrated using a rotary evaporator until it was blackish green in color. Identification of ecdysteron compounds in purslane leaves using LCMS (Liquid Chromatography Mass Spectroscopy) analysis. LC (Liquid chromatography) was used for quantitative analysis, while MS (mass spectroscopy) was used for qualitative analysis.

The ionizing source used in this test is ESI (Electrospray Ionization) positive ion mode [M+H] + so that the readable m/z is equivalent to the molecular weight plus 1 Da, while the mass analyzer used is a Quadruple analyzer. Measurement of ecdysteron hemolymph content was carried out using High Performance Liquid Chromatography (HPLC). The mobile phase used was a mixture of methanol: water

(85:15), a flow rate of 1 ml/minute with an injection volume of 20 µl. The detector used is a photodiodearray (PDA) with a wavelength of 366nm. Quantification of ecdysteroids compounds using standard 20-hydroxyecdysone (sigma).

The application of the hormone ecdysteron is by injection into the base of the fifth swimming leg using a 1 mL volume syringe with a 27 Gauge syringe size (Figure 1a). The treatment of giving ecdysteron hormone doses from purslane leaf extract to crabs was carried out based on previous research, Hasnidar (2018), it was found that the best dose of 20-Hydroxyecdysone hormone in stimulating mud crab molting was 1 µg/head. Crabs were fed mangrove snails (Telescopium) as much as 5% of body weight per day with a frequency of feeding twice a day ad libitum. Crab haemolimph collection was carried out before the purslane leaf extract injection treatment. 1 mL of hemolymph that has been taken is then stored in eppendoft and mixed with 1 mL of anticoagulant. The mixture is stored in the refrigerator at a cold temperature, the next step is extraction (Figure 1b). The test container is a plastic basket (crab box) measuring 15 cm x 18.5 cm x 20 cm in length, width and height. Observations were made every day (morningafternoon-evening) to see if there were any crabs that experienced molting after being treated.

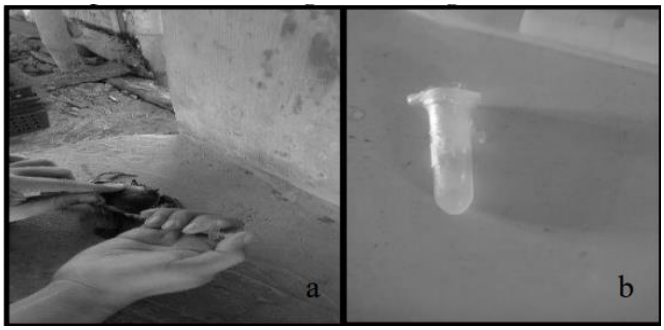

Figure 1. (a) Injecting *Portulaca oleracea* extract on mangrove crabs and (b) *Scylla serrate* hemolymph extract

Data Analysis

The data obtained from each treatment were then analyzed statistically using analysis of variance (ANOVA) according to the design used with a mean difference of significance at the 95% confidence level (α = 0.05). If from the data of varians it is known that F count > F table then to compare values between treatment groups it is continued with the DMRT test. The data was processed using the SPSS (Statistical Package for the Social Scientist) application version 25 for windows. Environmental parameter data were analyzed descriptively.

Result and Discussion

Result

Extraction Portulaca oleracea

The extraction process used purslane leaves with a wet weight of 2.40 kg, then dried using an oven at 40°C to obtain 120 grams of P. oleracea powder. The solvent used was 96% ethanol with a ratio between simplicia and solvent (1:3). The yield value of P. oleracea extract by maceration method was 13%. The result of maceration is a dry extract of ethanol.

Liquid Chromatography Mass Spectroscopy-Quadrupole Electroscopy (LCMS-QESI+) from Portulaca oleracea

The ethanol extract was then subjected to qualitative and quantitative analysis. The standard used is 20-hydroxyecdysone (sigma). MS spectogram results show that purslane leaf extract contains several phytoecdysteroid compounds with different molecular weights including 464.31m/z (Ecdysone and Ponasterone A), 480.30m/z (20-Hydroxyecdysone), 496.30m/z (Muristerone A) and 494.32m/z (Makisterone A). The results of LCMS show that the standard compound 20-hydroxyecdysone has a retention time (RT) value of 2.7 min, while the purslane extract sample did not quantitatively detect the target compound (20-hydroxyecdysone), in other words, purslane extract contains 20-hydroxyeccdysone compounds in very small amounts.

Table 1. LCMS Results from Portulaca Oleracea

Compounds	Molecular Weight (m/z)
Ecdysone	464.31
20-hydroxyecdysone	480.30
Makisterone A	494.32
Muristerone	496.30
Ponasterone A	464.31

High Performance Liquid Chromatography Analysis of 20-Hydroxyecdysone Compounds from Portulaca oleracea

HPLC analysis is carried out to determine the content of target compounds in purslane leaf extract, this needs to be done to support the results of LCMS tests that have been carried out previously. The results of HPLC analysis in 1 ppm contained 0.506 ppm of 20-hydroxyecdysone compounds with a retention time (RT) value of 2.823 minutes

Analysis In-Silico

Purslane leaf extract was analyzed using PyRx application to determine the binding affinity value and RMSD (root mean square deviation) value of the compound obtained from the Liquid Chromatography Mass Spectrometry (LCMS) test results. The docking method used can be said to be good or valid if the RMSD

value is less than two (RMSD<2), The ligands used include: 20-hydroxyecdysone (20-HE); Ecdysone; Ponasterone A; Makisterone A and Muristerone A, while the receptor used is EcR-RXR (Ecdysteroids Receptor - Retinoid X Receptor).

Table 2. LCMS Results from Portulaca Oleracea

Ligand	Target protein	Binding Afinity (kkal/mol)
Ecdysone	EcR-RXR	-6.80
20-hydroxyecdysone	EcR-RXR	-6.60
Makisterone A	EcR-RXR	-6.60
Muristerone	EcR-RXR	-6.10
Ponasterone A	EcR-RXR	-6

According to Friedman (2022), and Chatterjee et al. (2023), binding affinity is the ability of a compound to bind to the receptor, the smaller the value, the higher the affinity between the receptor and the ligand and vice versa. From the table above, it can be concluded that the best binding affinity value of the five compounds is the ecdysone compound of - 6.80 and the lowest is Ponasterone A of -6.0. As for the compound 20-hydroxyecdysone (20-HE), it gets a greater value than Ponasterone A and Muristerone A, which is -6.6. This also applies to Makisterone A because it has the same binding affinity value. Based on the research of Knigge et al. (2021), stated that ecdysone compounds synthesized by the Y organ are released into the hemolymph and then converted into 20-HE to bind to the EcR-RXR heterodimer complex. The RMSD value of the five ligands has the same result of 0, so it can be concluded that the docking method performed is valid (because it is less than 2) so that it can be used for docking 20-hydroxyecdysone (20-HE) compounds.

Effect of Phytoecdysterone Hormone on Mangrove Crab Molting Percentage

An image of a molting crab is presented in Figure 2. Based on the results of the analysis of variance showed that the dose of extract treatment had a significant effect ($p<0.05$) on the percentage of mangrove crab molting, while the results between groups did not significantly affect the percentage of mangrove crab molting.

The results of the DMRT further test found that treatments K, A and D were not significantly different in their effect on the percentage of molting, but they were different from treatments B and C (Figure 3). The two treatments were not significantly different in their effect on the percentage of molting of mangrove crabs. Both treatments are not significantly different in their effect on the percentage of mangrove crab molting, so the best treatment on the parameter of molting percentage is treatment B.

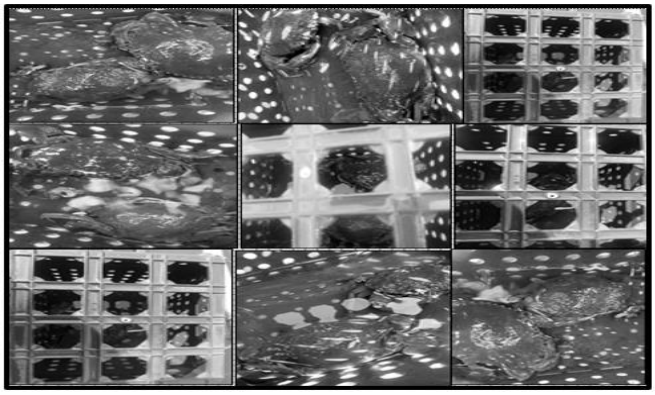

Figure 2. Mangrove crab molting in each group

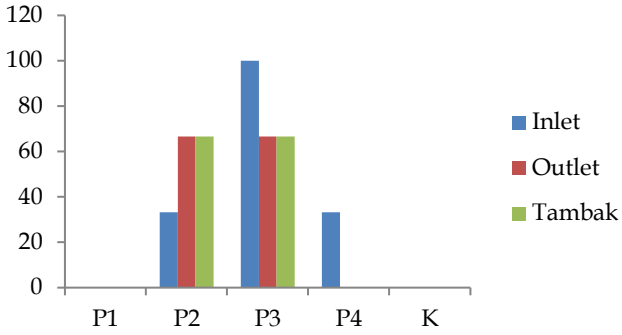

Figure 3. Percentage of molting in mangrove crabs after injection phytoecdisteroids hormone from purslane leaf extract

Effect of Phytoecdysterone Hormone on the Molting Period of Mangrove Crabs

The results of the analysis of variance showed that the molting period between groups was not significantly different while each treatment had significantly different results. This indicates that the dose of phytoecdisteroids hormone significantly ($p<0.05$) affects the molting period of mud crabs. The shortest molting period was obtained in the 24 µg/head dose treatment and was significantly different from the other treatments. The number of days required by the crabs to molt after the injection treatment of phytoecdisteroids hormone from purslane leaf extract is presented in Figure 4.

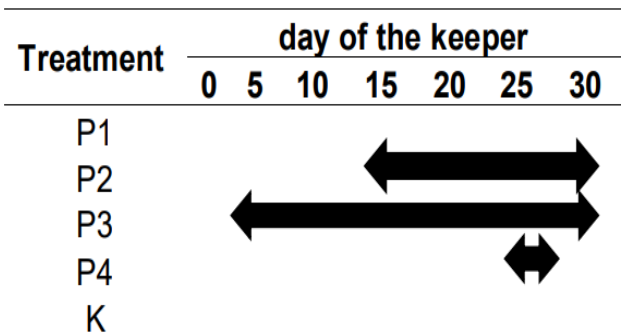

Figure 4. Molting period in mangrove crabs post injection phytoecdysteroid hormone from purslane leaf extract

Effect of Phytoecdysterone Hormone on Mangrove Crab Survival Rate

The results of the analysis of variance showed that the purslane leaf extract injection treatment significantly affected the survival rate of mud crabs, while the results between groups did not significantly affect the survival rate of mud crabs. Based on the DMRT further test, it was found that treatments D, B and C were not significantly different in their effect on the survival rate of mangrove crabs (Figure 5).

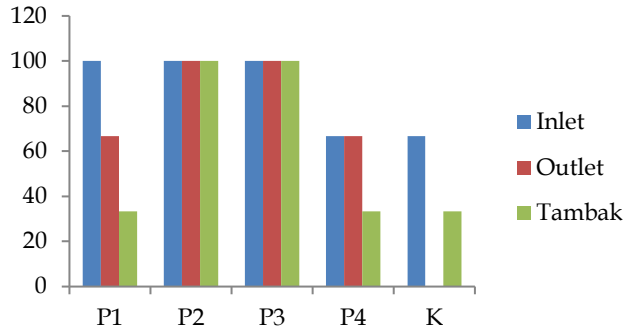

Figure 5. Survival rate of mangrove crab after phytoecdysteroid hormone injection from purslane leaf extract

Effect of Phytoecdysterone Hormone on Carapace Width and Body Weight Gain of Mangrove Crab

Based on the results of the analysis of variance, the injection of purslane leaf extract has a significant effect on the increase in carapace width and body weight of mangrove crabs. The results of the DMRT further test found that treatments K and A were not significantly different in their effect on the increase in carapace width and body weight, but were different from treatments B, C and D. The three treatments were not significantly different in their effect on the increase in carapace width and body weight of mangrove crabs. The highest increase in body weight and carapace width occurred in treatment C. Mangrove crab weight gain after molting ranged from 45 - 55gram or 64.30 - 71.42% (Figure 6), while carapace width gain ranged from 8 - 15mm or 13 - 17% (Figure 7).

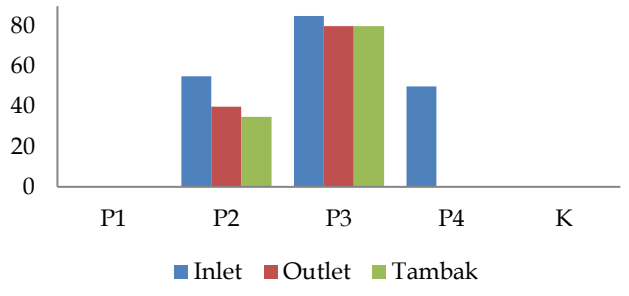

Figure 6. Weight gain of mud crab post injection

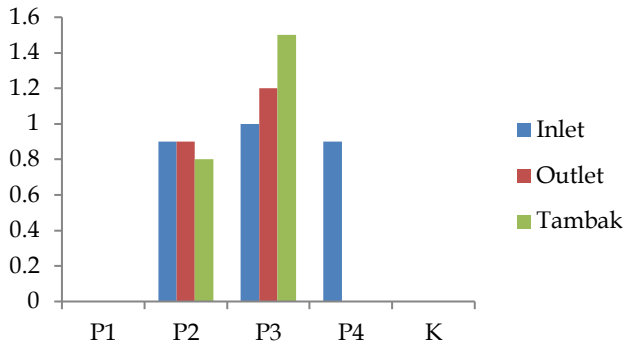

Figure 7. Post injection mud crab carapace width gain

Water Quality Parameters

The water quality parameters observed in this study are thought to greatly affect the concentration of hemolymph ecdysteroids and the molting activity of mud crabs (Table 3).

Table 3. Results of Water Quality Measurements during the Study

Parameters	Value Range	Literature
Temperature	26.40-33.80^{0}C	25-35^{0}C
pH	6.83-7.20^{0}C	6.80-8.20^{0}C
Salinity	20.70 – 26.70 ppt	15 – 30 ppt
DO	4 – 4.52 ppm	>5 ppm

Discussion

The highest percentage of molting occurred in the treatment of hormone dose of 24 µg/bb (treatment C) at 77.70% and not significantly different from the dose of 18 µg/bb (treatment B) at 55.50% followed by a dose of 30 µg/bb (treatment D) at 11.10%, while the treatment with the lowest results was treatment A and control, each at 0% or no molting occurred. The addition of phytoecdysteroid hormones from purslane leaf extract resulted in an increase in the content of ecdysteroids in the crab hemolymph. However, the increase in ecdysteroid content is not necessarily followed by an increase in the percentage of molting in crabs. This can be proven that the results of the percentage of molting in treatment D are lower than treatment C, a similar thing also occurred in the research of Liu et al. (2022), the addition of exogenous hormone 20-hydroxyecdysone (20-HE) which is getting higher can cause a negative feedback effect on the work of ecdysteroids in crab hemolymph to stimulate molting activity.

The negative feedback response carried out by the crab is to inactivate existing hormones and receptor performance decreases (Knigge et al., 2021). According to Gajula et al. (2023), inactivation can be done through the conversion of metabolites into more polar ones or making conjugate formations. In addition, a decrease in receptor performance will result in inhibition of protein

formation so that the process of growth and molting in crabs will also be inhibited. The shortest molting period occurred in treatment C, where molting occurred on days 4 to 29 post injection, followed by treatment B on days 13 to 29 post injection, followed by treatment D with a span of days 26 post injection. The longest molting period was in treatment A and control, which was more than 30 days. On the 17th day after injection, treatment C experienced simultaneous molting in each group. According to Indarjo et al. (2020), Fazhan et al. (2022), and Widigdo et al. (2017), stated that the length of time required by mangrove crabs is influenced by several factors including: size (weight), sex, species and hormones.

As with the results of the percentage of molting, the molting period parameter requires the appropriate dose of hormones to produce the shortest time. The highest survival rate results were found in treatment C and treatment B at 100%, while the lowest results were found in the control treatment which amounted to 33.30%. The survival rate in treatments A and D were 66.60% and 55.50%, respectively. The addition of purslane leaf extract in all treatments has better results than the control treatment, this can occur due to the function of the 20-hydroxyecdyson compound contained in purslane extract. Ecdisteroid compounds can increase body resistance so as to reduce stress and increase energy in the crab body (Lafont et al., 2021). Factors that affect the survival rate of crabs are external and internal factors (Eddiwan et al., 2021).

Internal factors themselves come from the test crabs, where the test crabs are obtained from the catch of fishermen whose places of origin are different, while external factors come from the environment such as water quality. Based on the results of the study, there were fluctuations in salinity and lack of dissolved oxygen levels in the maintenance waters. According to Li et al. (2024), suggesting that fluctuating conditions in the water will cause decreased appetite in crabs so that it will interfere with growth and even more extreme, namely death. The highest body weight gain and carapace width occurred in treatment C (Figure 8). Mangrove crab weight gain after molting showed differences with the results of previous studies using different natural extracts. Research by (Fujaya et al., 2020) which used mulberry leaf extract (Morus alba sp.) experienced growth of 25.44 grams, research by (Chen et al., 2022), using graying fern extract (Nephrolepis biserrata), obtained the highest growth of 33.75 grams.

Water quality parameters in this study are still in the optimum range. According to Addisie (2022), crabs need an optimum temperature range to grow around 25-35^{o}C. Temperatures that are too low result in decreased crab activity so that the crab's appetite level becomes low. Crabs will experience slow growth if their appetite level decreases (Syeed et al., 2023). According to Boscolo-Galazzo et al. (2018), Srijaya et al. (2014), and Thirukanthan et al. (2023), the higher the temperature causes the process of metabolism and decomposition to increase. The increase in these two things results in higher oxygen consumption by mangrove crabs. According to Susanto et al. (2015), stated that the optimum salinity for mangrove crab cultivation in ponds ranges from 15-30ppt. High and low salinity affects the level of osmoregulation, resulting in the utilization of feed energy for crab growth can be disrupted.

Figure 8. Mangrove crab body weight after molting

High salinity causes the crabs to become stressed. According to Mégevand et al. (2022), Ortega et al. (2022) mangrove crabs that experience stress in the body have a process of reallocation of metabolic energy from growth and reproduction activities to activities to improve homeostasis such as respiration, movement, hydro-mineral regulation and tissue repair. The results obtained during the study are still within the optimum range for mangrove crab cultivation in accordance with the research of Hastuti et al. (2019) the optimal value of acidity for mangrove crab rearing is 6.80-8.20. Factors that cause fluctuations in acidity are residual artificial feed, density and plankton population (Ortiz et al., 2024). This was also added by Ceyda-Irkin (2022), the value of acidity is considered important because it affects the process and speed of biochemical reactions in the crab's body. Based on research by Rinaldy et al. (2023), the DO value for mangrove crab needs is >4 ppm. Oxygen levels below 3 ppm will cause molting activity in crabs to be slow and if oxygen levels fall below 2 ppm, mangrove crabs do not perform molting activities (Karniati et al., 2021).

Conclusion

The content of 20-HE compounds from purslane leaf extract measured using High Performance Liquid Chromatography is known to be 0.5 ppm. Purslane leaf extract injection applied to mud crabs can increase the percentage of molting, survival rate, growth in length and body weight and accelerate the latent molting period of mud crabs. The dose that has the best results from several research parameters is treatment C, which is injecting purslane leaf extract at 24 µg/head, with the following details: The highest percentage of molting was 77.70%, the shortest molting period was day 4 to 29, the highest survival rate was 100%, while for growth there was no significant difference from each treatment

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Author Contributions
Conceptualization; G. A. I.; methodology.; M. S. W.; validation; A. R. F.; formal analysis; A. F. P.; investigation.; G. A. I; resources; M. S. W.; data curation: A. R. F.; writing—original draft preparation. A. F. P.; writing—review and editing: G. A. I.; visualization: M. S. W. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest
The authors declare no conflict of interest.

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