Larvae of Hermetia illucens Promotes the Immunocompetence of Haematology and Muscle Histopatology of Common Carp (Cyprinus carpio) Challenged with Aeromonas hydrophila

Febi Nadhila Nurin, Maftuch, Uun Yanuhar

Abstract: Immunostimulant of Hermetia illucens larvae was prepared for assessment on total leukocyte, differential of leukocyte and muscle histopathology of common carp. The extract of Hermetia illucens larvae was administrated in the diet at A (4%), B (6%), C (8%), and D (10%). The extract of Hermetia illucens larvae contained of flavonoid compounds, tannins/polyphenols and alkaloids. During 30 days of the feeding trial, total leukocyte was decreased as well as decreament of differential of leukocyte at dose C (8%) also at that dose was showed the best increament of immune system of Cyprinus carpio, average of total leukocyte post-infection results was shown 1,95 x 10^5 sel/ml and differential of leukocyte (lymphocytes, monocytes, neutrophils) was shown 81,55%; 16,42%; 0.33%. Furthermore, the histopathological of muscle before being fed the extract showed the oedema, vacuolisation, and necrosis. The average score of the lowest muscle damage was also shown 1.27%; 1.47%; and 1.4% at dose C (8%).

Keywords Cyprinus carpio, Aeromonas hydrophila, Hermetia illucens larvae, Immunostimulant

1 INTRODUCTION

Common carp is one of the most important fish species because it has a high protein source. Nevertheless, handling of the maintenance is very lack, especially on intensive system (1). Intensive system in aquaculture trigger stressful effect for the organism and trigger the various disease agents (2). One of the disease is the presence of bacteria Aeromonas hydrophila on the cultivation of common carp. A.hydrophila is a gram-negative bacteria, rod-shaped bacteria and found in many freshwater and brackish water. A.hydrophila is known as opportunistic bacteria because it happens when the fish is stressed. Clinical symptoms are generally characterized by the presence of hemorrhagic on the skin, gills, and oral cavity. Common clinical signs generally characterized by exophthalmia, ascites and swollen lymph and kidney (3), To avoid economic losses, veterinary drugs are commonly used in aquaculture to prevent or treat disease outbreaks, and the regular administration as additives in fish food or in baths and injections is a commonly used. However, the side effects of veterinary drugs in terms of both environmental and health safety have become a major concern. Immunostimulants are effective means of increasing the immunocompetency and disease resistance by enhancing both specific and non-specific defense mechanisms of fish and shellfish. One of the natural ingredient is larvae of Hermetia illucens (Diptera: Stratiomyidae). One of the function of larvae Hermetia illucens is as antimicrobial effect (4).

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In recent years, maggot or larvae of H. illucens has been known as an effective method for the treatment of chronic disease, necrotic, and wound infections, as well as to cure burns in humans (5). In addition, this study shows not only the effects of both 'in vitro' and 'in vivo' extract Hermetia illucens but also the various biological functions and effects of H. Illucens for immune system. The influence of active substances or compounds isolated from maggot and potential pharmacological functions has not been reported in various bacteria, cells, and animals. In a previous study, it was reported that extract of H. illucens contained of hexanedioic acid which is as antibacterial activity against gram negative bacteria (6). Therefore, to develop a potential immunostimulant for Cyprinus carpio to promote the benefits in aquaculture, the extract of larvae H. illucens was used to increased the immune system of C. carpio for 30 days of feeding trial.

2 MATERIAL AND METHOD

This study was conducted in October-December 2017. The implementation of the proximate test, conducted at the Processing of Fishery Products Laboratory, Faculty of Fisheries and Marine Science, University of Brawijaya, Indonesia.Iimmune testing of common carp, conducted at the Disease and Health Fish Laboratory, University of Brawijaya, phytochemical testing was conducted at UPT Material Medica, Batu, Malang, and FTIR testing was conducted at Chemistry Laboratory, Faculty of Chemistry, UIN.

Process of obtaining the extract of H. illucens

H. illucens that used in this research is H. Illucens in phase instar 6 and purchased from cultivators in Slipi, West Jakarta, Indonesia. H. Illucens was cleaned and then preserved in the freezer. Furthermore, H. Illucens dried up and keep out of the sun. Then, dried H. illucens was grind with a blender or grinder to obtain the H. Illucens powder (7).

Cyprinus carpio
Common carps, Cyprinus carpio, were obtained from commercial farm in Pasuruan, Malang, East Java, Indonesia, and acclimatized at room temperature for a week before the experiment began. The selected fish must be uniformly sized arround 7-9 cm, respectively. Common carps were fed immunostimulant diets at a rate of 5% body weight twice dialy. The common carps were maintanance for 30 days of the feeding trial with the immunostimulant.

Phytochemicals Analysis
Phytochemicals analysis for H. Illucens were performed into simplicia according to Standard Operating Procedure (SOP) of "UPT Materia Medica Batu, Malang, East Java, Indonesia".

Spectophotometry Fourier Transform Infra Red (FTIR) Analysis
FTIR analysis for H. illucens were performed into simplicia according to Standard Operating Procedure (SOP) of Laboratory of Compound, Universitas Islam Negeri Malang, East Java, Indonesia.

Lethal Dose (LD_{50}) of A. hydrophila
A. hydrophila was obtained from "Balai Karantina Ikan Kelas I", Surabaya ,East Java, Indonesia with the results of specimens $2.726x10^9$ cells/ml. The LD (Lethal Dose) was performed with a bacterial density of 10^6, 10^7, 10^8 cells / ml.

Process of Obtaining the Immunostimulant
Simplicia of H. illucens were substituted on the commercial feed. The dose of simplicia was determined from previous research (7). Further, this study used dose 4%, 6%, 8% and 10% of simplicia H. Illucens was administrated in commercial feed.

Total of Leukocyte
The total leukocytes was measured by taken of blood fish as much as 0.5 µl using leukocyte pipette. Then diluted with turk solution in leukocyte pipette up to 11 µl. Removed two drops of the mixture and the third drop was placed on the haemocytometer and then covered with a cover glass and observed using a light microscope by calculating the total leukocyte in all leukocyte boxes (8). the formula of total leukocyte (9);

SDP = (A/N) x (1/V) x Fp
= (A/4) x {1 / (1x1x0.1)} x 20
= A / 0.4 x 20
= A x 50

Which is :
SDP = Total of leukocyte
A = The amount of leukocyte cell
N = The amount of haemocytometer
V = Volume of haemocytometer
Fp = Dilution factor

Muscle Hstophatology Analysis
The preparation of histopathology was performed according to Susanto (2008) (10). The percentage of damage per field area is calculated based on the number of damaged cells by the formula (11):

$$\text{Damage Percentage} = \frac{\text{Total of damage cell}}{\text{Total of analysis cell}} \times 100\%$$

Table 1. *Percentage of scoring (11).*

Score	Damage Percentage (%)
1	0-5%
2	6-25%
3	26-50%
4	>50%

Statistical Anaysis
A one way analysis of variance (ANOVA) was used to analyze the data. Multiple-comparison (Tukey's) test was conducted to examine significant differences among treatments using SPSS vers. 16 computer software (SPSS, Chicago, IL, USA).

3 RESULTS AND DISCUSSION

Phytochemical Analysis

Table 2. *The Results of Phytochemical Analysis*

Compounds	Result	Positive Features
Flavonoid	+	Dark red
Tanin/Polifenol	+	Green black
Saponin	-	Foam permanently
Alkaloid (P. Meyer)	-	White precipitate
Alkaloid (P. Dragendrof)	+	Orange precipitate

Phytochemicals analysis were used to determine the presence of compounds such as flavonoids, tannins, saponins, and alkaloids. The phytochemical results of the H. illucens seen in Table 2, it was found that the H. illucens contains of flavonoid, tannin/ poliphenol, and alkaloid. The compounds capable to inhibit the bacterial growth, the favonoid used for antibacterial by three mechanisms: inhibit bacterial nucleic acid synthesis, inhibit bacterial cell membrane function and inhibit bacterial energy metabolism. The mechanism of action of tannins as antibacterial agents, through the destruction of bacterial cell membranes due to toxicity of tannins and the formation of complex bonds metal ions. In otherwise, alkaloids are also able to destruct the peptidoglycan components in bacterial cells (12).

Spectophotometry Fourier Transform Infra Red (FTIR) Analysis
According to FTIR analysis, the result showed that the extract of H. illucens obtained the 5 frequency that showed the kind of bonds and compounds

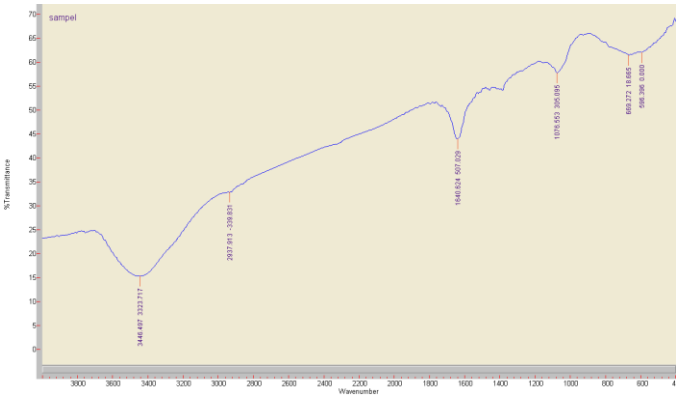

***Figure 1**. Frequency of FTIR Analysis*

***Table 3**. The Result of FTIR Analysis*

Wavelenght	Functional Groups
3446.497	OH bonded
2937.913	CH_2
1640.624	Phenols
1076.553	CH_2OH
669.272	Phenols
696.396	Phenols

Lethal Dose (LD_{50}) of A. hydrophila

The LD_{50} analysis was performed with bacterial density of 10^6 cells/ml, 10^7 cells/ml, and 10^8 cells/ml, it was found that LD_{50} of 10^7 cells/ml for 8 hour . Thus the bacterial density used in the research is 10^7 cells/ml.

Total of Leukocyte

***Table 4**. Total of leukocyte before infected with A. hydrophila*

Treatment	Replicated			Total x 50	Average (10^5)
	1	2	3		
A	5380	5357	4345	754100	2,51[b]
B	4990	4,825	4,976	739550	2,46[b]
C	3858	2928	2285	453550	1,51[a]
D	4213	1613	4494	516000	1,72[a]
K	2250	2999	3017	413300	1,37[a]

***Table 5**. Total of leukocyte after infected with A. hydrophila*

Treatment	Replicated			Total x 50	Average (10^5)
	1	2	3		
A	6766	5913	5984	933150	3,11[c]
B	4508	6436	5472	820800	2,74[b]
C	4708	5745	1259	585600	1,95[a]
D	5308	3502	4702	675600	2.25[b]
K	2250	3984	3517	487550	1,63[a]

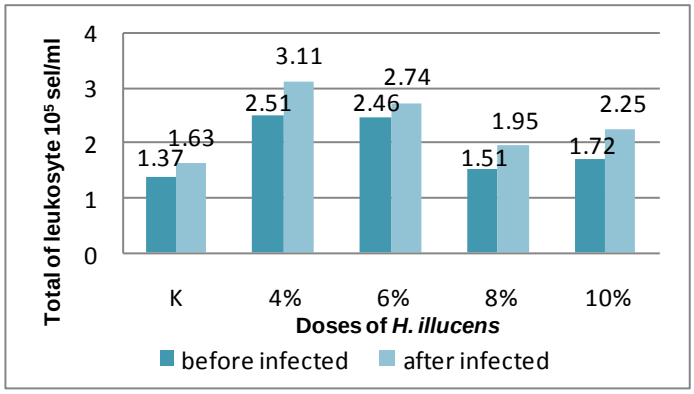

***Figure 2**. Graph of total leukocyte before and after infected with A. hydrophila*

The graph showed that the highest total leukocyte before and after bacterial infection at dose A (4%), nevertheless, the lowest total leukocytes at the dose C (8%), this result showed that the treatment at dose C (8%) observed the best immune system than any other treatment. Leukocyte is a blood cell that plays a role for immune system because prevent the various infectious diseases and foreign materials (13), Thus, if the result showed the increament of total leukocyte likewise the increament of infectious diseases and foreign materials were resisted.

Differential of Leukocyte

***Table 6.** The Average of Limphocyte*

Treatment	Before Infected		After Infected	
	Total	Average	Total	Average
A	236	78.42	273.83	91.28[b]
B	227	75.67	249.67	83.22[a]
C	241	80.33	244.66	81.55[a]
D	227	75.23	247.17	82.39[a]
K	216	72.00	226.98	75.66[a]

***Table 7**. The Average of Monocyte*

Treatment	Before Infected		After Infected	
	Total	Average	Total	Average
A	73	24.33	25.29	8.43[a]
B	74	24.67	51.25	17.08[c]
C	59	19.67	52.08	17.36[c]
D	65	21.67	49.26	16.42[b]
K	84	28.00	65.91	23.97

***Table 8**. The Average of Neutrophil*

Treatment	Before infected		After infected	
	Total	Average	Total	Average
A	0.00	0.00	3.00	1.00^{bc}
B	0.00	0.00	2.00	0.67^{ab}
C	0.00	0.00	1.00	0.33^{a}
D	0.00	0..00	1.00	0.33^{a}
K	0.00	0.00	7.00	2.33

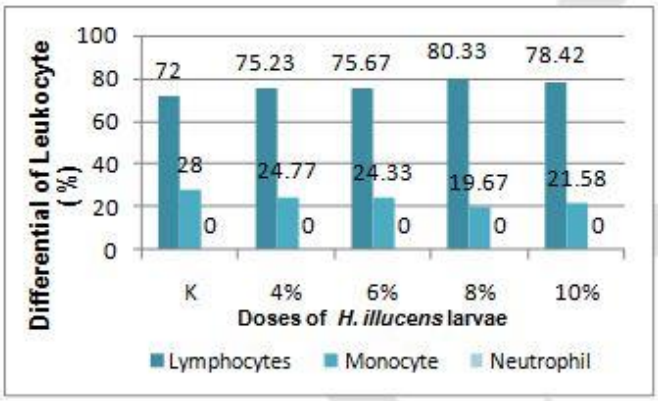

***Figure 3**. Graph of Differential Leukocyte before infected with A. hydrophila*

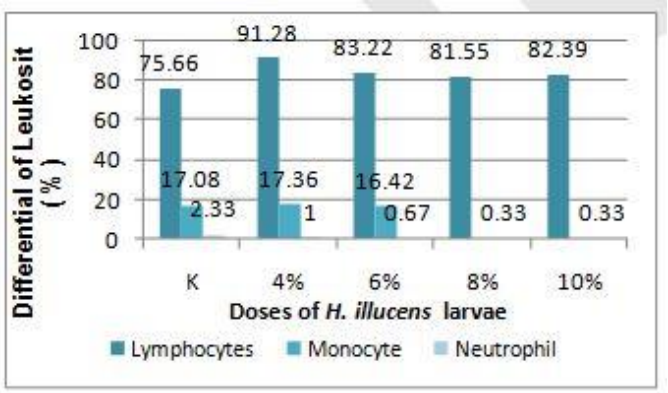

***Figure 4**. Graph of Differential Leukocyte After Infected with A. hydrophila*

The percentage of lymphocytes was higher than monocytes and neutrophils, nevertheless, neutrophils not found in all treatments before infection. The main function of leukocytes is for enhance the immune system. The fluctuation percentage of leukocytes in blood circulation interpreted that organism invaded the disease agents, inflammation, autoimmune diseases or allergic reactions. (13) Monocytes plays role for prevent swallowing and destroying cells, microorganisms and foreign objects that are pathogenic (14). The percentage of heterophile increase when bacterial infect the organism (15). Lymphocytes plays role responding to antigens (foreign materials) by forming antibodies and development of immunity (16). Therefore the resluts of differential leukocyte showed that immune system of C. carpio will be increase and plays important role for prevent the disease.

Muscle Histophatology

Based on the results, the condition of C. carpio muscle histology showed the different forms on each treatment after infected with A. hydrophila.

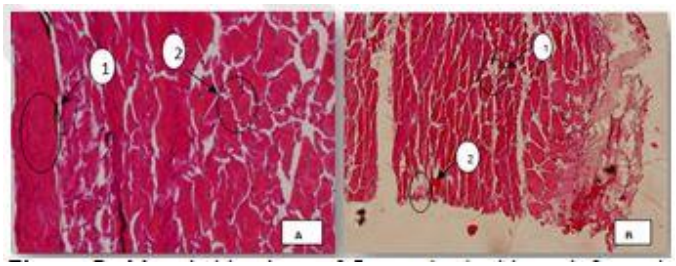

***Figure 5.** Muscle histology of C. carpio a) without infected with A. hydrophila. (1) smooth muscle, (2) striated muscle. Structure of histophatolofy (b) control (-). (1) necrosis (2) Oedema. Magnifation of the microscope 400 times.*

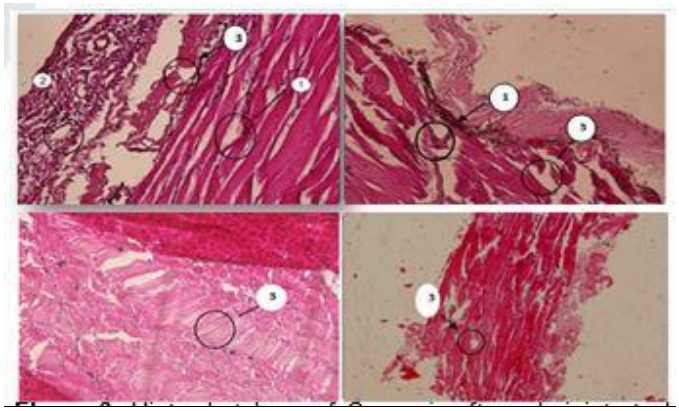

***Figure 6** . Histophatology of C. carpio after administrated the imunostimulant and infected with A. hydrophila. (A). 4%, (B). 6%, (C). 8% and (D) 10%. (1) Oedema, (2) Vakuolisation and (3) Necrosis. Magnifation of the microscope 400 times.*

The muscle histopathological of C. carpio after infected with A. hydrophila is oedema, vakuolisation, and necrosis. Oedema is an abnormal fluid accumulation in the body or in the interstitial space of the tissues and organs that cause swelling, whereas, necrosis is the death of the cells or tissues. Characteristics of necrotic tissue, which has more pale color than normal color, loss of spanning power (tissues becomes brittle and easily torn), or pale consistency (17). The scoring of muscle damage seen in the table 9:

***Table 9**. Scoring value of muscle histology after infected with A. hydrophila*

Treatment	The Average of Scoring		
	Oedema	Vakuolisation	Necrosis
A	3.07^{c}	3.13^{c}	3.47^{c}
B	2.33^{b}	2.67^{b}	2.73^{b}
C	1.27^{a}	1.47^{a}	1.4^{a}
D	1.53^{a}	1.67^{a}	1.73^{a}
K	1^{a}	0.87^{a}	0.93^{a}

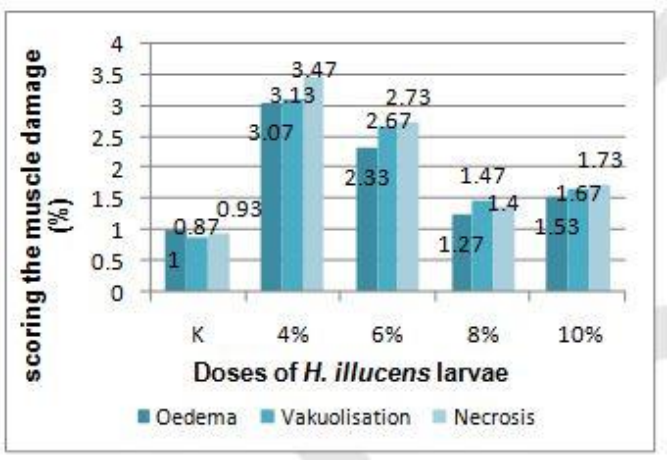

***Figure 7**. Graph of scoring the muscle damage in C. carpio*

The graphic showed that the treatment of C (8%) was the lowest score of muscle damage. Treatment C (8%) also showed that the average of scoring was close to positive control or without infected with A. hydrophila, but the result of treatment D (10%) was not significantly differences. That's might be because at that dose showed the best immune response. Recovery of tissue in the muscle, influenced by substitution of immunostimulant from H. illucens on commercial feed that has been given. Different doses also affect the different tissue recovery. The immunostimulant increasing the immune response and develop the protection against pathogen infection. Provision of immunostimulant at concentrations below the minimum value for the immune response will not have an effect (18). Based on the explanation and the percetage of scores concluded that the best dose that can reduce the level of damage in muscle tissue of common carp is 8%.

4 CONCLUSION

Based on the results, the best immune system enhance on dose C (8%) when reviewed from the results of total leukocyte, differential leukocyte, and histopathology of muscle.

References

[1] Shirali, S., Erfani Majd, N., Mesbah, M., Seif, M.R., 2012. Histological studies of common carp ovarian development during breeding season in Khuzestan province, Iran. World J. Fish Mar. Sci. 4. 159–164.

[2] Roohi,. M. Reza, I. 2015. The Efficacy Of The Oils Of Spearmint and Methyl Salicylate as New Anesthetics and Their Effect on Glucose Levels in Common Carp (Cyprinus carpio L., 1758) Juveniles. Aquaculture 437: 327–332.

[3] Irianto, A., Hernayanti dan Budiyono. 2004. Pengaruh Suplementasi Probiotik AS-51 dalam Pakan terhadap Makrofag Ginjal Ikan Mas (Cyprinus carpio) dalam Prosiding Pengendalian Penyakit pada Ikan dan Udang Berbasis Imunisasi dan Biosecurity. Seminar Nasional Penyakit Ikan dan Udang. Purwokerto. 151–154.

[4] Wangko, S. 2014. Hermetia Illucens Aspek Forensik, Kesehatan, dan Ekonomi. Jurnal Biomedik (JBM), 6 (1): 23-29.

[5] Feng, X., Cheng, G., Chen, S.Y., Yang, H., Huang, W., 2010. Evaluation of the burn healing properties of oil extraction from housefly larva in mice. Jurnal Ethnopharmacol. 130, 586–592.

[6] Choi, W.H., Yun, J.H., Chu, J.P., Chu, K.B., 2012. Antibacterial effect of extracts of Hermetia illucens (Diptera: Stratiomyidae) larvae against Gram-negative bacteria. Entomol. Res. 42.

[7] Sugianto, D. 2007. Pengaruh Tingkat Pemberian Maggot Terhadap Pertumbuhan dan Efisiensi Pemberian Pakan Benih Ikan Gurame (Osphronemus gouramy). Thesis. Institut Pertanian Bogor. 45 pages

[8] Bijanti, R. 2005. Hematologi Ikan Teknik Pengambilan Darah dan Pemeriksaan Hematologi Ikan. Bagian Ilmu Kedokteran Dasar Veteriner. Fakultas Kedokteran Hewan. Universitas Airlangga. Surabaya.

[9] Svobodova, Z., Vykusova, B. 1991. Diagnostics, prevention and therapy of fish diseases and intoxications. Research Institute of Fish Culture and Hydrobiology Vodnany, Czechoslavakia. 270 pages.

[10] Susanto, D. 2008. Gambaran Histopatologi Organ Insang, Otot, dan Usus Ikan Mas (Cyprinus carpio) di Desa Cibanteng. Thesis. Fakultas Kedokteran Hewan. Institut Pertanian Bogor. Bogor. 49 pages.

[11] Raza'I, T. S. 2008. Analisis Histopatologi Organ Insang dan Usus Ikan Kerapu Lumpur (Epinephelus coloides) yang diberi Khamir Laut (Marine Yeast) Sebagai Immunostimulan. Thesis. 95 pages.

[12] Rahman, F, A., Tetiana Haniastuti, Trianna Wahyu Utami. 2017. Skrining okimia dan aktivitas antibakteri ekstrak etanol daun sirsak (Annona muricata L.) pada Streptococcus mutans ATCC 35668. Vol 3 No 1. 1-7.

[13] Lestari, S.H.A., Ismoyowati, dan M. Indradji. 2013. Kajian jumlah leukosit dan diferensial leukosit pada berbagai jenis itik lokal betina yang pakannya di suplementasi probiotik. Jurnal Ilmiah Peternakan 1 (2): 699-709.

[14] Isroli, S. Susanti, E. Widiastuti, T. Yudiarti, dan Sugiharto. 2009. Observasi beberapa variable hematologis ayam kedu pada pemeliharaan intensif. Seminar Nasional Kebangkitan Peternakan, Semarang. 548-557.

[15] Napirah, A., Supadmo dan Zuprizal. 2013. Pengaruh penambahan tepung kunyit (Curcuma domestica Valet) dalam pakan terhadap parameter hematologi darah puyuh (Coturnixcoturnix japanica) pedaging. Peternakan 37 (2): 114-119.

[16] Bikrisirna, S.H.L., L.D. Mahfudz, dan N. Suthama. 2013. Ketahanan tubuh ayam broiler pada kondisi tropis yang diberi jambu biji merah (Psidium guajava) sebagai sumber antioksidan. Agromedia 31 (2):46-57.

[17] Pazra, D.F. 2008. Gambaran histopatologi insang, otot dan usus pada ikan lele (Clarias Spp.) asal dari daerah bogor. Insitut Pertanian Bogor. Thesis. 64 pages.

[18] Agung, M. 2007. Penelusuran efektifitas beberapa bahan sebagai kandidat antibakteri dalam mengatasi penyakit vibriosis pada udang windu. Fakultas Perikanan Dan Ilmu Kelautan Padjadjaran. Jatinangor. 36 pages.