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Potential extracts of wedge sea hare (*Dolabella auricularia*) as immunostimulators in comet fish (*Carassius auratus auratus*) infected by *Aeromonas hydrophila*

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Abstract. *Nurhalisa N, Nur I, Suryani S. 2022. Potential extracts of wedge sea hare (*Dolabella auricularia*) as immunostimulators in comet fish (*Carassius auratus auratus*) infected by* Aeromonas hydrophila*. Biodiversitas 23: 1884-1893.* Microencapsulation is an innovative method to protect the active compound of pharmaceutical material from environmental impacts or unwanted conditions. This study aims to determine the active compound content and appropriate concentration of wedge sea hare extract (*Dolabella auricularia*) using the microencapsulation method to treat *Aeromonas hydrophila* bacterial which infected in comet fish (*Carassius auratus auratus*). The treatment consisted of three concentrations of *D. auricularia* extract (6.3, 7 and 7.7 g/kg feed) and commercial feed as a positive control in triplicates. *C. a. auratus* fed with experimental diets twice a day (8.00 a.m and 4.00 p.m) for 30 days of culture period before being challenged with an injection of *A. hydrophila*. Blood profile, weight gain, survival and relative survival of *C. a. auratus* were observed before and after the challenge test. The characteristics and types of active compounds in the extract were observed qualitatively, as well as the morphology and particle size distribution of the microcapsules. The results showed that *D. auricularia* extract contained phenol, tannins, steroids, terpenoids, alkaloids, and saponins. The microcapsules in all treatments were a spherical shape. The average diameter was similar to all extract treatments. The addition of *D. auricularia* extract microcapsules in the feed produced significantly different results on the blood profile, weight gain and survival. The highest of total leukocytes, total erythrocytes, hematocrit and blood hemoglobin were obtained in addition to 7.7 g *D. auricularia* extract/kg of feed with respective values of 7.35±0.94×104 cell.mm-3, 2.83±0.03×106 cell.mm-3, 22.43±0.03% and 6.72±0.02 g.dL-1, respectively. The highest weight gain was found in fish-fed diets contained of 7.7 g of *D. auricularia* extract (3.37 g). The highest relative survival and survival were found in the treatment of 7 and 7.7 g extract/kg of feed, which was 100%. This study concluded that microencapsulated with *D. auricularia* extract at dose 7 g extract/kg of feed was the optimum dose for *C. a. auratus* infected with *A. hydrophila*.

Keywords: Immunostimulant, microcapsule, wedge sea hare

INTRODUCTION

One of the diseases that may infect freshwater fish is Motile Aeromonas Septicemia (MAS), caused by *Aeromonas hydrophila* bacteria (Nasrullah et al. 2020). Bacterial infection by *A. hydrophila* causes 80% of the deaths of cultured fish at various stages. The disease is characterized by the presence of gill and fin lesions, as well as skin ulceration (Pękala-Safińska 2018). At a more severe level of infection, it causes flatulence, ulcers, and hemorrhagic (Kartikaningsih et al. 2020). Internally, accumulation of ascitic fluid in the fish and it can damage to fish organs, especially the kidneys and liver, can be observed (Baumgartner et al. 2017). Histopathologically, MAS is characterized by the appearance of necrosis in the spleen and kidney (Rozi et al. 2018).

In some ornamental fish, it has been found that the bacteria *Aeromonas* spp. could be resistant to various types of antibiotics (Saengsitthisak et al. 2020). Therefore, a disease control strategy is thus needed to achieve a cultivation system that is safe, environmentally friendly, and will not prompt any bacteria-resistant effects. Such strategy can be enforced through the use of marine biological material compounds mostly found in marine invertebrate groups such as sponges, tunicates, bryozoans, soft corals and mollusks (Proksch et al. 2002). For example, *Holothuria* sp. is a sea cucumber that possesses an active compound (Azlan et al. 2021) known to have antibacterial (Sulardiono et al. 2020), antifungal (Mohammadizadeh et al. 2013), and antioxidant activities (Nobsathian et al. 2017). Administration of *H. scabra* ethanol extract in fish feed can induce potential immunostimulant effect in the prevention of *A. hydrophila* infection.

Similarly, gastropods are a category of marine biota that can be used as an antibacterial as it produces secondary metabolites. Bioactive compounds produced by gastropods are candidates for new drug sources (Fisch et al. 2017). One kind of gastropod that has yet been widely known for its potential in inhibiting bacterial growth is the wedge sea hare (*Dolabella auricularia*). It is usually found in shrimp ponds as a pest for shrimp culture (Sofian et al. 2021). However, it has potential for biomedical and economic benefits. As reported in Gao et al. (2021), an active

component of mollusk *D. auricularia*, well-known name as Dolastin 10 was found to have antimitotic activity for cancer and tumor cells.

Extracts of biomedicals can be administered into feed in various ways, such as spraying (Hussain et al. 2017) and the extract is directly formulated into feed ingredients (das Neves et al. 2022). However, high temperatures and mechanical stress, which are used in the processing of formulated feed, cause the deterioration of bioactive compounds (De Cruz et al. 2015). In such cases, the active compounds of the extract will not accomplish their optimal benefits towards the target organs. It follows that a technique is needed to maintain the active compound of the *D. auricularia* extract from environmental influences or unwanted conditions.

To address the challenges in administrating the extract, the technology of microencapsulation can be used. Microencapsulation is a material coating technique whereby material can thus be protected from unfavorable environmental influences such as heat and chemicals (Victor and Heldman 2001). Microencapsulation technique has been used in pharmaceuticals for humans and can help mask bitter tastes, protect drugs from environmental conditions (moisture, light, heat, and oxidation), prevent gastric irritation, and prolong drug release time. This method is advantageous in that it can protect the encapsulated molecules and maintain their activity during encapsulation (Mardikasari 2020).

Microcapsules application in aquaculture has been ventured in the synbiotic administration in shrimp feed (Yunarty et al. 2016). Additionally, microencapsulation of crude garlic extract as a feed substitute was found to have demonstrated an effect on the growth, proximate composition, immunological and antioxidant status of rainbow trout (*Oncorhynchus mykiss*) juveniles (Adineh et al. 2020). If the right dose is given, the immunostimulatory compounds contained in the microcapsules can work optimally to increase the body's immune system (Rhamadhani et al. 2015).

Accordingly, this study aims to determine the active compounds contained in *D. auricularia* extract as well as determine the best potency and dose to be used in the form of microencapsulated extract in feedthrough blood profile, growth, the survival rate of comet fish (*Carassius auratus auratus*) infected with *A. hydrophila* bacteria. The preparation of *D. auricularia* bioactive through the microcapsule method is expected to be effective in controlling fish disease and subsequently will support aquaculture health management.

MATERIALS AND METHODS

Location of study

Dolabella auricularia organisms were collected in three marine water locations (Toronipa, Tapulaga, and Tanjung Tiram Village) in Southeast Sulawesi, Indonesia. The extraction process, phytochemical screening, extract microencapsulation, bacterial infection, and fish rearing were carried out in three laboratories, each with different research activities, namely the Laboratory of Analytical Chemistry Unit, Laboratory of Pharmacy, and Laboratory of Aquaculture, Halu Oleo University, Kendari, Indonesia.

Sampling

Sampling of *D. auricularia* was carried out by hand in habitats with small currents, seagrass, sand, and mud at low tide. Sampling was carried out at night because the animal is nocturnal (Sofian et al. 2021).

Extract preparation

The solvent used in the extraction of the *D. auricularia* was ethyl acetate as it can extricate various important compounds such as tannins, saponins, and steroids. The ratio of the material and solvent used was 1:2. Samples of wet *D. auricularia* meat and offal were cut into small pieces and then mashed. The sample was macerated and the extracted solution obtained was filtered to separate the filtrate and residue. The filtrate was evaporated with a rotary evaporator at a temperature of 40°C and calculated for the yield obtained.

Qualitatively screening for active compounds

The qualitative screening for the compounds in the extract was carried out by the colorimetric method to detect the presence of secondary metabolites. The screening entailed the standard method of identification for phenols, tannins, flavonoids, steroids, terpenoids, alkaloids and saponins (Yadav and Agarwala 2011).

Microcapsule preparation

Three microcapsules formulas of the *D. auricularia* extract at different concentrations were prepared. The microcapsules were produced by dissolving the extract at different concentrations in a 1% NaOH solution. In a different container, a solution was made by dissolving 1% sodium alginate that was mixed with the extract until homogeneous using a magnetic stirrer. The mixture was taken using a 22 G syringe and dropped into a 2% $CaCl_2$ solution to form the microcapsules. The obtained microcapsules were left submerged in $CaCl_2$ solution for ±10 minutes and then filtered using filter paper. The microcapsules were then dried using an oven at a temperature of 30-40°C.

Figure 1. The morphology of wedge sea hare (*Dolabella auricularia*). Bar: 2 cm

Microcapsule morphological examination

The formed microcapsule morphology was observed using a microscope and the sample was measured for its size.

Preparation of test feed

The test feed used was commercial pellet feed with a protein content of 22%. The feed was mixed with the microencapsulated extract using egg white, as much as 2% of the weight of the commercial feed used, as a binder.

The test feed during the experimental period was given to the test fish at a feeding frequency of 2 times a day and as much as 5% of the fish body biomass.

Fish testing and maintenance

A total of 108 *C. a. auratus* with lengths of 8-15 cm and an average weight of 14.09 ± 0.96 g were kept separately in 12 unit aquariums with a size of 35 x 35 x 40 cm. The fish had previously been acclimatized for 1 week in a fiber tank with a diameter of 150 cm and a height of 80 cm. Tank was filled with tap water half of its volume.

As the best dose obtained from the in vitro test in the previous study was equivalent to 7 g/kg (as yet unpublished), this study was structured with a completely randomized design of 4 treatments and 3 replications, namely: fish group given commercial feed without *D. auricularia* extract (control), the fish group given 6.3 g extract/kg feed (Treatment B), the fish group given 7 g extract/kg feed (Treatment C), and fish group given 7.7 g extract/kg feed (Treatment D). All treatments, including control, were challenged with 0.1 mL bacteria with a concentration of 107 CFU/mL based on the lethal dose 50 (LD50) determination.

Blood sampling

Blood sampling was carried out on the 30th day of treatment and after the bacteria test period (37th day). The process entailed the use of a syringe rinsed with a 3.8% Na-citrate anti-coagulant. Fish blood was taken from the caudal vein and then the blood was put into the blood collection box for observation.

Parameters observed

The *D. auricularia* extract yield and qualitative identification of active compounds were characterized. In addition, a blood profile was observed for the total leukocytes, erythrocytes, hematocrit, and hemoglobin. Likewise, weight gain and relative survival were also observed.

Extract yield was calculated using the following formula below. Screening for the compounds contained was aimed to identify secondary metabolites.

$$Yield\ (\%) = \frac{Extract\ mass}{Starting\ material\ mass} \times 100$$

Blood samples were taken three times, namely on day 0 (before treatment), 31 (after treatment), and 37 (after bacterial test or infection). Fish blood sampling was carried out for each treatment group by taking 3 test animals (replication) from each aquarium.

The calculations of total leukocytes and total erythrocytes were carried out according to Blaxhall and Daisley (1973), while the calculation of hematocrit level followed Anderson and Siwicki (1993) and the hemoglobin level followed Vutukuru (2005). Measurement of the hematocrit level was done by comparing the volume of blood cells with the total volume of blood after being centrifuged, expressed in percent. The calculation is as follows:

$$Hematocrit\ (\%) = \frac{T}{t}\ x\ 100\ \%$$

Where, T is the height of the tube containing the red blood cells, t is the height of the tube containing the whole blood.

The weight gain of fish was be calculated using the following formula:

$G = W_t$ (final mass) – W_0 (initial mass)

Relative percent survival (RPS) was calculated using the formula from Amend (1981) as follows:

RPS (%) = 1- (Number of dead fish in the treatment group/number of dead fish in the control group) x 100%.

Observation of clinical symptoms was carried out after the bacterial test through observations of physical changes (external) and changes in behavior. The physical changes discerned consist of red spots, peeling scales, damaged fins, protruding eyes (exophthalmia) and stomach bulging (dropsy), whereas the behavioral changes consist of response to feeding, response to swimming motion, and response to shock or reflex.

Data analysis

Blood profile data (total leukocytes, total erythrocytes, hematocrit and hemoglobin levels), weight gain, and relative percent survival of the *C. a. auratus* were tested with One Way ANOVA at 95% confidence level to determine the effect of treatment between groups. Before carrying out the ANOVA test, the data must first meet the requirement of the normality and homogeneity assumption, then they were checked by Kolmogorov-Smirnov test. Whether the result of the ANOVA test showed that there was a significant difference between treatments, then it was continued with the Duncan's Multiple Range Test (DMRT) test to find differences between treatments and single out the best treatment. Statistical analysis was performed with SPSS version 23.0.

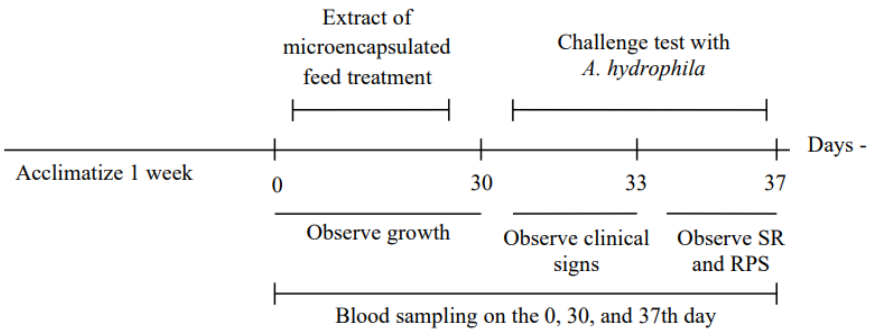

Figure 2. Timeline of research procedure

RESULTS AND DISCUSSION

Extract characteristics

Yield and compound contents of Dolabella auricularia *extract*

The yield from the ethyl acetate *D. auricularia* extract obtained was 0.32%. The qualitative test for the compound content of the *D. auricularia* extract showed that the *D. auricularia* extract contains phenolic compounds, tannins, steroids, terpenoids, alkaloids, and saponins.

Extract microcapsules and pellets characteristics

The *D. auricularia* extract microcapsules had granule form with a microcapsule particle size of ±1.5 mm. Observation of the morphology of the *D. auricularia* extract microcapsule particles revealed a spherical shape. Additionally, no apparent difference was observed in the average diameter of the extract microcapsules between treatments. The microcapsules in all treatments were a spherical shape. The average diameter was similar to all extract treatments, 36.71, 36.57 and 36.5 µm, respectively.

Blood profile

Total leukocytes

On the 30 days of fish rearing after the administration of microencapsulation, the total number of leukocytes of *C. a. auratus* had shown an increase (Table 1). Also, when the fish infected with *A. hydrophila*, the total number of leukocyte increases was also observed.

Total erythrocytes

Similarly, with total leukocyte, the highest of total erythrocyte was observed in the fish fed with 7.7 g extract/kg feed. Data on total erythrocytes in *C. a. auratus* presented in Table 2 show that the average number of erythrocytes, after the administration of microcapsules extracts for 30 days, had shown an increase. This was as well the case even after being infected with *A. hydrophila* bacteria.

Hematocrit level

Similar to two data above, the *C. a. auratus* fed with a diet contained of 7.7 g extract *D. auricularia* /kg feed had the highest of hematocrit level compared to other treatments. Data shown in Table 3 suggest that the average hematocrit level, after the administration of microcapsules extracts for 30 days, had increased. Despite being the *C. a. auratus* infected with *A. hydrophila* bacteria increased level was still observed.

Hemoglobin

The fish fed with feed containing of 7.7 g extract/kg feed had the highest of hemoglobin content compared to other treatments. Data in Table 4 shows that the average hemoglobin level, after the administration of microcapsules extracts for 30 days, had increased, and so was the case even being infected with *A. hydrophila* bacteria.

Weight gain

Data of weight gain of *C. a. auratus* presented in Figure 2 shows that the highest of weight gain was found in fish fed with the feed contained 7.7 g extract/kg feed, followed by the treatment of 7 g extract/kg feed, and lastly, 6.3 g extract/ kg feed. The lowest of weight gain was found in the control treatment group.

Relative Percent Survival (RPS)

The RPS data of *C. a. auratus* shown in Table 5 demonstrate that the highest of RPS values were found in treatments 7 and 7.7 g extract/kg feed. The lowest RPS value was found in the treatment of 6.3 g extract/kg feed.

Table 1. The total leukocytes in *Carassius auratus auratus* before, and after the administration of microcapsules *Dolabella auricularia* extract, and after injection with *Aeromonas hydrophila* bacteria

Day	Leukocytes (× 10^4 cell.mm^{-3})			
	Treatment of microcapsules (g extract/kg feed)			
	Control feed	6.3	7	7.7
0	4.75±1.06	4.75±1.06	4.75±1.06	4.75±1.06
30	2.13±0.23^a	4.76±0.15^b	5.05±0.16^b	6.33±0.99^c
37	1.66±0.34^a	4.03±0.47^b	7.03±0.74^c	7.35±0.94^c

Table 2. The total erythrocytes in *C. a. auratus* before, and after the administration of microcapsules *D. auricularia* extract, and after injection with *A. hydrophila* bacteria

Day	Erythrocytes (× 10^6 cell.mm^{-3})			
	Treatment of microcapsules (g extract/kg feed)			
	Control	6.3	7	7.7
0	1.79±0.62	1.79±0.62	1.79±0.62	1.79±0.62
30	1.12±0.12[a]	1.79±0.13[b]	2.31±0.05[c]	2.67±0.002[d]
37	0.99±0.13[a]	1.48±0.24[b]	2.44±0.04[c]	2.83±0.03[d]

Table 3. The hematocrit level in *C. a. auratus* before, and after the administration of microcapsules *D. auricularia* extract, and after injection with *A. hydrophila* bacteria

Day	Hematocrit (%)			
	Treatment of microcapsules (g extract/kg feed)			
	Control	6.3	7	7.7
0	11.77±1.59	11.77±1.59	11.77±1.59	11.77±1.59
30	1.12±0.12[a]	9.29±0.26[a]	15.30±0.74[c]	16.12±0.64[c]
37	0.99±0.13[a]	10.46±1.22[b]	16.07±0.04[c]	22.43±0.03[d]

Table 4. The hemoglobin level in *C. a. auratus* before, and after the administration of microcapsules *D. auricularia* extract, and after injection with *A. hydrophila* bacteria

Day	Hemoglobin (g.dL^{-1})			
	Treatment of microcapsules (g extract/kg feed)			
	Control	6.3	7	7.7
0	3.27±0.63	3.27±0.63	3.27±0.63	3.27±0.63
30	2.51±0.10[a]	3.73±0.76[b]	5.69±0.04[c]	5.98±0.04[c]
37	2.22±0.15[a]	3.60±0.44[b]	6.64±0.04[c]	6.72±0.02[c]

Table 5. The mortality and relative percent survival of fish after conducting the challenge test with *A. hydrophila* bacteria

Parameters	Treatment of microcapsules (g extract/kg feed)			
	Control	6.3	7	7.7
Mortality (%)	29.63	22.22	0	0
RPS (%)	-	25.01	100.00	100.00

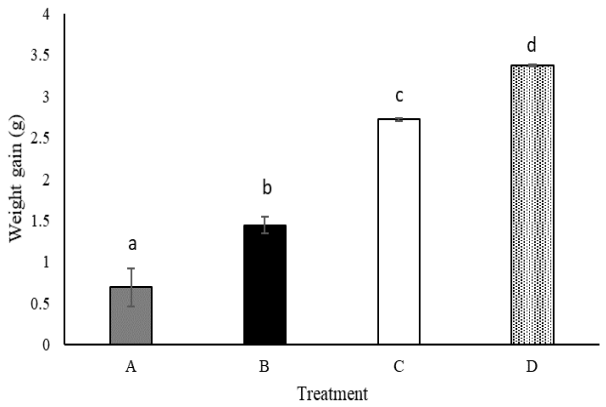

Figure 3. The weight gain of *Carassius auratus auratus* during research. Treatment A (control); B (6.3 g extract/kg feed); C (7 g extract/kg feed); Treatment D (7.7 g extract/kg feed)

Discussion

The selection of gastropod molluscs, especially the *D. auricularia* species as an immunostimulant in fish with consideration of its benefits, namely the content of active ingredients, its amount which is very abundant in nature so that it is easy to be obtained, these do not compete with human needs because these organisms are included in the organism which has not been utilized by humans, they are safe to use, and have no economic value. This is in line with the research of Ashari et al. (2021) and Azlan et al. (2021) that in some marine biota, for example, the sea cucumber, *Bohadschia* sp. and *H. scabra* are good to be used for aquaculture because they have the active compounds containing certain doses, they are not toxic so that they are safe to be used. The parameters of blood profile and fish survival after challenge with bacteria, it was shown that immunostimulation with extracts in the form of microencapsulated is possible to be applied in the control of bacterial diseases.

To ensure the active compound does not change due to the extraction process so that it is optimal in its use as an immunostimulant, in the preparation step, it needs to be considered carefully. In this study, the whole body of the sea hare organism was used without removing the viscera. In addition, the preparation process was not done by heating or drying process, but maceration was done in the wet condition of organisms. This phenomenon was explained by Çoklar and Akbulut (2017) that the phenol compound obtained was obtained at a low level in the drying method under the sunshine. Then, it was also explained by Luximon-Ramma et al. (2002) that phenol compound had properties which that and it was sensitive to heat treatment. Furthermore, it was also explained by Manulang (2016) that in the selection of maceration method, it needs to be considered that the effectiveness of the dissolved substance in the sample from the influence of heat during the maceration process. Likewise, the type of solvent in the maceration process will determine the yield of the extract. Wang et al. (2011) explained that the different types of solvent and temperature influenced the amount of extract produced. The total amount of ethyl acetate solvent obtained in this analysis was smaller than that of methanol solvent. Each solvent has a different level of polarity to attract bioactive compounds, hydrogen bonds also influence the types of compounds that could be dissolved. The bioactive compounds could dissolve in solvents with the same polarity.

In this research, the results of the compound content of sea hare extract qualitatively using ethyl acetate solvent were detected containing some compounds: steroid, terpenoids, alkaloids, tannins, phenolics and saponins. For comparison, the qualitative analysis result of the bioactive compound on mollusk *Discodoris* sp, the extract was alkaloid, steroid, saponin, and flavonoid. The content was found very diverse depending on the type of solvent and the body part of the biota, meat or viscera exact (Nurjanah et al. 2012).

Ethyl acetate is a semi-polar solvent so it can attract polar and semi-polar compounds. Polar compounds are alkaloids, tannins, phenolics, and saponins, while steroids

are non-polar to semi-polar and terpenoids are semi-polar. Alkaloids, saponins, and tannins can be attracted by the ethyl acetate fraction because of the presence of resonant electrons in the benzene ring, which causes the decrease of the polarity of these compounds so that they are more attracted with semi-polar ethyl acetate.

Steroids can detain the secretion of pro-inflammatory cytokines, including IL-12 p40, IL-6, and TNF-α and can prevent and treat inflammatory diseases (Thao et al. 2013). Furthermore, in vivo test, which was done in goldfish, it showed that the active ingredients in sea cucumbers caused a better blood profile and also, they were potential as an immunostimulant (Azlan et al. 2021).

Terpenoid has function as antibacterial. According to Lu et al. (2012) that terpenoid, which is from the rhizome genus Curcuma has anticancer activity, in terms of in vitro, all compounds of curcumin show anti-inflammatory and antioxidant properties (Pintatum et al. 2020). Saxena et al. (2013) stated that terpenoid, which is from various plants, has antiulcer and antimicrobial properties.

Alkaloid compound is an organic substance that is not vital for the organisms which produce them, but it is possible that this alkaloid component comes from the food consumed. Although alkaloids are the most abundant substance in higher plants, they are also secondary metabolites produced by a large variety of organisms. Both from the *Litsea cubeba* plant and the mollusk sea slug (*Discodoris* sp.), the extract of alkaloid compounds has been proven to have antioxidant activity because they can efficiently stop the free radical chain reaction (Nurjanah et al. 2012).

Tannins are high molecular weight phenolic compounds that are commonly found both in terrestrial plants and sea plants (Cuong et al. 2019). Most likely why tannin compounds are also obtained from sea hare extract is because it comes from its food. This study used all parts of the body of the sea hare, including the viscera. Tannin can be used as a natural substance that functions as antimicrobials (Fiori et al. 2013) because it can detain metabolic processes in microbes. Tannin from the extract of *Centella asiatica* plants has been proven to overcome the problem of bacterial pathogens in aquaculture (Rukisah et al. 2019). Phenolic is also a compound that functions as an antioxidant. This is in accordance with Nobsathian et al. (2017) that the phenol component has functioned as an antioxidant by reducing free radicals. Saponin has functions as an antioxidant and antibacterial. This is in accordance with research done by Akinpelu et al. (2014), which states that saponins can prevent damage caused by free radicals and infection caused by pathogenic bacteria.

Different extraction results may be obtained from the same organism, this is predicted due to differences in the environmental conditions that have an impact on the type and amount of secondary metabolites contained in a material (Iswantini et al. 2011). This is in accordance with the statement of Neugart et al. (2018) that environmental and agronomic variables influence secondary plant metabolites. Wang et al. (2011) mentioned that different types and levels of solvent polarity could produce different yields of extracts and bioactive components. Sompong et al. (2011) stated that different species and growing places produce different bioactive components.

The reasons for using the microencapsulation technique are to increase stability, reduce side effects and toxic effects of drugs or to protect the digestive tract, especially the stomach, from irritation caused by the active ingredients of the drug, and control the time of drug release (Poshadri and Kuna 2010). The making process of microcapsules can be done with different materials and methods. The main component in the microencapsulation process is a polymer. Natrium alginate is a polymer that the ability to protect the active components from environmental factors that affect stability (Mardikasari 2020). Natrium alginate was chosen as a polymer which commonly used in the ionic glass method, it is because alginate can produce a good shape (Lee and Mooney 2012). Natrium alginate has the ability to form water-insoluble gels with the presence of divalent cation, which was the basis for the use of natrium alginate in the drug coating process (Manz et al. 2003). The polymers which used in the ionic glass method besides alginate were chitosan and carrageenan (Liouni et al. 2008). The process of making microencapsulation with the ionic glass method using cross-link Ca and alginate can also reduce the evaporation rate of the oil (Soliman et al. 2013) and increase the bioavailability of plant extract (Suryani et al. 2016). Sea hare extracts with different concentrations and used natrium alginate polymer with the same concentration in each treatment were done in the preparation of microcapsules in this study. According to Mardikasari et al. (2020), these microcapsules granules can be formed due to a cross-linking reaction between the polymer of natrium alginate polymer and $CaCl_2$ solution, which has a role as a cross-linking agent. Cross-linking occurs when natrium alginate droplets are dropped into the $CaCl_2$ medium. When natrium alginate is dropped into a solution containing calcium ions, calcium ions will displace natrium ions in the polymer to form a three-dimensional gel tissue and it is described as an "egg-box" model.

Methods in making microcapsules include the use of ionic glass, air suspension, separation of co-conservation phase, spray drying and freezing, coating in a pan, multi-hole centrifugal process, and method of solvent evaporation. The choice of ionic glass method was done based on the fact that this method was relatively simple and could avoid harmful organic solvent. Therefore, this method has the ability to protect the encapsulated molecule and maintain its activity during encapsulation which was its main advantage. The ionic glass method was in great demand because it had good biocompatibility properties, it was easy to apply, and did not require a large amount of organic solvent, so the cost was relatively cheap (Mardikasari et al. 2020).

The results of the study on total leucocytes can be seen in Table 1, in general, before the addition of the extract of microencapsulated sea hare to the feed, the total leukocyte in the *C. a. auratus* in all treatments were in the normal range. According to Setyaningsih et al. (2019), the normal number of leukocytes in *C. a. auratus* is around 1.8 × 104 cells.mm-3. However, when the fish fed with diet

contained the extract of *D. auricularia* in the feed, the total leukocyte of the fish comet was increased. This value indicated that the total leukocytes were in the normal range. The increase in the number of leukocytes was caused by the sea hare extract containing active compounds which were able to stimulate the immune system, antibacterial, anti-inflammatory, antiulcer and so on.

The observation results of total leukocytes on the 37th day or after the challenge test using *A. hydrophila* bacteria increased at the treatment dose of 7 g extract/kg feed and 7.7 g extract/kg feed, but it was different results with the lowest dose treatment. It was because of its relation to the concentration of the extract given, if the dose given were right at that concentration, then the immunostimulatory compound contained in the sea hare extract could work optimally to improve the immune system of fish, and as a result, *A. hydrophila* bacteria could not easily attack the immune system of fish. According to Azlan et al. (2021) that the increase in leukocytes after infection is an indication that the defense system of fish gives responses to pathogens that enter the body. Leukocytes are one of the blood components that have functioned as an innate defense that will localize and eliminate pathogens through the process of phagocytosis (Biller and Takahashi 2018). The stimulation of the immune system of fish and its ability to respond to bacterial infection is predicted because the sea hare extract contains phenolics, steroids, terpenoid, alkaloid, saponin, and tannin. Those compounds have a function as immunomodulators, antiviral, antimicrobial, antioxidant, and antibacterial besides the sea hare meat contains the essential amino acid, one of which is arginine 1.61%, which works to influence the function of T cells. According to Alexander and Supp (2014), arginine can influence the secretion of growth hormones in terms of accelerating wound healing. Lim et al. (2006) stated that hydrolyzed tannins could detain the occurrence of cell biosynthesis on the walls and membranes of microbial cells so that they can be used as antimicrobials. Nobsathian et al. (2017) explained that the phenol component has function as an antioxidant by reducing free radicals. Akinpelu et al. (2014) stated that saponin could prevent damage caused by free radicals and infection caused by pathogenic bacteria. Jantan et al. (2015) stated that phytochemicals such as lactones and alkaloids have a role in stimulating the immune system or as an immunomodulator.

Based on the data in Table 2, the total erythrocytes of *C. a. auratus* before administration of the extract of microencapsulated sea hare in feed showed that the total erythrocytes were in normal condition. This is in accordance with the research of Setyaningsih et al. (2019) that the number of erythrocytes in normal *C. a. auratus* ranges from 1.3 to 1.7×106 cells.mm^{-3}. The increase in total erythrocytes in *C. a. auratus* was predicted because of the content of calcium, potassium, magnesium, sodium, and phosphorus of the extract, where these minerals could influence blood pressure and the content of lysine which could strengthen the circulatory system. This is in accordance with a research of Manulang (2016) that chemical analysis of sea hare meat contained I-leucine 0.46% leucine 0.90%. The mineral content which was in sea hare meat (*D. auricularia*) contained some substances, like calcium (Ca) 68100 mg/kg, potassium (K) 1000 mg/kg, magnesium (mg) 7600 mg/kg, natrium (Na) 8200 mg/kg, and phosphorus (P) 1200 mg/kg. Then, it is also added by Ovie and Eze (2010) that lysine assists the absorption of calcium and mends the broken tissues. Lack of lysine can cause anemia. According to Behradmanesh and Nasri (2013) that there is a significant inverse relationship between serum calcium and diastolic blood pressure. The cooperation between magnesium and calcium is very useful in maintaining a normal heart rhythm by relaxing and contracting the heart muscle. Potassium and natrium are a pair of minerals that work together to maintain fluid, electrolyte, and acid-base so that these two minerals influence blood pressure regulation.

The observation results of total erythrocytes after the challenge test using bacteria *A. hydrophila* on the 37th day, the erythrocyte level increased in the treatment of 7 g extract/kg feed and 7.7 g extract/kg feed, while in the treatment 6.3 g extract/kg feed decreased. According to Azlan et al. (2021) that the increase in erythrocytes after infection shows that it is still in normal number, which indicates that the process of hematopoiesis still occurs in fish. According to Kumar et al. (2016) stated that *A. hydrophila* is responsible for the decrease in the number of erythrocytes. Harikrishnan et al. (2010) explained that *A. hydrophila* could cause mass destruction of red blood cells, which are known as septicemia.

The results of the study of the hematocrit level of *C. a. auratus* before giving the microcapsules of sea hare extract in the feed can be seen in Table 3. The value shows that *C. a. auratus* are in healthy condition. A high hematocrit level indicates the presence of contaminant and causes that fish to experience stress, it is different if the hematocrit level is low, it illustrates that fish lack vitamins, protein or fish are being infected, some fish that experience anemia have a high value of hematocrit as much as <10% (Witeska 2013; Destiani et al. 2019).

Hematocrit levels in *C. a. auratus* showed an increase on the 30th day or after administration of microcapsules sea hare extract in the feed. The highest hematocrit level was found in the treatment of 7.7 g extract/kg feed, which indicated that the fish did not get stressed. The results of the observation of hematocrit level on the 37th day or after the challenge test using *A. hydrophila* bacteria increased in treatments 7 and 7.7 g extract per kg of feed, while in the treatment of 6.3 g extract per kg of feed, it showed a decrease in hematocrit level when compared to the 30th day. The increase and decrease showed that *C. a. auratus* were still in good health. The hematocrit level in the treatment of 7.7 g extract per kg of feed on the 37th day was higher than the other treatments. A decrease in hematocrit level indicated that the level of erythrocyte also decreased and vice versa. This is in accordance with a research of Baba et al. (2016) that the administration of *Avena sativa* extract, which was a preventive effort in controlling *A. hydrophila* infection, showed a higher hematocrit than the control.

The results of research related to the hemoglobin level of *C. a. auratus* before microcapsules were given to sea

hare extract in feed (Table 4). After administration of microcapsules of wedge sea hare extract in feed, it showed that the hemoglobin level was in normal condition. This is in accordance with the statement of Destiani et al. (2019) that normal hemoglobin level was supported by high erythrocytes. This statement is also supported by Fazio et al. (2015), which stated that the increase of erythrocyte levels in the blood indicated that hemoglobin level increased and it also indicated that the stress in fish might be due to splenic contraction. Parrino et al. (2018) state that environmental stress impact on hemoglobin oxygen-binding properties. From the results of observation, the treatment of 7.7 g of extract per/feed was the best treatment compared to the other treatments. It could indicate that the utilization rate at the concentration, especially the doses given in the feed for *C. a. auratus*, was better than other doses.

The observation results of hemoglobin level on the 37th day or after the challenge test using *A. hydrophila* bacteria increased in both treatments, namely 7 g extract per/feed and 7.7 g extract per/feed. This condition showed that hemoglobin level was in the normal range, whereas in the treatment of 6.3 g extract per kg of feed, there was a decrease in hemoglobin level when compared to the 30th day. This value showed that the hemoglobin level was in an abnormal condition. Low hemoglobin level or outside the normal range indicated that the process of hematopoiesis in *C. a. auratus* started to be disrupted or hampered because of the interference after being tested by a challenge test with *A. hydrophila* bacteria.

The results of research in terms of the weight gain in *C. a. auratus* showed that the addition of microcapsules of sea hare extract in the feed gave a significantly different influence between treatments (Figure 3). Based on these data, it could be indicated that *C. a. auratus* were not in stressful conditions so that the energy for their growth could be used properly. It was supported by the statement of Bastien and Benjanim (2019) that stress was related to growth. Deane and Woo (2009) explained that stressed fish caused the growth of hormones to decrease.

Based on that issue, it was indicated that the addition of microcapsules of sea hare extract in the feed could increase the weight gain of *C. a. auratus*. The value of weight gain was influenced by the sea hare meat which contained 12.49% protein, essential amino acid, such as arginine 1.61%, leucine 0.90%, valine 0.54%, threonine 0.50%, I-leucine 0.46%, phenylalanine 0.44%, lysine 0.34%, methionine 0.20%, histidine 0.07%, and contained the non-essential amino acid, such as glycine 3.02%, glutamic acid 2.78%, aspartic acid 1.59%, alanine 1.11%, serine 0.95%, tyrosine 0.41%. This is in accordance with Ovie and Eze (2010) that lysine has a function to maintain the growth of the normal cells. Lysine deficiency can create stunted growth.

The relative percent survival value was obtained by comparing the number of death of fish in the treatment and control groups. The observation results of relative percent survival (RPS) showed that both treatments 7 extract/kg feed and 7.7 g extract/kg feed were the treatments with the highest RPS value (Table 5). According to Grisez and Tan (2005), a successful vaccination has a minimum RPS value of 60% in fish. Based on these results, those two treatment doses were included in the range of more than 60% so that they were decent to be the best dose. It also proved that those two doses of treatment could work effectively in *C. a. auratus* to prevent diseases caused by *A. hydrophilla* bacterial infection.

In this research, the highest total mortality data was found in the control treatment. It was because this group was not treated in the form of microcapsules of sea hare extract, which has functioned as an immunostimulant so that it was not resistant to the attack of *A. hydrophila* bacteria. The external clinical symptoms in the form of a sore on the back, red spots or bleeding (haemorrhage), peeling scales, flaking on the caudal fin, reddish areas of infection, lesions in the head area, exophthalmia (protruding eyes), purulent (white eyes), and abdomen. On the other hand, there were no fish that died or were infected with *A. hydrophila* bacteria in both treatments of 7 g extract/kg feed and 7.7 g extract/kg feed, it was proven by the condition of fish which were in good health and without clinical symptoms. Overall, it also proved that the sea hare extract contained compounds that function as immunostimulants that could induce the resistance of the body toward the attack of *A. hydrophila* bacteria.

The results of this study may provide information about the extract of *D. auricularia* contains secondary metabolites in the form of phenolic compounds, tannins, steroids, terpenoid, alkaloid, and saponin, which show their ability to stimulate fish health. Administration of microencapsulated extract as much as 7 g/kg of feed was the optimum dose to stimulate the immunity of *C. a. auratus* against infection with *A. hydrophilla* bacteria as indicated by better clinical symptoms of fish, blood profile, relative percent survival, and weight gain. In conclusion, our results suggest that *D. auricularia* biota can be developed to be used in controlling disease in aquaculture. Further research is recommended to mix the extract of microcapsules into formulated feed ingredients so that they are easy to be used, especially in the feed industry. In addition, it is necessary to make efforts to cultivate *D. auricularia* to be developed.

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