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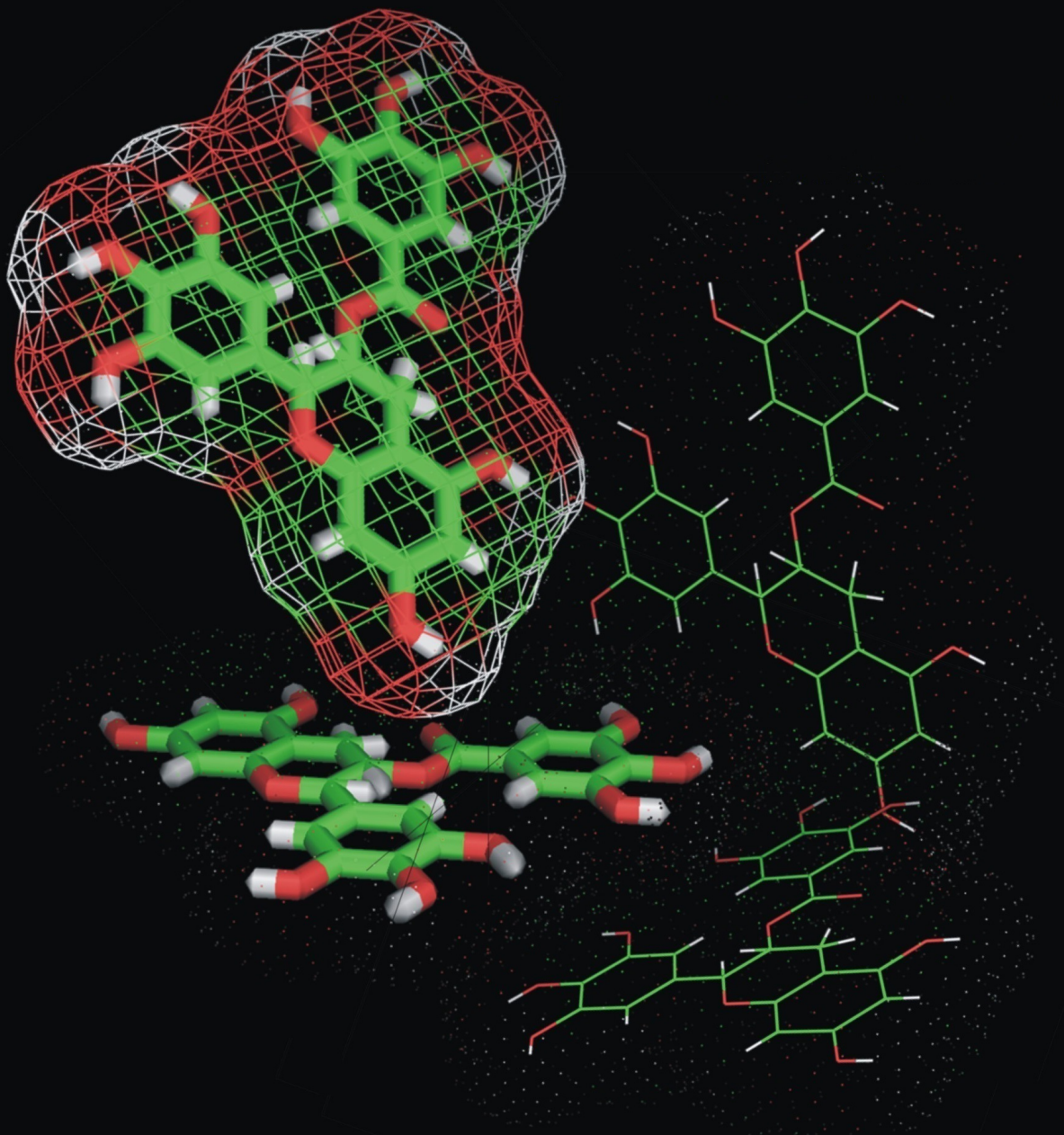

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The Abnormality of Spermatozoa Goat after Freezing on -80°C Using Tris Diluent Added Combination Hatching Egg Yolk and Amniotic Fluid

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Abstract

This study aims to determine the combination of the hatching egg yolk (HEY) and amniotic fluid (AF) in Tris diluent to the type of goat spermatozoa abnormalities after frozen at temperature -80°C. This study used four treatments and 6 replications. The semen is diluted using tris extender and added treatment, there are control group (T_0) (Tris egg yolk 20%), T_1 (15% HEY + 5% AF), T_2 (10% HEY + 10% AF), and T3 (5% HEY + 15% AF). After dilution, the semen was equilibrated for 2 hours, then stored using *Mr. Frosty* and frozen at temperature -80°C in the ultra-freezer for 24 hours. The type of spermatozoa abnormalities type was evaluated on fresh semen and after thawing. The data were analyzed using ANOVA ($P<0.05$). The results showed that there is no significant difference among treatments. The primary abnormalities in fresh semen, there were macro-cephalic, amorphous, and the secondary spermatozoa abnormalities such as detached head, mid-piece defect, coiled tail, loosehead, shoe-hook tail, and a broken tail. The secondary abnormalities in post-thawing were found both in the control group and treatment, which are detached head, loosehead, coiled tail, shoe-hook tail, and broken tail. The conclusion of this study was the percentage of secondary abnormalities after thawing was increasing both in the control and treatment groups.

Keywords: Amniotic Fluid, Hatching Egg Yolk, *Mr. Frosty*, Type of Spermatozoa Abnormality.

INTRODUCTION

The advancement of biotechnology in livestock reproduction is very useful to increase the quality and quantity of livestock. Reproduction biotechnology that is widely developed, is artificial insemination technique [1]. Artificial insemination generally uses frozen semen from cryopreservation [2]. Cryopreservation generally uses liquid nitrogen. However, the availability of liquid nitrogen in some areas cannot be obtained easily [3], and the price is expensive. To overcome this obstacle, it is necessary to find an alternative way to replace the freezing media by freezing in the ultra-freezer. The freezing and storage of semen in the ultra-freezer have some advantages than using liquid nitrogen. There are large semen storage capacity, shorter equilibration processes, easy sample handling, no need to change the media places regularly, and the cost is cheaper [3].

During the cryopreservation process, the spermatozoa are susceptible to damage due to cold shock and ice crystals formation [4]. Previous studies showed the quality of spermatozoa post-thawing was decreasing. One of the most common parameters for examining the quality of spermatozoa is the morphology. Based on the previous study showed the percentage of post-thawing spermatozoa abnormality was increasing. This is caused by the changing of osmotic pressure in diluents [1,5,6].

To prevent the low quality of frozen semen, it needs a diluent that can maintain the quality of spermatozoa during storage. Commonly the used diluent has main components which contain nutrient, buffer, cryoprotectant, and antibiotic [7]. Another diluent component that is usually added is egg yolk because it has a role as an anti-cold shock component. Egg yolk has a composition of lipoproteins that can serve as extracellular cryoprotectant [8].

In this study, the diluent was added by amniotic fluid and egg yolk from chicken embryo in 8-day incubation. This based on the pH in egg yolk from 8-day incubation has a neutral pH (6.92) than fresh egg yolk [9] and it is suitable for addition to the diluent because goat semen has a normal pH (6.8-7.0) [1,10]. Amniotic fluid from chicken embryo has several amino acids such as histidine, serine, arginine, glycine, aspartame acid, glutamic acid, threonine, alanine, proline, lysine, tyrosine, valine, isoleucine, leucine, phenylalanine, methionine and various proteins [11]. Amino acid administration can prevent damaging during pre-freezing and post-thawing [12]. This study aims to determine the abnormalities of goat spermatozoa after freezing at -80°C using tris diluent which added

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with the combination of egg yolk from 8 days incubation and amniotic fluid from chicken.

MATERIALS AND METHODS

Research Material

Three healthy male goats between the age 1-2 years and the weight 88-93 kg were used in this experiment. Each goat was individually fed by *indigofera, odot* grass, and concentrate about 7-9 kg every day. Goats are maintained at Center for Artificial Insemination (BBIB) Singosari Malang. Semen goat was ejaculated using an artificial vagina and collected two times a week.

Hatching Egg yolk and Amniotic Fluid Preparation

Egg yolk and amniotic fluid were collected from six chicken eggs aged 8 days. The collection was conducted by using a syringe and stored at 4°C before used.

Research Methods

This study used 4 treatments and 6 replications each. The fresh semen was evaluated by the volume, concentration, pH, motility, viability and abnormality type. Fresh semen was diluted using tris diluent [13] (diluent A) composed of 2.96 g tris-aminomethane, 1.65 g citric acid, 2 g fructose, 0.1 g of penicillin, 0.1 g of streptomycin and 80 ml of aqubides. The Hatching Egg Yolk (HEY) and Amniotic Fluid (AF) was added to the diluent with the following comparison:

T_0 (control group) : tris diluent + 20% egg yolks
T_1 (treatment group 1) : tris diluent + 15% HEY + 5% AF
T_2 (treatment group 2) : tris diluent + 10% HEY + 10% AF
T_3 (treatment group 3) : tris diluent + 5% HEY + 15% AF

The addition of diluent after calculating the concentration of spermatozoa, and we used 100 million.mL^{-1}. The diluted semen was equilibrated in cool top at 4°C for 2 hours and added with diluent B (diluent A + glycerol 6.5%). After equilibration, the sample was put into cryofial then stored in *Mr. Frosty*, and frozen at -80°C in ultra-freezer. The abnormalities of spermatozoa were observed after 24 hours of freezing. The spermatozoa abnormalities was observed based on the method of Susilawati [14].

RESULTS AND DISCUSSION

The Quality of Fresh Semen

The qualities of fresh semen were examined in a macroscopic and microscopic parameter. The macroscopic examination includes volume and pH. The microscopic examination includes concentration, individual motility, viability, and abnormality type. Table 1 shows the macroscopic and microscopic parameter of fresh semen.

Table 1. Macroscopic and Microscopic Parameter of Fresh Goat Semen

Parameter	Average ± SD
Volume (mL)	1.8 ± 0.75
Concentration (Million.mL^{-1})	2244 ± 0.43
pH	6.8 ± 0.40
Individual motility (%)	45.8 ± 3.76
Viability (%)	77 ±8.29
Abnormality	4.66 ± 0.55

Note: SD = Standard Deviation

The semen volume in this study is higher than the previous study which the semen of Peranakan Ettawah goat is 0.86 mL.ejaculate^{-1} [15] and 1.03 mL.ejaculate^{-1} [16]. The differences of semen volume that was produced by each male goat can be influenced by animal age, collecting frequency, and individual variation of livestock [17]. The pH of semen in this study is normal. The normal pH of semen was 6.4-7.8 [18]. The pH variation of semen in each individual can be influenced by the concentration of lactic acid produced by spermatozoa metabolism [7].

Individual motility of goat semen in this study is 45.8%. This semen is still can be used for further process because the previous study which using motility 43% showed that the post-thawing motility is 15-26% [19]. The post-thawing motility 20% still can be used for insemination [20].

The viability in this study is 77% and this semen is categorized as good semen. It is based on the statement of Ducha et al. that state the viability cell is >70% is categorized as good semen and can be used for further processing [21].

The abnormality is 4.62%, and this percentage is lower than the semen in the previous study that the abnormality is 9.57% from Ettawah crossbreed goat [15]. The semen that used in this study is categorized as good semen because it is based on the statement of Hafez that the abnormality <20% indicates it is as good semen [22]. The observation of spermatozoa abnormality is necessary because it is associated with spermatozoa fertility, if the number of spermatozoa abnormalities is high then the spermatozoa's fertility will decrease [23].

The Spermatozoa Abnormalities in Post-thawing

The percentage of spermatozoa abnormalities after added by tris diluent combination egg yolk hatching and amniotic fluid in post-thawing evaluation can be seen in table 2. The statistical test of the spermatozoa abnormality in this study shows no significant difference in each treatment. The percentage of spermatozoa abnormalities after thawing in this study is lower than the previous study is 16-34% in Ettawah crossbreed goat [24], and 8.4-13.9% in Boer goat [25].

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Table 2. The Percentage of Spermatozoa Abnormalities in Post-Thawing

Treatment Group	Post-thawing Abnormality (% ± SD)
T_0	7.36 ± 1.47
T_1	5.86 ± 0.75
T_2	7.00 ± 2.00
T_3	8.83 ± 3.06

Notes:
SD = standard deviation
T_0 (control group) = tris diluent + 20% egg yolks
T_1 (treatment group 1) = tris diluent + 15% egg yolk + 5% amniotic fluid
T_2 (treatment group 2) = tris diluent + 10% egg yolk + 10% amniotic fluid
T_3 (treatment group 3) = tris diluent + 5% egg yolk + 15% amniotic fluid

The result of this study indicates that there is an increase in spermatozoa abnormalities from fresh semen to post-thawing. Treatment group 1 showed the percentage of spermatozoa abnormalities is lowest compared to the other treatment groups. The increased spermatozoa abnormalities after thawing may be caused by cold shock during freezing-thawing [22]. The other factors that cause increased abnormality are the temperature changes extremely and the dilution is not isotonic.

Hatching egg yolk contains lipoprotein and lecithin that can act as an extracellular cryoprotectant. Lipoprotein and phospholipid will cover the surface of the spermatozoa membrane by maintaining the phospholipid bilayer configuration of the spermatozoa membrane [26,27]. Thus, cold shock can be prevented during the cryopreservation process. Amniotic fluid contains amino acids that can act as antioxidants such as proline, histidine, glycine, and alanine. Based on the previous study, the addition of proline to the diluent can maintain the quality of horse spermatozoa during the cryopreservation process [28]. Amino acids can act as cryoprotection in keeping the spermatozoa membrane from the damage during freezing and thawing, and also protects spermatozoa during calcium uptake [29].

In the treatment group 3 (T_3) shows the highest percentage of abnormality is 8.83%. This is caused in 5% hatching egg yolk has fewer lipoprotein and lecithin so it's less optimum to act as extracellular cryoprotectant and buffer. This causes a decrease in membrane integrity in this treatment group. The composition of egg yolk in Tris diluent which can maintain the quality of spermatozoa is 10-20% [30].

Type of Fresh Semen Abnormalities and Post-Thawing

Spermatozoa abnormality was observed after the sample was stored inside the ultra-freezer at temperature -80°C for 24 hours. The types of spermatozoa abnormalities in fresh semen and post-thawing can be seen in Table 2.

Spermatozoa abnormalities are classified into two type, namely primary and secondary abnormality. Primary abnormality can occur during spermatogenesis in the seminiferous tubules. The type of primary abnormalities such as pyriform, tapered head, undeveloped, abnormal contour, narrow, macrocephalus, microcephalus, double head, and double tail [7,14]. The secondary abnormality occur when the spermatozoa through the epididymis, ejaculation, and processing sample during cryopreservation [31]. Secondary abnormalities such as midpiece droplets, loss of acrosome cover, tail folded, broken tail and coil tail [32].

Table 2. Types of Spermatozoa Abnormalities in Fresh Semen and Post-Thawing

Abnormality type	Fresh semen (% ± SEM)	Post thawing (% ± SD)			
		T_0	T_1	T_2	T_3
Tapered head	-	0.33 ± 0.51	0.50 ± 0.54	-	-
Detache head	0.33 ± 0.51	1.66 ± 1.21	1.83 ± 0.75	3.16 ± 0.30	3.16 ± 1.72
Macrocephalic	0.16 ± 0.40	-	-	-	-
Pyriform head	-	-	0.16 ± 0.40	-	-
Amorphous	0.16 ± 0.40	0.16 ± 0.40	0.50 ± 0.83	0.16 ± 0.16	0.16 ± 0.40
Double tail	-	-	-	-	0.16 ± 0.40
Droplet midpiece	0.66 ± 0.81	0.50 ± 0.54	-	-	0.16 ± 0.40
Coiled tail	1.66 ± 1.03	1.33 ± 1.50	0.66 ± 1.21	1.00 ± 0.81	1.00 ± 1.26
Bent tail	1.33 ± 0.81	1.66 ± 1.50	1.16 ± 1.47	0.83 ± 0.47	2.83 ± 1.32
Shoehook tail	0.16 ± 0.40	1.00 ± 0.89	0.20 ± 0.40	1.00 ± 0.25	0.33 ± 0.51
Broken tail	0.16 ± 0.40	0.33 ± 0.81	0.16 ± 0.40	0.50 ± 0.34	0.33 ± 0.51
Loose head	-	0.16 ± 0.40	0.50 ± 0.83	0.33 ± 0.21	0.66 ± 0.81
TOTAL	**4.66 ± 0.55**	**7.36 ± 1.47**	**5.86 ± 0.75**	**7.00 ± 2.00**	**8.83 ± 3.06**

Notes:
SD = Standard Deviation
T_0 (control group) = tris diluent + 20% egg yolks
T_1 (treatment group 1) = tris diluent + 15% egg yolk + 5% amniotic fluid
T_2 (treatment group 2) = tris diluent + 10% egg yolk + 10% amniotic fluid
T_3 (treatment group 3) = tris diluent + 5% egg yolk + 15% amniotic fluid

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In this study the primary abnormality in fresh semen such as detached head, macro-cephalic and amorphous. The secondary abnormality such as midpiece droplets, coiled tail, bent tail, shoe-hook tail and broken tail. According to Rodriguez-Martinez and Barth, the morphological abnormalities of spermatozoa are always found in every ejaculation [33].

The results of this study indicate an increase in secondary abnormalities after thawing. This result is supported by the previous study in human spermatozoa, there is an increase of abnormality in spermatozoa tail after cryopreservation [34]. The study of Ozkavucku et al. also showed an increased abnormality in detached head, loose head and coiled tail. Abnormality in the tail, especially coiled tail, are commonly caused by membrane damage, osmotic pressure and exposure by chemicals in diluents such as cryoprotectant glycerol [35]. When cryoprotectants replace the position of intracellular fluid it is causing changes in the composition of water during freezing, this causes coiled tail [36]. Another researcher suggested that the increase of spermatozoa abnormality post-thawing has the closely related to DNA damage. The cryopreservation process can cause the morphological change in spermatozoa and plasma membrane thus it influenced the chromatin structure spermatozoa [37,38].

CONCLUSION

The percentage of spermatozoa abnormalities was increased after thawing. Most of post-thawing spermatozoa abnormalities in the control group and treatment groups was a secondary abnormality. The spermatozoa abnormalities were found such as detached head, loosehead, coiled tail, shoe-hook tail, and broken tail.

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Expression of Hsp70 and β-actin Genes as The Immune Response against Viral Nervous Necrosis that Infected Asian Seabass (*Lates calcalifer*)

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Abstract

The viral nervous necrosis (VNN) is betanodavirus of the family Nodaviridae with acute infection and associated with high levels mortality up to 100% of numerous larval-stage marine and freshwater fish species. The danger signals of viral infection that are capable of activating APCs (Antigen Presenting Cells), furthermore produced molecules protein receptors such as interferon and heat shock proteins. Stimulating interferon type I (IFN I) induce several antiviral molecules, further binding the actin cytoskeleton to reach the site of infection. This study demonstrated a correlation between increased levels of Hsp70 (heat shock proteins) and actin filamentous (β-actin) within invasion wild-type isolate of ssRNA VNN from Asian Seabass (*L. calcalifer*) juvenile. Furthermore, expression of Hsp70 and actin as an indicator or biomarker of stressed states in fish. The reverse transcriptase polymerase chain reaction (RT-PCR) method used to finding expression Hsp70 and β-actin. Whereas nested RT-PCR used for VNN genes. The up-regulation of Hsp70 was observed on the brain tissue higher than eye tissue of sample positive infected VNN. Whereas different expression of β-actin, β-actin receptor expression tends to be stable in the organs of the brain and eye, both invaded VNN or normal, while on the eye, the ratio slightly increased based on the intensity of the band. The research shows that altered expression of heat shock protein 70 and beta-actin gene receptors in the target organs that response to invasion VNN at Asian seabass (*L. calcalifer*) juvenile. Alteration expression of heat shock protein 70 and beta-actin can be used as an indicator or biomarker of stress cells, especially from VNN invasion at fish.

Keywords: Asian seabass, β-actin, Hsp70, Immune response, Viral nervous necrosis.

INTRODUCTION

Since outbreak at 1997 in Indonesia sea bass (*Lates calcalifer*) aquaculture [1] and for past three decades, the Viral Encephalopathy and Retinopathy (VER) or Viral Nervous Necrosis (VNN) has been frightening spectre to the sea bass aquaculture industry in Asian Marine Culture [2,3]. VER is Betanodavirus of the family Nodaviridae with acute infection [4], and they are virus associated with high levels mortality up to 100% of numerous larval-stage marine fish species [5,6]. In every cases of VER, fingerlings and juvenile fish affected exhibit erratic swimming patterns (whirling) with up swimbladder, skin darkness, anorexia (poor appetite), solitary or clusters near the side of a pool, lordosis or scoliosis, exophthalmia, and a range of neurological abnormalities, including vacuolization and cellular necrosis in the central nervous system and retina [6,7,8].

Based on phylogenetic analysis, including host range, optimum temperature, and geographic distribution gene of nervous necrosis virus (NNV) can be categorized into four genotypes: Red-Spotted Grouper Nervous Necrosis Virus (RGNNV), Barfin Flounder Nervous Necrosis Virus (BFNNV), Tiger Puffer Nervous Necrosis Virus (TPNNV), and Striped Jack Nervous Necrosis Virus (SJNNV). The phylogenetic analysis on the similarity variable T4 region (partial RNA2) which encode the Cp (coat protein) genes. RGNNV was found in warm water fish, TPNNV and BFNNV were found in cold water fish, and SJNNV were found in warm and cold water fish. The optimal range temperatures for viral replication in the different strains such as RGNNV: 25–30°C, TPNNV: 20°C, BFNNV: 15–20°C, and SJNNV: 20–25°C [9].

In some cases of viral infections, viral proteins can target effector molecules by encoding homologous cytokines or cytokine receptors, having acquired such genes through modification or capture of host cellular genes, or inhibit the complement-mediated killing of virus-infected cells [6]. An external pathogen (such as virus) is first recognized by pattern-recognition receptors

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(PRRs), and this induces diverse intracellular signals that provide pathogen defense [10].

The danger signals of viral infection that are capable of activating APCs (antigen presenting cells), in this model are either molecules protein receptor produced by stressed cells such as interferon and heat shock proteins [11,12]. The Hsp (Heat shock protein) genes are highly conserved and have been marked in a wide range of organisms. The heat shock response is an evolutionarily conserved mechanism for maintaining cellular homeostasis following sublethal noxious stimuli. The heat shock protein affects diverse a part of fish physiology, belonging growth and aging, stress physiology and endocrinology, immunology, environmental physiology, stress endurance and acclimation [13]. The heat shock proteins have constitutive functions in unstressed cells, and that is necessary for protein metabolism [14].

Viral infection causes stressed aquatic organism, and as a consequence of such as cellular stress of protein synthesis, particularly causing defect and disruption in protein homeostasis. The defect in protein homeostasis can trigger deviate protein aggregation. As an antigen, viral infection stimulates expression of intracellular Hsp (Heat shock protein) especially Hsp70. The previous study explained that Hsp and MHC (Major Histocompatibility Complex) are two part of peptide binding protein in immune response [18]. Peptide non-native from the viral antigen, bound by Hsp70. Hsp70 polypeptide binding and release is regulated through cycles of ATP hydrolysis and exchange. Misfolding protein caused by viral infection is managed, in part, through the action of Hsp70 molecular chaperones. However, a non-native polypeptide is bound by an Hsp40 co-chaperone (co-factor of Hsp70) for degradation by the ubiquitin-proteasome system (*poly-ub*) [39]. The Hsp70 chaperones are present in most cell compartments and are essential in several processes, folding of polypeptides, refolding of misfolded proteins, translocation of intracellular proteins, and binding of misfolded proteins for degradation [15]. The folding of nascent polypeptide chains acts as a molecular chaperone and mediates the repair and degradation of altered or denatured proteins facilitated by Hsp70 [16].

Fish are an eminent vertebrate specimen to inquiry the physiology, function, and arrangement of Hsps. It is because in their natural environment fish are unprotected to thermal and other stressors. The connection between Hsp synthesis and the establishment of thermotolerance has been investigated by some researcher [17]. Studies on Hsp70 are the most extensive and have demonstrated that the regulation of Hsp70 gene expression occurs mainly at the transcriptional level [14]. The modifications in environmental salinity, disease, and chemical exposure are known to differ the expression and the arrangement of Hsps. *Vibrio anguillarum* acute infection induced an increase in Hsp70 (Heat shock protein 70) in rainbow trout liver and head kidney as examined on immunoblots [19]. Enhancement of hepatic Hsp70 expression in sea bream caused by the *Vibrio alginolyticus* infection at one-day subsequent pathogen administration [14,18].

At the mechanism of innate immune response, viral infection causes stimulating Interferon Type I (IFN I) and induce IFN-Stimulated Gene (ISG). ISG has several antiviral molecules, such as ISG56 or as known protein 56 (P56). P56 protein binds to the actin cytoskeleton component to reach the site of infection for viral elimination [40]. Actin is the others receptor-mediated immune response. The actin cytoskeleton plays pivotal roles in cell shape, cell migration and signaling [20]. Functionally, actin determines the shape and replacement of cells, as well as phagocytosis, intracellular communication and the distribution of organelles [21].

The actin cytoskeleton is important for lymphocyte antigen receptor signaling. The actin cytoskeleton regulates a number of cellular functions via polymerization and depolymerization. Polymerization and depolymerization are a process that includes dynamic changes between monomer Globular (G-actin) and Filamentous or polymerized actin (F-actin) [38]. At the immune response, especially for B-cell activation depends on the polymerization of the actin cytoskeleton [22]. Actin is associated with all three eukaryotic RNA polymerases and it is directly involved in gene transcription [23].

This study has demonstrated a correlation between increased levels of Hsp70 (heat shock proteins) and actin filamentous (β-actin) within invasion wild-type isolate of ssRNA VNN from Asian Seabass (*L. calcalifer*) juvenile. We highlight some of the interactions that are promoted by viral proteins which redirect the function of the actin cytoskeleton and Hsp70. Furthermore, the author use of Hsp70 and actin as an indicator or biomarker of stressed states in fish that infected viral ssRNA VNN.

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MATERIALS AND METHODS

Fish and Materials

The Asian seabass juvenile (*Lates calcalifer*) approximately 10-12 cm in length and 15-20 g in weight were obtained from the fish hatchery Situbondo Brackishwater Aquaculture Development Center, Ministry of Marine Affairs and Fisheries. The lethal sampling method was used for fish sampling. The visual methods were corresponding to the appearance of clinical signs. The type of clinical sign of viral nervous necrosis (VNN) such as blackening, solitary, anorexia, whirling, and exophthalmia. Ten Asian Seabass juvenile were collected randomly from tanks with 32-35 ppt salinity and 28-30°C temperature where the outbreak occurred.

The Asian Seabass juvenile were preserved in live condition. Further, the sample was carried to the laboratory for shortly processing and extraction of RNA. At the laboratory, the live fish were killed and dissected by sharp scissors to severance of spinal cord. Brain and eye were collected as the tissues target for infection of VNN, frozen immediately and stored at -25°C until RNA extraction.

Isolation of VNN, Heat shock protein (Hsp) and β-actin cDNA

The total RNA was isolated from the samples of all untreated fish (based on clinical sign; healthy and infected fish) : 25 mg of tissue from each the brain and eye of *L. calcalifer* was used for extraction of total RNA by using Trizol (Invitrogen, USA) and treated with RNase-free DNase I (Qiagen, USA) to remove any remaining genomic DNA, according to the manufacturer's instructions.

The reverse transcription was carried utilizing the MyTaq $^{(TM)}$ One-Step RT-PCR kit (Bioline UK Cat.No. Bio-65049), following the manufacturer's manual. The reaction system at a final volume of 20 µL contained 7 µL H_2O (NFW), 12,5 µL GoTaq®Green Master Mix, 0.5 µL Tetro reverse transcriptase enzyme, and 4 µL total RNA (500 ng). The reaction was carried out at 45°C for 45 min, and 94°C for 2 min.

Nested RT-PCR and RT-PCR amplification and Measurement of the VNN, Heat Shock Protein (Hsp) and β-actin

Lates calcalifer VNN, Hsp70, and β-actin were amplified using primers designed based on sequences in GenBank and analysis by using the NCBI BLAST. The primer at Table 1. The PCR amplification for VNN genes was carried out under the following conditions: pre-denaturation at 94°C for 2 min; 25 cycles of 94°C for 40 s, 55°C for 40s, extension at 72°C for 40s, and final extention at 72°C for 5 min. The second nested RT-PCR for VNN genes under the following condition : pre-denaturation at 94°C for 2 min; 25 cycles of 94°C for 40s, 50°C for 40s, extension at 72°C for 40s, and the final extention at 72°C for 10 min. Furthermore, the PCR amplification for HSP 70 and β-actin genes was carried out under the following conditions: pre-denaturation at 94°C for 2 min; 35 cycles of 94°C for 30s, 54°C for 30s, extension at 72°C for 30s, and final extention at 72°C for 10 min. The PCR product was examined by electrophoresis with 1.5% agarose gels containing sybersafe. The ImageJ software were used for semi quantitative digital analysis DNA band PCR [24,25].

Table 1. List Primers for amplification RT-PCR

No	Gene	Primer sequences	size (bp)
1.	Actb F	TACCACCGGTATCGTCATGGA	150
	Actb R	CCACGCTCTGTCAGGATCTTC	
2.	Hsp70 F	CGTAAGAGGTGGAAACGCCA	562
	hsp70 R	CAGCGTTGGACACCTTTTGG	
3.	VNN F2	CGTGTCAGTCATGTGTCGCT	420
	VNN R3	CGAGTCAACACGGGTGAAGA	
4.	VNN F'2	GTTCCCTGTACAACGATTCC	294
	VNN R'3	GGATTTGACGGGGCTGCTCA	

Source: from various scientific journals such as : 1. [26]; 2. NCBI get primer menu; 3 and 4 [27].

DNA Sequencing

Positive template of PCR product contains cDNA VNN, Hsp70, and β-actin packed for sequencing. Sequencing done at First Base Laboratories (molecular biology service) Malaysia.

RESULTS AND DISCUSSION

VNN Infection in Asian Seabass

The previous survey of Betanodavirus illustrated that the clinical sign mass mortality of the Asian Seabass (*L. calcalifer*) at 18–21 dph (days post-hatch) typical clinical manifestations such as anorexia, blackening, lack of swimming coordination and settling to bottom [28]. According to the visual observations of VNN that infected juvenile Asian Sea Bass (*Lates calcalifer*) show the same typical clinical manifestation with the previous survey, in addition fish that infected VNN show exophthalmia, solitary or clusters near the side of a pool, lordosis or scoliosis, and erratic swimming patterns (whirling) (Fig.1).

Another study explaining that clinical symptoms are not always altered in infected fish [7]. Based on the nested RT-PCR test, the results on the test showed that two of six samples

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identified positive VNN did not show clinical sign (Fig.1 and 2). To make the infection and replication, virus requires access to the host intracellular environment such as the capacity of the host to develop a proper immune response, the velocity of virus replication, cytopathogenicity and the spread of infection within and between organs, which again may or may not depend on the presence of specific cellular receptors [29]. Several studies further elucidate the absence of clinical symptoms of viral attack, the possibility of the virus invading the host cell through the lysogenic cycle [21].

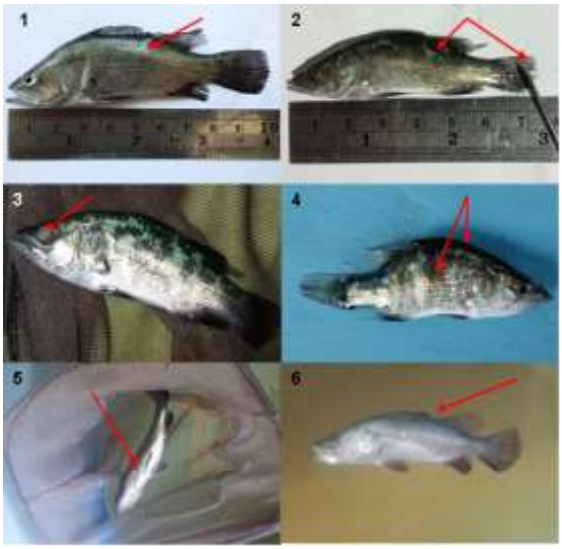

Figure 1. Clinical Symptoms Fish Infected with VNN.
Description: 1. Blackening, 2. Damage fin, 3. Exophthalmia, 4. Petichae, 5. Whirling, 6. Solitary.

Several studies explained that VNN invasion of fish can occur with vertical transmission through broodfish to the eggs in the broodstock offspring [4,28,30] and horizontal transmission of infected fish to non-infected, fellow fish in one water area infected with the virus or contaminated through waters, and through natural live feed [9]. The review found that fish infected through horizontal transmission, from the environmental factor such as the temperature. In these studies, the water temperature of fish tanks between 28-30°C. This temperature is optimal temperature of RGNNV, one type of VNN development in the fish host [7,9]. Optimal temperature of RGNNV development that can cause outbreaks between 25-30°C.

VNN Genes Expression

The Partial cDNA band of VNN genes that infected Asian Seabass (*L. calcalifer*) showed single fragments of 294 base of a nucleotide sequence for VNN genes were determined. The band of all fish samples was compared among genes from healthy and infected fish. In this study, VNN genes were identified in 6 samples which positive infected VNN from 10 observed samples.

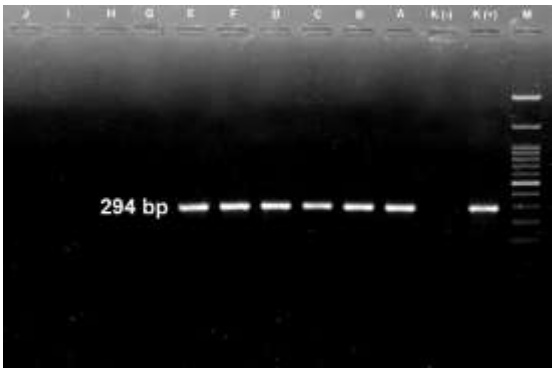

Figure 2. nestedRT-PCR test for VNN that infect Asian Seabass.
Description:
Line M = marker until 1000 bp
K (+) = positive control of VNN genes
K (-) = negative control (nuclease free water)
Line A – J = *L. calcalifer* sample
Line A and F = positive detected VNN genes at 294 bp
Line G – J = negative from VNN genes

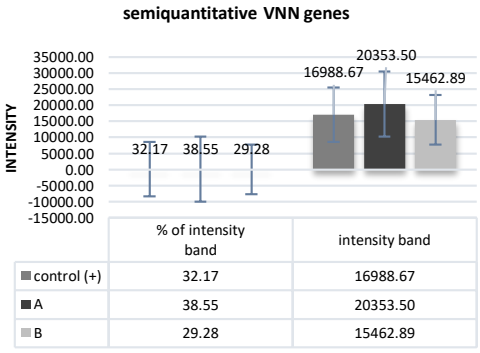

Figure 3. Semiquantitative VNN genes using ImageJ software. Based of fig.2, 3 nested RT-PCR band of VNN genes was analysis using software, and result intensity of band.
Description:
Control (+) = gene template (+) VNN gene
A = the brain tissue positive (+) VNN gene
B = the eye tissue postive (+) VNN gene

The expressions of the VNN genes was observed in brain and eye tissues in infected fish of this study. However, the expressions levels in different tissues varied. The emergence of this band indicates the presence on a copy of VNN genomes in the target sample of organs.

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Whereas the sample that did not appear the PCR band indicated that the target organ had no viral genomes or possibly a very small volume of VNN viral genome that could not be duplicated and visualized by the nested RT-PCR method. The brain tissues showed a higher expressions band level of the VNN genes than eye tissues (based on the semiquantitative analysis using imageJ software Fig.3). The difference in the copy of the viral genome level of VNN may be caused to the distribution and replication of the viral genome on the host's target organs [31].

Profile of β-actin and Hsp70

Reverse transcriptase PCR (RT-PCR) method was used to determine the profile of β-actin and Hsp70 bands in each target organ such as the brain and eye. Effect of VNN invasion on the expressions of the Asian Seabass Hsp70 and β-actin genes (Fig.4).

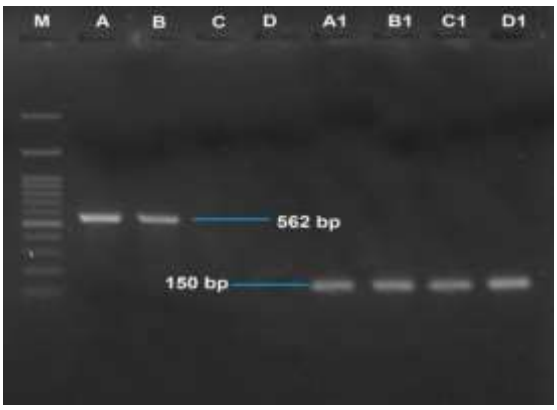

Figure 4. Different light band of RT-PCR test in infected fish and negative fish band of Hsp70 at 562 bp, and β-actin at 150 bp.
Description of Line:
M = Marker until 1000 bp band;
A = Hsp70 at the brain positive (+) sample of VNN genes
B = Hsp70 at the eye positive (+) sample of VNN genes
C = Hsp70 at the brain negative (-) sample of VNN genes
D = Hsp70 at the eye negative (-) sample of VNN genes
A1 = β-actin at the brain positive (+) sample of VNN genes
B1 = β-actin at the eye positive (+) sample of VNN genes
C1 = β-actin at the brain negative (-) sample of VNN genes
D1 = β-actin at the eye negative (-) sample of VNN genes

According to Figure 4, the two target organs of VNN invasion express their respective β-actin and Hsp70 receptors. In a sample detected negative VNN also expresses the β-actin receptor. The interaction between receptors and viruses is an early interaction of virus invasion to the host.

The receptor is a surface molecule acting as a key for initiating the viral infection process, which is capable of directly triggering the penetration of virions attachment on cell membranes (such as endosome and plasma membranes) into specific cellular regions and leading to subsequent infections [32]. Expression of actin as a protein and polymer that form cytoskeleton abundant in eukaryotic cells that have important functions such as motility, growth, cytokinesis, endocytosis and intracellular transport [10,33]. The brain tissues show higher expressions band level of the Hsp70 genes than eye tissues (based on the semiquantitative analysis using imageJ software Fig.5).

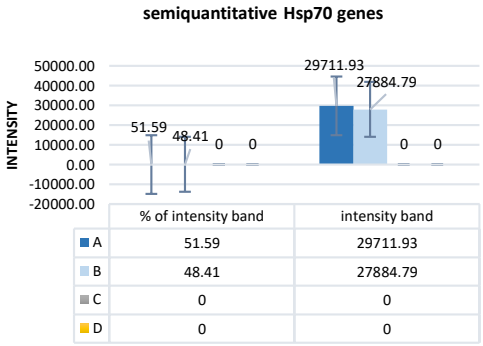

Figure 5. Semiquantitative VNN genes using ImageJ software. Based of fig.4, 2 RT-PCR band of positive (+) Hsp70 genes was analysis using software, and result intensity of band.
Description:
A = Hsp70 at the brain positive (+) sample of VNN genes
B = Hsp70 at the eye positive (+) sample of VNN genes
C = Hsp70 at the brain negative (-) sample of VNN genes
D = Hsp70 at the eye negative (-) sample of VNN genes

VNN invasion of the CNS (Central Nervous System), the brain and the eyes, provides an enhanced immune response of receptor Hsp70 in the brain and eye organ to virus degradation. The Hsp70 up-regulated and expression on tumor cell surface and is not expressed in normal cells [34]. VNN invasion caused an Hsp70 stimulation, plays a role in protein aggregation, refolding damaged proteins, protein degradation, immune signal transduction, converting proinflammation to immune cells (cytokines), and producing variations in immune responses [35]. Study at the tilapia (*Oreochromis niloticus*) shows the

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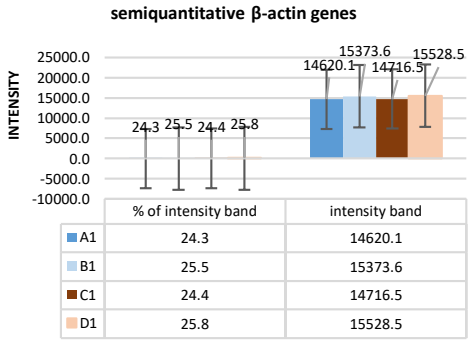
conservative Hsp characteristics of the Hsp70 family, and the introns Hsc70-1, Hsc70-2, and Hsc70-3, have a significant increase of the Hsp family in the liver, kidney (head kidney), lymph, and gills after 6 hours infected with *Streptococcus agalactiae*. According to an *Oreochromis niloticus* Hsp70 research, a significant up-regulation in Hsp70 transcripts was stimulated infection of *Streptococcus agalactiae* [36].



	% of intensity band	intensity band
A1	24.3	14620.1
B1	25.5	15373.6
C1	24.4	14716.5
D1	25.8	15528.5

Figure 6. Semiquantitative VNN genes using ImageJ software. Based of fig.4, 4 RT-PCR band of positive (+) β-actin genes was analysis using software, and result intensity of band.

Description:

A1 = β-actin at the brain positive (+) sample of VNN genes

B1 = β-actin at the eye positive (+) sample of VNN genes

C1 = β-actin at the brain negative (-) sample of VNN genes

D1 = β-actin at the eye negative (-) sample of VNN genes.

Figure 6 explains that β-actin receptor expression tends to be stable in the organs of the brain and eye, both invaded VNN or normal, while the ratio on the eye organ was slightly increased based on the intensity of the band. The propensity for stable actin gene expression is also explained by previous research which elucidate that actin gene expression has the highest stability compared to 7 other encoding gene expressions such as DNA directed RNA polymerase II subunit I (DRP2), elongation factor-1 alpha (EF1α), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PD), hypoxanthine phosphoribosyl transferase (HPRT), ribosomal protein L8 (RPL8), and 18S ribosomal RNA (18S) in the exposed of Cadmium metal at rainbow trout growth phase [37].

CONCLUSION

The research shows altered expression of heat shock protein 70 and beta-actin gene receptors in the target organs that response to invasion VNN at Asian Seabass (*L. calcalifer*) juvenile. Alteration expression of heat shock protein 70 and beta-actin can be used as an indicator or biomarker of stress cells, especially from VNN invasion at fish. The research elucidates that the intracellular stress response is likely to be essential in increasing the survival and health of the infected fish. Measurements of intracellular Hsp70 and β-actin could be a helpful application, particularly when considering minimal invasive methods for diagnostic, acquiring the influence of stress which related to the immune response for ssRNA VNN infection.

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The Effect of *Morinda citrifolia* L. Fruit Extract Supplemented in to Diluent Tris-Egg Yolk Toward the Abnormality of Goat's Spermatozoa after Freezing at -80°C

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Abstract

The aim of this research is to know the effect of diluent Tris-egg yolk medium added with *Morinda citrifolia* L. extract towards the sperms abnormality after storage at -80°C for 24 hours. The extracts added in diluent Tris-egg yolk medium were 0% (P_0), 10% (P_1), 20% (P_2), and 30% (P_3). The result showed that the percentage of sperms abnormality in P_0, P_1, P_2, and P_3 were respectively 8.67%, 6.67%, 5.67%, and 10%. P_2 was the lowest percentage, while P_3 was the highest percentage of sperms abnormality. In addition, this research found twelve kinds of sperms abnormality divided into categories: major abnormality included the broke tail, absent head, detached head, *dag* defect, tapered head, proximal droplet, round head and abaxial; and minor abnormality included the coiled tail, shoe hook tail, bent tail, and distal droplet. It has been concluded that the allotment of 20% *Morinda citrifolia* L. extracts in Tris-egg yolk medium able to maintain the sperm morphology at -80°C storage for 24 hours.

Keywords: goat, *Morinda citrifolia* L., post-thawing, spermatozoa.

INTRODUCTION

Goat, cattle and its production are very potential to be developed for filling domestic and export market. Big population in Indonesia increases the level of lamb consumption every year. Besides, goat's milk has a good prospect to replace cow's milk which is majority used in society [1]. Increasing of goat population through reproduction efficiency with biology reproduction technology providing in Livestock Research Center or other institution is one of the methods to fill goat consumption needs [2]. Sperm cells cryopreservation is the method to keep sperm cells in freeze circumstance, thus it can use anytime to support the application of reproductive technology for livestock [3].

The obstacle of cryopreservation is a freezing and thawing process decreasing the quality of spermatozoa [4]. The result of the previous study has been showed that post-thawing reduces motility, viability and enhances the abnormality in spermatozoa of cat [5], sheep [6], cow [7], and donkey [8]. Thawing causes drastic temperature alteration, thus it increases metabolism, augmenting the concentrate of free radicals [9]. Free radicals cause lipid peroxidation, thus damage spermatozoa cells and induce an autocatalytic reaction, which destructs the double bond of an unsaturated fatty acid constituent of phospholipid plasma membrane of spermatozoa [10]. The effect of the freezing process toward the increment of free radicals has been confirmed in 2001 using cow's spermatozoa; it showed the increasing of ROS during sperm production until post-thawing process [11]. Moreover, research in 2016 showed that a number of lipid peroxidation in post-thawing semen is higher than fresh semen [5]. Awada and the relatives also presented that the effect of free radicals induced the rise of hydrogen peroxidation, lipid peroxidation, and reduce motility spermatozoa of Boer goat [12].

Lipid peroxidation has influenced the damage of structure and metabolism of spermatozoa so it can increase the abnormality of spermatozoa [13]. The drastic temperature alteration when freezing could affect the physiological condition of sperm cells like tail and the middle of the body around the head [14]. Based on Gogo, morphology is the important indicator to determine the quality of spermatozoa [15]. A previous study has been shown that there was a correlation between freezing with the increasing of spermatozoa abnormality [16].

Antioxidant addition in a diluent process can be applied to minimize damage due to free radical activity [17,18]. Some researchers have

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been indicated that antioxidant addition such as vitamin C, gluation, and ß-caroten in the diluent process could maintain the quality of freezing sperm cells through prevention of cell membrane damage and acrosome areas [18,19]. *Morinda citrifolia* L., a medical plant that is commonly found in Indonesia. The extract of *M. citrifolia* L. fruit has antioxidant activity [22]. It contains bioactive compounds like flavonoid, alkaloid, saponin, and triterpenoid [23]. In addition, it also contains 29.29 $mg.mL^{-1}$ ascorbic acid [24]. Ascorbic acid is capable to give the hydrogen atom to the free radicals, secondary antioxidants. This mechanism is like with vitamin E work as an antioxidant and it can prevent the occurrence of chain reactions [23]. Ascorbic acid also plays to overcome free radicals, thus the spermatozoa membrane still save and reduce the abnormality [25]. Flavonoid alkaloid, saponin, triterpenoid, and ascorbic acid belongs to strong antioxidant compounds [23]. Because of the reasons above, this research was conducted to evaluate the abnormality of post-thawing spermatozoa in various dosages of *M. citrifolia* L. fruit extract in diluent n Tris-egg yolk medium.

MATERIALS AND METHODS

Goat's Semen

Goat's semen was collected from five goats aged 2-5 years old from The Center of Artificial Insemination Singosari, Malang. Semen collection was conducted two times in a week using Artificial Vagina (AV). First, we analyzed the quality of fresh semen before the semen was frozen. Animal procedures in this research were allowed with an ethics commission from Research Ethics Committee University of Brawijaya No. 875-KEP-UB.

Morinda citrifolia L. Fruit Extract

Fruit extract of *M. citrifolia* L. was made from simplicia fruit powder obtained from Material Medika, Batu-Malang. The extraction process was conducted using the maceration method based on Zin et al. [22]. Total of 200 g simplicia fruit powder, 400 mL hexane, 1,600 mL methanol, and ethyl acetate was used to make the extraction. From twice maceration process, we obtained 13 g crude extract of *M. citrifolia* L. fruit.

Formation of Diluent Medium

The making of diluent Tris-egg yolk medium was according to the work instruction of BBIB Laboratory, Singosari. Total of 100 mL diluent contained basic constituent of tris aminomethane 1.5%, citric acid 0.9%, lactose 1.4%, egg yolk 20%, raffinose 2.5% streptomycin 0.1 $g.100mL^{-1}$, aquadest 80%, and penicillin 0.1 $g.100mL^{-1}$. The diluent added into semen was divided into 2 groups: diluent A and diluent B. Diluent A consisted of Tris-egg yolk medium and *M. citrifolia* L. fruit extract, while diluent B consisted of diluent A with glycerol 7%. In this research, the dosage of the extracts added into diluent A were composed of 0% (P_0), 10% (P_1), 20% (P_2) and 30% (P_3). The volume of diluent A and B added into the semen were determined by work instruction of BBIB laboratory as the formulation below:

$$V\ tot = \frac{vol.semen\ x\ semen\ concentration\ x\ vol.cryovials}{number\ of\ \ spermatozoa\ per\ crythe\ ovials}$$

Thus,

$$V\ diluent\ \ B = \frac{V\ tot}{2} V\ diluent\ \ B = \frac{V\ tot}{2},$$

$$V\ diluent\ A = V\ diluent\ B - V\ diluent\ \ A$$

$$V\ diluent\ A = V\ diluent\ B - V\ diluent\ \ A$$

Freezing Process

Freezing process, equilibration, thawing and spermatozoa abnormality evaluation were conducted based on work instruction of BBIB Laboratory, Singosari. The diluent was conducted in two steps. First, adding diluent A into fresh semen, this method was carried out in water bath at 37°C. Second, adding diluent B when equilibration, equilibration was done after diluent A was added. Semen was equilibrated in cool top at 5°C for 2 hours and followed by diluent B addition twice: 1 hour after equilibration and 1 hour later after the first equilibration. Then the semen of the equilibration result was filled into 1 mL cryovials and put into Mr Frosty™ for keeping in ultrafreezer with -80°C. Thawing was conducted in water bath 37°C for 30 seconds after 24 hours of the storage.

Analysis of Spermatozoa Quality

Evaluation of spermatozoa quality was carried out in twice: fresh semen evaluation and past thawing semen evaluation. The evaluation of fresh semen included: volume, concentration, pH, individual motility, viability, and abnormality, while post-thawing semen evaluation included: abnormality evaluation. Volume evaluation was carried out by observing directly in the scale of tube used when collecting the semen. Concentration evaluation was done by mixing 8 µL semen with 4 µL NaCl and then poured into cuvet to

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analyze using a spectrophotometer. Evaluation of pH was conducted by dripping 5 µL of semen on the litmus paper. The result of the litmus paper was matched with the provided color scale. Individual motility evaluation was performed by dripping 5 µL NaCl and 5 µL semen on the preparation object glass and then observed using a microscope. Viability evaluation was carried out by dripping 10 µL semen and 10 µL eosin-nigrosin on the preparation object glass and then it was mixed, swabbed, and left until dry. After that, we counted the spermatozoa with the light microscope and hand counter. Spermatozoa that absorbed the color was dead, whereas the spermatozoa that did not absorb color was alife. Abnormality evaluation in fresh semen was done by adding 5 µL semen and 5 µL eosin-nigrosin, whereas the evaluation of post-thawing semen was carried out by adding 10 µL semen and 10 µL eosin-nigrosin; the mixture of semen and eosin-nigrosin then swabbed and left. The observation was performed using a light microscope (Olympus, Tokyo, Japan) with magnification at 400x for evaluation of motility and viability, whereas magnification at 1000x was used to evaluate abnormality. Abnormal spermatozoa counting was conducted as many as 200 cells using hand counter [26].

Data of fresh semen obtained were analyzed statistically with one-way ANOVA. If there was significant differentiation, it was continued by Least Significant Difference (LSD) and showed in average and deviation standard, while data of abnormality kinds were showed descriptively.

RESULTS AND DISCUSSION

Quality of Fresh Sperm Cells

Fresh semen was analyzed in macroscopic and microscopic way for knowing the first quality of semen that would determine the degree of dilution. The result showed in Table 1.

Table 1. Evaluation of Fresh Semen

Parameter	Average
Volume (mL)	1.87 ± 0.24
Concentration (10^6.mL^{-1})	2.543 ± 796.39
pH	6.6 ± 0.08
Individual motility (%)	43.33 ± 4.08
Viability (%)	74.67 ± 2.50
Abnormality (%)	5.33 ± 0.44

According to the fresh semen evaluation, pH and viability were belong to good fresh semen criteria [27]. The result of volume, concentration, and abnormality of fresh semen evaluation was indicated as the value that can be processed to frozen. However, individual motility evaluation had a low percentage. The previous study in 2016 mentioned only fresh semen has a volume between 1.0 and 2.0 mL with concentration more than 2.5 x 10^9 spermatozoa.mL^{-1}, motility > 75% and normal spermatozoa > 85% used for cryopreservation [28]. Nevertheless, based on the buffalo sperm research conducted in 2006, the freezing process decreased the spermatozoa motility 22.67% [29], the motility has also decreased by 12% in grouper [30]. However, the post-thawing spermatozoa motility 20% can be used for artificial insemination [31]. The motility of fresh semen in this research still could be used in the next process.

Morphology Structure of Post-Thawing Semen

Abnormal morphology of spermatozoa related with the fertility of livestock. Moreover, freezing process caused cells damage and the ability of the fertilization was lower than fresh semen [32]. Table 2 showed the different of fresh semen abnormality between post-thawing semen given extract of *M. citrifolia* L. fruit. According to Table 2, the percentage of the abnormality increased from fresh to post-thawing semen, P_0, P_1, P_2, and P_3, respectively. The comparison of post-thawing semen presented that P_2 had a low abnormality of 5.67%, whereas P_3 had a high abnormality of 10%. The lowest abnormality rate in P_2 had a value which was not much different from the fresh semen abnormality value with a percentage of 5.33%.

Table 2. Spermatozoa Abnormality in Fresh Semen and Post-thawing Semen in Various *M. citrifolia* L. Fruit Extract Dosage

Spermatozoa Abnormality (%)				
Fresh semen	Post-thawing Semen			
	P_0	P_1	P_2	P_3
5.33±0.44	8.67±0.50	6.67±0.58	5.67±0.35	10.00±0.90

Abnormality percentage of spermatozoa increased after the freezing process. The increment of abnormality percentage could be caused by cold shock [33]. Garner and Hafez also mentioned that the enhancement of the processes during the storage and the reducement of the temperature quickly increase the percentage of abnormality [34]. Cold shock caused by the freezing and rethawing process gain free radicals that could cause lipid peroxidation decreasing spermatozoa quality [12]. The increment of free radicals during the process freezing and thawing also decreased the spermatozoa motility [11]. There was a negative correlation between spermatozoa motility and

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abnormality; high abnormality reduced the spermatozoa motility [35]. In addition, the effect of the freezing process toward the reducement of spermatozoa abnormality has been shown in previous researches in *Baung* [35], cat [5], donkey [8], and cow [7].

From the post thawing evaluation, there was reducement of abnormality percentage in P_0 to P_3 treatment. It indicated that semen with extract of *M. citrifolia* L. fruit had lower abnormality than semen without *M. citrifolia* L. fruit extract. P_2 treatment had the lowest abnormality, it meant that 20% *M. citrifolia* L. fruit extract effective to reduce abnormality because of free radical increment during the process.

Fruit extract of *M. citrifolia* L. contains antioxidant compounds such as vitamin C, flavonoid, alkaloid, saponin, triterpenoid, and phenol that can prevent lipid peroxidation. Antioxidant compounds like flavonoid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) can give hydrogen atom to reduce DPPH radical. Next aryl radical of flavonoid resonate and give back hydrogen atom to DPPH radical. The complex between antioxidant and DPPH balance the stability and potential reaction of its molecule structure [23]. Based on the mechanism, antioxidant compounds has a stable characteristic in radical structure [36]. In addition, Nugraheni et al stated that constitute vitamin C in *M. citrifolia* L. fruit extract has played as an antioxidant to overcome free radicals so it could save the cell membrane of spermatozoa and reduce the abnormality [25].

The rule of vitamin C in *M. citrifolia* L fruit extract as the antioxidant compound can donor an electron to form semi dehydroascorbate which is not reactive, then it disproportionate to form dehydroascorbate which is not stable. Dehydroascorbate will be degraded to form oxalic acid and treonate acid [37]. Previous studies have confirmed that the addition of antioxidant compound could reduce the free radical and lipid peroxidation in sheep. In Boer goat, it was approved that the value of Malondialdehyde (MDA) in semen added with antioxidant compounds (ascorbic acid, BHT, hypotaurine, and cysteine) was lower than semen without antioxidant. It was also mentioned that antioxidant addition could suppress spermatozoa abnormality [18].

There was an increment of abnormality percentage in P_3 treatment. It meant that the addition of fruit extract dosage increased the abnormality. It might indicate that high dosage could give a negative impact on the spermatozoa. It was related to Rahardhianto et al. that adding the high concentrate mixture was not suitable with spermatozoa medium because spermatozoa could metabolize maximum if the diluent was in isotonic [39]. The cell membrane has semipermeable characteristic so both hypertonic and hypotonic will influence the water transportation through membrane cell and damage the integrity of the cell [40]. Cahyadi et al have mentioned that high dosage of addition bioactive compounds like flavonoid and alkaloid reduced the quality of spermatozoa [33] Moreover, a high concentration of ascorbic acid could accelerate the fructolysis and lactic acid accumulation and reduce pH [41]. Besides, lactic acid also had a toxic effect on the spermatozoa and furthermore caused spermatozoa morphological damage [42].

Table 3. Types of Abnormalities in Fresh Semen and Post-thawing Semen

No.	Fresh semen	Post-thawing semen			
		P_0	P_1	P_2	P_3
Major abnormality					
1	Broke tail	Broke tail	Broke tail	Broke tail	Broke tail
2	-	Deteched head	Deteched head	Deteched head	Deteched head
3	-	Absent head	Absent head	Absent head	Absent head
4	*dag* defect	-	*dag* defect	-	-
5	-	Tapered head	-	Tapered head	-
6.	Proximal droplet	Proximal droplet	-	-	-
7.	-	Round head	-	-	-
8.	-	Abaxial	-	-	-
Minor abnormality					
9.	Coiled tail	Coiled tail	Coiled tail	Coiled tail	Coiled tail
10.	Shoehook tail	Shoehook tail	Shoehook tail	Shoehook tail	Shoehook tail
11.	Bent tail	Bent tail	-	-	-
12.	Distal droplet	-	-	Distal droplet	-

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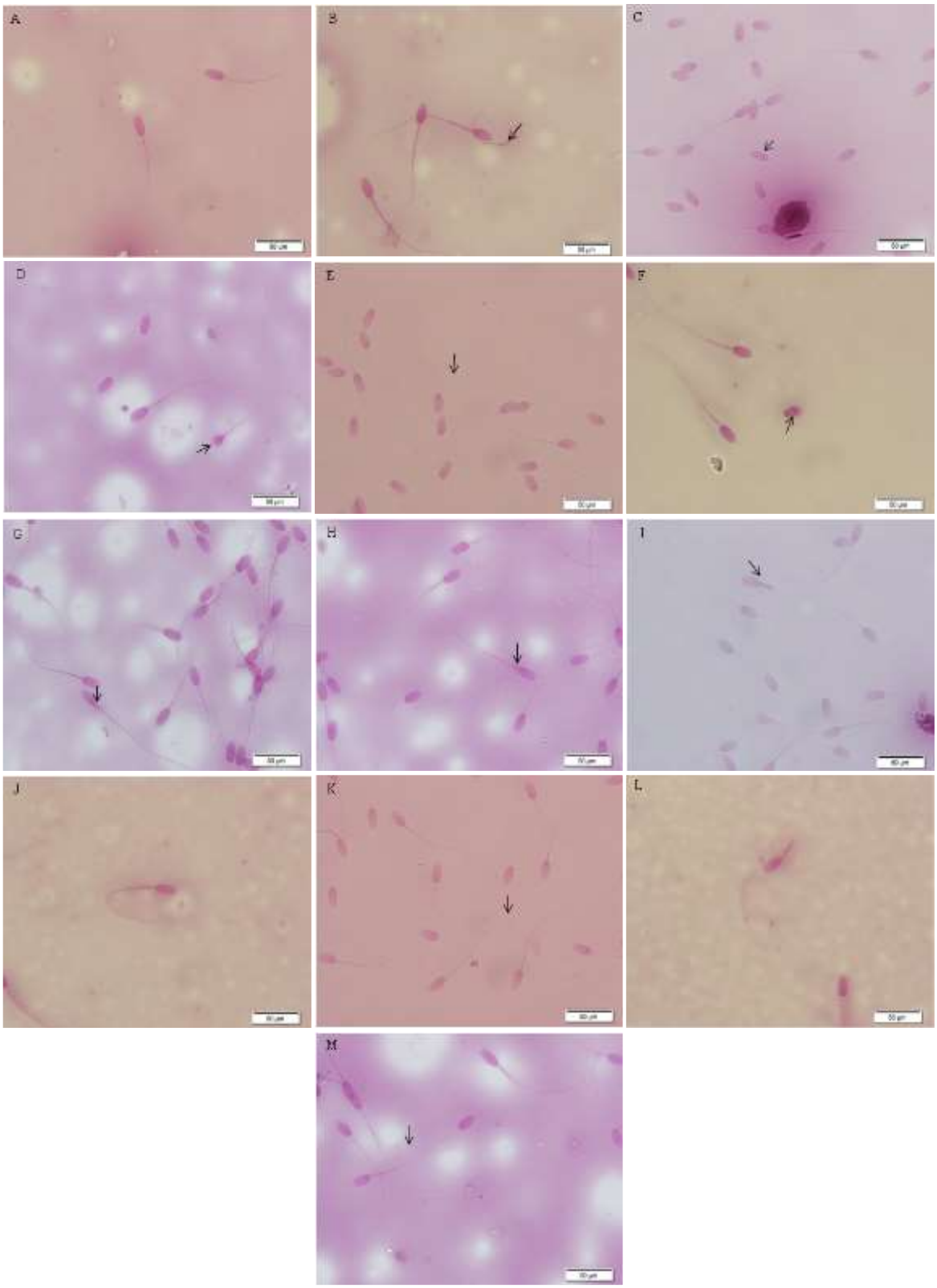

Figure 4. The Observation of Morphology Structure of Goat's Spermatozoa using Olympus BX53 Microscope with magnification at 1000x : (A) normal structure of spermatozoa (B) absent head, (C) tapered head, (D) round head, (E) broke tail, (F) deteched haid, (G) abaxial, (H) proximal droplet, (I) *dag* defect. Structure of minor abnormalities : (J) shoehook tail, (K) coiled tail (L) bent tail (M) distal droplet.

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The addition of antioxidants compounds in high dosage have been reported that could be prooxidant causing oxidation inside the body [41,42]. The reaction between unsaturated fatty acid of spermatozoa cell membrane with excess oxygen has caused irreversible damage to the cell membrane [45]. The broken cell membrane increase abnormality of spermatozoa [46]. The last study using pome fruit extraction has also been showed that the addition of 50% fruit extract in the diluent medium could enhance the abnormality [47].

According to Table 3, there were 12 abnormalities in goat's sperm cells, and it was divided into two groups: major abnormality like broke tail, absent head, detached head, *dag* defect, tapered head, proximal droplet, round head, and abaxial; and minor abnormalities like coiled tail, shoe hook tail, bent tail, and distal droplet [5]. Each of abnormalities could be seen in Figure 4. P_0 had highest abnormality variations, 10, while P_3 was in lowest position with 5 variations. P_0 also had the highest number of major variation with 7 variations, while P_3 was also in the lowest position with 3 variations. In minor variation term, P_0 and P_3 were in the highest and lowest position respectively. Abnormalities variations like broke tail, coiled tail, and shoe hook tail were mostly found in all of the samples.

P_0 had the most number of abnormality variations. It might be P_0 could not obtain the additional antioxidant maintaining the plasma membrane structure from free radicals that came from cold shock during the freezing process. Freezing and thawing process in the cryopreservation process have increased the spermatozoa morphology abnormality [4,35]. According to the result, broke tail, detached head, coiled tail, and shoe hook tail were found in all post-thawing treatments. This result was in line with the last study that the damage after freezing was bent tail, coiled tail, cracked (broken) tail, and damage (detached head), and it also has shown that abnormalities mostly found after cryopreservation was major abnormalities [7]. Even though P_3 had the lowest abnormality variations (Table 3), but the intensity was highest is spermatozoa samples (Table 2).

Base on Chenoweth, the grouping major and minor abnormalities was based on their impact on male fertility. Major abnormality has big effected fertility, while minor abnormality has a small effect on fertility [48]. According to Table 3. P_1, P_2, and P_3 treatment (added by *M. citrifolia* L. fruit extract) had a smaller number of major abnormality than P_0 (without *M. citrifolia* L fruit extract). It indicated that the addition of *M. citrifolia* L. fruit extraction diluent medium could maintain the spermatozoa rather than without *M. citrifolia* L. fruit extract.

The average abnormality percentage generated in each treatment was still belonged to normal, not more than 10%, and still could be used for fertilization. Susilawati has mentioned that the percentage of normal morphology in the semen sample appropriate to the percentage of the motility [32]. According to Garner and Hafez percentage of good spermatozoa abnormalities for artificial insemination was 5 - 20% [34].

CONCLUSION

There were 12 types of abnormalities found in this research, it was divided into 2 groups: major abnormality included broke tail, absent head, detached head, *dag* defect, tapered head, proximal droplet, round head, and abaxial; and minor abnormality included coiled tail, shoe hook tail, bent tail, and distal droplet. The highest percentage of post-thawing abnormality was P_3, while the lowest was P_2, thus 20% *M. citrifolia* L fruit extract addition in diluent Tris-egg yolk medium was the best concentration to maintain the normal morphology goat's sperm cells post-thawing.

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Screening of Rhizosphere Bacteria from Clove (*Syzygium aromaticum*) in Tidore Island as Plant Growth Promoting Rhizobacteria

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Abstract

Tidore Island in North Maluku Province is one of the clove (*Syzygium aromaticum*) producing regions. Clove plant fertility is maintained even though it is not given organic and inorganic fertilizers. This study aims to explore bacteria in the rhizosphere of the clove trees planted in Tidore island as candidate of biofertilizer agents have potency in Indol-3-Acetic Acid (IAA) production, phosphate solubilization and ammonium production. Bacteria were isolated from the rhizosphere of clove trees. Screening of the isolates was done according to qualitative and quantitative methods. Data collection of the qualitative method were based on medium color changes for each variable while that the quantitative method (i.e. the concentrations of produced IAA, solubilized phosphate, and produced ammonium) were obtained based on spectrophotometry. The results obtained 110 bacterial isolates, nineteen bacteria were potential as biofertilizer agents, of which isolate R11, R8P, and A1N showed the highest in IAA production (8.71 μgL^{-1}), phosphate solubilization (8.37 μgL^{-1}), and ammonium production (11.71 μgL^{-1}), respectively. The three isolates respectively have similarities to the genus of *Enterobacter, Burkholderia*, and *Stenotrophomonas.*

Keywords: Biofertilizer, Clove, Rhizobacteria, Rhizosphere.

INTRODUCTION

Total area of clove plantation in Tidore Island is 1,029 ha with the productivity of 500 $kg.ha^{-1}$ [1]. The clove trees on Tidore Island grow naturally and the soil has never been supplemented with either organic or chemical fertilizers. However, crop productivity is naturally preserved. Clove commodity is selected because the plantation area of this commodity estimates increase. In addition, increases of soil fertility without contamination by chemical fertilizers and the need of other perspectives in order to exploit the potential of spice crops also became reasons. It has been reported that the bacteria from the clove rhizosphere were tested in-vitro capable of producing Indole-3-acetic acid [2] but have not revealed the potency of bacteria in the production of ammonium and phosphate solubilization. On the other side of field observes no research report yet on the use of clove rhizosphere bacteria as a biofertilizer agent.

Generally, crop productivity is supported by external factors such as climate and soil microorganism, especially bacteria. The climate of Tidore is a humid tropical region influenced by sea breeze surrounded by the sea of Halmahera, Seram, and Maluku. Observations in this study recorded the type of soil in clove plantation commodity dominated by Inceptisol soil or Alluvial, although in some altitudes there is Mollisol soil. Bacteria found in the plant rhizosphere are called Plant Growth Promoting Rhizobacteria (PGPR). The bacteria use plant exudates as a source of nutrients for their growth [3].

The existence of PGPR on plant roots is beneficial for the plants. Some bacteria can support plant growth or act as agents of biocontrol for plant diseases [4]. Bacteria in the rhizosphere can stimulate the growth of plants through direct or indirect mechanisms. Direct mechanisms involve bacteria as a biofertilizer to stimulate the growth of plants, and as an agent for preventing plant stress, while indirect mechanisms include reducing the intensity of plant diseases, producing antibiotics, inducing systemic resistance, and competing for nutrition and space [5]. The main roles of PGPR are plant growth promoters including nitrogen fixation, production of plant growth hormones such as auxin and cytokinin, and solubilizing phosphate and iron from non-soluble form [6].

The use of PGPR as a biofertilizer agent was developed as an effort to maintain environmental health and prevent environmental damage due to the extensive use of synthetic

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chemical fertilizers. The use of PGPR has several advantages including 1) not polluting the environment, 2) not containing ingredients cause residues in the food chain; 3) not requiring repeated application as microbes can grow with a supportive environment; 4) as a companion for indigenous microbial plants, 5) can increase plant resistance against pathogens [7].

Some special genera of microbes have been found to be useful as PGPR. Anandaraj and Dinesh [8] reported several genera of bacteria as PGPR including *Acinetobacter, Alcaligenes, Azospirillium, Azotobacter, Bacillus, Beijerickia, Burkholderia, Enterobacter, Erwinia, Flavobacterium Rhizobium* and *Serratia.* Several studies have reported the positive effect of PGPR on plant growth such as PGPR has a positive effect on wheat crops [9], fruits [10], and vegetables [11].

Due to the enormous potential of PGPR in extensive plantations and plant health, in this study, exploration of the potential bacteria in the rhizosphere of clove plantation in Tidore Islands, North Maluku as biofertilizer- functioning PGPR was carried out.

MATERIAL AND METHOD

Soil Sampling

Soil sampling from a clove plantation on Tidore Island,was carried out at three location on different altitude. The rhizosphere soil samples were taken from several clove plant roots at a depth of 0-20 cm from the surface. Soil chemical compounds such as organic matter, C/N ratio, soluble phosphate, nitrogen content were analyzed in Faculty of agriculture, University of Brawijaya.

Isolation of Potential PGPR

Twenty-five grams of soil samples were suspended in 225 mL of 0.85% NaCl solution and shaken on rotary shaker for 60 min at 27 ^{o}C 120 rpm. Samples were diluted and transferred on Tryptic Soy Agar (TSA) medium containing 200 mg.L^{-1} L-tryptophan for the production of Indole-3 acetic acid (IAA), and Pikovskaya (PVK) agar medium for phosphate solubilization assay containing Glucose 10g; NaCl 0.2g; Ca_3PO_4 2.5g; KCl 0.2g; $MgSO_4$. $7H_2O$ 0.1 g; $MnSO_4$ 0.025 g; $FeSO_4$. $7H_2O$ 0.025 g; $(NH_4)_2SO_4$ 0.5g; Yeast Extract 1 g; and Bacto Agar 15 g in 1000 mL of Aquades. Semisolid Nfb medium [12] was used for nitrogen-fixing bacteria assay containing K_2HPO_4 0.5g; $MgSO_4$. $7H_2O$ 0.2g; $FeCl_2$. $6H_2O$ 0.015g; NaCl 0.1g; DL-Malic Acid 5g; KOH 4.8g; Yeast Extract 1 g; 0.1% Bromothymol blue 5 mL; Bacto Agar 3 g in 1000 mL Aquades [13]. Total plate count (TPC) was performed on each growth medium to determine the number of cells of each bacterial isolate.

IAA Production Assay

Production of IAA by bacteria was tested using Tryptic Soy Broth (TSB) and Salkowski reagents (1.05 g $FeCl_3.6H_2O$; 60 mL absolute H_2SO_4; 100 mL aquades). The bacteria were cultured in TSB medium containing L-tryptophan 200 mg.mL^{-1} and incubated with a rotary shaker at 120 rpm in the dark at room temperature for 3 days. Then the culture was centrifuged at 12,000 rpm for 15 min to pellet cells and two mL of supernatant were added to 3 mL Salkowsky reagent and incubated in the dark for 30 minutes at room temperature. The intensity of pink color, indicating positive IAA production, was measured using a UV-VIS spectrophotometer at a wavelength of 535 nm [14]. From the standard curve prepared with known IAA concentrations, the quantity of IAA in the culture is determined and expressed as mg.mL^{-1} [15].

Phosphate Solubilization Assay

All bacterial isolates were first tested on the Pikovskaya agar medium to observe the phosphate solubilization index as described by Gaur [16]. Quantitative analysis of phosphate solubilization was done in liquid medium as described by King [17]. Isolates were cultured in 25 mL of Pikovskaya broth and incubated for 24 to 72 hours at room temperature 28 ± 2°C. Bacterial culture was centrifuged at 12,000 rpm for 15 min to pellet cells. One mL of the supernatant was mixed with 5 ml of chloromolybidic acid and added 0.25 mL clorostannous. The bluish gray absorbance was read at 690 nm. The amount of dissolved phosphorus was calculated based on the KH_2PO_4 standard curve. The pH of medium was measured to determine the correlation of phosphate concentration to the medium acidity.

Nitrogen Fixation (Ammonium Production) Assay

The concentration of ammonium was estimated using Serra Ammonium detection Kit. The isolates were cultured in 25 mL NFb broth medium. After centrifuging the liquid culture at 12,000 rpm for 10 min at room temperature, one mL of supernatant was transferred into a test tube. One drop of $NH_4$1, $NH_4$2, and $NH_4$3 was added to the sample and mix well and the sample was left at room temperature for 5 min. The

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ammonium concentration was estimated using the provided color chart [18].

Bacterial isolates were tested for the production of ammonia in peptone water. Bacterial isolates were inoculated in 20 mL Nfb broth medium without Bromothymol Blue in each tube and incubated for 48-72 hours at 25 ± 2^{o}C. One mL of the Nessler reagent was then added in each tube. Development of yellow color is a positive indication for ammonia production [19]. The absorbance of the developing color was read at 425 nm. The quantity of ammonium production was calculated according to the NH_4Cl standard curve.

Bacterial Identification

Identification was performed on bacterial isolates that produced the highest concentrations in IAA production, phosphate solubilization, and ammonia production (NH_3). The extraction of genomic DNA from bacterial cells harvested from a 24-hour culture on NA medium was performed using the i-genomic DNA Extraction Kit (Intron) according to the manufacturer's protocol.

DNA amplification of 16S rDNA sequence was performed by Polymerase Chain Reaction (PCR) using primers 27F (5 '-GCC TAA CAC GTC ATG CAA GA-3') and 1496r (3 '-CGT AAC ATT CGG GGC TGC TGG TGC-5'). The PCR process was performed according to Zarei et al. [20] with 35 cycles consisting of denaturation at 94°C 30 s annealing 55°C for 30 s and extension of 72°C for 65 s. The PCR products were visualized using 1% agarose gel to present the DNA band and then aligned using the O'genRulerTM Ladder Mix DNA.

The correct size of PCR products was sent to 1st BASE Malaysia for sequencing. The obtained sequences were aligned using the Nucleotide BLAST to find the similarity to GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The results of BLAST and the reference strain were aligned and constructed by phylogenetic trees consisting of evolutionary relationships according to Saitou and Nei [21], Felsenstein's [22]. While similarity percentage with Tamura and Nei [23]. Evolutionary distances are shown using MEGA 6 [24].

Statistical Analysis

Quantitative data from each assay were subjected to analysis of variance (ANOVA) and means were compared by Tukey test α 0.05 using the SPSS ver. 16.

RESULT AND DISCUSSION

Soil Chemical Properties

Soil chemical properties of the sampling location (i.e. organic matter, total nitrogen, C/N ratio, available phosphate, organic matter, and acidity) are presented in Table 1. According to Table 1, Tdr_1C soil samples contain low organic matter (1.98%), moderate total N content (0.21%) and very low dissolved phosphate content (2.25 mg.kg^{-1}). Tdr_2R contains moderate soil organic matter (4.08%), moderate total N content (0.4), and low dissolved phosphate (3.1 mg.kg^{-1}). Tdr_3A contains high soil organic matter (6.5%), very high total N content (0.77%) and very low dissolved phosphate content (0.81 mg.kg^{-1}). Variation of chemical soil properties is influenced by soil nutrient cycle in nature, the process in this cycle includes physical, chemical and biology, soil formation, chemical elements that bind to soil molecules and immobilization of soil microorganisms in the process of soil nutrient formation [25].

Table 1. Chemical properties of soil sample from clove plantation

Code of Soil Sample	Content of soil chemistry								
	C Organic (%)	N. Total (%)	Categories*	Ratio of C/N	P. Bray1 (mg.kg^{-1})	Categories*	Organic Matter	Categories*	pH
Tdr_1	1.98	0.21	moderate	9	2.25	very low	3.42	low	5.9
Tdr_2	4.08	0.4	moderate	10	3.1	low	7.05	moderate	5.4
Tdr_3	6.5	0.77	very high	8	0.81	very low	11.25	high	5.1

Note: *The categories according to CSR/FAO 1983 [26]

Total Plate Count of Rhizosphere Bacteria

Estimation of the rhizosphere bacterial population was done using total plate count (TPC) and MPN methods. The TPC and MPN results were analyzed using one way ANOVA with significance (α <0.05) according to the Tukey test. The MPN method used semisolid NFb medium to observe the activity of bacteria in nitrogen fixing. This activity is characterized by white ring-shaped on the surface of medium (Fig. 2c). The highest number of colonies shown on semisolid Nfb medium was in the range of 6.8 x 10^4-7.03 x 10^7 cells.g^{-1}, TSA showed total bacteria in the range of 6.5 - 6.7 x 10^5 CFU.g^{-1} and the Pikovskaya medium shows the number of colonies in the range of 6.1 – 7.1 x 10^5 CFU.g^{-1} per gram (Fig. 1).

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The highest number of bacteria colonies were found in the Tdr_3A soil samples which is correlated with the abundance of N total content.

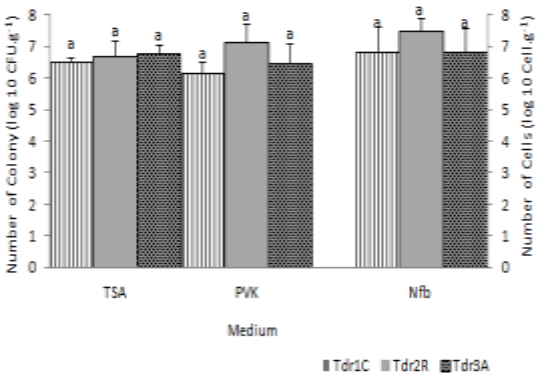

Figure 1. The bacteria population on the soil samples grouped by type of media TSA, PVK and Nfb. The same letter shows no significant different ($P < 0.05$) based on Tukey's test.

Qualitative and Quantitative Assay of PGPR Bacteria as Biofertilizer

IAA Production Assay

Qualitative assay of IAA were carried out using TSB medium containing L-tryptophan with a concentration of 200 $mg.L^{-1}$ as a precursor of IAA synthesis. The Salkowsky reagent added to the supernatant of bacterial cultures resulting in red color indicated the production of IAA (Fig. 2a). The results of the screening, obtained 7 isolates able to produced IAA.

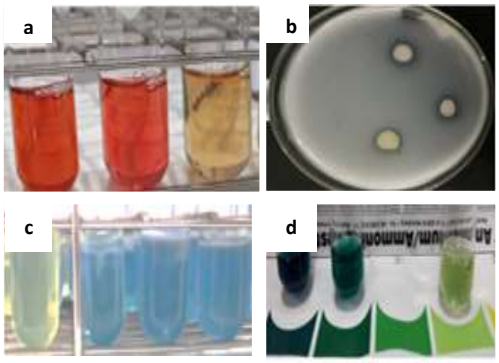

Figure 2. Qualitative and Quantitative Test of PGR.
a. Results of qualitative test of IAA Production
b. qualitative test of Phosphate solubilization
c. the indicator of Nitrogen-Fixing bacteria, and
d. qualitative test of ammonium production.

Isolation of Potential PGPR

A total of 110 rhizospheric bacteria isolates have been obtained from all soil samples. In TSA medium was obtained 65 isolates, Pikovskaya medium was 37 isolates and Nfb medium was 9 isolates. Qualitative and quantitative selection resulted 7 potential isolates as IAA producing, 7 isolates as phosphate solubilization and 5 isolates as nitrogen fixation (Table 2).

The seven isolates were then tested quantitatively based on the IAA standard curve. The results of the analysis obtained different concentrations of IAA for each isolate. The highest yielding IAA isolate was R11 with a concentration of 8.71 $\mu g.L^{-1}$, while the lowest in IAA production was A3 isolate with IAA concentration of 3.26 $\mu g.L^{-1}$ (Fig. 3).

Table 2. Number of Bacterial Isolates has Potentcy as Biofertilizer

Code of Soil Sample	Medium	Number of Isolates	Number and Code of Potential Isolates		
			IAA Production	Phosphate solubization	Nitrogen-Fixation (NH_3)
Tdr_1 C	Tryptic Soy Agar	23	2 (C4, C8)		
	Pikovskaya	12		2 (C2.2P, C2.3P)	
	Nfb Medium	3			2 (C8N,C3N)
Tdr_2 R	Tryptic Soy Agar	23	2 (R8, R11)		
	Pikovskaya	14		2 (R4P, R8P)	
	Nfb Medium	1			1 (R9N)
Tdr_3 A	Tryptic Soy Agar	19	3 (A2, A3, A10)		
	Pikovskaya	11		3 (A4.2P, A4.3P, A6P)	
	Nfb Medium	3			2 (A1N, A5N)
Total Number of Isolates		**110**	**7**	**7**	**5**

Source: Research Analysis

Ahmad et al. [27] reported that there were different concentrations of IAA production in each type of bacteria with tryptophan at different concentrations. The using of 200 $mg.L^{-1}$ concentration as the optimal precursor for IAA synthesis in this study, the presence of tryptophan consumption will affect the high levels of IAA concentrations produced by bacteria. Glickmann and Dessaux [15] reported the use of 2.5 $mg.L^{-1}$ L-tryptophan which led to a decrease in IAA production. Kesulya et al also reported 100 $mg.L^{-1}$ L-tryptophan obtained a maximum concentration of 5.82 $mg.L^{-1}$ [28].

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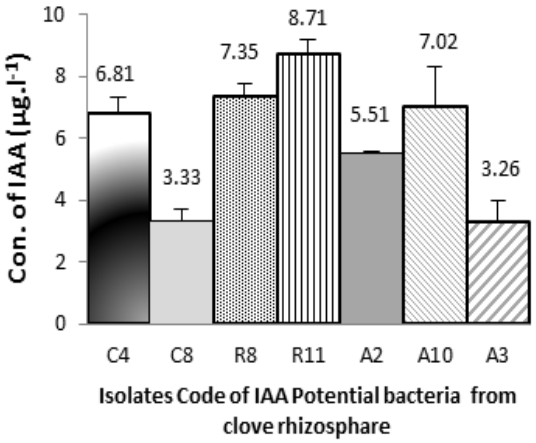

Figure 3. IAA producing by Some Isolates from Rhizospheric Soil of Clove Plantation

Some microorganisms require tryptophan to produce IAA from the IAA biosynthesis mechanism [29]. PGPR is known to affect plant growth because it can produce phytohormones, such as the auxin or in this study called IAA that has been known to stimulate cell lengthening and cell division differentiation [27] and gene regulation [30]. The results of molecular identification using the 16S rDNA gene showed that the rhizosphere bacterium isolate R11 had similarities to the *Enterobacter hormaechei* species. The construction of phylogenetic trees showed a similarity of 98.5% (Fig. 4). It has been reported that bacteria from the soil of rhizosphere have similarities with *Enterobacter sp.* and capable of producing IAA (5.09 mg.L^{-1}) as a biofertilizer agent [31].

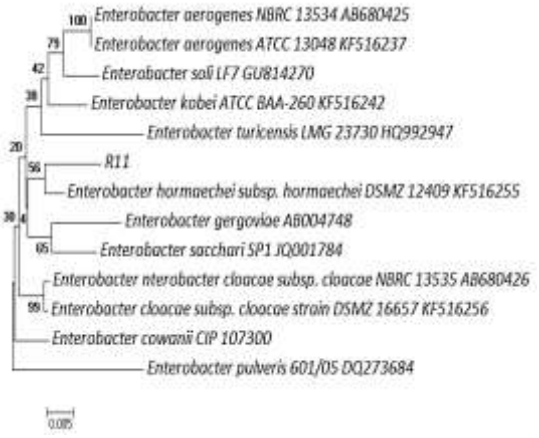

Figure 4. The Phylogenetic Tree of R11 Isolate based on 16s rDNA Gene Sequence

Phosphate Solubilization Assay

The clear zone around bacterial colonies on Pikovskaya agar medium containing Tricalcium phosphate (Ca_3PO4) shows the presence of phosphate dissolving activity. From qualitative selection, there were 7 isolates of bacteria showing solubilization activity of phosphate. Each isolate showed a phosphate solubilization index that varied in the range of 10 to 13 mm. Tukey test analysis with significance α 0.05 showed that each isolate showed significantly different in solubilization of phosphate (Table 3). The phosphate solubility index was obtained based on the clear zone around the bacterial colony. Clear zone formation is caused by the production of organic acids, the production of polysaccharides, or the activity of phosphatase enzyme from phosphate solubilizing bacteria [32]. Quantitative tests with Pikovskaya broth medium at pH 7.0 were performed to measure the concentration of solubilized phosphate (PO_4) based on a standard KH_2PO_4 curve.

Table 3. Phosphate Solubilization Index of Bacteria Isolates Isolates from Rhizospheric Soil of Clove Plantation

Isolat Code	Solubilization Index ± SD
C2.2 P	13.68 ± 3.15^{c}
C2.3P	13.02 ± 2.18^{bc}
R4P	10.34 ± 0.7^{a}
R8P	11.45 ± 1.07^{ab}
A4.2P	12.89 ± 1.78^{bc}
A4.3P	12.69 ± 2.55^{d}
A6P	13.75 ± 3.28^{c}

The assay results showed that pH medium varied in each isolate (Fig. 5). In the isolate R8 there was a correlation between the higher PO_4 concentration (8.37 µg.L^{-1}) followed by a decrease in the pH of the media to 4.09.

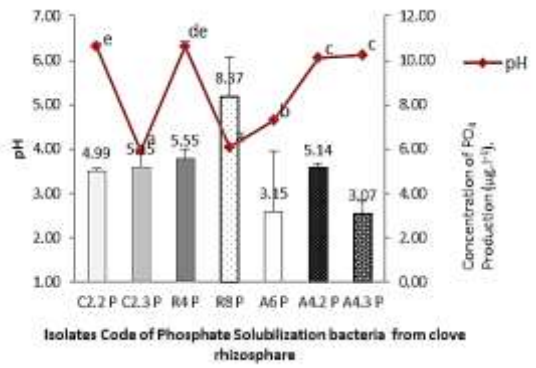

Figure 5. Correlation of pH and PO_4 Concentration

Other isolates showed significant variations based on statistical analysis. Acid levels in bacterial media indicate the production of organic acids which are common mechanisms of phosphate dissolution [32,33]. Molecular identification was performed on isolates with the

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highest phosphate concentration in R8P isolates. Identification using a sequence of 16S rDNA genes showed that R8P isolate had similarities with *Burkholderia multivorans* at 95.5% (Fig. 6). *B. multivorans* WS-FJ9 has been known to dissolve phosphate and decrease pH from initial pH [34].

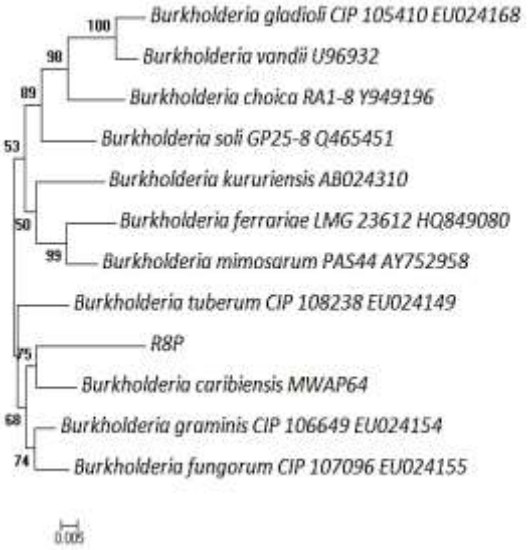

Figure 6. The phylogenetic tree of A1N isolate based on 16s rDNA gene sequence

Nitrogen Fixation (Ammonium Production) Assay

The nitrogen fixation assay shows the change of media color from green to blue and has a white follicle on the sub-surface medium (Fig. 2c). The TPC results showed that nitrogen fixation bacteria had the highest number of cells in the range of 3.5 x 10^5-6.5 x 10^6 cfu per gram of soil (Fig. 1). The result of the qualitative test using Serra ammonium test obtained 5 isolates of nitrogen fixation bacteria produce ammonium with varying concentration that is blue and dark green color showed high ammonium content while light green contains low ammonium concentration (Fig. 7). Qualitative test of ammonium concentration based on NH_4Cl standard curve obtained 5 isolates with different concentration. The isolate that produces the highest concentration is A1N.

Results of molecular identification based on the sequence of 16s rDNA gene showed that A1N isolate had a similarity of 98.7% with *Stenotrophomonas maltophilia* species (figure 8). It is known that *S. maltophilia* bacterial strains are capable of producing ammonium with various carbon sources [18].

Bacteria associated with clove plants in the rhizosphere are able to improve nitrogen fixation that has been widely reported as a plant growth promoter [35]. The activity of nitrogen fixation organisms is generally active in plant roots.

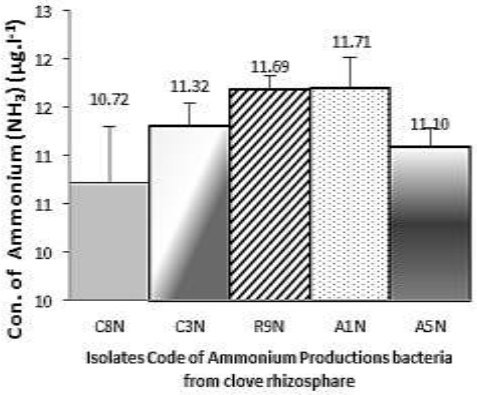

Figure 7. NH_3 Production of Bacterial Isolates

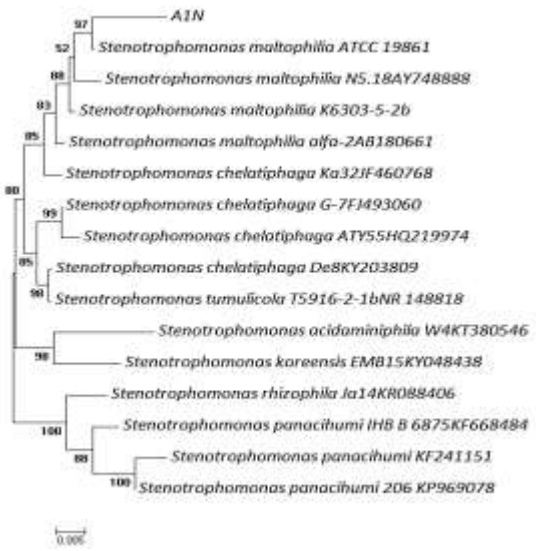

Figure 8. The phylogenetic tree of R8Pisolate based on 16s rDNA gene sequence

CONCLUSION

This study obtained *Enterobacter hormaechei*, *Burkholderia multivorans* and *Stenotrophomonas maltophilia* that are potential for biofertilizer agents. The recommendation for further research is Application of synergism or antagonism then the isolates perform as consortium and prepare for biofertilizer and apply to plants of three isolates in a greenhouse to determine their effect on crops, without exception to commodity crops such as cloves. In addition, a biochemical and physiological characterization and identification of encoding genes are necessary to support the identified molecular data of bacterial species.

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Comparing Vector-host and SEIR models for Zika Virus Transmission

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Abstract

Some mathematical models to describe Zika virus transmissions have been analyzed. In this study, we construct two models of Zika virus transmission. The first one is the vector-host model. It considers the human population as host and mosquito's population as the vector. The second model is where there is only infected human population who act as transmitter without the existence of infected mosquitoes in the population. The impact of modeling assumption of Zika virus is studied by analyzed the reproduction number using Next Generation Matrix (NGM) method. Formerly, we compare the dynamics of the two models by interpreting the reproduction number of each model. Biologically, the two models cause a similar effect. If the reproduction number is less than one, then the disease is extinct. Otherwise, an endemic condition exists. The numerical simulation also used to explain the comparison of two models. The recovery and the transmission period are solved to compare these two cases.

Keywords: comparison, mathematical model, reproduction number, SEIR, Zika Virus.

INTRODUCTION

Zika virus is spread by mosquito bites which are *Aedes aegypti* and *Ae. albopictus*. It is also transmitted by sexual intercourse, and blood transfusion. Zika virus can be moved horizontally from a pregnant woman to her embryo. It causes a certain birth defect when it is infected pregnant women. Based on the latest update of CDC, Zika virus also can be transported to animals, namely monkeys and apes. No vaccine or medicine exists to prevent Zika virus [5].

Zika virus will become a serious problem when it infects pregnant women because it relates to congenital abnormalities including microcephaly, spontaneous abortion, and intrauterine growth restriction. Further, for non-pregnant women also for men, it causes Guillain-Barre Syndrome. Transmission between humans and *Ae. Aegyepti* mosquitoes can increase the epidemics of Zika [1,3].

The CDC is reporting that Zika virus has been transmitted around the United States. Women and men, who travel to the United States, should be aware and follow steps to prevent mosquito bites to reduce their risk for Zika. For at least 6 months, men should using a condom or not having sex, and women also using a condom or not having sex for at least 2 months from August 29, 2017 [6].

Some mathematical models have been studied Zika virus epidemiology, also the vector-host Zika transmission. Andayani analyzed the dynamics of Zika virus transmission with the vector-host model, which is SIR model of host and SI model for the vector [8]. The existence of R_0 (reproduction number) is used to identify the stability of the system. If R_0<1 the DFE is stable locally and globally, otherwise the END is stable locally and globally. It is appropriate with CDC report, which is Zika does not cause death [8].

Pandey [4] investigated the comparison of vector-host and SIR model of dengue transmission and fit the models to data for validates. Then estimate the parameter by using Markov chain Monte Carlo (MCMC). Jafaruddin [2] constructed a host-vector for West Nile Virus transmission model in mosquito chicken populations. The dynamics analysis investigated by interpreting the reproduction number. In this paper, we investigate the impact of modeling assumptions on epidemiological for Zika Virus by using reproduction number interpretation. In the final section, the two models are compared by numerical simulations.

MATHEMATICAL MODELS

We construct two mathematical models of Zika virus transmission. First, in which the mosquito is existed in the area and spread Zika virus to human, it is a vector-host model. Other

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we assume there exist mosquito, and the human is infected by traveling from Zika transmits area.

Vector-Host Model

The vector-host model of Zika virus transmission represented by the following nonlinear differential equations:

$$\begin{cases} \frac{dH_S}{dt} = m_1 - c_1 H_S V_I - c_2 H_S H_E - \mu_H H_S, \\ \frac{dH_E}{dt} = c_1 H_S V_I + c_2 H_S H_E - \alpha H_E - \mu_H H_E, \\ \frac{dH_I}{dt} = \alpha H_E - \gamma H_I - \mu_H H_I, \\ \frac{dH_R}{dt} = \gamma H_I - \mu_H H_R, \\ \frac{dV_S}{dt} = m_2 - c_3 H_I V_S - \mu_V V_S, \\ \frac{dV_I}{dt} = c_3 H_I V_S - \mu_V V_I, \end{cases} \quad (1)$$

where $H_S, H_E, H_I, H_R, V_S, V_I$ stand for suspected human, exposed human, infected human, recovery human, suspected vector, and infected vector, respectively. In this case, we assume all parameters are positives. The parameter m_1 denotes the growth rate of the human; m_2 denote the growth rate of mosquito. Then c_1 denote the rate of direct transmission of the virus, c_2 denote the rate of transmission from exposed to a suspected human, and c_3 denote the rate of transmission from infected human to suspected vector. The parameter α is per capita infection rate of the exposed human, and γ is per capita recovery rate of the infected human. The parameter μ_H means the death rate of human, and μ_V means the death rate of the vector (mosquito).

Biologically, the trajectories of the system (1) can be explain by identified the reproduction number of the system. The reproduction number of system (1) can be compute by using Next Generation Matrix (NGM) method [1], which is

$$R_0 = \frac{(\mu_V c_2(\mu_H + \gamma) + \alpha c_1 c_3 m_2) m_1}{\mu_H \mu_V^2 (\mu_H + \alpha)(\mu_H + \gamma)}. \quad (2)$$

Based on equation 2, it identified that all parameters influenced the value of reproduction number. The rate of direct transmission of virus(c_1), the rate of transmission from exposed to a suspected human, and the rate of transmission from infected human to suspected vector (c_3) which causes the mosquitoes infecting virus, increase the value of reproduction number. When the parameters of the rate of direct transmission of virus, the rate of transmission from exposed to suspected human, and the rate of transmission from infected human to suspected vector are increasing, then the reproduction number also increases. In addition, when the growth rate of human (m_1) and the growth rate of mosquito (m_2) are increasing, then the reproduction number also increase. It is suitable for real cases. If the number of population is greater, then the interaction between human and mosquito will be greater than usual. This factor causes advances the spread of infection.

SEIR Model

The SEIR model of Zika virus transmission represent by the following nonlinear differential equations:

$$\begin{cases} \frac{dH_S}{dt} = m_1 - c_1 H_S H_I - c_2 H_S H_E - \mu_H H_S, \\ \frac{dH_E}{dt} = c_1 H_S H_I + c_2 H_S H_E - \alpha H_E - \mu_H H_E, \\ \frac{dH_I}{dt} = \alpha H_E - \gamma H_I - \mu_H H_I, \\ \frac{dH_R}{dt} = \gamma H_I - \mu_H H_R, \end{cases} \quad (3)$$

where H_S, H_E, H_I, H_R, stand for suspected human, exposed human, infected human, recovery human, respectively. The parameter m_1 denotes the growth rate of the human. The parameter c_1 means the rate of direct transmission of virus from infected human, and c_2 denote the rate of transmission from exposed to suspected human. The parameter α denotes infection rate of the exposed human, γ denote recovery rate of the infected human, and μ_H means the death rate of the human.

The interpretation of the system (3) is determined by analyze the reproduction number R_0, which is computed by using Next Generation Matrix (NGM) method. The following equation is the reproduction number for system (3), such as

$$R_0 = \frac{(\mu_H c_2 + \alpha c_1 + c_2 \gamma) m_1}{\mu_H (\mu_H + \alpha)(\mu_H + \gamma)}. \quad (4)$$

All parameters of the SEIR model are influenced with the value of reproduction number. The rate of interaction among human, which is the rate of infected and suspected human, and the rate of transmission is from exposed to a suspected human, are directly proportional with reproduction number. It is due to the more frequent interaction between human and mosquito then the chances of infection become greater. Moreover, the number of

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infection spread can be influenced by the number of population which contact with the growth rate of the human. When the growth rate of the human is increasing, then the probability of interaction which is caused infection also increased.

SIMULATION AND RESULTS

The simulation of these systems also needs to see the trajectory of the system. In this study the simulation of the system are analyzed using software Matlab. At the first case, we choose the value of parameters are

$c_1 = 0.1;\ c_2 = 0.1;\ c_3 = 0.01;\ \alpha = 0.1;\ \mu_H = m_1 = 0.00004;\ \mu_V = m_2 = 0.05;$

and $\gamma = 0.3$. Based on the chosen parameter we have $R_{0VH} > 1$ and $R_{0SEIR} > 1$. Then the simulation of the system (1) and (3) are shown by the following :

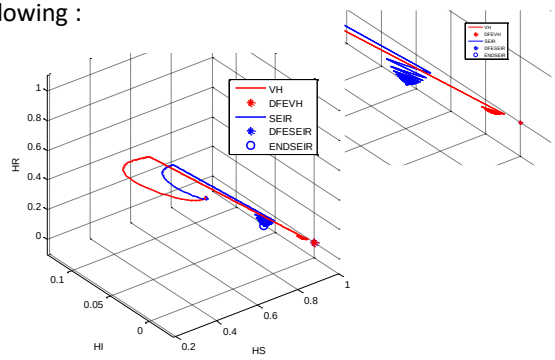

Figure 1. The Simulation of Case 1

The second case of these simulations is when the reproduction numbers are less than one. Then choose the parameter values are

$c_1 = 0.1;\ c_2 = 0.01;\ c_3 = 0.1;\ \alpha = 0.09;\ \mu_H = m_1 = 0.00004;\ \mu_V = m_2 = 0.05;$

and $\gamma = 0.3$. Then we have the following simulation:

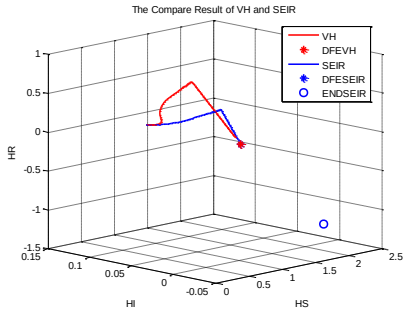

Figure 2. The Simulation of Case 2

Based on figure 2, we can see that by using the parameter values on cases 2 the trajectories of the system (1) and (3) are tends to the disease free equilibrium. Biologically this case means according to that parameters of the case 2, the populations will be free from Zika virus.

The third case is when $R_{0VH} < 1$ and $R_{0SEIR} > 1$. Then choose the parameter values are $c_1 = 0.1;\ c_2 = 0.01;\ c_3 = 0.2;\ \alpha = 0.1;\ \mu_H = m_1 = 0.00004;\ \mu_V = m_2 = 0.05;$

and $\gamma = 0.3$. The simulation of the system (1) and (3) are in the Figure 3.

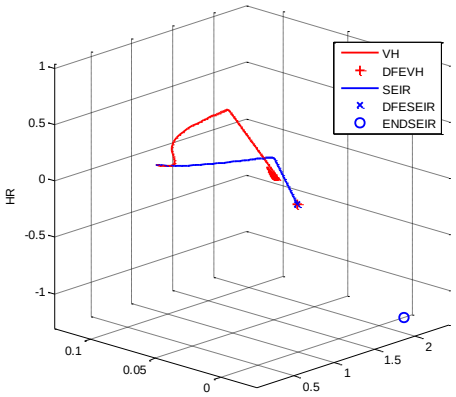

Figure 3. The simulation of case 3

By the figure 3 we can see the trajectories of system (1) will tends to endemic and the system (3) will tends to disease free. It is consistent with the previous simulation which is epidemic Zika virus in case $R_0 > 1$, otherwise the Zika virus will be free in case $R_0 < 1$.

Finally, the comparison of the vector-host and SEIR models are describe by the following table:

Table 1. The Comparison of Vector-Host and SEIR Models

Reproduction number	Characteristic	Vector-host model (days)	SEIR model (days)
$R_0 > 1$	Recovery time	32	20
	Epidemic	21	31
$R_0 < 1$	Recovery time	25	40
	Epidemic	20	40

CONCLUSION

According to the previous explanation, we have the following conclusion.

1) In generally, Zika virus will be free in the area when the reproduction number is less than one. Otherwise, the Zika virus will epidemic.
2) If reproduction number is greater than one, the recovery time of vector-host model is 32 days and the SEIR model is 20 days. Moreover, the Zika virus will be epidemic in the area in 21 days for the vector-host model, and 31 days in the SEIR model.

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3) If reproduction number is less than one, the recovery time of vector-host model is 25 days and the SEIR model is 40 days. Additionally, the Zika virus will be epidemic in the area in 20 days for the vector-host model, and 40 days in the SEIR model.

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Isolation and Identification of Antagonistic Bacterium against Pathogens of Bacterial Tuber Rot of *Amorphophallus muelleri*

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Abstract

Rhizosphere bacteria have the ability to protect the host plants from the infection of pathogenic microorganisms. This study aimed to identify rhizosphere bacteria that were capable of inhibiting the growth of bacterial isolates that cause tuber rot of *Amorphophallus muelleri*. Rhizosphere bacteria were isolated using Nutrient Agar medium by pour plate method. Isolates were subjected to antagonistic assay against several bacterial isolates from the rotten tuber of *A. muelleri* using dual culture method. The potential isolate was identified based on 16S rDNA sequence. Isolate R7 showed the strongest inhibition to the growth of bacterial isolates from rotten tuber with an inhibition zone diameter of 19.66 mm. The 16S rDNA sequence of isolate R7 R7 was 99.7% similar to *Delftia tsuruhatensis* PCL1755. The isolate was potential to be developed as phytopathogen control agent.

Keywords: *Amorphophallus*, antagonistic bacteria, rhizosphere bacteria, rotten tuber, 16S rDNA.

INTRODUCTION

Tubers of *Amorphophallus muelleri* Blume. contain a high concentration of glucomannan. Glucomannan is a starch that can be used as a thickener agent in foods such as noodles [1]. Glucomannan in *A. muelleri* tubers has a high economic value and is expected to improve Indonesian economy. Therefore, it is necessary to increase the production of *A. muelleri* tuber. Nowadays, the problem is pathogenic microorganisms that attack the tubers either under the ground or even post-harvest. The common pathogenic microorganisms that attack the tubers are *Erwinia caratovora* and *Pectobacterium caratovora* on *Amorphophallus konjac* tuber [2,3] and *Dickeya dadantii* on *Amorphophallus rivieri* [4,5].

To control the tuber rotting, farmers use chemicals such as pesticides. Continuous application of synthetic pesticides caused negative impacts on the environment [6]. The residue of the pesticides in the soil as well as on the plant parts (fruits, leaves, and tubers) [7,8] were indirectly or directly toxic to humans [9,10].

Therefore, biological agents are required as antagonistic agents to control the growth of bacterial rot pathogens. One alternative that can be used as an ecologically safe and effective antagonistic agent against pathogens is the rhizosphere bacteria [11,12,13]. Rhizosphere bacteria are present in the soil around plant roots. They have many benefits for plants such as promoting nitrogen fixation, phosphate and potassium solubilization, production of phytohormones and antibiotics [14,15,16].

Some rhizosphere bacteria that act as antagonistic agents are *Bacillus*, *Pseudomonas*, *Pantoea*, and *Lactobacillus*. One of the antagonistic bacteria that can inhibit the growth of *E. caratovora* (one of the causes of tuber rot bacteria) is *Bacillus subtilus*. It is able to produce antibiotic compounds such as bacitracin, bacillin, bacillomycin B, difficidin, oxydifficidin, lecithinase, and subtilisin. These compounds cause shrink in cells so that bacterial cells of *E. carotovora* will lose water and experience plasmolysis [17,18]. In addition, *Bacillus amyloliquefaciens* is also able to inhibit the growth of *Erwinia* bacteria which causes postharvest tuber rot [19]. This research aims to analyze the potency of isolated rhizosphere bacteria to inhibit *A. muelleri* tuber rot bacteria and to identify the potential rhizosphere bacteria based on 16S rDNA sequence.

MATERIAL AND METHOD

Isolation of Rhizosphere Bacteria

Soil samples were obtained from the rhizosphere of *A. muelleri* from Rejosari Village, Bantur City, East Java Province, Indonesia. The soil was taken at a depth of 5-10 cm of topsoil and kept in plastic bags in the isotherm box [20]. At each sampling point, abiotic factors including

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the ambient and soil temperature, and light intensity were measured directly at the field; while moisture, pH, and organic matter of soil were measured in the Laboratory of Microbiology and Laboratory of Ecology, University of Brawijaya. The data of abiotic factors were analyzed using ANOVA and Tukey test with five percent significant differences.

Twenty five grams soil sample was diluted at 10^{-1} - 10^{-7} in sterile physiological saline solution. Each suspension of 0.1 mL was transferred into Nutrient Agar (NA) medium in the Petri dishes according to pour plate method and incubated at room temperature for 72 hours. Each bacterial colony was enumerated and purified according to the spread plate method. The pure culture of rhizosphere bacteria in the NA medium was stored at 4°C [21]. The diversity of bacterial communities was determined based on the Simpson Diversity index according to the equation 1 [22-25].

$$D = 1 - \{\sum n\,(n-1) \,/\, N\,(N-1)\} \quad \text{.....(1)}$$

Description:
D = Simpson Diversity Index
n = Number of individual types of i
N = Total number of individuals

Isolation of Pathogenic Bacteria from Rotten Tuber of *A. muelleri*

Bacterial pathogens of *A. muelleri* tubers were isolated according to Ashmawy et al. [26] with modifications. The rotten of tuber was cut into a dimension of 1.0 x 0.5 x 0.2 cm^3. It was sterilized by soaking in 1.0% NaOCl solution for two minutes and rinsed two times with sterile ddH_2O. The pieces of sterilized rotten tuber were weighed to 25 g. They were blended with 225 mL sterile physiological saline solution and diluted at 10^{-1} - 10^{-7}. Sample suspension of 0.1 mL was inoculated into NA medium according to pour plate method and incubated at room temperature for 48 hours. The bacterial colony was purified according to the spread plate method and pure cultures were stored at 4°C.

Antagonist Assay of Rhizosphere Bacteria Against *A. muelleri* Tuber Rot Bacteria

The antagonistic assay among bacterial isolates was done using dual culture method [27]. The 100 µL suspension of isolated tuber rot bacterium with 10^6 cells.mL^{-1} density was spread on NA medium and directly incubated at 4°C for 4 hours. The NA agar plates were perforated to make 6 mm wells. The wells were inoculated with 60 µL of 10^7 cells.mL^{-1} density of antagonistic rhizosphere bacteria. The cultures were incubated at room temperature for 72 hours. The growth inhibition of tuber rot bacteria was indicated by the clear zone around the well. The diameter of the inhibition zone were measured and the data was analyzed using ANOVA and Tukey test with five percent significant differences.

Identification of Potential Rhizosphere Bacteria

Rhizosphere bacteria with the highest potency to inhibit tuber rot bacteria was identified based on phenotypic and phylogenetic characters. Phenotypes of bacteria were characterized based on Bergey's Manual of Systematic Bacteriology [28,29]. The phenotype of bacteria consists of the colony and cell morphology, biochemical, and physiological characters. Phylogenetically, the bacteria isolate was identified based on 16S rDNA sequence similarity. The genomic DNA of the selected isolate was extracted using Heat Treatment method [30]. The sequence of 16S rDNA was amplified using universal primer of:

27f (5'-AGAGTTTGATCCTGGCTCAG-3'), and
1492r (5' CTACGGCTACCTTGTTACGA-3')

The composition of 50 µL PCR reaction was 25 µL PCR master mix, 19 µL ddH_2O, 2 µL of each primer, and 2 µL of DNA template. The components were homogenized and 16S rDNA was amplified using the PCR program at 35 cycles includes: predenaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s; and followed by post extension at 72°C for 5 minutes.

The amplicon of 16S rDNA was verified with electrophoresis on 1.5 % agarose gel. The amplicon of 16S rDNA was purified and sequenced at First Base, Malaysia using Automatic Sequencer Analyzer ABI 3130. The sequence of 16S rDNA was edited using the Sequence Scanner V.1 program and the sequences were combined using the BioEdit V.7.2.5 program. The 16S rDNA sequence of the isolated bacteria was aligned together with 16S rDNA reference that obtained from the NCBI database. The phylogenetic tree was constructed based on Neighbor-Joining with bootstrap 1000 using the MEGA 6.00 program [31,32,30].

RESULT AND DISCUSSION

Density and Diversity of *A. muelleri* Rhizosphere Bacteria

A total of isolates of *A. muelleri* rhizosphere bacteria were obtained from three locations.

Based on Simpson's diversity index, community diversity of the rhizosphere bacteria was in the range of 0.84 – 0.87 (Fig. 1). It indicated that the community was highly diverse and there were no dominant species [25]. However, the density of rhizosphere bacteria was relatively low in the range of 3.54 - 3.56 Log 10 CFU.g^{-1} (30.0 – 59.0 x 10^2 CFU.g^{-1} soil (Fig. 2). The low density might be caused by the low organic matter and low moisture of the soil. Since those parameters are limiting factors for the growth of several rhizosphere bacteria. Soil bacteria require a minimum of 2% soil organic matter and 60% soil moisture for support of the optimal growth [33,34], while in this experiment the organic matter and soil were less than 0.2 and 32%, respectively.

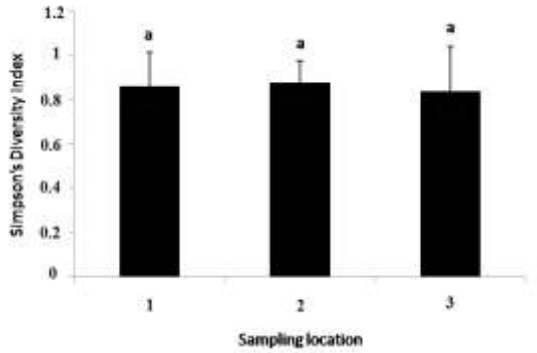

Figure 1. The Bacterial Diversity at rhizoSphere of *A. muelleri* Plantation. The same notation show diversity index does not significantly different among the sampling location (p> 0.05).

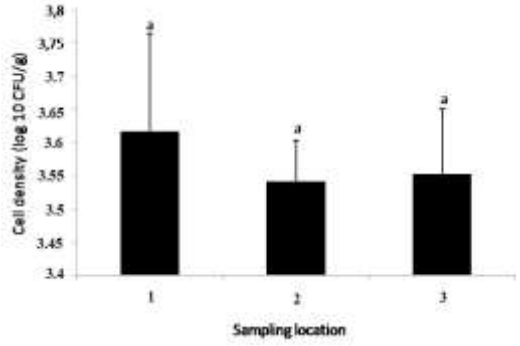

Figure 2. The Bacterial Density at Rhizosphere of *A. muelleri* Plantation. The same notation show cell density does not significantly different among the sampling location (p> 0.05)

Environmental for the three sampling locations were presented in Table 1. The soil parameters especially plant and soil type, and farm practice affects the diversity and density of soil microorganisms and plants growth [34]. The low nutrient and water availability in the soil may inhibit metabolism and growth of micro-organisms. Soil organic matter plays an important role in soil structure and texture, microaggregate stability, soil moisture and pH, nutrient availability, and microorganism density and diversity [25,35]. In all locations, soil moisture was low due to low content of organic matter. Organic matter will increase soil moisture and decrease soil pH. The increase of soil organic matter will increase the content of organic carbon which utilized by bacteria as carbon and energy source [36]. Soils of *A. muelleri* plantation were acid, with pH value 3.78 – 4.13. In general, bacteria grow in the pH range 5-7 as optimum conditions [37]. The acidity of the soil may be caused by contamination of metals derived from the use of chemicals (fungicides and pesticides), pollution, organic fertilizers, and household waste disposal [38].

Table 1. Enviromental Parameters at Sampling Locations

Parameter	Location 1	Location 2	Location 3
Soil pH	4.13 ± 0.12	3.79 ± 0.03	4.01 ± 0.01
Soil Humidity (%)	30 ± 1	30.7 ± 1.53	30 ± 1.73
Soil organic matter (%)	0.16 ± 0.02	0.17 ± 0.03	0.16 ± 0.02

Antagonistic Potency of Rhizosphere bacteria

Rhizosphere bacteria consisting of nine isolates with a density of 10^7 CFU.mL^{-1} were tested for their inhibition against three tuber rot bacteria of *A. muelleri* (PT4, PL9, and PR11). The rhizosphere bacterial isolates had different potency in inhibiting and only four isolates can inhibit tuber rot bacteria (Fig. 3). Isolate R3 was not able to inhibit isolate PT4 but it inhibited isolate PL9 and PR11 with inhibition zone diameter 12 and 10 mm, respectively. Isolate R5 was able to inhibit PT4, PL9, and PR11 with inhibition zone diameter of 2.07, 5.93, and 6.96 mm respectively. Isolate R7 was able to inhibit the three isolates of tuber rot bacteria, PT4, PL9, and PR11 isolate with inhibition zone diameter of 19.66, 11.24, and 12.42 mm. Isolate R9 was only able to inhibit isolate PT4 and PL9 with inhibition zone diameterof 2.00 and 6.21 mm, respectively.

Isolate R7 had the highest inhibition potency among the other *A. muelleri* rhizosphere bacteria. The isolate was able to inhibit the three isolates of tuber rot bacteria and categorized in the high potential with inhibition zone more than > 10 mm [39]. Based on previous experiment [40], *Bacillus circulans* rhizosphere bacterium was able to inhibit *Escherichia coli*, *Bacillus subtilis,* and *Serratia marcescens* with inhibition zone diameter of 11, 12, and 6 mm, respectively.

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Antagonistic bacteria have a mechanism to inhibit the growth of pathogens. The inhibition is performed by producing antimicrobial compounds such as enzymes capable of attacking the main cell components of pathogens [41,42]. Antimicrobial compounds produced by antagonistic bacteria cause damage to the cell membrane and shrink the cell. Furthermore, the activity of the bacteria becomes disturbed and causes it to die. Another antibiotics compounds are responsible for inhibition of protein synthesis process. The synthesis is inhibited when exposed to antibiotic compounds and cause cell death of pathogens [43,44].

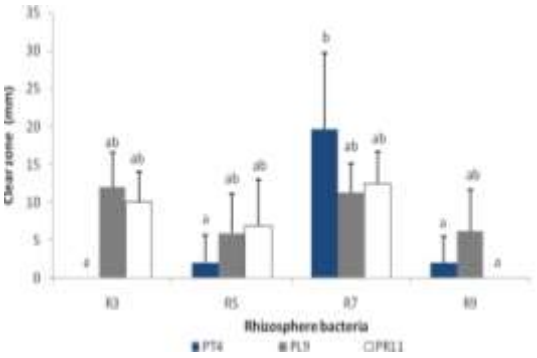

Figure 3. The Potency of *A. muelleri* Rhizosphere Bacteria to inhibit tuber rot bacteria (PT4. PL9, and PR11). The same notation show diversity index does not significantly different among the sampling location (p> 0.05)

The inhibition mechanism of tuber rot bacteria by isolate R7 was antibiosis. Antibiosis is the ability of antagonistic isolates to produce secondary metabolites of antibiotics, siderophores, and several enzymes such as chitinase, protease, and cellulase enzymes that inhibit the growth of target cell [45,46]. The activity of antibiosis was determined by clear zones; it proved that rhizosphere bacteria could produce antibiotics that inhibited the growth of tuber rot bacteria [47,48]. In addition, inhibition potency of antagonistic bacteria may also be caused by antagonism such as competition of root colonization and nutrient [49-52]. Antagonism is the ability of antagonistic microorganisms to produce antibiotics that can kill pathogenic microorganisms [53]. Competition of space and nutrients cause limitation of nutrition and space for growth of pathogens [37,54,55,56].

Identification of *A. muelleri* Rhizosphere Bacterium as Antagonist of Tuber Rot Bacteria

The isolate R7 (Fig. 4) had a 99.7% similarity of 16S rDNA sequence with *Delftia tsuruhatensis* PCL1755. The phenetypic data of isolate R7 was used as additional data to support the results of phylogenetic identification (Table 2). The strain is isolated from various soil types such as eggplant, tomato, pepper, and avocado and it showed a widespread inhibition to the growth of *Fusarium oxysporum f. sp. radicis lycopersici* [57,58].

The *D. tsuruhatensis* is one of the rhizosphere bacteria that act as a biocontrol agent or plant growth promoting rhizobacteria (PGPR) [59]. Some strains of these bacteria have the ability to degrade the inorganic pollutants [60]. The natural habitat of these bacteria are dispersed in soil, activated sludge, and also in contaminated environments. Bacteria *D. tsuruhatensis* was first isolated from active sludge and acted as degradation of terephthalate or plastic (environmental pollutants) [57,58,61]. One strain of *Delftia* is *D. tsuruhatensis* HR4 had the ability to control disease in rice caused by *Xanthomonas oryzae, Rhizoctonia solani*, and *Pyricularia oryzae* [60,62,63]. In some studies, although it has the ability as an antagonist agent against pathogens, the mechanisms of synthesis and antimicrobial compounds owned by these bacteria is still unclear. This is due to the lack of research on these bacteria. The strain of *D. tsuruhatensis* MTQ3 has the ability to inhibit the growth of *Ralstonia solancearum* and *Phytophtohora nicotinae* [58].

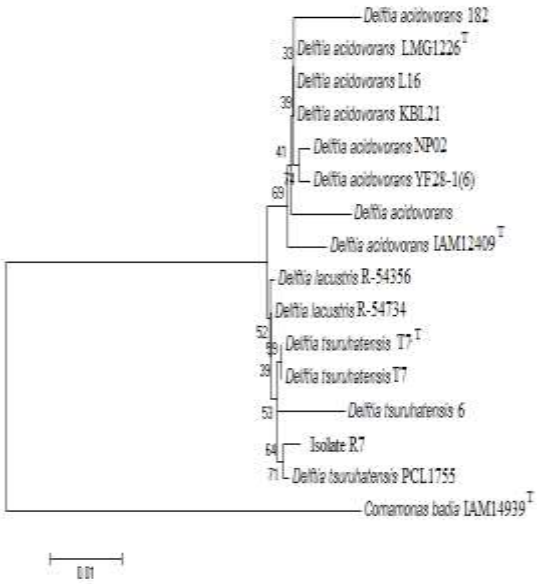

Figure 4. Phylogeny Tree of Rhizosphere Bacteria and Reference Isolates Based on 16S rDNA Sequence according to Neighbor-Joining Algorithm

Table 2. Phenotypic Characteristics of Rhizosphere Bacterium Isolate R7

Characteristics of phenotypes	Isolate R7
Colony	
Shape	Irregular
Colony Elevation	Convex
Configuration	Wave
Texture	Smooth
Consistency	Like butter
Colors	Cream
Cell	
Gram staining	Negative
Cell Shape	Rod
Catalase	Negative
Nitrate Reduction	Positive
Simmon Sitrat	Positive
Methyl Red Test (MR)	Negative
Voges Proskauer Test	Negative
Sugar fermentation	
- Glucose	Positive
- Sucrose	Positive
- Lactose	Negative
- Mannitol	Negative
- Maltose	Positive
Ability to live in salinity	
- 0%	Positive
- 5%	Positive
- 10%	Negative
Aerobic growth	Positive

CONCLUSION

The rhizosphere bacteria isolate R7 of *A. muelleri* had the highest potency as antagonist of tuber rot bacteria. The isolate R7 had 99.7% similarity with *Delftia tsuruhatensis* PCL1755 base on 16S rDNA sequence. This isolate is potential to be developed as biological control/biopesticide agent against tuber rot bacteria of *A. muelleri*.

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White Spot Syndrome Virus (WSSV) Detection at Traditional Ponds of *Lithopenaeus vannamei* in Pasuruan District

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Abstract

Vaname shrimp (*Litopenaeus vannamei*) is a type of shrimp that widely cultured in Indonesia. Pasuruan is one of the districts where there are many ponds that culture Vaname shrimp (*L. vannamei*) traditionally. The occurrence of a decrease in production due to White Spot Syndrome Virus (WSSV) virus attacks that cause a lot of losses for farmers, thus it needs a preventive effort by doing early warning and monitoring on the existence of the virus. The study was conducted from April to May 2018 with the aim of obtaining data on the presence of WSSV virus and its prevalence at traditional Vaname shrimp (*L. vannamei*) farms in Pasuruan District. The sampling location is located in Pasuruan District consisting of three locations namely Bangil, Kraton, and Rejoso with each has 10 ponds (total of 30 ponds sites). Detection of WSSV was using Nested PCR with shrimp bodyparts taken are swimming foot, road leg, and tail. PCR results in 848 bp and 333 bp indicated the presence of WSSV infection in the Bangil and Rejoso ponds where the prevalence rate of WSSV attack in each region differs, i.e. Bangil 0 - 15%, Kraton 0% and Rejoso 0 - 15%.

Keywords: PCR, Prevalence, Shrimp, *Vannamei*, WSSV.

INTRODUCTION

Vaname shrimp (*Litopenaeus vannamei*) is one of the many shrimp species cultivated in Indonesia. This type of shrimp dominates farming in Indonesia both intensively and traditionally. Pasuruan is one of the districts that cultivate many traditional Vaname shrimp (*L. vannamei*). The cultivation of Vaname shrimp (*L. vannamei*) developed in Pasuruan District is located in Sub-districts of Bangil, Kraton, and Rejoso [1].

But in these sub-districts, there was a decrease in Vaname shrimp production caused by White Spot Syndrome Virus (WSSV) attack on Bangil, Kraton and Rejoso. The virus is very malignant and very difficult to stop with a mortality rate of 100% in the shrimp age of cultivation between 40-60 days in just 3 to 10 days from the clinical symptoms appear. It was causing a lot of losses for the farmer so WSSV became the most serious pathogen for shrimp farming and has destroyed the shrimp industry in various countries [2-4].

Based on the above explanation, it is necessary to do a preventive effort, i.e. by doing early warning and monitoring of the existence of the virus in the environment of traditional ponds during the cultivation period. This study aims to obtain data on the presence of WSSV virus and its prevalence in traditional Vaname shrimp (*L. vannamei*) farm in Pasuruan District.

MATERIALS AND METHODS

Sampling Sites

The method used in this research is survey method with an epidemiological and observational approach. This research was conducted from April to May 2018 at traditional Vaname shrimp (*L. vannamei*) farm in Pasuruan District consisting of three locations of Sub-districts, i.e. Bangil, Kraton and Rejoso.

The number of traditional ponds sampled in Bangil sub-district are 10 ponds, Kraton Sub-District 10 ponds, and Rejoso Sub-District 10 ponds. Thus, the total samples taken are 30 ponds sites.

Shrimp sampling

Shrimp's size taken is four to seven cm long, weighing between six to 10 grams, sample age was one to two months. The number of shrimp taken from each pond is two. The sample is then preserved using 75% alcohol, put into Styrofoam/ cool box then take it to the laboratory.

WSSV tested using Nested Polymerase Chain Reaction (PCR)

Detection of WSSV was using Nested PCR with the shrimp bodyparts taken is the foot pool, foot path, and part of the tail. PCR analysis was performed in the following way:

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Extracting DNA

The 10-20 mg Vaname shrimp tissue was cut of tissue into small pieces or can also be crushed, after which it is placed on a 1.5 mL microcentrifuge tube with 180 µL ATL Buffer, plus 20 µL of K. Protein material was mixed by Vortex and incubated at a temperature of 56°C in 2-3 hours or until all of the tissue was lysis perfectly. The sample at the time of incubation can be mixed occasionally by vortex or can also be placed on thermomixer, rocking plate or water bath shaker. After vortex-mixed for 15 seconds, it added with 200 µL absolute ethanol (96-100%), vortex-mixed again until it mixed properly. The mixture in the third way is taken by using the pipette including with the sediment into the Rneasy Mini Spin column.

The mixture/sediment/pellet was placed into 2 mL collection tube. After that, the mixture/ sediment/pellet in the tube was centrifuged with a speed of 8000 rpm for approximately one minute. Then collect the tube and the solution in the tube can be removed. Then Rneasy Mini Spin collum is placed on collection tube of two mL size, after which added 500 µL buffer AW2, centrifuged for three minutes with speed 14000 rpm to dry Rneasy membrane. Collection tubes and solutions are discarded. Rneasy Mini spin column placed on a microcentrifuge tube (1.5-2 mL) added 200 µL AE Buffer for one minute and later centrifuged for one minute at 8000 rpm.

Amplification

This step was using ICP 11 Primer. The primer of mix, positive control, and negative control was placed on the cold box. Then centrifuge is done so that all the liquid collected at the bottom of the tube, which can also reduce aerosol. Each tube is placed in the thermal cycler preheat lid and set the temperature to reach 105°C. PCR is programmed to detect WSSV in order to produce a DNA Band of 848 bp at step 1 and 333 bp at step 2.

Electrophoresis

We mixed 4 µL (pipette size) in each test sample with 1 µL of diluted SYBR® Green as well as 4 µL marker plus one µl SYBR® Green of 5 µL and injected at each well at agarose carefully and slowly. Electrophoresis was conducted on 100 V for 40-60 minutes. The tool for taking electrophoresis images is a BioDoc System Imaging tool.

RESULTS AND DISCUSSION

WSSV Detection with Nested PCR

The result of the test using nested PCR found that there is WSSV in both sub-districts, Bangil and Rejoso. DNA WSSV shown with white band (as in positive control) at step 1 or band 848 and at step 2 or band 333 (Fig. 1).

Sampling and observation results with PCRs found no infection in step 1 where the positive controls were 848 bp. However, in step 2 with 333 bp positive control found some samples containing or positively has WSSV.

A healthy shrimp sample has a characteristic such as active swimming and good eating. If analysed with one step PCR, it will produce negative results. However, after testing using a nested PCR, the sample of healthy shrimp can be suspected with positive results of WSSV [5]. Detection of WSSV DNA on suspected shrimps and WSSV shrimps carriers (pests and diseases) will be more sensitive if using PCR [6].

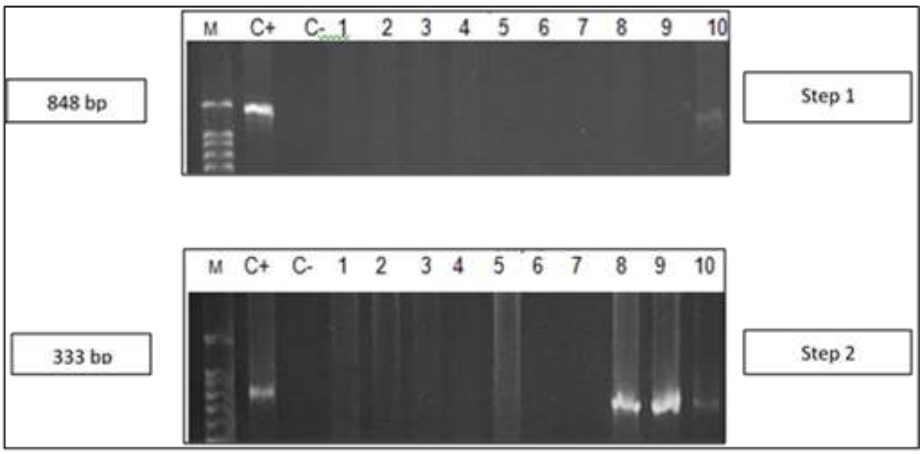

Figure 1. Results of Electrophoresis + Control at 848 bp for step 1 and 333 bp at step 2. DNA samples from shrimp in traditional farm. **Description:** Line M = Marker, C+ = Control Positive, C- = Control negative, 1 = Sample 1, 2 = Sample 2, 3 = Sample 3, 4 = Sample 4, 5 = Sample 5, 6 = Sample 6, 7 = Sample 7, 8 = Sample 8, 9 = Sample 9, 10 = Sample 10.

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White patches are a clinical symptom that the Vaname shrimp samples were attacked by the WSSV. This occurs because it is a specific lesion of WSSV [7]. This white patches assumed to be caused by the deviation on calcium metabolism accumulated in the cuticle layer on shrimp [8]. Initial diagnosis WSSV is characterized by polymorphic white spots meaning development of WSSV attacks [9]. WSSV that attacks the organs at the ectodermal and mesodermal tissues, e.g. in lymphoid, intestine, gill, skin. Whereas invaded part of the endodermal tissue is hepatopancreas [7]; in an attack on cells, the WSSV virion will use a sheath on the protein. The shell will blend with the endosome and nucleocapsid transported through the nucleus, thus attacking the membrane of the nucleus and then releasing the WSSV genome in the nucleus [10]. The WSSV gene will replicate in the cytoplasm followed by the mitochondrial damage.

The length of the WSSV DNA bands in some samples in PCR step 2 was suspected come from carrier shrimp. Through this step also found a long band of WSSV DNA bands (range of 200 bp) under positive control that we used; where the shrimp immune system contains hemocytes on shrimp hemolymph [11]. Infected WSSV has different body resistance, in which ICP 11 is a dominant gene to encode WSSV and begin expression in weaker shrimp, otherwise, shrimp with immunity to protect against disease attacks is stronger in resisting the expression of ICP 11 [12]. Factors that affect, among others, antiviral activity derived from hemocyanin which is a natural immune response to WSSV where trying to delay viral infection and also will inhibit the replication of the virus [13].

Prevalence of White Spot Syndrome Virus

Bangil Sub-district in 2018 with the number of samples as many as 20 infected shrimp samples, there are 2 positive samples of WSSV with 15% prevalence value. Whereas in Kraton Sub-district with 20 samples, the infected sample findings are 0 positive samples, thus 0% WSSV prevalence value and Rejoso Sub-district with 20 samples have the findings of infected sample 2 positive samples of WSSV, and 15% prevalence value.

Prevalence of WSSV in Bangil and Rejoso means that potential occurence of the disease was 15% while in Kraton was 0% (Table 1). The percentage of these occurrence may increased or decreased depending on factors such as water quality and environmental conditions of shrimp culture. WSSV infection in shrimp culture was triggered by fluctuations in temperatures of 3-4^{o}C, low salinity below 15 ppt, and high amounts of *Vibrio* [14].

Table 1. Prevalence Data of WSSV Presence on *Vannamei* Shrimp during the Study

Location	Total of Sample	Positive WSSV	Prevalence (%)
Bangil	20	2	15
Kraton	20	0	0
Rejoso	20	2	15

CONCLUSION

Based on the research that has been done in Bangil Sub-district, Kraton and Rejoso in Vaname Shrimp pons in Pasuruan, it is concluded that WSSV spread in Bangil and Rejoso Sub-districts during the research can be detected by positive samples marked by checking using Nested PCR with positive control used at 848 bp and 333 bp. Whereas the prevalence rate of WSSV attack on each region is as follows: Bangil 0 - 15%, Kraton 0% and Rejoso 0 - 15%.

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Immunostimulant Activity of Neem Leaf (*A. indica* A. Juss) Ethanol Fraction on Tilapia (*Oreochromis niloticus*)

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Abstract

Intensive cultivation of Tilapia with high stock density and continuous artificial feeding will decrease the water quality and increase the growth of pathogenic bacteria. That situation led to the fish stressed so that the fish become the target of bacterial pathogen attacks. Alternatively, the environmental friendly treatment using herbal immunostimulant to solve that problem is required. The herbal in this study used neem leaf (*Azadirachta indica* A. Juss). The aim of this study was to evaluate ethanol fraction of neem leaf on immunostimulant activity of *Oreochromis niloticus*. In this study, the extraction method used maceration. Fractionation methods were using Thin Layer Chromatography (TLC) and Column Chromatography (CC). The biological component was analyzed using Phytochemical, FTIR and GC-MS methods. TLC test showed that ethyl acetate: n-hexane (1:1, v/v) result in brightest luminescence colors on the plates of silica gel under UV light (365 nm). The chromatography column from the ethanol extract of neem leaf (*A. indica* A. Juss) showed that separation of the fraction was done based on the formation of color, which obtained 5 (five) types of fractions. The results of the phytochemical test for the fraction 4 of neem leaf contains two compounds, i.e. flavonoids (+) and triterpenoids (++). The FTIR test showed that fraction 4 of neem leaves contain a class of terpenoid and flavonoid compounds. GCMS test results on fraction 4 of the neem leaves (*A. indica* A. Juss) obtained the high peak, which has 4 terpenoid compounds, 1 compound of flavonoid, and 1 type of lauric acid. The total leukocyte of the fish test increased after treated with fraction 4 of neem leaf (*A. indica* A. Juss) compared with control on day 7 after injection, i.e. 25.29 x 10^4 cells.mL^{-1}.

Keywords: fraction, leukocyte, Neem Leaves, Tilapia, Phytochemicals.

INTRODUCTION

Production targets of tilapia fish are still below the expectation. Production target of the fish at 2016 was 1,882,200 tons while it only realized for 1,187,812 tons, and the target until 2019 is 2,500,600 tons.$year^{-1}$ [1]. To meet the target of production, cultivation was done intensively with the application of high stock density and artificial feeding continuously. As the effects, the quality of culture media will decrease due to the accumulation of organic material on the bottom of the ponds, which is favorable for the growth of pathogenic bacteria. With the bad condition of the cultivation media, the fish become stress so that the fish become the target of bacterial pathogen attacks that grow fast in the maintenance media resulting in the failure for reaching the production target [2,3,4].

Treatment is the last alternative in cultivation activities. Thus before the fish attacked by the disease, the maintenance activities were given immunostimulants with the aim to increase the fish body's endurance if there is a situation that exceeds the normal limit of the environment. Alternative ingredients that are environmentally friendly and safe for health for immunostimulants are using plant herbal ingredients [2,5-9].

One of the potential herbal ingredients as an immunostimulant for fish is neem leaves. This is because neem leaves have been widely used to overcome the attack of pathogenic bacteria. Neem leaves are used as an antimicrobial because the extract of neem leaf can inhibit the growth of pathogenic bacteria both for gram-positive and gram-negative bacteria [10,11,12]. Terpenoids or triterpenoids increase the total leukocytes of carp that attacked by Koi Herpes Virus (KHV) virus as a body defense because terpenoids have inhibitory activity against bacteria, fungi, virus, and protozoa [13]. The increase in total leukocytes in the fraction-4 treatment of the neem leaves makes the immune response more readily available to detect any attack of foreign substances entering the fish body. An increase in the number of leukocytes will make the phagocytosis process in the antigen will be better and increased the antibodies [14].

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There is an immunomodulatory effect of neem leaf (*A. indica* A. Juss) ethanol extract on the peritoneal macrophage cell number in mouse-induced BCG vaccine in which macrophages are innate immunity that serves as an initial defense against infection by phagocytosis process. It also plays an important role as the Antigen Presenting Cell (APC) that initiates and directs immunity to the cellular immune system [15]. Based on these facts in the framework of the cultivation development activities, especially for tilapia, it is necessary to evaluate the ethanol fraction of neem leaf (*A. indica* A. Juss) on immunostimulant activity of tilapia *Oreochromis niloticus*.

MATERIAL AND METHOD

Flouring and Extraction

Flouring done by grind 100 g of neem leaf (*A. indica* A. Juss) shade-dried. The extraction was performed by maceration method for once. Total of 100 grams of neem leaf flour soaked with ethanol solvent for 2 x 24 hours and stirred every 4 hours, and the ratio of the material to the solvent was 1:3, b/v [16].

Fractionation

Fractionation characterization of neem leaf ethanol extract was analyzed by Thin Layer Chromatography (TLC) and Column Chromatography (CC) [17]. TLC analysis was performed to determine the ratio of solvent (eluent) which can draw the active ingredient compound of the extract to be performed on CC. The TLC analysis utilized a silica gel phase. The silica gel with a mobile phase comparison n-hexane: ethyl acetate (15:1, 5:1, 9:1, 1:1; v/v) and chloroform: methanol (1:1; v/v). The action steps of TLC Analysis are as follows:

- TLC plate cut as needed with the width of 1 cm and height 10 cm, at the top of the limit using a thin pencil 0.5 cm and the bottom 1 cm, at the bottom of the fitting middle given the point as a midpoint and then the plate is inserted into the oven with temperature of 100-120°C about ± 15 minutes to evaporate the water content;
- The extract is dissolved (ethanol) sufficiently. Then taken ± 5 µL with microsyringe and bottle on TLC plate 1 cm from the lower edge with 5 mm diameter 20 times the bottle;
- TLC plates inserted upright into 250 mL chamber containing ± 10 mL eluent solvent then covered with cling wrap to avoid evaporation;
- If the eluent has reached the upper limit then the TLC plate is taken with tweezers. The appearance of the stain used is ultraviolet (UV) rays. When irradiated with UV distillation (254 nm) and distant UV (366 nm), then Rf value is calculated for polyphenol isolation (tannin) in column chromatography according to Retardation factor (Rf) value on TLC plate.

Column chromatography is the second stage in the fractionation process after thin layer chromatography (TLC). Liquid-solid adsorption chromatography based on the packing of a column with absorbent of silica gel, and neem leaf extract (*A. indica* Juss) in elution with a solvent, i.e. ethyl acetate: n-hexane (1:1). It is based on the gravitational flow through the column, where the gravitational force push the solution down and through the column. The steps of Column Chromatography are as follows:

- Weigh 20 g of silica gel G60, then put in the oven with temperature 100°C ± 15 minutes then put in distillation until cool;
- Silica gel G60 suspended into the n-hexane motion phase: ethyl acetate (1:1, v/v) ± 100 mL and stirred with a magnetic stirrer for 1 hour so that when the silica gel is inserted the column not cracked;
- At the bottom of the column (above the valve of the column) is filled with glass wool as a silica holder, silica is inserted slowly. To avoid the breakage of silica in the column, shaking and suspending until the silica compaction has been established for ± 12 hours (in the column there must be eluent);
- Weigh the extract as much as 1 g and dissolve it in 2 mL eluent, eluent in the column is removed until it remained 0.5 cm above silica then the dissolved extract is slowly inserted in the column and tap down the column in the open position;
- If the liquid extract in the column is running low do gradual addition of eluent;
- Separate fractions formed based on the color difference. The fractionation results are accommodated in different glass bottles according to the fraction color difference. The resulting fractional was arranged in order to obtain the fraction in a dry state fraction, concentrated in nitrogen evaporator at a temperature of 40-45°C. The fraction is ready for further testing.

Phytochemical Test and FTIR Analysis

The phytochemical test based on Harbone method [18]. The available composition of the

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fraction obtained from Column Chromatography (CC) analysis. In phytochemical tests, there are flavonoids, alkaloids, tannins, terpenoids, and saponins. Phytochemical identification was performed on candidate of neem leaf ethanol fraction (*A. indica* A. Juss) using Harborne method [18].

GC-MS Analysis

The candidate of neem leaf fraction (*A. indica* A. Juss) is best analyzed by Gas Chromatography–Mass Spectrometry (GC-MS) to know the compounds in the fraction. The GC-MS specification used is Gas chromatogram-HP 6890, equipped with capillary model of Agilent 1909 1 S-433 HP-5 MS (5% Phenyl Methyl Siloxane), 250 (µm) in diameter, 0.25 (µm) thickness and 30 m capillary length with a flow rate of 1 $mL.min^{-1}$. Set the oven temperature to 80°$C.min^{-1}$ - 325°C per 15 minutes. The carrier gas is helium with a flow rate of 19.9 $mL.min^{-1}$ with a pressure of 9.32 psi. The injector temperature is 300°C.

Treatment

Treatment with ethanol fraction of neem leaf by injection intraperitoneal consists of 2 phase. Phase 1, with a dose of 25 ppm done to determine the best fraction that can increase the number of leukocytes and erythrocytes of tilapia.

Phase 2, fish treated with the best fraction of neem leaf base on phase 1 with doses of 0 ppm, 75 ppm, 100 ppm and 125 ppm, with the espectation it can increase the number of leucocytes and erythrocytes of tilapia. Blood sampling is done at the beginning of maintenance (day 0) as data before treatment, day 3 and day 7 after injection.

RESULT AND DISCUSSION

Thin Layer Chromatography Analysis (TLC)

Eluent determination on Thin Layer Chromatography (TLC) was performed by experiment. It was conducted by combining some solvents which can produce the most color sparking in the stationary phase of silica gel plate 60F254 [18,19] seen in Figure 1.

TLC analysis is the first step in the fractionation process because it is the stage of determining eluent that can produce fractions of the tested extract. TLC analysis step is very important in relation to the isolation of small-scale pure compounds to be produced. The more precisely the solvent-solvent mixtures by virtue, the better the pure compounds can be isolated. Thus, to obtain the pure compound of the fractionation process, TLC analysis was done to determine the best eluent [19].

Based on the results of the TLC analysis on the solvent ratio, the eluent which is capable of producing the most color sparks in the 60F254 silica gel plates (Fig. 2) irradiated by UV rays (365 nm) is the mixing of the ethyl acetate solvent: n-hexane (1:1, v/v). So, this yield is called fraction of ethyl acetate: n-hexane.

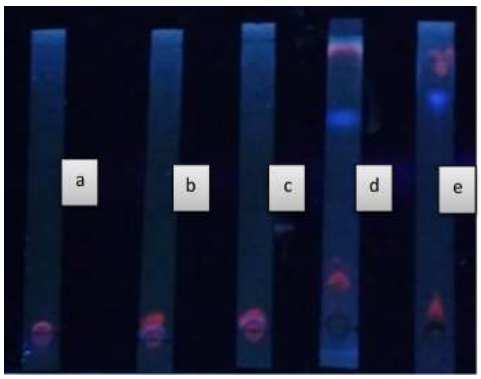

Figure 1. Thin Layer Chromatography (TLC) Analysis for Eluent Determination.
Description:
a = N-Hexane : Ethyl Acetate (15 : 1),
b = N-Hexane : Ethyl Acetate (5 : 1)
c = N-Hexane: Ethyl Acetate (9 : 1)
d = N-Hexane : Ethyl Acetate (1 : 1)
e = Chloroform : Metanol (1 : 1).

Column Chromatography

Column chromatography was performed to produce an abundant amount of ethanol leaf fraction using eluent obtained from the Thin Layer Chromatography (TLC) test, i.e. eluent ethyl acetate: n-hexane (1:1; v/v) as the mobile phase and the stationary phase using silica gel powder 60F254. The result of column chromatography (CC) of neem leaves (*A. indica A. Juss*) ethanol fraction is seen based on the formation of color. The number of fraction of neem leaf ethanol seen from the formation of color obtained 5 (five) kinds of fraction/color (Fig. 2).

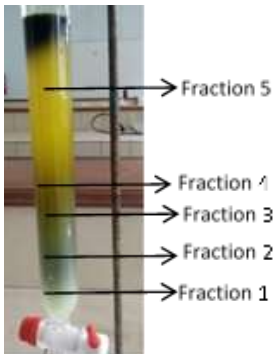

Figure 2. Column Chromatography (CC) Analysis of the Neem Leaf Ethanol Extract (*A. indica* A. Juss)

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Ethanol Fraction Test of Neem Leaf on Tilapia Fish Leukocyte Response

The neem leaf fraction test (*A. indica* A. Juss) was carried out to extract one of the best fractions of the 5 fraction types resulting from Column Chromatography (CC). The CC analysis obtained by referring to the *In Vivo* nonsteroidal non-specific immune respiration (leukocytes) of tilapia (*O. niloticus*). The result of the determination of the fraction from neem leaf (*A. indica* A. Juss) is shown in Table 1.

Table 1. Leukocyte Response (*O. niloticus*) againts neem leaf extract *(A. indica* A. Juss*)*

Fraction	Leukocyte response (x 10^4 cell.mL^{-1})	
	Day-3	Day-7
1	15.83 ± 0.45[b]	16.25 ± 1.71[b]
2	15.30 ± 0.59[b]	15.82 ± 0.90[b]
3	17.15 ± 0.25[c]	17.93 ± 0.15[b]
4	**24.09 ± 1.35[d]**	**25.29 ± 1.21[c]**
5	15.33 ± 0.43[b]	16.44 ± 0.20[b]
Control	10.01 ± 0.26[a]	10.42 ± 0.18[a]

Notes: Day - is after injected by neem leaves fraction

From the observation of tilapia (*O. niloticus*) leucocytes, the fraction 4 was significantly different from the number of produced leukocytes compared to the other fractions and control, where for the leukocyte count of fraction 4 on day 7 after injection was 25.29 x 10^4 cells.mL^{-1}. The high value of leukocytes in fish exposed to fraction 4 may be due to fraction 4 contained antigen material that can affect the leukocytes of the test fish. Increased number of leukocytes in fish due to exposure of a substance indicates that the fish responds to protect the body against incoming foreign matter [21]. Therefore, fraction 4 is the best candidate fraction for testing the non-specific immune response of tilapia (*O. niloticus*) at the next stage.

Fraction-4 on Leukocyte

The results of observation on leukocyte values in tilapia were significantly different (Table 2) between fish treated with fractional doses of Fraction-4 neem leaf (75, 100 and 125 ppm) with Control (0 ppm or without treatment). At the beginning of the treatment, the leukocyte content at each concentration increased as the given fraction concentration was also increased, compared to the control. Increasing number of leukocytes with the fraction-4 neem leaf is suspected because of the terpenoid compounds. Terpenoid is secondary metabolites composed by isoprene structure that is good for the health.

The increase of leukocyte value by giving fraction-4 neem leaves make the immune response more ready to detect any foreign body attack that enter into the fish body. An increase in the number of leukocytes will make the phagocytosis process in the antigen will be better and increased the fish body's antibodies [14].

Table 2. Leukocyte of Tilapia (*O. niloticus*)

Doses (ppm)	Leukocyte (x 10^4 cell.mL^{-1})		
	Day-0	Day-3	Day-6
0	14.23 ± 0.46[a]	14.49 ± 0.26[b]	13.51 ± 0.34[a]
75	14.40 ± 0.57[a]	23.40 ± 0.36[c]	21.32 ± 0.21[b]
100	15.14 ± 0.09[a]	25.54 ± 0.23[d]	23.47 ± 0.31[c]
125	15.40 ± 0.42[a]	27.40 ± 0.03[e]	24.71 ± 0.12[d]

Notes: Day - is after injected by neem leaves fraction

On day 6, there was a decrease in the number of leukocytes in the treatment of doses of 75, 100 or 125 ppm. This is supposedly the fish's body defense system is working in recognizing the existence of foreign objects that enter the body. In a research explained leukocytes are blood cells that play the role of antibody system functions in the fish body [22]. When the body already recognizes the foreign object, the leukocyte level will decrease indicating that the fish is back to normal condition.

Fraction-4 on Erythrocytes

The results of observation on erythrocyte values in tilapia during the study increased after the treatment of fraction-4 neem leaf (*A. indica* A. Juss). Treatment of fraction dose (75, 100 and 125 ppm) was significantly different from Control treatment (0) without fraction; where total erythrocyte values increase along with increasing doses (Table 3).

In each treatment of dose fraction-4 neem leaves (*A. indica* A. Juss), the erythrocyte level increased compared to Control (0) without fraction. The increase is higher along with the increase of dose fraction of neem leaves (*A. indica* A. Juss). It is predicted that neem leaves fraction (*A. indica*) can increase the erythrocyte content after 3 days of administration. Increased levels of erythrocytes are assumed to be an effect from the activity of the neem leaves.

Table 3. Erythrocyte of Tilapia (*O. niloticus*)

Doses (ppm)	Erythrocyte (x 10^5 cell.mL^{-1})		
	Day-0	Day-3	Day-6
0	20.14 ± 0.51[a]	20.27 ± 0.57[a]	21.13 ± 0.55[a]
75	20.30 ± 0.32[a]	20.80 ± 0.20[a]	24.03 ± 0.49[b]
100	19.87 ± 0.46[a]	21.87 ± 0.75[a]	26.50 ± 0.75[c]
125	20.10 ± 0.53[a]	23.10 ± 0.70[b]	27.76 ± 0.66[c]

Notes: Day - is after injected by neem leaves fraction

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Terpenoids flavonoids are compounds that capable of increasing erythropoiesis (the process of erythrocyte formation) in the bone marrow [23]. Erythrocyte function is for supplying feeding to cells, tissues, and organs. If the amount of erythrocytes is low then the process of fish metabolism will be hampered [18].

Phytochemical Analysis of Fractions-4 Neem Leaf (*A. indica* A. Juss)

Phytochemical tests on the fraction-4 neem leaf (*A. indica* A. Juss) included flavonoids, alkaloids, saponins, tannins, terpenoids and polyphenols [23]. The result of phytochemical analysis of fraction-4 neem leaf (*A. indica* A. Juss) ethanol solvent can be seen in Table 4.

Table 4. Phytochemistry of Neem Leaf (*A. indica* A. Juss) Fraction 4

Phytochemistry	Result	Note
Flavonoid	+	Formed pink
Alkaloid		
Meyer	-	No white precipitate formed
Dragendroff	-	No orange precipitate formed
Tannin	-	No color changes
Saponin	-	Absence of permanent foam
Terpenoid	++	Absence of permanent foam
Polyphenol	-	No blackish-green color

The result of the phytochemical test showed that fraction-4 neem leaf is positively contains 2 compounds namely flavonoids (+), terpenoids (++). It can be concluded that fractionation using Column Chromatography (CC) method can separate the existing compounds from a material simplicia that can be used for subsequent application. The insight on the method of analysis and solvent used on a material will affect the secondary metabolite content produced [24].

FTIR analysis of Fraction 4 leaves of neem (*A. indica A.* Juss)

Results of FTIR spectrophotometric analysis of fraction-4 neem leaves (*A. indica* A. Juss) shows the frequency region as 8 types of bond in a compound. With FTIR spectra patterns, it is shown that fraction-4 contains several functional groups, each of the functional groups provides different absorptions in each specific area. Based on the wavelength obtained, the fraction-4 of neem leaves contains the dominant functional groups in the spectra 3401.543 cm^{-1} and 1391.103 cm^{-1}, i.e. carboxylic acid (RCO_2H) and alcohol (ROH); spectra 2928.039 which is a CHO functional group with alkane, aldehine and ketones and spectra 1062.567 cm^{-1} (OH) which is a hydroxyl functional group. The fraction bands and clusters at fraction-4 neem leaf can be seen in Table 5.

Table 5. Fraction and Force Absorption Bands in Fraction-4 Neem Leaves

Bandwidth Numbers and Compounds	Bandwidth Numbers (cm^{-1})	Cluster	Intensity	Functional Groups
4000 – 3200	3401.543	O-H RCO_2H	Great widened	Alcohol (H-bounded) Carboxylic acid
2940 – 2915	2928.358	CHO	medium, sharp	Alkanes, Aldehydes, Ketones
1870 – 1550	1645.358	C-H Aliphatic	Medium, sharp	Alkanes
1490 – 1150	1391.103	O-H RCO_2H	Medium, sharp	Alcohol (H-bounded) Carboxylic acid
1090 – 1020	1062.567	OH	Tall, sharp	Hydroxyl

Based on the spectra formed on the FTIR test, the fraction-4 neem leaves consists of terpenoids and flavonoids. According to Harborne, terpenoid compounds are compounds containing alcohols, aldehydes, and carboxylic acids, while for flavonoids can be found as mono-, di- or triglycosides in which the hydroxyl (OH) group in the flavonoid molecule is bound by sugar [25].

Gas Chromatography Spectrophotometric Mass Analysis (GC-MS) Fraction-4 Neem Leaf

The GC-MS analysis is a continuation identification of the FTIR test, which in FTIR test, the compound contained has not been ascertained in the test isolate. The GC-MS analysis aims to measure the type and content of compounds in a sample both qualitatively and quantitatively. GC-MS analysis detected fraction-4 neem leaves (*A. indica* A. Juss) contained 10 active compounds. Existing active compounds are identified by comparison of retention time and mass spectrum with library databases (Fig. 3). GC-MS is a method of separation for organic compounds using two methods of analysis, namely Gas Chromatography (GC); to quantitatively analyze of compounds and Mass Spectrometry (MS) to analyze the molecular structure of compounds [26]. The results of the identification are several highly active compounds. Based on the database of MS

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libraries, this compound formed by molecules α-Cubebene/Copaene, Caryophyllene, Benzofuranone, Azulene-Cycloprop, Neophytadiene, and Dodecanoic.

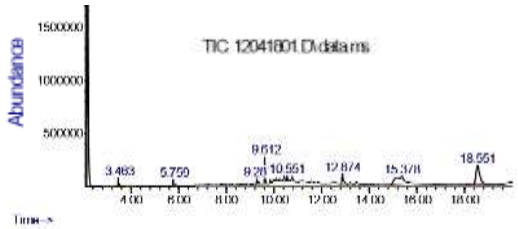

Figure 2. Fraction-4 GC-MS analysis of Neem Leaf (*A. indica* A. Juss)

The highest content of compound obtained from fraction-4 neem leaves (*A. indica* A. Juss) is formed by terpenoid compounds. It is indicated by the highest peak with 4 terpenoid compounds, 1 flavonoid compound, and 1 lauric acid. The compounds obtained from the GC-MS analysis can be seen in Table 6.

Table 6. GC-MS analysis of fraction 4 of neem Leaf (*A. indica* A. Juss)

Compounds	Groups
α-Cubebene/Copaene	Sesqueterpene /Terpenoid
Caryophyllene	Sesqueterpene /Terpenoid
Benzofuranone	Quercetin /Flavonoid
Azulene-Cycloprop	Sesqueterpene /Terpenoid
Neophytadiene	Diterpenoid /Terpenoid
Octadecanoic acid, Dodecanoic	Lauric Acid/Lipid Group

Terpenoid group compounds and lauric acid flavonoids are organic compounds that are very useful for health as anti-inflammatory, antibacterial, antioxidant, and even as a material to increase endurance. Terpenoid compounds, flavonoids contained in herbs, are capable of destroying the bacterial membrane by destroying the outer membrane of Gram-negative bacteria [27,28].

CONCLUSION

The fraction-4 neem leaves compounds contains terpenoid, flavonoids, and lauric acid. These compounds are potential as immunostimulant ingredients for tilapia because it can increase the number of erythrocytes and leukocytes, which are parameters for fish health.

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Anti-hyperglycemic and Immunomodulatory Activity of a Polyherbal Composed of *Sesbania grandiflora*, *Salacca zalacca* and *Acalypha indica*

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Abstract

Diabetes has become a serious global public health problem due to its high prevalence and mortality. Unfortunately, current anti-diabetic drugs are having some limitations and adverse effects. Therefore, searching for a new anti-diabetic agent is an urgent challenge. In this research, we examined the effectiveness of a traditional anti-diabetic polyherbal composed of *Sesbania grandiflora* seeds, *Salacca zalacca* leaves and *Acalypha indica* roots (2:1:1). The study was aimed to explore the anti-hyperglycemic effect of the polyherbal in STZ-induced diabetic mice and to investigate the immunomodulatory activity involved in the process of controlling hyperglycemia. Our results showed that the polyherbal water extract (150 mg.kgBW^{-1}) could suppress blood glucose elevation and preserve pancreatic islet of diabetic mice. Moreover, the polyherbal treatment could normalize the relative amount of activated $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells. The polyherbal extract also stimulated the production of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) which is known to play an important role in diabetes control. In addition, polyherbal treatment also increased the relative amount of anti-inflammatory cytokines IL-10 and TGF-β. These results revealed that the polyherbal extract has an anti-hyperglycemic and immunomodulatory activity that may provide beneficial function in diabetes healing.

Keywords: Antidiabetes, Immunomodulator, Polyherbal, Regulatory T cell.

INTRODUCTION

Diabetes is a disease characterized by hyperglycemia [1]. It occurs due to inadequate peripheral tissue response to levels of insulin in the body, and/or by overall insufficient insulin production [2]. Diabetes can cause some serious health problems including of the damage of blood vessels, heart, kidneys, eyes, nerves, as well as increases the risk of stroke. Diabetes and related complications are also a prime cause of death [3]. The International Diabetes Federation (IDF) estimated that the total number of people with diabetes in the world was 415 million in 2015 and predicts this figure will increase to 642 million people in 2040. Because of its high rate of prevalence and mortality, diabetes has become one of the global health emergencies of the twenty-first century [4].

Currently, there are some approved drugs available to clinically treat diabetes. However, the use of those drugs has been reported to lead to undesirable side effects [5]. Moreover, these medicines do not significantly improve β cell function, so they cannot cure diabetes completely in a patient [6,7]. Contrastingly, many people have claimed to have recovered from diabetes by consuming plant-based herbal medicines as prescribed in traditional remedies. Unfortunately, most of their claims have not yet been supported by scientific evidence. Therefore, it is necessary to scientifically investigate anti-diabetic activity resulting from plant-based herbals used in traditional diabetes remedies. This investigation is essential if there are to be discoveries of potential anti-diabetic agents that are more effective and safer than drugs available today.

Studies on diabetes have revealed the close link between immune system and diabetes pathology. It has been reported that pro-inflammatory markers were increase in diabetic patients [8,9]. Inflammatory cytokines also contributes to the onset of insulin resistance [10] and diabetic complications [11]. Based on these findings, a new stategy for diabetes treatment namely immunomodulatory theraphy was developed by using anti-inflammatory or other immunomodulatory agents [12]. Recent data revealed that immunomodulatory treatments could give beneficial effects on glycemia, β-cell function, and insulin resistance [13]. In accordance with this research, compounds

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contained in traditional anti-diabetic herbals could be expected to have immunomodulatory properties that may modulate immune system and further ameliorate diabetes symptoms.

In this study, we investigated scientific evidence of the effectiveness of an Indonesian traditional polyherbal composed of *Sesbania grandiflora* seeds, *Salacca zalacca* leaves and *Acalypha indica* roots (2:1:1) which has been used to combat diabetes by local people in Malang City, Indonesia. Originally, this polyherbal mixture was made by a traditional herbal practitioner who has more than 20 years of experience in composing various plant-based medicines. The study was aimed to investigate the anti-hyperglycemic effects of the polyherbal treatment in STZ-induced diabetic mice and to analyze the immune system modulation involved in the process of controlling hyperglycemia.

MATERIAL AND METHOD

Materials

Sesbania grandiflora (local name: turi, English name: hummingbird tree) seeds were purchased from a local agricultural seed supplier. *Salacca zalacca* (local name: salak, English name: snake fruit) leaves were taken from private local gardens in Pasuruan City, Indonesia (-7°38'43.08" S 112°54'27.00" E). *Acalypha indica* (local name: anting-anting, English name: Indian nettle) roots were taken from local private gardens in Malang City, Indonesia (7º55'23.61" S 112º38'4.62" E). The plant names of materials used in this study have been checked with plant list species provided in http://www.theplantlist.org.

Animals

Animals used in this experiment were 10-weeks old male BALB/c mice (*Mus musculus* Linnaeus) with an average body weight of 30 $\pm$ 2 g. The mice were contained in individual cages at the animal experimentation laboratory of Biosains Institute, Brawijaya University.

Induction of Diabetes

Induction of diabetes was conducted by the method of Furman [14] with modification at the dose of STZ. The healthy non-diabetic mice were injected intraperitoneally with streptozotocin solution (Bioworld, USA) in a dose of 140 mg.kg^{-1}. STZ solution was prepared immediately before injection by dissolving an appropriate amount of STZ powder with 10 mM Na-citrate buffer (pH 4.5) at a final concentration of 20 mg STZ mL^{-1}. In order to optimize STZ absorption, the mice were fasted for 4 hours prior to injection. At six days post-injection, blood glucose levels of the mice were measured with a glucometer device. Mice with glucose levels higher than 200 mg dl^{-1} were considered as diabetic mice.

Preparation of Water Extract of Polyherbal

The polyherbal mixture ingredients were washed with fresh water and left to dry outdoor for 24 hours. The materials were then dried in an oven (set at 120°C) for 1 hour, and then each component was ground finely into a powder. The powders were then mixed in the ratio of *S. gradiflora*: *S. zalacca*: *A. indica* = 2:1:1 as prescribed in the traditional polyherbal preparation.

Traditionally, the polyherbal decoction was prepared by pouring hot water into the powdered polyherbal mixture, and, once the mixture had cooled and the solid materials had settled, the decoction was consumed immediately. In this experiment, the polyherbal extract was prepared by dissolving the polyherbal powder into boiled water (at a ratio of 1:10) and then kept at room temperature for 24 hours. The solid sediment was then separated from aqueous extract. The liquid extract was then evaporated using a freeze dryer.

Treatments

Animals were divided into three groups. The first group was comprised of only normal mice (Non-diabetic Mice, NDM), the second group contained diabetic mice who were not being given treatment (Untreated Diabetic Mice, UTDM), and the third group was made up of diabetic mice who were administered with the polyherbal extract (Polyherbal-treated Diabetic Mice, PTDM). Six experimental mice were used for each group. The extract was administered once a day for 24 days orally using the gavage technique at a dose of 150 mg kg^{-1} BW.

Blood Glucose Measurement

The blood glucose level was measured with a glucometer (General Electric, USA) every six days. At the blood glucose readings, the tip of each mouse's tail was carefully snipped and then massaged until a small bead of blood had formed. The blood was then put on the test strip which was inserted into the glucometer device.

Histology of Pancreatic Islet

On day 25 of the study, the mice were sacrificed, then their pancreas was harvested and fixed in 10% formalin solution. The fixed pancreas specimens were then immediately sliced, processed and embedded into paraffin

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blocks. The blocks were cut into 4 µm paraffin sections by a rotator microtome and stained with Hematoxylin and Eosin (H&E) [15]. The histological observation was done by using a light microscope.

Isolation of Splenocytes and Flow Cytometry Analysis

Isolation of splenocytes and flow cytometry analysis were performed according to the method of Rifa'i and Widodo [16] with modification in the type of antibodies. Harvested mouse spleens were washed with sterile PBS twice and placed on a petri dish containing additional sterile PBS. The spleens were then pressed using a syringe holder. A single cell solution was filtered with a sterile wire and placed into a 15 mL polypropylene tube. PBS was added to this suspension till the 10 mL mark and then centrifuged at 2,500 rpm at 4°C for five minutes. The supernatant was then discarded, and the obtained pellet was resuspended in 1 mL of sterile PBS. The single cell suspension containing around 2-3 × 10^6 cells was washed with PBS and stained with FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8, PE-conjugated anti-mouse CD25, PE/Cy5-conjugated anti-mouse CD62L.

Intracellular cytokine staining was performed with a Cytofix/Cytoperm kit (BD-Biosciences Pharmingen) according to the protocol provided by the manufacturer. Pellets with approximately 2-3 × 10^6 cells were stained with FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD25 for 30 min. After incubation, the suspension was washed, and the pellet was resuspended in cytofix buffer (200 µL) for 20 min in the dark at 4°C, then resuspended in 1 mL wash-perm and centrifuged again at 2500 rpm at 4°C for 5 min. The supernatant was discarded, and the obtained pellet was subjected to intracellular staining with PerCP anti-mouse FoxP3, PerCP anti-mouse interleukin-10 and PE/Cy5 anti-mouse TGF-β for 30 minutes.

Data Analysis

The data were analyzed using the one-way analysis of variance (ANOVA) to determine the significance of the difference between the means of the groups. A post hoc analysis was conducted according to Fisher's Least Significant Different (LSD) test at 95% of confidence level. The ANOVA and LSD tests were performed using software Genstat 18th Edition (VSN International Ltd., UK).

RESULT AND DISCUSSION

Effect of Polyherbal Administration on Blood Glucose of Diabetic Mice

Figure 1 showed that the blood glucose of untreated diabetic mice increased to 487 ± 38 mg.dL^{-1} after 24 days of treatment. In the diabetic mice receiving polyherbal treatment, the blood glucose level was 313 ± 19 mg.dL^{-1} which were very significantly lower than that in diabetic control. This result indicates that polyherbal administration was able to prevent the blood glucose elevation in diabetic mice.

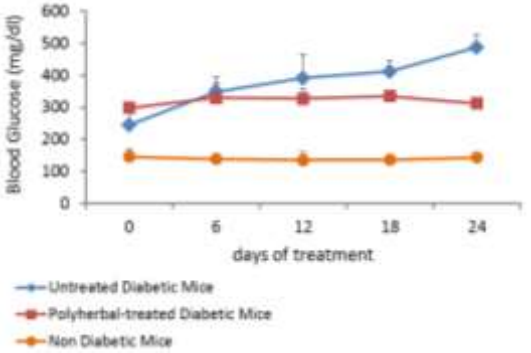

Figure 1. Blood Glucose (mg.dL^{-1}) of Experimental Mice after 24 Days of Treatment.

The anti-hyperglycemic activity of polyherbal extract showed in this study was also supported by the histological observation of the pancreatic islet. The pancreas samples of normal mice showed a granular islet cells with a smooth edge (white arrow), while untreated diabetic mice showed an irregular shaped of a damaged islet which was red in color due to blood infiltration. On the other hand, the pancreas of diabetic mice receiving polyherbal extract treatment was partially recovered. A granular islet can be seen in the pancreas samples from polyherbal-treated mice, though the size was smaller than those from normal mice (Fig. 2).

Figure 2. Photomicrograph of the Pancreas Sections. (A) Non-diabetic Mice, (B) Untreated Diabetic Mice and (c) Polyherbal-treated Diabetic Mice, stained by Hematoxylin and Eosin.

The ability of the polyherbal extract to prevent blood glucose elevation might be resulted from its immunomodulatory properties

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that contribute to pancreatic islet preservation after beta cells was destroyed by STZ toxicity. Previous studies showed that STZ could destroy pancreatic islet through necrosis mechanism [17, 18] followed by islet-specific autoreactivity after cellular debris from β cells were presented on antigen presenting cells [19]. Interestingly, immunomodulatory therapy could restore self-tolerance, causing the suppression of islet-specific autoimmune responses and prevention of β-cell destruction [20]. Moreover, therapy with immuno-modulatory agents is considered as a new prospective strategy that could give beneficial effects in the diabetes treatment [21, 12,13]. The immunomodulatory activity of polyherbal extract in this experiment are discussed in more detail in the subsections below.

Effect of Polyherbal Administration on Relative Amount of Activated T Cells in Diabetic Mice

The relative amount of $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells in the diabetic mice were 73.55% and 71.24%, respectively (Fig. 3). These values were significantly higher than that in the healthy mice (52.29% and 47.69%, respectively). The increase of $CD4^+CD62L^-$ and $CD8^+CD62L^-$ T cells relative amount in the diabetic mice indicates that hyperglycemia stimulates activation of both T helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) which were characterized by lost of their L-selectin ($CD62L^-$) from their surface. This result is similar to that found by Rifa'i and Widodo [16] showing an increase of activated T cells in the diabetic mice group. In addition, it has been found that diabetic patients have more pro-inflammatory markers than healthy people [10,11].

Mature T cells are developed in the thymus which is immunologically considered as naïve T cells [22]. After naïve T cells are released from the thymus gland, they circulate between the blood and lymph, making intermediate stops in the secondary lymphoid organs such as the spleen and lymph nodes. In secondary lymphoid organs, naïve T cells will be activated when they encounter mediators for T cell activation. Once T cells are activated, they lose their adhesion molecules L selectin (CD62L) from their surface and induce other immune cells leading to inflammatory state [23,24,25,26].

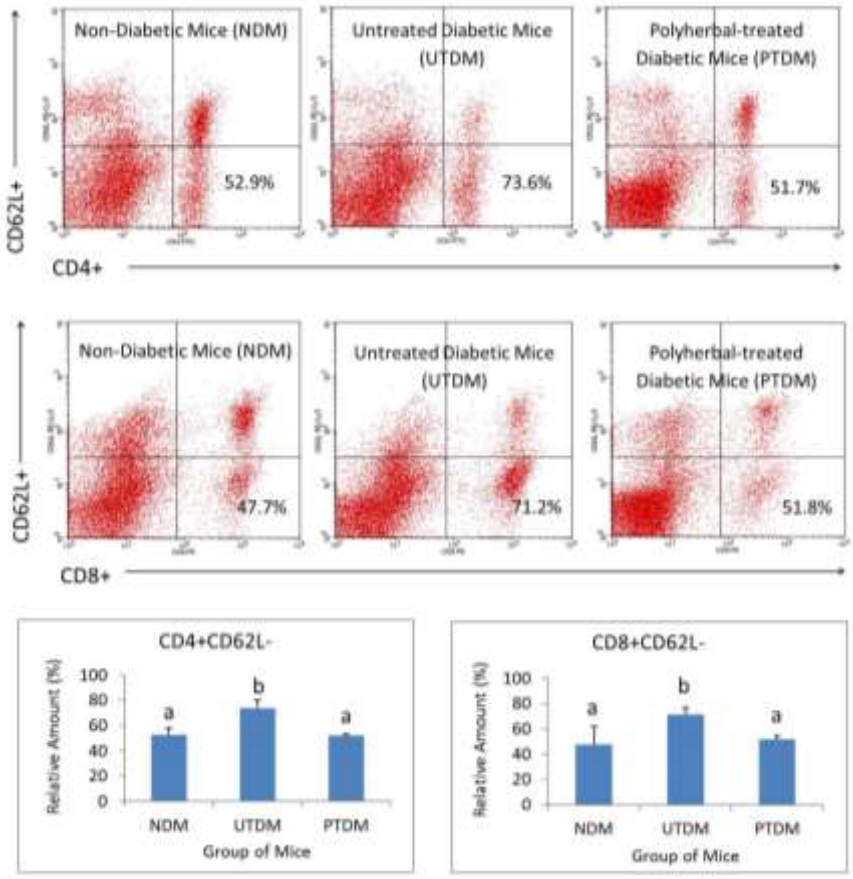

Figure 3. Relative Amount of Activated $CD4^+$ and $CD8^+$ T Cells in Experimental Mice after 24 Days of Treatment

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In the diabetic state, T cells might be activated through hyperglycemia-induced oxidative stress [27] or by exposure of islet-specific antigens [19,28]. Although the precise mechanism is debatable, involvement of T cells auto-reactivity leading to β cell self-destruction is evidenced both in mice and in human [29]. Based on this understanding, some trials have been conducted by targeting of T cells to induce β cell-specific tolerance for diabetes treatment [29,30].

Interestingly, administration of the polyherbal extract could lower the population of activated T cells in diabetic mice to as low as the numbers in healthy mice (Fig. 3). This finding indicates that the polyherbal extract used in this study is a potential source of an immunomodulator, which may lead to benefit in the treatment of diabetes. Possibly, polyherbal suppress T cell activation by decreasing oxidative stress facilitated by antioxidant compounds contained in the polyherbal extract. Alternatively, compounds in the polyherbal extract might stimulate the production of endogenous biological substances that act as immune suppressors such as regulatory T cell (Treg) and anti-inflammatory cytokines.

Effect of Polyherbal Administration on Relative Amount of Regulatory T Cells in Diabetic Mice

Recently, regulatory T cells (Tregs) have become widely accepted as a new tool for understanding DM1 pathogenesis as well as giving new prospects in prevention and treatment of the disease [31]. Earlier studies [32,33] have shown that the balance between effector Th cells and Tregs plays a role in diabetes progression. After the onset of diabetes, autoimmunity progression continuously increases as the increase of ratio between effector Th cells and Tregs occurs within an inflamed pancreas [34].

Tregs are characterized by a high constitutive surface expression of the IL-2 receptor alpha chain (IL2RA), which is also commonly called Cluster of Differentiation (CD)25. In addition, the expression of intracellular forkhead box protein 3(FOXP3) transcription factor is accepted as being the best marker of Treg cells [31,35].

In this study, the polyherbal administration was found to increase the relative amount of $CD4^+CD25^+$ regulatory T cells (Tregs) in STZ-induced diabetic mice. Specifically, $CD4^+CD25^+FoxP3^+$ Tregs level increased more than two-fold when compared to the diabetic control group (Fig. 4). These results indicate that polyherbal extract could stimulate the production of Treg cells. Regarding the important role of Tregs in T1D, boosting Tregs number may provide an effective aspect of diabetes treatment [36,37].

The role of Tregs in diabetes treatment is correlated with its immunomodulatory function which suppresses the excessive responses of immune cells in both innate and adaptive immune systems. Previous research has revealed that CD^+CD25^+Treg could suppress $CD4^+$ and $CD8^+$ T cells proliferation and their pro-inflammatory cytokines production in addition to suppressing their effector activities such as $CD8^+$ T cell cytotoxicity [38]. Various immune cells that are suppressed by Tregs include T cells, B cells, natural killer, macrophage, neutrophils, dendritic cells and mast cells [39]. In this study, the contribution of Tregs to the suppression of T cell activation could be seen in polyherbal-treated diabetic mice as shown in Figure 3.

To our knowledge, this is the first study that showed stimulation of Tregs induction by using polyherbal composed of *S. grandiflora*, *S. zalacca,* and *A. indica*. However, several studies have been done on Tregs induction by using other medicinal herbals. Licorice (the root of *Glycyrrhiza* species) extract, and its two constituents, isoliquiritigenin, and naringenin have been proved to effectively promote Treg cell production both *in vitro* and *in vivo* [40]. Other studies also reported that some medicinal plants and their derivatives, including *Astragalus membranaceus*, *Pterodon emarginatus* Vogel, *Hypericum perforatum*, hyungbangpaedok-san, matrine, Bu Shen Yi Sui Capsule, resveratrol, and curcumin could induce Tregs production and increase its functional activities [41].

Effect of Polyherbal Administration on Anti-inflammatory Cytokines Production

Several mechanisms have been proposed as being explanations of the suppressive effect of regulatory T cells. One of these Tregs suppressive mechanisms is done by anti-inflammatory cytokines secretion. Based on cytokine production, Tregs have been classified as follows: (1) Th3 cells which are characterized by TGF-β production; (2) Tr1 cells which produce IL-10, and (3) Tr35 cells which produce IL-35 [42]. In this study, IL-10 and TGF-β produced by $CD4^+CD25^+$ regulatory T cells in polyherbal treated diabetic mice showed an increase relative to those in the untreated diabetic control and normal control groups (Fig. 5). This result is

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consistent with the finding of an overall increase of Tregs in polyherbal-treated diabetic mice as described above (Fig. 4).

In addition to its suppressive effect, IL-10 also plays a role in the process of cell regeneration. The ability of IL-10 to promote regenerative healing is most likely a result of its synergistic multiple actions including regulation of inflammatory response, endothelial progenitor cells, fibroblast cellular function, and extracellular matrix [43].

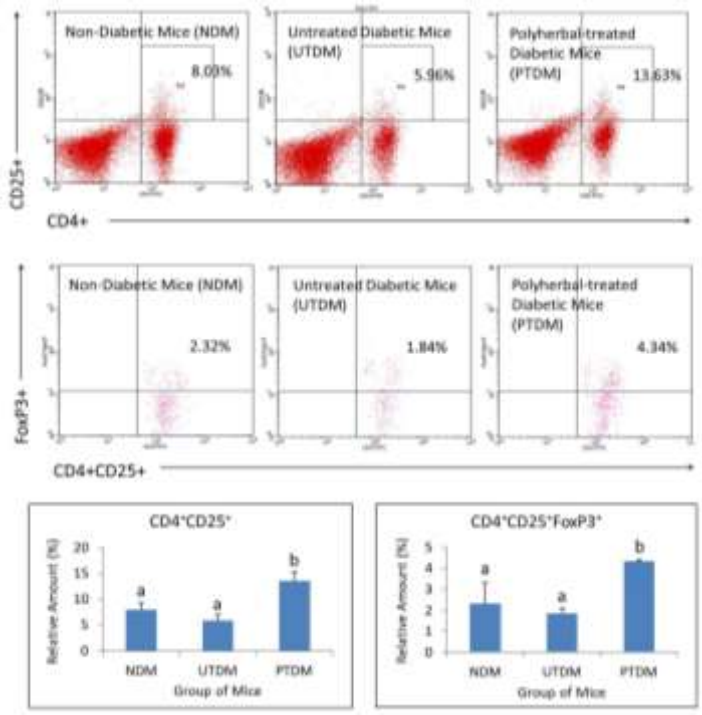

Figure 4. Relative Amount of Regulatory T Cells in Experimental Mice after 24 Days of Treatment

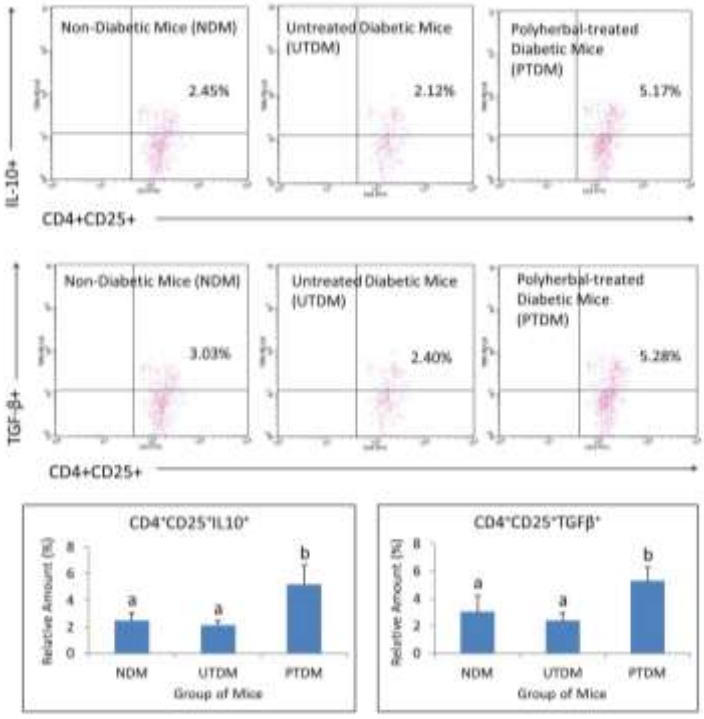

Figure 5. Relative Amount of Pro-inflammatory Cytokines in Experimental Mice after 24 Days of Treatment.

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Endogenous IL-10 limits the severity of fibrosis and glandular atrophy as well as regulates cell regeneration in experimental chronic pancreatitis [44]. Therefore, we suggest that IL-10 might help the process of pancreas regeneration after STZ destruction so that polyherbal- treated diabetic mice could partially recover their islet cells as shown in Figure 2 above. However, more in-depth research is needed to prove this speculation.

CONCLUSION

Water extract from a polyherbal mixture containing *Sesbania grandiflora* seeds, *Salacca zalacca* leaves and *Acalypha indica* roots (2:1:1) was proven to be able to prevent the progression of blood glucose elevation in STZ induced diabetic mice. The pancreatic islet of polyherbal-treated diabetic mice was also found to be partially recovered from the damage of diabetes induction. Furthermore, this study highlighted the significant increase of regulatory T cells and anti-inflammatory cytokines production after polyherbal administration which contributes to lowering the relative amount of activated T cell in diabetic mice. These results revealed that the polyherbal extracts have an anti-hyperglycemic effect and that the polyherbal treatment could modulate the immune system of diabetic mice contributing to the process of controlling hyperglycemia.

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ETHICAL APPROVAL

This experiment has been reviewed and legalized by the Ethics Committee of the Brawijaya University, Indonesia. All procedures performed in this experiment were in accordance with the guidelines and ethical standards of the Ethics Committee of Brawijaya University.

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The Morphological Characters of the Malayan Pit Viper *Calloselasma rhodostoma* (Kuhl, 1824): on The Cephalic Scalation and Distribution Status in Indonesia

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Abstract

The examination on variations of morphological characters among 35 specimens of *Calloselasma rhodostoma* (Kuhl, 1824) from four different populations in Indonesia has been completed in this study. Univariate and multivariate analyzes allowed us to recognize the clustering of four populations through morphological diagnosis. The results of the average body size (Total Length) showed that the largest male is from Kangean Island (579.33 mm), while the largest female is from Java (841.07 mm). Comparison of meristic analysis represented three clusters from Principal Component Analysis (PCA) which is considered to be independent population. Here we also described three types of cephalic scalation variation that called small accessories scales and their distribution in Indonesia.

Keywords: *C. rhodostoma*, Indonesia, Meristic, Morphometry, Viperidae.

INTRODUCTION

Vipers (Viperidae), a family of venomous snake, have been divided into two major subfamilies, Crotalinae (Pit viper) and Viperinae (Pitless viper) [1]; comprised of 242 species, and 100 of which are distributed across all continents except Australia and Antarctica [1,2]. Malayan Pit Viper (*Calloselasma rhodostoma*: Crotalinae) is pit viper originated from tropical Southeast Asia [3,4]. This species has been described by Kuhl in 1824 as *Trigonocephalus rhodostoma* (Boulenger, 1896), and was the sole species included in the monotypic genus. *Calloselasma rodhostoma* is also known as *Leiolepis rhodostoma* (Dumeril & Bibron, 1854), *Agkistrodon rhodostoma* (De Roij, 1917), *Ancistrodon annamensis* (Angel, 1933), *Agkistrodon annamensis* (Bouret, 1936) [2], *Tisiphone rhodostoma* (Peters, 1862) [5].

Malayan Pit Viper is widely distributed in South East Asia, including Cambodia (Phnom Nam Lyr Mountain and Cardamom Mountain) [6], Thailand (Lop Buri, Sakon Nakhon, Korat, Chiang Mai, Nakhon Si Thammarat, Trang and Phuket) [7], Malay Peninsula (Alor Star in North Kedah), Bangnara (Patani) [8]. In Indonesia, this snake could be found in three islands; Java, Karimunjawa, and Kangean [4]. But in 1912, a single female specimen with six juveniles was collected from North Borneo, which deposited in Museum Zoologicum Bogoriense-Bogor, Indonesia (MZB 428). This species inhabits various habitat types indicating that this species is well adapted in lowland forest, plantations, shrubs, and rocky areas [4].

The morphological differences on snakes are due to several factors, including geographical variation, environmental factor (ecology, adaptation, microhabitat), and genetic [9,10], such as shown in *Tropidolaemus* complex [11]. Studies on *C. rhodostoma* has been conducted related to its spatial distribution in Southeast Asia [14-18], ecological study [3,19,20], molecular systematic [21,22], diet and venom evolution [23]. The morphological character of *C. rhodostoma* is presented in cephalic scalation, dorsal scalation, ventral scalation, and body coloration [4,5,6,12,13]. However, previous studies are only limited in the comparison of snake's morphology inter-island. Therefore, the purpose of this study is to investigate the morphological variations among population of *C. rodhostoma* from Java, Karimunjawa Island, and Kangean Island.

MATERIAL AND METHOD

Object Study

In this study, we examined a total of 35 preserved specimens consisted of 14 males and 21 females. All specimens were deposited in Museum Zoologicum Bogorinse (MZB-LIPI Indonesia). We divided samples into their origin (geographic consideration), to be specific, under

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3 Operational Taxonomic Unit (OTU) [25,26]. The population were chosen as follows: OTU 1 (6♂, 15♀): Java (East Java, Central Java, West Java, and other part of Java); OTU 2 (4♂, 3♀): Karimunjawa Island (Legon Moto, Legon Lele); OTU 3 (3♂, 3♀): Kangean Island (Paliat Island). We also examined type specimens deposited in Rijksmuseum van Natuurlijke Historie (RMNH-Leiden, Netherland) and Natural History Museum (NHM-London, United Kingdom) (the list of specimens is showed in appendix I).

Morphometric and Meristic Characters Measurement

The measurement of morphometric and meristic characters in this study followed Vogel et al. [11], with modification as listed in Table 1. A total of 37 morphological characters, consisted of 19 morphometric and 18 meristic characters, were examined for each specimen. We used a slide-caliper to the nearest 0.05 mm for morphometric measurements, except Snout Vent Length (SVL) and Tail Length (TaL) which was measured to the nearest 1 millimeter. The counting of ventral scales, body scales, and subcaudal scales followed Dowling [23]; the terminal scute of subcaudal was excluded in this counting; while the counting of dorsal scales row followed Vogel et al. [11].

To prevent juvenile specimens be examined, we determined the size of Total Length (TL) as 400 mm for adult specimens. Pattern and coloration of the body were observed from fresh preserved specimens [11]. Here, we also examined all specimens for the variation on cephalic scalation identified as small accessories scale and the distribution in Indonesia. The small accessories scale that we identified as scale that touched to internasal, prefrontal, nostril and loreal scale.

Data Analysis

Univariate analysis was conducted. Each sample was classified into three groups recognized as Java, Karimunjawa, and Kangean population as presented in Table 2. For multivariate analysis, morphometric data were classified into male and female, since snakes exhibited sexual dimorphism. Data analysis were conducted in 6 characters with bootstrap 999 for Principle Component Analysis (PCA) using PAST3 program after standardizing all characters. The standardization was done by dividing all characters with SVL and converting into percentage. The converted data was transformed to log 10 values.

Table 1. List of Morphological Characters Used in This Study and Their Abbreviation

Number	Abbreviation	Character
Morphometric		
1	SVL	Snout-vent length
2	TaL	Tail length
3	TL	Total length
4	HL	Head length
5	HD	Head width
6	ED	Eye diameter (horizontal)
7	IOD	Inter orbital distance
8	SnL	Snout length
9	D E-nostril	Distance between the anterior eye margin and the nostril
10	Tal/TL	Ratio tail length/Total length
11	SnL/HL	Ratio snout length/Head length
12	HL/SVL	Ratio head length/Snout-vent length
13	SnL/ED	Ratio snout length/Eye diameter
Scalation		
14	VEN	Ventral plates
15	SC	Subcaudal plates
16	SL	Supralabial scales
17	IL	Infralabials scales
18	NVEN	Number of ventral plates
19	NSC	Number of subcaudal plates
20	NASR	Number of dorsal scales rows behind head
21	NMSR	Number of dorsal scales rows at midbody
22	NPSR	Number of dorsal scales rows before vent
23	TNSL	Total number of supralabial scales

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Table 2. Summary of Morphometric Measurements of *C. rhodostoma* from Three Different Localities. Data are shown as the mean and standard deviation, followed by the range in parentheses. All measurements are shown in mm.

Morphological Character	Java		Karimunjawa		Kangean	
	♂ n : 7	♀ n : 15	♂ n : 4	♀ n : 3	♂ n : 3	♀ n : 3
SVL	446.57±23.39 (399-467)	743.87±77.69 (613-892)	409.75±30.25 (382-452)	481.67±32.08 (451-515)	465.67±9.07 (459-476)	614.67±15.18 (601-631)
TaL	109.29±7.83 (96-119)	97.20±10.10 (82-116)	89.00±7.39 (82-98)	68.33±6.66 (61-74)	113.67±4.51 (109-118)	76.00±7.81 (71-85)
TL	555.71±30.20 (495-568)	841.07±85.07 (704-1008)	498.75±34.88 (474-550)	550.67±35.56 (514-585)	579.33±12.74 (571-594)	690.67±10.79 (683-703)
HL	28.90±1.41 (27-30.7)	45.00±3.53 (39.1-52)	26.55±1.47 (25.4-28.7)	28.53±4.24 (23.7-31.6)	29.80±1.06 (28.6-30.6)	40.10±1.22 (39.3-41.5)
HW	16.59±1.23 (14.7-18.2)	30.36±4.27 (23.3-36.1)	17.08±0.88 (16.1-18)	20.83±3.97 (16.5-24.3)	16.43±0.75 (15.7-17.2)	26.57±1.32 (25.4-28)
DE-Nostril	5.89±0.65 (5.1-7.1)	8.62±0.60 (7.6-9.9)	5.05±0.72 (4-5.6)	5.93±1.07 (4.7-6.6)	5.60±0.20 (5.4-5.8)	7.43±0.15 (7.3-7.6)
SnL	7.24±0.83 (6.4-8.7)	10.77±0.91 (9.4-12.7)	6.40±0.50 (5.8-6.9)	6.83±1.53 (5.1-8)	8.50±0.20 (8.3-8.7)	10.27±0.40 (9.9-10.7)
ED	3.99±0.21 (3.8-4.3)	4.73±0.64 (3.1-5.8)	3.98±0.22 (3.7-4.2)	3.77±0.06 (3.7-3.8)	4.13±0.06 (4.1-4.2)	4.53±0.15 (4.4-4.7)
TaL/TL	0.20±0.01 (0.19-0.20)	0.12±0.01 (0.09-0.13)	0.18±0.01 (0.17-0.19)	0.12±0.01 (0.12-0.13)	0.20±0.00 (0.19-0.20)	0.11±0.01 (0.10-0.12)
SnL/HL	0.25±0.02 (0.23-0.30)	0.24±0.01 (0.22-0.26)	0.24±0.02 (0.22-0.26)	0.24±0.02 (0.22-0.26)	0.29±0.01 (0.27-0.30)	0.26±0.02 (0.24-0.27)
HL/SVL	0.06±0.00 (0.06-0.07)	0.06±0.0 (0.05-0.07)	0.07±0.01 (0.06-0.07)	0.06±0.01 (0.05-0.07)	0.06±0.0 (0.06-0.07)	0.07±0.0 (0.06-0.07)
SnL/ED	1.81±0.15 (1.56-2.02)	2.32±0.42 (1.91-3.55)	1.61±0.11 (1.48-1.72)	1.82±0.43 (1.34-2.16)	2.06±0.03 (2.02-2.07)	2.27±0.14 (2.11-2.38)

Notes: n = number of specimen, ♂ = male specimen, ♀ = female specimen

RESULT AND DISCUSSION

In this study, univariate statistical analysis consists of mean, standard deviation, minimum and maximum values of Java, Karimunjawa, and Kangean population as presented in Table 2 for all characters examined. The largest and smallest average males by TL character were 579.33 mm and 498.75 mm from Kangean Island and Karimunjawa Island, respectively. Whereas the largest and smallest average females were 841.07 mm and 573 mm from Java and Karimunjawa Island, respectively.

The meristic comparison that consists of the number of ventral, subcaudal, supralabial, infralabial, and dorsal scale, is presented in Table 3. According to the range of ventral scale number in males, Karimunjawa population shows higher range than Java and Kangean population. That result is in contrast to females, which shows that Karimunjawa and Kangean population is in the range of Java population. Meanwhile, according to subcaudals scale number, Kangean population has a higher range than Java and Karimunjawa population, both in male and female specimens. The range of dorsal scales number in female specimens shows that Java population has the highest range than other populations.

Several characters indicate that *C. rhodostoma* exhibits sex dimorphism (see Table 2), including SVL, HL, HW, DE-Nostril length, SnL, ratio TaL/TL, ratio SnL/ED, number of ventral scales, and subcaudal scales. The ratio of TaL/TL (males *x*: 498.75-579.33, females *x*: 550.67-841.07) shows that females have longer size than males [27,28]. The ratio of TaL/TL (males: 0.18-0.20, females: 0.11-0.12) strongly shows that sexual dimorphism has occurred in *C. rhodostoma*. The benefits of sexual dimorphism in species with few or no parental care are related to its adaptive ability.

Generally, larger females would produce larger and higher number of offspring. Larger offspring would have higher survival rates as it is able to store more yolk for their development. In males, an individual with a smaller size will have a positive impact including enhancing their ability to find and court female. In addition, the smaller size refines the chemosensory senses, higher mobility, inconspicuous to the predator, and early maturation [29-33]. The tail length differences between males and females are due to the influence of copulatory organs (hemipenes) inside the tail, which is the size of tail length affecting male mating success [28].

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Table 3. The Comparison of Number of Ventrals, Subcaudals, Supralabials, Infralabials, and Dorsal Scales of *C. rhodostoma* Specimens between Localities.

Region	Ventrals		Subcaudals		Supralabials		Infralabials		Dorsal Scales	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Java ♂ : 7, ♀: 15	144-149	148-156	48-54	31-42	7-8	7-9	10-12	11-13	22-21-17	(22/23/24)-21-17
Karimunjawa ♂ : 4, ♀: 3	148-152	150-155	48-52	36-39	7-8	7-8	11-12	12-13	22-21-17	23-21-17
Kangean ♂ : 3, ♀: 3	141-147	149-152	53-56	38-43	7-8	7-8	11	11-12	22-21-17	(22/23)-21-17

Notes: ♂ = male specimen, ♀ = female specimen

Principal Component Analysis (PCA)

Factor loadings of the first two components from Principal Component Analysis (PCA) are presented in Table 4. Principal Component Analysis in males and females are shown in Figure 1. The two first scores of factor loadings of principal component (PC) males explain a moderate total variability. All variables show positive score loadings in the first PC, with TaL and DE-Nostril Length as the most positive loaded, in contrast with ED which is lower. The second PC shows three variables that are positively loaded, and the other three are negatively loaded. The variables of SVL and TL are the most positively loaded, in contrast to the lower load of HL. The PCA results of males' *C. rhodostoma* are shown in Figure 1. (A), in this graph, plot of the two first PC show three groups: Cluster II, Cluster III, and Cluster I. Based on the locality, Cluster III is separated with Cluster II and I. However, our results show a little overlap among these two clusters (Cluster II and I). The single specimen of OTU 3 (Cluster II) is placed inside of Cluster I.

Table 4. Factor loading on the two first components extracted from the correlation matrix of 6 characters for males and females of *C. rhodostoma*

Characters	Male		Female	
	PC 1	PC 2	PC 1	PC 2
SVL	0.31562	0.4161	0.50773	0.049582
TaL	0.62882	0.24665	0.17259	0.8832
TL	0.3563	0.38977	0.45884	0.15948
HL	0.26703	-0.00029	0.4112	-0.3043
DE-Nostril	0.54955	-0.74247	0.29035	-0.30293
ED	0.068625	-0.25068	0.2964	0.087715
Eigenvalue (97.5%)	87.345	74.045	84.587	80.149
% variance	73.525	16.285	75.166	12.059

The first and second score factor loadings of PC females explain a moderate total variability which is similar to males. All variables show positive score loadings in the first PC with SVL and HL as the highest and TaL as the lowest. The second PC shows strongly structured by variables related to body characters, SVL, TaL, TL, and ED, all with positive loads. In contrast, variables related to cephalic scalation showing the highest negatively loads are HL and DE-Nostril Length. As in males graph (Fig. 1 B), the plot of the two first PC show 3 groups: Cluster II, Cluster I, and Cluster III. Cluster II and Cluster I were separated independently among Cluster III. Cluster I and III are showed close than Cluster II.

The PCA results in males and females (Fig 1. A and B, respectively), show in both cases the occurrence of three clusters of plots, identified as Cluster I to III on the graph. According to the OTUs defined above, these clusters are generated by specimens according to the following scheme. These specimens are discussed below:

Cluster I : OTU 1 (Java population)
Cluster II : OTU 2 (Karimunjawa population)
Cluster III : OTU 3 (Kangean population)

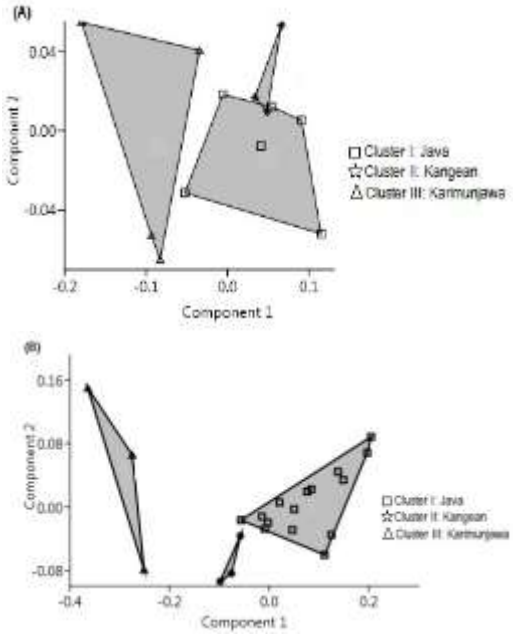

Figure 1. Plot of component 1 (PC1) versus component 2 (PC2) for the principal component analysis of males and females in three populations of *C. rhodostoma*. (A) Males; (B) Females.

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Description Specimen of *C. rhodostoma*

The maximal total length among our samples is 1008mm (SVL 892mm, TaL 116mm) for a female specimen from Java (Bogor, West Java) (MZB Oph 3861) and brown. Mouth is white with black dots distributed evenly. The forehead is dark brown with white stripes on both edges from the snout to the neck. The tail is slender than the body and short, can be distinguished clearly from body parts. The tail coloration on the juvenile is yellowish. The eye is small size with oval-shaped pupils (vertical).

Dorsal Scales: males specimens 22-21-17, females specimens (22/23/24) (23 in 2 specimens, 24 in 2 specimens)-21-17, with smooth scales both in males and females. Ventral scales: VEN (1-2 paraventrals) males: 141-152, females: 148-156; with SC: 48-56 and 31-43 respectively and all paired, entire anal shield. Cephalic scalation: single rostral and visible from above, 2 Internasal contact to nostril, 2 prefrontal touching the loreal pit, single frontal, 2 supraocular above the eye, 2 parietal contact with temporal scale. Supralabial 7-8 in males, 7-9 in females with 3rd, 4th, contact with the eye. Infralabial 10-12 in males, and 11-13 in females. The number of the dark brown series triangular pattern on dorsal is 19-31 [5].

Head Scalation

Based on the variation of head scalation, we found a small accessory scales (Fig. 2). A small accessory scale is a small scale bordered by internasal, prefrontal, nostril, and loreal scale. Variation on the presence or absent of a small accessory scale revealed three different types in *C. rhodostoma* that can be described as follows (Table 5):

1. **Type 1**: a small accessory scale is absent on both sides of head scalation.
2. **Type 2**: a small accessory scale is presence on both sides of head scalation.
3. **Type 3**: a small accessory scale is presence on the right side of head scalation.

In the small accessories scales, we found Type 1 as many as 44 individuals, 5 individuals for Type 2, and Type 3 as many as 5 individuals. Figure 3 showed the distribution of *C. rhodostoma* based on the variation of the accessory scales type. Type 1 has the most widespread distribution that was found in Thailand, Malaysia, Sumatra, Java, Karimunjawa Island, Kangean Island, and Kalimantan. Type 2 was distributed on Kalimantan and Java islands, while type 3 was only distributed in Java Island.

Figure 2. Head scalation of specimen of *Calloselasma rhodostoma* (MZB 470) from Bogor, West Java, showing the position of a small accessory scale directly adjacent to four other scales (as: accessory scale, in: internasal, pf: prefrontal, l: loreal, n: nasal).

The different variation on head scalation of *C. rhodostoma* may be affected by the snake's adaptation process with its ecological environment. Various habitats resulted in a variety of adaptation processes that lead to morphological differences in *C. rhodostoma*. The same location with different ecological conditions is also able to result in different ecogenesis processes [34]. In this research, several types of accessories scales were found in Java Island. However, the variation represented different geographical region for example Type 2 was found only in West Java, while Type 1 and Type 3 were found in Central Java and East Java.

CONCLUSION

Morphological variation in chepalic scalation, including three types of small accessories scale between population of *C. rhodostoma* in Java, Karimunjawa, Kangean Island can be used as one of the supporting data to determine the taxonomic status of the species. However, this finding needs to be tested using molecular studies for a further determination on the taxonomic status of these populations.

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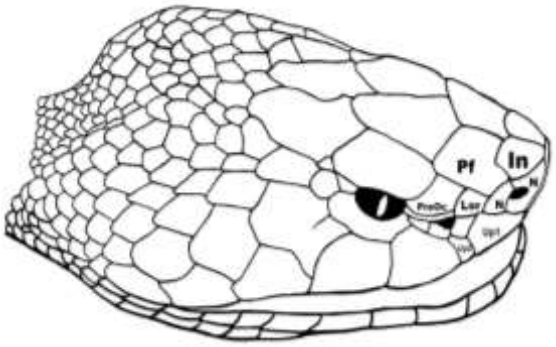
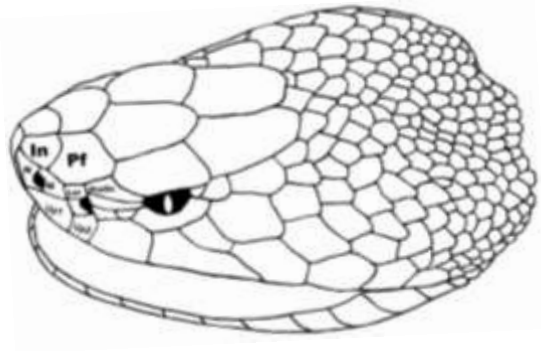
Table 5. Three types of small accessories scale of C. rhodostoma in Indonesia

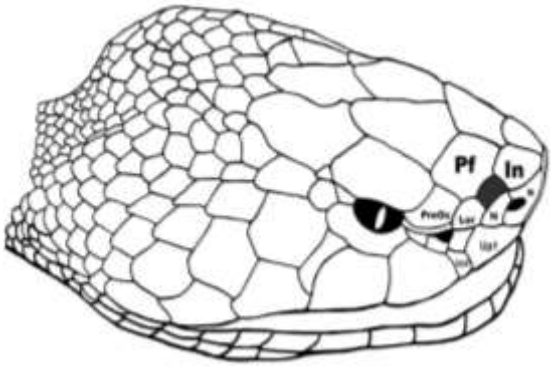


A.

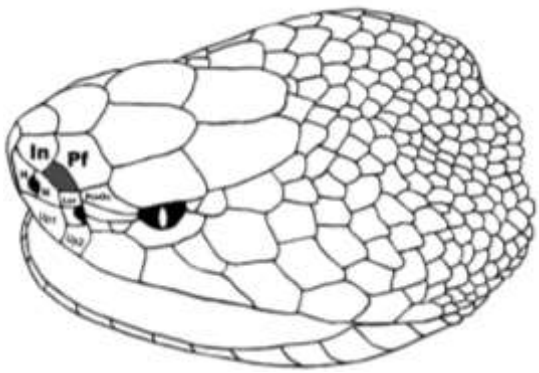


B.

Type 1. Cephalic scalation shows the absence of small accessories scales on both sides of the head. (A). Right side of head, (B). Right side of head, (In: Internasal, Pf: Prefrontal: Up1: Upperlabial ke-1, N: nostril, Lor: Loreal, PreOc: Preocular.

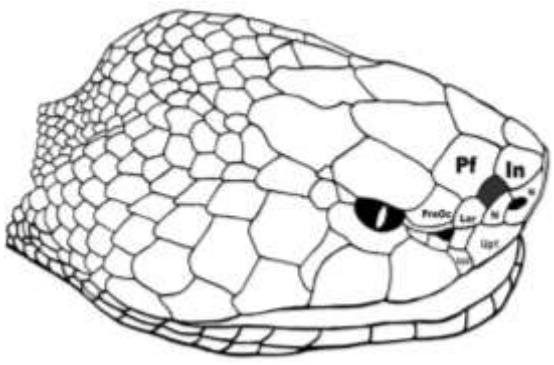


C.

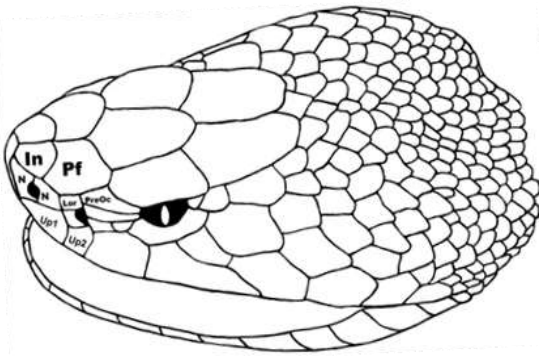


D.

Type 2. Cephalic scalation shows the presence of small accessories scales on both sides of the head. (A). Right side of head, (B). Right side of head, (In: Internasal, Pf: Prefrontal: Up1: Upperlabial ke-1, N: nostril, Lor: Loreal, PreOc: Preocular.



E.



F.

Type 3. Cephalic scalation shows the presence of small accessories scales on right sides of the head. (A). Right side of head, (B). Right side of head, (In: Internasal, Pf: Prefrontal: Up1: Upperlabial ke-1, N: nostril, Lor: Loreal, PreOc: Preocular.

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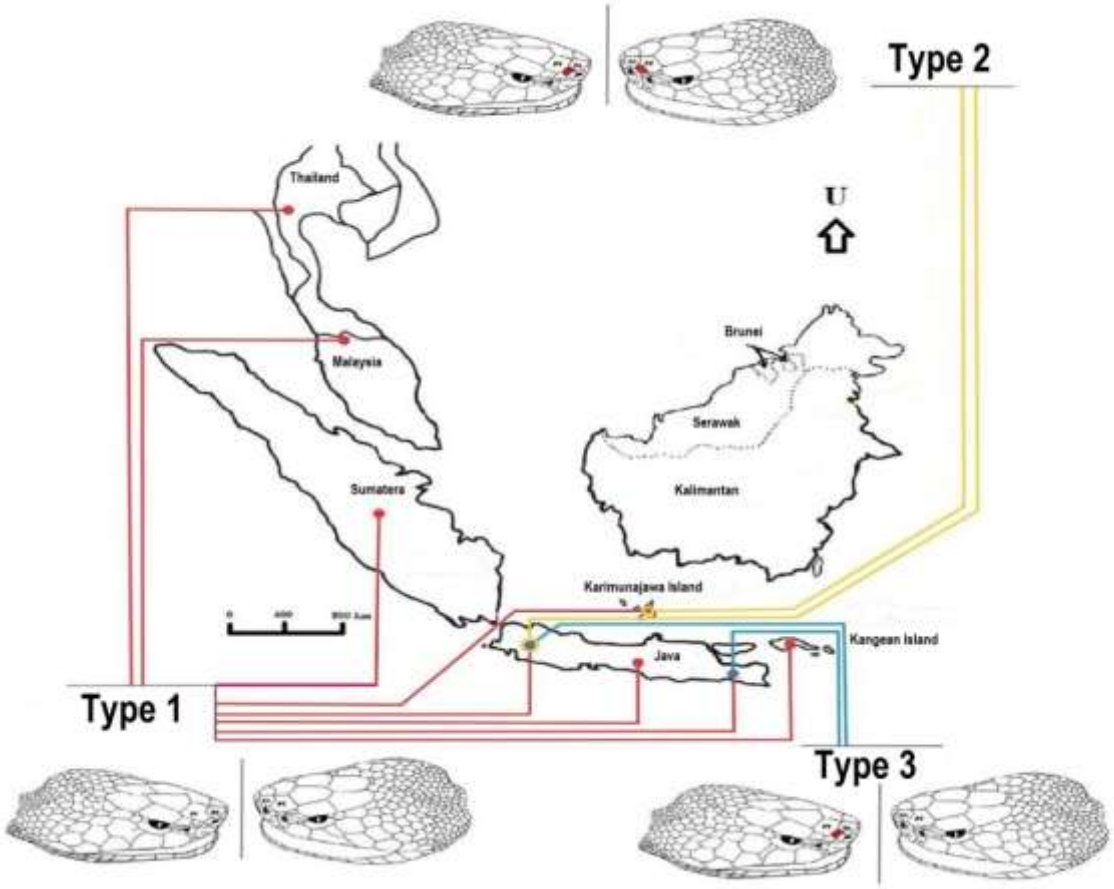

Figure 3. Relative Amount of Pro-inflammatory Cytokines in Experimental Mice after 24 Days of Treatment.

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APPENDIX I. Specimens material used for examine of *C. rhodostoma*from Indonesia and sorroundings area

Java: RMNH. 1510 – Holotype of *C. rhodostoma*; NHM. 66.8.14.316; ZMA. RENA. 13844, 21576; RMNH.10134, 1509; BMNH. 44.2.22.47. **Banten.** Pandeglang: MZB. 3759 (382), 3760 (384), 3081; Tanggerang: MZB. 27766; Serang: MZB. 2125. **West Java.** Cimahi: MZB. 898; Cibinong: MZB. 4438; Bogor: MZB. 470, 452, 1229, 3861, 451; Lebak: ZMA. RENA. 21575; Ujung Kulon: MZB. 3818. **Central Java.** MZB. 4539;Karimunjawa: MZB. 432, 3017, 3047 (289), 3132 (203), 1109, 3133 (215), 3134 (216).**East Java.** Surabaya: MZB. 1228, Kangean Island: MZB. 3802 (2758), 3799 (2962), 3800 (2963), 3801 (2759); Malang: RMNH. RENA. 10319a, 10319b, MZB. 942; Kediri: NHM. 1885.12.31.31. **Sumatera.** ZMA. RENA. 21576. **Thailand.** NHM. 1937.2.1.13.

APPENDIX II. Museum label abbreviations.
MZB: label of collection for Museum Zoologicum Bogoriense, Cibinong, Indonesia; NHM: Natural History Museum, London, United Kingdom (BMNH: British Museum and Natural History); RMNH.RENA: label of reptiles and amphibians collection for Naturalis Biodiversity Center, Leiden, Netherlands (formerly RMNH: The Rijksmuseum van Natuurlijke Historie); ZMA.RENA: label of zoology collection for Zoological Museum Amsterdam, Netherlands

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Numerical Simulation of Leslie-Gower Predator-Prey Model with Stage-Structure on Predator

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Abstract

In this paper, we introduce Leslie-Gower predator-prey model with a stage-structure population on the predator. This model consists of two populations, that are prey and predator populations. Here, we divide predator into two stages. Thus, we have three classes of population in this model that are prey, juvenile predator, and mature predator. The focus of this paper is to know the interaction between the population that is affected by stage-structure in predator population in the model and to study numerically the effects of stage-structure in predator population on the interaction of prey and predator. It is found that the trantition rate from juvenile to mature predator is a very important parameter which may determine the long-term behavior of both prey and predator.

Keywords: Leslie-Gower model, predator-prey model, stage-structure.

INTRODUCTION

Ecology is one branch of science from biology that is still frequently studied. One of the things studied in ecological problems is the interaction between organisms. The interaction between organisms cannot be separated from the problem of predation. Predation can be modeled into a mathematical model called a predator-prey model. By solving the model, we can predict the population behavior of both prey and predator in the future. One of the famous prey-predator models is the Leslie-Gower model. Leslie-Gower [1] introduced a predator-prey model that assumes that predator grows logistically with its carrying capacity comparable to the number of prey populations.

Many studies have been produced using Leslie-Gower model. Aziz-Alaoui and Okiye [2] added environmental protection parameter to the Leslie-Gower model for the survival of predator. In this model, they use Holling Type-II functional response which states that predation depends only on the number of prey populations alone. In 2014, Yu [3] assumed that predation depends not only on prey population but also on predator due to interference between predator during predation. So he uses the Beddington-DeAngelis functional response on this model. In 2016, Indrajaya [4] considered that the Allee effect is one of the factors that influence the interactions between prey and predator.

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Leslie-Gower model has been studied by many researchers, but the age of the population is often be ignored. But, many cases in life consider that the population dynamics depend on the stage-structure, e.g. juvenile and mature population. This case is commonly encountered in mammals. In 1990, Aiello and Freedman [5] studied the model of one species undergoing two stage. This model assumes that the average age of the mature population is expressed as a delay time constant, this implies the late birth of juvenile population and reduced juvenile population that turn into mature population. In 2006, Cui and Yasuhiro [6] introduced predator-prey models with periodic functions with age structure on prey population. Then Cai and Song [7] studied predator-prey models whose predator population is divided into two stages.

In this paper, we modify Yu [3] model's and construct a Leslie Gower predator-prey model with stage-structure population on the predator, because the age of population can influence the evolution of the population. After that, we investigate the behavior of the model numerically and we will compare the behavior of model with or without the stage-structure on predator population.

MATERIAL AND METHOD

Construction Model

In this section, Leslie-Gower predator-prey model in Yu [3] is modified into Leslie-Gower predator-prey model with stage-structure on the predator. We modify Yu's predator-prey model [3] into a predator-prey model of Leslie-Gower with stage-structure on predator by adding the assumption that predator can be divided into two

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stages, that are juvenile predator and mature predator.

Determining Equilibrium Points

The equilibrium point is the solution of a model that has a constant value, which can be determined by solving:

$$\frac{dx}{dt} = \frac{dy_1}{dt} = \frac{dy_2}{dt} = 0.$$

Numerical Simulation

Numerical simulations are performed to present the interaction of each population in the system (1) and (2). In this paper, several different transition rate values (β) are used to determine the effect of stage-structure on the model. Simulations are done by solving the model using the 4th order Runga Kutta method [8].

RESULT AND DISCUSSION

Mathematical Model

Leslie-Gower's predator-prey model on [4] is expressed as follows

$$\begin{aligned}\frac{dx}{dt} &= r_1 x\left(1-\frac{x}{k_1}\right) - \frac{\alpha xy}{a+bx+cy},\\ \frac{dy}{dt} &= r_2 y\left(1-\frac{y}{x+k_2}\right).\end{aligned} \quad (1)$$

In this model, it is presumed that the prey grows following the logistic growth rate with the birth rate of the prey (r_1) and carrying capacity (k_1). Predator can grow logistically with birth rate (r_2) and carrying capacity depending on the number of prey populations and environmental protection (k_2) for the survival of the predatory population. Predator can attack prey populations with predation rates modeled by the Beddington-DeAngelis functional rensponse. This functional rensponse assumes that predation not only depends on the prey population but also on the predator population.

In this paper, we add the assumption that predator experiences stage-structure, that are mature predator and juvenile predator. We add the assumption that the birth rate of mature predator is derived from a juvenile that turn into mature predator with rate denoted by β. While mature predator has a natural rate of death that is symbolized by γ.

We obtain Leslie-Gower predator-prey model with stage-structure on predator as

$$\begin{aligned}\frac{dx}{dt} &= r_1 x(t)\left(1-\frac{x}{k_1}\right) - \frac{\alpha xy_2}{a+bx+cy_2},\\ \frac{dy_1}{dt} &= r_2 y_1(t)\left(1-\frac{y_1}{x+k_2}\right) - \beta y_1,\\ \frac{dy_2}{dt} &= \beta y_1 - \gamma y_2,\end{aligned} \quad (2)$$

with $r_1, r_2, a, b, c, k_1, k_2, \alpha, \beta, \gamma > 0$.

Equilibrium Points

Model (2) has four equilibrium points, namely

1. Trivial equilibrium point $E_0 = (0,0,0)$,
2. Predator-free equilibrium point $E_1 = (k_1, 0, 0)$,
3. Survival of predator equilibrium point $E_2 = \left(0, k_2\left(1-\frac{\beta}{r_2}\right), \frac{\beta}{\gamma}k_2\left(1-\frac{\beta}{r_2}\right)\right)$,
4. Interior equilibrium point $E_3(x^*, y_1^*, y_2^*)$ where $y_1^* = \left(1-\frac{\beta}{r_2}\right)(x^*+k_2)$, $y_2^* = \frac{\beta}{\gamma}\left(1-\frac{\beta}{r_2}\right)(x^*+k_2)$ and x^* are positive solution of quadratic equation $(b\gamma r_1 r_2 + c\beta r_1 r_2 - c\beta^2 r_1)x^{*2} + (c\beta^2 k_1 r_1 - c\beta k_1 r_1 r_2 - \alpha\beta^2 k_1 + \alpha\beta k_1 r_2 + c\beta k_2 r_1 r_2 + a\gamma r_1 r_2 - c\beta^2 k_2 r_1 - b\gamma k_1 r_1 r_2)x^* + c\beta^2 k_1 k_2 r_1 - a\gamma k_1 r_1 r_2 - c\beta k_1 k_2 r_1 r_2 + \alpha\beta k_1 k_2 r_2 - \alpha\beta^2 k_1 k_2 = 0.$

Numerical Simulation

In this section, some numerical simulations are carried out to see the interaction between the population in model (1) and (2). Numerical simulations are performed using parameters in Table 1.

Table 1. Parameters Value

Parameters	r_1	r_2	k_1	k_2	α	γ	a	b	c
Value	1.6	1.5	1.72	1.2	1	0.05	0.7	1	0.1

Using parameters as in Table 1, we obtain a solution for model (1) as plotted in Figure 1. We can see that in this case, the two populations can live together and stable toward a certain value.

Next we perform simulation using the same parameters as in Table 1, but with varying the value of β to observe the effect of stage-structure on the model. The result is shown in Figure 2. We get that for a relatively small β

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value, for example $\beta = 0.75$ or $\beta = 1.25$ then the population of prey may be extinct, while the predator (juvenile and mature) will survive. If we set $\beta = 1.48$, then the three population will live together towards a certain value. And when $\beta = 1.75$ and $\beta = 2$, then the prey population will survive while the predator (juvenile and mature) will go to extinction. This shows that the value of transition rate β is very important in this model.

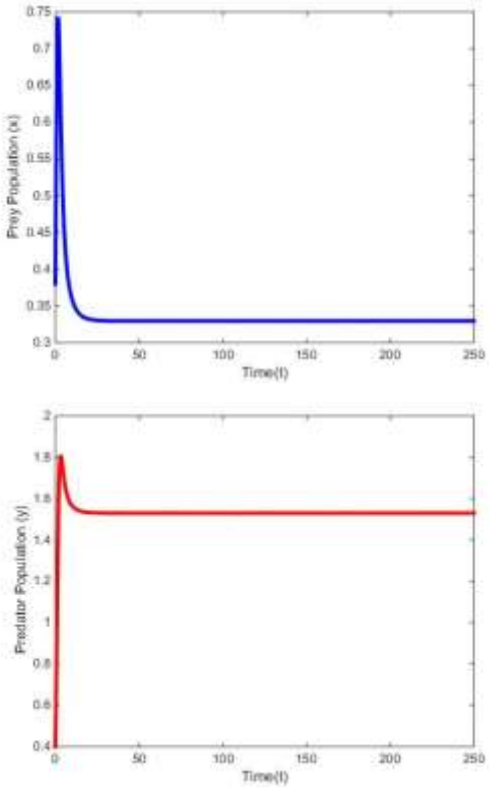

Figure 1. Numerical Solution of Model (1) for Prey and Predator Population.

To see the effect of β on the behavior of the system, we perform simulation using $\beta \in [0.5, 2]$ and it is found that for $\beta \in [0.5, 1.2]$ shows the same behavior as $\beta = [1.2, 1.4458]$. Therefore, we plot of the convergent solution was carried out for $\beta \in [1.2, 2]$ to see better results. The plot of the convergent solution shown in Figure 3. Notice in Table 1 that the birth of the predator is $r_2 = 1.5$. If $\beta > r_2$, then the predator (juvenile and mature) will extinct, while the prey survive. This indicates that the solution converges to the equilibrium point E_1.

While if $\beta < r_2$, then the predator will not extinct, and the prey may be extinct if the β are relatively small. Such behavior indicates that the solution converges to the equilibrium point E_2. However, if β are large enough but $\beta < r_2$, then all three populations can live together, indicating that the solution converges to the equilibrium pointE_3.

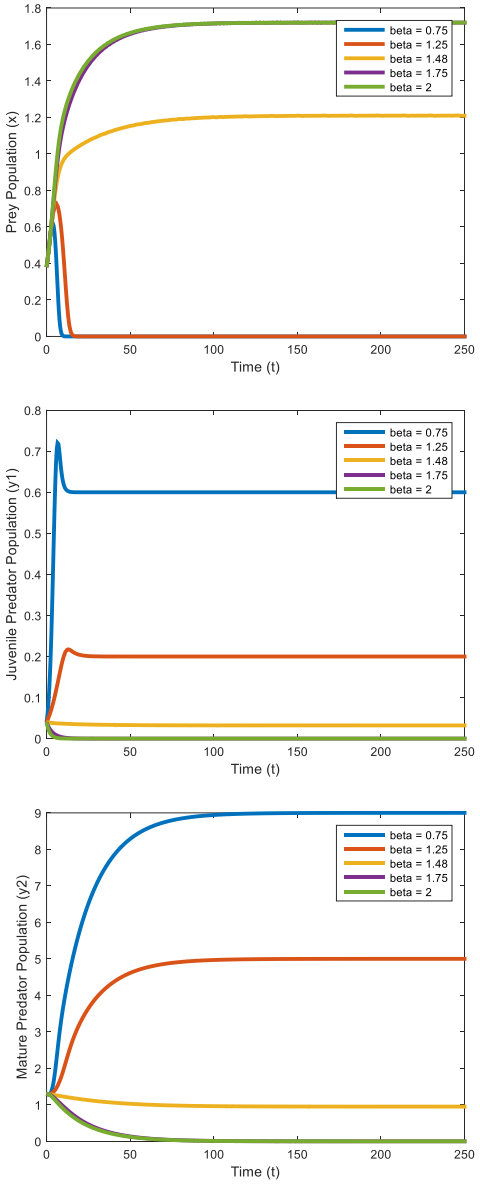

Figure 2. Numerical Solution of Model (2) for Prey, Juvenile Predator, and Mature Predator Population.

We can see from Figure 3, there is an interval in β value where the solution does not converge to any equilibrium point. In more detail, it is obtained numerically that if $0.5 < \beta < 1.4458$ then the solution will converge to E_2, if $1.4546 < \beta < 1.4955$ then the solution will converge to E_3, and if $1.5081 < \beta < 2$ then the solution will converge to E_1. If

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$1.4458 < \beta < 1.4546$ or $1.4955 < \beta < 1.5081$, then the solution is not convergent. For example, if we take $\beta = 1.45$, the solution of model to (2) shown in Figure 4. It appears that the three populations live together with the periodically changing population.

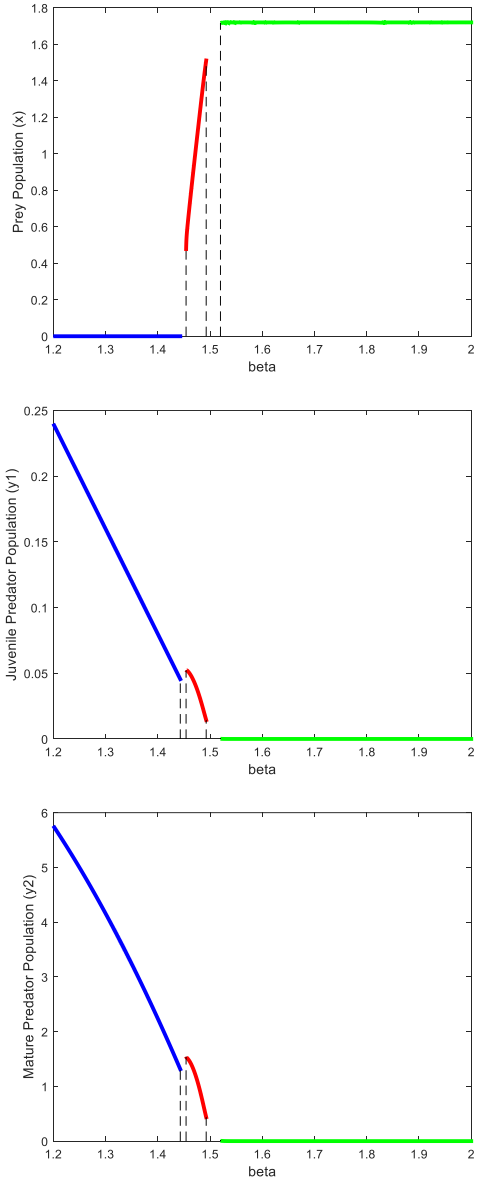

Figure 3. Convergent Solution of Model (2).

In mathematics, this condition indicates the presence of Hopf bifurcation. That is, there is a stability change in the model (2) because of the change in a parameter. In this case, the bifurcation parameter is β.

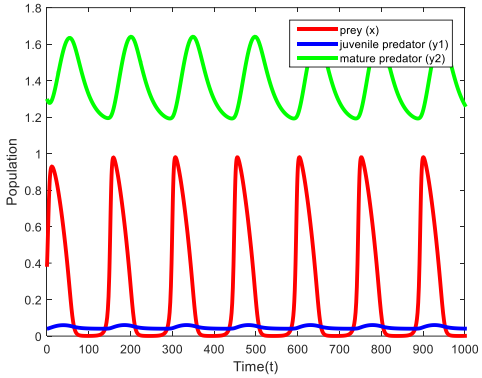

Figure 5. Solution of model (2) with $\beta = 1.45$

CONCLUSION

In this paper, we proposed Leslie Gower predator-prey model with stage-structured on predator using the Beddington-DeAngelis functional response. We obtained the solution of the model numerically shown in Figure 2. Then we compare the solution behavior that occurs in the model with the solution behavior in the Leslie-Gower predator-prey model without stage-structure. It is shown that we have four equilibrium from model (2), that are E_0, E_1, E_2, and E_3. We get that if we set $\beta > r_2$, then the predator (juvenile and mature) will extinct, while the prey survive or the solution converges to E_1. But if we set $\beta < r_2$, then the predator will not extinct, and the prey may be extinct if the β are relatively small or the solution converges to the equilibrium point E_2. However, if β are large enough but $\beta < r_2$, then all three populations can live together which indicate that the solution converges to the equilibrium point E_3.

And if we compare the solution of model (2) with the solution behavior in the Leslie-Gower predator-prey model without stage-structure, it can be seen that with stage-structure making the behavior of the model more variable depending on the value of β and it makes β is very important parameter which may determined the long-term behavior of both prey and predator.

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Proximate Composition of Some Common Fish Feed Flour Substitute

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Abstract

Indonesia has very large territorial water, so it supports the development of the economy in the field of fisheries, one of which is through aquaculture. Recently, fish meal has become the most expensive protein ingredient in aquaculture feeds. We aim to proximately analyse of some common alternative flour as fish feed ingredient i.e. fish flour, shrimp head flour, soybean flour, and bran flour to get best-recommended flour for the type of cultured fish. Fish flour, shrimp head flour, soybean flour, and bran flour was purchased from the fish farmer in Malang who use alternative fish feed flour for their aquaculture. Proximate analysis carried out by protein content, moisture content, fat and fiber. Proximate analysis was conducted in the Laboratory of Fishery Products Engineering, University of Brawijaya on April 2018. The results showed that shrimp head flour had higher protein content than others flour that is about 48.71%, fat and carbohydrate content about 7.99%, and 18.34% respectively. Soybean flour contains protein, fat and carbohydrate about 31.29%, 20.75%, and 30.45% respectively. Carnivorous fish culture can use high protein flour as an alternative ingredient for feed such as fish flour and shrimp head flour; balance proximate composition for omnivorous fish cultures such as soy flour, and high carbo flour such as bran flour for herbivorous fish culture.

Keywords: Bran flour, proximate analysis, shrimp head flour, soybean flour.

INTRODUCTION

Indonesia has very large territorial waters, thus it supports the development of the economy in the field of fisheries, e.g. aquaculture. In aquaculture activities, the feed is a major factor that greatly affects fisheries production. In this case, the availability of nutrients for fish growth [1].

Commercial fish feed commonly used in aquaculture activities. High prices are one of the main problems for fish farmers. One of the causes of high feed prices is due to the availability of expensive ingredients, such as protein from fish which is needed as one of the main composition of feed production.

For commercial culture of fish, the formulation of low-cost balanced diet using locally available agro-industry byproducts is needed. Recently, fish meal has become the most expensive protein ingredient in aquaculture feeds. Many studies have shown considerable success in partially replacing fishmeal with soybean meal and other soybean products in the diet for various fish species [2-4]. Shrimp head waste silage has been reported as a protein source to formulate the feed of African catfish (*Clarias gariepinus*) [5]. Bran flour and fish meal have also been reported to be alternative fish feed formulations [6].

Based on the previous description, it is necessary to do a proximate test of some of these materials. Thus, we will know the suitability of the need for fish feed formulations with nutritional content which includes protein, fat, carbohydrate, ash, and crude fiber content in each ingredient. We aims to proximately analyse some common alternative flour as fish feed ingredient, i.e. fish flour, shrimp head flour, soybean flour, and bran flour to get best recommended flour, based on the type of cultured fish.

MATERIAL AND METHOD

Material Preparation

Fish flour, shrimp head flour, soybean flour, and bran flour was purchased from fish farmer in Malang who use alternative fish feed flour for their aquaculture.

Proximate Analysis

Proximate analysis consisted of crude protein content, ash content, crude fat content, moisture content, and crude fiber according to AOAC [7]. Explained as follows :

Protein and Ash Content Analysis

Protein content analysis was carried out by the Kjeldahl methods described in Chang [8].

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Moisture Content Analysis

This analysis uses a weigh bottle that has been oven at 105°C for 4 hours, and cooled in a desiccator for 30 minutes, before being weighed. In the weighing bottle, 2 grams of selected flour was added and re-ovened at 105°C for 4 hours. Then cooled again in the desiccator for 30 minutes, and then weighed. Repeat this treatment for all selected flours, until you find the desired fixed weight. Moisture content can be calculated using the following formula:

$$Moisture\ Content = \frac{Fresh\ Weight\ (g) - Dry\ Weigh\ (g)}{Fresh\ Weight\ (g)} \times 100\%$$

Fat Content Analysis

The filter paper that has been provided is cut to a length of 10 cm and a width of 8 cm, then oven at a temperature of 105°C for 12 hours. The filter paper is weighed (a) and weighs 0.5 grams of sample (b). The sample is wrapped in filter paper and put into soxhlet. 150 mL of ether petroleum was put into soxhlet flask for extraction at 40-60°C for 5-6 hours, then the sample was put in oven at 105°C for 12 hours, and cooled in a desiccator for 30 minutes before being re-weighed (c). Fat content is calculated using the following formula:

$$Fat\ Content = \frac{Weight\ of\ Extracted\ fat\ (a+b) - c}{dryweight\ (b)} \times 100\%$$

Fiber Content Analysis

Dry the tools and ingredients by oven, then the tool is put in a desiccator and weighed. As much as 2.5 - 5 grams of dried sample wrapped in filter paper. The sample was extracted with diethyl ether for 6 hours using soxhlet. The sample was put into a 600 mL erlenmeyer, added to the sample 200 mL of boiling H_2SO_4 solution, and then boiled again for 30 minutes. The suspension obtained is filtered with filter paper and the residue left in the erlenmeyer or on the filter paper is washed with boiling water.

The residue was washed again with 200 mL of NaOH solution with the same treatment as the treatment of H_2SO_4 solution. The residue was filtered again with known weight filter paper, and washed with 10% K_2SO_4 solution, boiling water, and then using 95% alcohol. Filter paper used was oven at 110°C, and cooled in a desiccator then weighed. The weight of the residue is the weight of the crude fiber. Coarse fiber is calculated using the following formula:

$$Fiber\ Content = \frac{Crude\ fiberweight}{Initial\ Sample\ Weight} \times 100\%$$

RESULT AND DISCUSSION

The results of the analysis obtained the proximate content of the fish meal, shrimp head flour, soybean flour, and bran flour showed in Table 1. The highest protein content other than the fish meal is shrimp flour which is 48.71% and the lowest is bran flour 10.01%. Protein is important for the fish body because on average 65-75% of the fish's dry body weight is protein [3]. So that fish with protein eat can maintain body cells and can reproduce. The need for protein in freshwater pomfret that have ranged from 25-37% [9].

Base on figure 1, the highest fat content was found in soybean flour 20.75% and the lowest in the fish meal was 2.99%. Fat plays a very important role as a source of energy in fish feed [10]. The levels of fat present in the feed are generally in the range of 6.89% [11]. Fat content in shrimp head flour, soy flour, and bran flour, has been able to meet the standard fat requirements for fish growth.

The highest fiber content in Figure 1 is on soy flour by 12.22% and the lowest in shrimp flour head is 6.10%. The high percentage of coarse fiber causes the fish difficult to digest food [12]. High crude fiber content is less utilized by fish, especially for omnivorous fish but more carnivorous tendencies such as catfish, thus less effectively digesting polysaccharides than species that tend to herbivores such as tilapia [13-15]. Digestive functions of some carnivorous fish less able to digest carbohydrates. This is because of α-amylase levels on carnivorous fish are very low [16].

Ash is a residue produced by the combustion of organic matter in the form of inorganic materials in the form of oxides, salts, and minerals. Based on the results of the proximate analysis in Table 1, the highest content of ash content was the fish flour of 21.26% and the lowest of soybean flour by 5.28%. Mineral content or ash content is very important for teething and scales [17]. However, the high content of ash cannot be used as feed ingredients. The ash content in the feed represented the mineral content of the feed, with corresponding rate was 3-7% [18]. Based on the table, fish flour and shrimp flour have ash content that exceeds the needs of fish while bran meal and soy flour are still in the range of fish needs.

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Table 1. Proximate Analysis of Fish flour, Shrimp Head Flour, Soybean Flour, and Bran flour

Material	Protein (%)	Fat (%)	Fiber (%)	Ash (%)	Carbo (%)
Fish Flour	55.78 ± 1.4	2.99 ± 1.2	9.38 ± 2.0	21.26 ± 1.3	10.55 ± 1.4
Shrimp Head Flour	48.71 ± 0.8	7.99 ± 1.7	6.10 ± 2.2	18.84 ± 0.5	18.34 ± 2.1
Soy Flour	31.29 ± 2.1	20.75 ± 2.2	12.22 ± 1.4	5.28 ± 0.8	30.45 ± 2.5
Bran Flour	10.01 ± 1.9	11.00 ± 1.3	8.88 ± 0.7	7.16 ± 1.6	62.92 ± 0.9

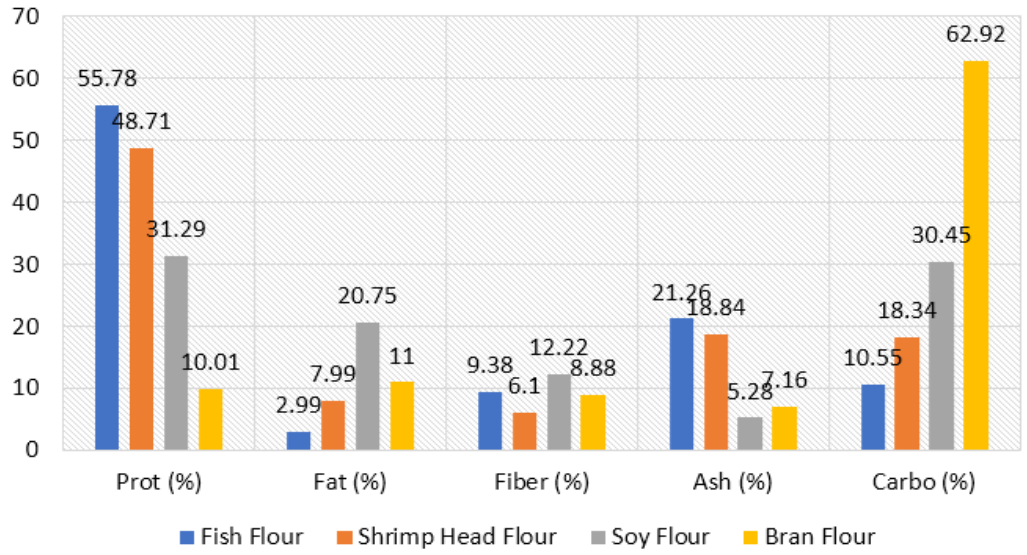

Figure 1. Proximate analysis of Fish Flour, Shrimp Head Flour, Soybean Flour, and Bran Flour

Carbohydrates in the fish feed are present in the form of crude fiber and Free-Nitrogen extract (FNE), but crude fiber has low nutritional value [19]. Fish used up to 45% carbohydrate feed for growth [20]. The highest BETN content was found in bran flour of 62.92% and the lowest in fish flour of 10.55%. The need for carbohydrate feed content is varied to each group of fish in sizes and species [21]. The comparison between carbohydrates and proteins in feed affects the protein utilization for fish tissue formation [22]. If the carbohydrate content is not sufficient as a source of energy, fish will use protein as a source of energy for tissue formation and as a driving force for the body. Digestive functions capable of hydrolyzing a greater variety of carbohydrate-containing feedstuffs have developed in herbivorous and omnivorous fish, which in contrast to carnivorous fish [23].

The fish use energy for growth, daily metabolism, and life-keeping activities. Energy requirements for fish are usually associated with protein requirements. The high protein content of feed is good for fish. The habit of eating freshwater pomfret belongs to the omnivorous fish group, but it is also mentioned that this fish tends to be a carnivore that is visible from its sharp teeth which, while still seed-sized, likes plankton and aquatic plants. So that can be said this pomfret can utilize protein, fat, and carbohydrate for their energy needs.

CONCLUSION

Shrimp head flour had contents of protein, fat, and carbohydrate about 48.71%±0.8, 7.99%±1.7, and 18.34%±2.1, respectively. Soybean flour contains protein, fats, and carbohydrates about 31.29%±2.1, 20.75%±2.2, 30.45%±2.5, respectively. Fish flour had contents of protein, fat, and carbohydrate about 55.78%±1.4, 2.99%±1.2, 10.55%±1.4, respectively. Carnivorous fish culture can use high protein flour as an alternative ingredient for feed such as fish flour and shrimp head flour. Balance proximate composition is for omnivorous fish culture such as soy flour, and high carbo flour such as bran flour for herbivorous fish culture.

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MANUSCRIPT SUBMISSION

FOCUS AND SCOPE

Journal of Experimental Life Science (JELS) is scientific journal published by Graduate Program of Brawijaya University as distribution media of Indonesian researcher's results in life science to wider community. JELS is published in every four months. JELS published scientific papers in review, short report, and life sciences especially nanobiology, molecular biology and cellular biology. JELS is scientific journal that published compatible qualified articles to academic standard, scientific and all articles reviewed by expert in their field.

Journal of Experimental Life Science (JELS) have vision to become qualified reference media to publish the best and original research results, and become the foundation of science development through invention and innovation on cellular, molecular, and nanobiology rapidly to community.

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[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang, Indonesia
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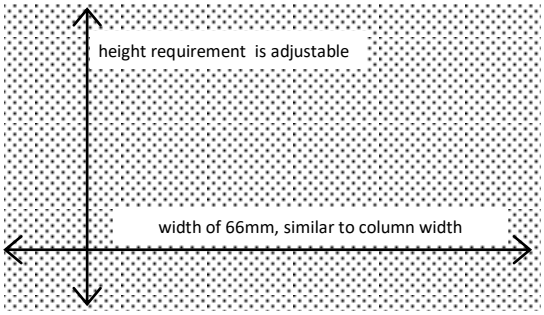

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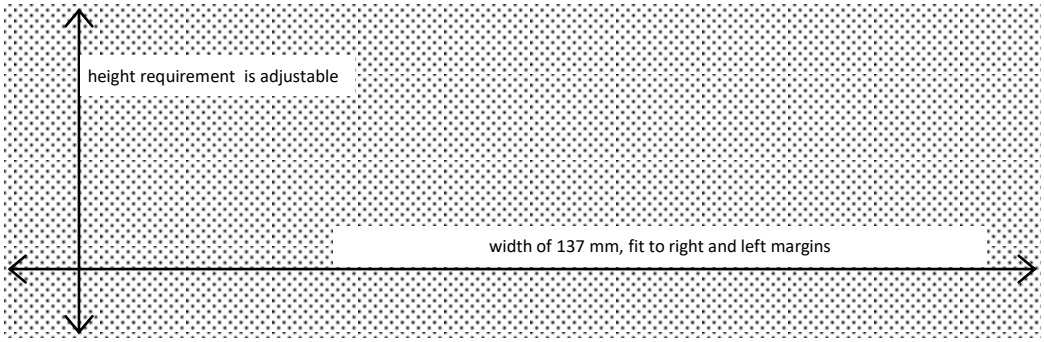

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CONCLUSION (Calibri 10 Bold, Left, Capslock)

Conclusion of the study's findings are written in brief, concise and solid, without more additional new interpretation. This section can also be written on research novelty, advantages and disadvantages of the research, as well as recommendations for future research.(Calibri 10 Justify)

ACKNOWLEDGEMENT (Calibri 10 Bold, Left, Capslock)

This section describes gratitude to those who have helped in substance as well as financially. (Calibri 10 Justify)

REFERENCES (Calibri 10 Bold, Left, Capslock)

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